ADENOSINE AND THE CORONARY VASCULATURE IN NORMOXIC AND POST-ISCHAEMIC HEARTS

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STATEMENT OF ORIGINALITY

The work presented within this thesis was performed in the Heart Foundation Research Centre, Griffith University. The research was carried out under the supervision of Associate Professor John P. Headrick.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself. This work has not previously been submitted for a degree or diploma in any other university.

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ABSTRACT

While previous research into the pathogenesis of ischaemic and reperfusion injuries has focussed on the cardiac myocyte, there is increasing evidence for a crucial role for coronary vascular injury in the genesis of the post-ischaemic phenotype [1-3]. Post-ischaemic vascular injury may be manifest as a transient or sustained loss of competent vessels, impairment of vascular regulatory mechanisms, and ultimately as the "no-reflow" phenomenon (inability to sufficiently reperfuse previously ischaemic tissue despite the removal of the initial obstruction or occlusion). It is now appreciated that the earliest distinguishing feature of various forms of vascular injury (including atherosclerosis and infarction) is "endothelial dysfunction", which is the marked reduction in endothelial-dependent relaxation due to reduced release or action of endothelial nitric oxide (NO). Importantly, vascular injury may worsen myocardial damage *in vivo* [4,5], significantly limiting tissue salvage and recovery.

The pathogenesis of post-ischaemic vascular injury and endothelial dysfunction is incompletely understood, but has generally been considered to reflect a cardiovascular inflammatory response, neutrophils playing a key role. However, while blood-borne cells and inflammatory elements are undoubtedly involved in the 'progression' of vascular injury [6,7], accumulating evidence indicates that they are not the primary 'instigators' [8]. It should be noted that a wealth of controversial findings exists in the vascular injury literature and mechanisms involved remain unclear. Indeed, multiple mechanisms are likely to contribute to post-ischaemic vascular injury.

Adenosine receptors are unique in playing a role in physical regulation of coronary function, and also in attenuating injury during and following ischaemia. While the adenosine receptor system has been extensively investigated in terms of effects on myocardial injury [9,10], little is known regarding potential effects of this receptor

system on post-ischaemic coronary vascular injury. This thesis initially attempts to further our understanding of the role of adenosine in normal coronary vascular function, subsequent chapters assess the effect of ischaemia-reperfusion on vascular function, and adenosine receptor modification of vascular dysfunction in the isolated asanguinous mouse heart.

Specifically, in Chapter 3 the receptor subtype and mechanisms involved in adenosine-receptor mediated coronary vasodilation were assessed in Langendorff perfused mouse and rat hearts. The study revealed that A_{2A} adenosine receptors (A_{2A}ARs) mediate coronary dilation in the mouse *vs.* A_{2B} adenosine receptors (A_{2B}ARs) in rat. Furthermore, responses in mouse involve a sensitive endothelial-dependent (NO-dependent) response and NO-independent (K_{ATP}-mediated) dilation. Interestingly, the ATP-sensitive potassium channel component predominates over NO-dependent dilation at moderate to high agonist levels. However, the high-sensitivity NO-dependent response may play an important role under physiological conditions when adenosine concentrations and the level of A_{2A}AR activation are low.

In Chapter 4 the mechanisms regulating coronary tone under basal conditions and during reactive hyperaemic responses were assessed in Langendorff perfused mouse hearts. The data support a primary role for K_{ATP} channels and NO in mediating sustained elevations in flow, irrespective of occlusion duration (5-40 s). However, K_{ATP} channels are of primary importance in mediating initial flow adjustments after brief (5-10 s) occlusions, while K_{ATP} (and NO) independent processes are increasingly important with longer (20-40 s) occlusion. Evidence is also presented for compensatory changes in K_{ATP} and/or NO mediated dilation when one pathway is blocked, and for a modest role for A_{2A}ARs in reactive hyperaemia.

In Chapter 5 the impact of ischaemia-reperfusion on coronary function was examined, and the role of A_1 adenosine receptor (A_1AR) activation by endogenous adenosine in modifying post-ischaemic vascular function was assessed in isolated buffer perfused mouse hearts. The results demonstrate that ischaemia does modify vascular control and signficantly impairs coronary endothelial dilation in a model devoid of blood cells. Additionally, the data indicate that post-ischaemic reflow is significantly determined by A_{2A}AR activation by endogenous adenosine, and that A_1AR activation by endogenous adenosine protects against this model of vascular injury.

Following from Chapter 5, the potential of A_1, A_{2A} and A_3AR activation by exogenous and endogenous agonists to modulate post-ischaemic vascular dysfunction was examined in Chapter 6. Furthermore, potential mechanisms involved in injury and protection were assessed by comparing effects of adenosine receptors to other "vasoprotective" interventions, including anti-oxidant treatment, Na^+/H^+ exchange (NHE) inhibition, endothelin (ET) antagonism, and 2,3-butanedione monoxime (BDM). The data acquired confirm that post-ischaemic endothelial dysfunction is reduced by intrinsic A_1AR activation, and also that exogenous A_3AR activation potently reduces vascular injury. Protection appears unrelated to inhibition of ET or oxidant stress. However, preliminary data suggest A_3AR vasoprotection may share signalling with NHE inhibition. Finally, the data reveal that coronary reflow in isolated buffer perfused hearts is not a critical determinant of cardiac injury, suggesting independent injury processes in post-ischaemic myocardium *vs.* vasculature.

Collectively, these studies show that adenosine has a significant role in regulating coronary vascular tone and reactive hyperaemic responses via NO and K_{ATP} dependent mechanisms. Ischaemia-reperfusion modifies vascular control and induces significant endothelial dysfunction in the absence of blood, implicating neutrophil

independent injury processes. Endogenous adenosine affords intrinsic vasoprotection via A_1AR activation, while adenosinergic therapy via exogenous A_3AR activation represents a new strategy for directly protecting against post-ischaemic vascular injury.

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ABBREVIATIONS

5'-AMP	adenosine 5' monophosphate
5'-NT	5'-nucleotidase
A_1AR	A_1 adenosine receptor
A_{2A}AR	A_{2A} adenosine receptor
A_3AR	A_3 adenosine receptor
AC	adenylyl cyclase
ACh	acetylcholine
AD	adenosine deaminase
Ado	adenosine
ADP	adenosine 5' diphosphate
AIF	apoptosis inducing factor
AK	adenosine kinase
ANT	adenine nucleotide translocase
ATP	adenosine 5' triphosphate
AV node	atrioventricular node
BK_{Ca}	large conductance
cAMP	cyclic adenosine monophosphate
CBF	coronary blood flow
cGMP	cyclic guanosine monophosphate
DAG	diacylglycerol
DFF	DNA fragmentation phase
DNA	deoxyribonucleic acide
EDHF	endothelium-derived hyperpolarising factor

EDRF	endothelium-derived relaxing factor
eNOS	endothelial nitric oxide synthase
EET	epoxyeicosatrienoic acids
ET	endothelin
$G_{i/o}$	guanine nucleotide binding inhibitory protein
GRKs	G-protein-coupled receptor kinases
G_s	Guanine nucleotide binding stimilatory protein
HEP	high-energy phosphate
I_{Ca}	L-type calcium channel current
$I_{KACh/Ado}$	inwardly-rectifying potassium channel current
I_{KATP}	ATP-sensitive potassium channel current
IK_{Ca}	intermediate conductance
IP_3	inositol-1,4,5,-triphosphate
ISF	interstitial fluid
I_{TI}	transient inward current
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	Ca^{2+} - sensitive potassium channels
K_d	dissociation constant
LAD	left anterior descending artery
Mito K_{ATP}	mitochondrial ATP-sensitive potassium channel
MPT	mitochondrial permeability transition
mPTP	mitochondrial permeability transition pore
n	number of observations
NAD	nicotinamide adenine dinucleotide

NCE	Na^+/Ca^{2+} exchange
NHE	Na^+/H^+ exchange
NO	nitric oxide
nt	nucleoside transporter
O_2^{-*}	superoxide anion
OH^*	hydroxyl radical
$ONOO^-$	peroxynitrite
pEC_{50}	negative logarithm of EC_{50} (concentration inducing haf-maximal response)
PFK	phosphoructokinase
PGI_2	prostacyclin
P_i	inorganic phosphate
PKC	protein kinase C
PLC	phospholipase C
PMN	polymorphonuclear neutrophils
PO_2	partial pressure of oxygen
PS	phosphatidylserine
ROS	reactive oxygen species
SA node	sinoatrial node
SAH(H)	s-adenosylhomocysteine (hydrolase)
Sarc K_{ATP}	sarcolemmal ATP-sensitive potassium channel
SEM	standard error of the mean
sGC	soluble guanylyl cyclase
SK_{Ca}	small conductance
SR	sarcoplasmic reticulum

TNF-α	tumour necrosis factor – alpha
XD	xanthine dehydrogenase
XO	xanthin oxidase

COMPOUND ABBREVIATIONS

2-CAD	2-chloroadenosine
8-SPT	8-(p-sulfophenyl)-theophylline
ADP	adenosine diphosphate
ATA	aurintricarboxylic acid
BDM	2,3-butanedione monoxime
BIIB-513 MS	benzamide,N-(aminoiminomethyl)-4-[4-(2-furanylcrbonyl)-1-piperazinyl]-3-(methylsulfonyl), methanesulfonate]
Bkn	bradykinin
CGS 21680	2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethyl carboxamidoadenosine
CHA	N^6-cyclohexyladenosine
Cl-IB-MECA	2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide
CSC	8-(3-chlorostyryl)caffeine
DMSO	dimethylsulfoxide
DPCPX	1,3-dipropyl-8-cyclopentylaxanthine
H_2O_2	hydrogen peroxide
L-NAME	N^6-nitro-*L*-arginine methyl ester
L-NOARG	N^G-nitro *L*-arginine
MPG	*N*-2-mercaptopropionyl glycine
MRS 1220	9-Chloro-2-(2-furanyl)-5-phenylacetyl)amino][1,2,4]- triazolo[1,5-c]quinazoline

NaOH	sodium hydroxide
NECA	5’-N-ethylcarboxamidoadenosine
PD 142893	N-Acetyl-β-Phenyl-D-Phe-Leu-Asp-Ile-Ile-Trp
R-PIA	R(-)-N^6-(2-phenylisopropyl)adenosine
SCH 58261	7-(2-phenylethyl)-5-amino-2(2-furyl)-pyrazolo-[4,3,e]-1,2,4-triazolo-[1,5-c]pyrimidine
SNP	sodium nitroprusside
SOD	superoxide dismutase

CHAPTER 1

LITERATURE REVIEW

Preface: This project focuses on the role of adenosine in the coronary vasculature under physiological conditions and in the modulation of vascular injury during ischaemia-reperfusion. Thus the following review will initially cover general aspects of adenosine and its role in coronary regulation, followed by a detailed review of post-ischaemic injury mechanisms in both myocardium and vasculature. Furthermore, this review will highlight the significance of coronary vascular injury on myocardial salvage, and will explore the potential of adenosinergic therapy to combat post-ischaemic vascular dysfunction.

I. ADENOSINE

Adenosine is classified as a purine nucleoside and is comprised of adenine and ribose, coupled by a glycosidic bond [11]. Adenosine is an autocoid released tonically by myocytes, endothelium, and neutrophils, and generated in increasing quantities when constituent cells are "stressed" or subjected to injurious stimuli [10]. Many of the cells that produce adenosine (including myocardial, vascular, fibroblast and inflammatory cells) also express adenosine surface receptors. When adenosine receptors are activated they can elicit a range of effects which often result in an improved balance between work performed or oxygen consumption and energy supply or delivery [10,11]. For example, in the heart adenosine is metabolically linked to the regulation of coronary blood flow (CBF), cardiac conductance and heart rate, substrate metabolism, and adrenergic sensitivity [10-15]. Therefore, adenosine is considered a "retaliatory metabolite" as it can mediate a decrease in cellular work in a negative feedback manner in order to protect the myocardium and prevent excessive breakdown of ATP [16]. Importantly for this study, adenosine may play a role as an endogenous determinant of ischaemic tolerance [17-21].

In outlining the role of adenosine in ischaemia-reperfusion I will first describe adenosine formation, receptors and effectors. I will then outline the role of adenosine in coronary regulation, before focusing on the impact of ischaemia-reperfusion on myocardial and vascular tissues and how adenosine might regulate injury.

Adenosine Formation, Metabolism and Transport

Formation

Adenosine formation is enhanced due to high consumption of adenosine triphosphate (ATP), which ultimately exceeds its supply, resulting in an increase in adenosine diphosphate (ADP) and adenosine 5' monophosphate (5'-AMP), the latter providing substrate for adenosine formation via 5'-nucleotidases (5'-NT) [10] (Fig. 1.1). According to the myokinase reaction, AMP accumulation will proceed as a function of free $[ADP]^2$ [10,11,22]. Numerous studies have identified a strong correlation between free cytosolic [5'-AMP] and myocardial adenosine levels [23-29]. However, it is now apparent that adenosine formation generally increases much more than 5'-AMP [22,27,28,30,31]. Adenosine formation via additional paths, allosteric activation of 5'-NT (eg. by Mg^{2+} and ADP) [25,32], and/or inhibition of adenosine catabolism may account for this non-linear relationship [10].

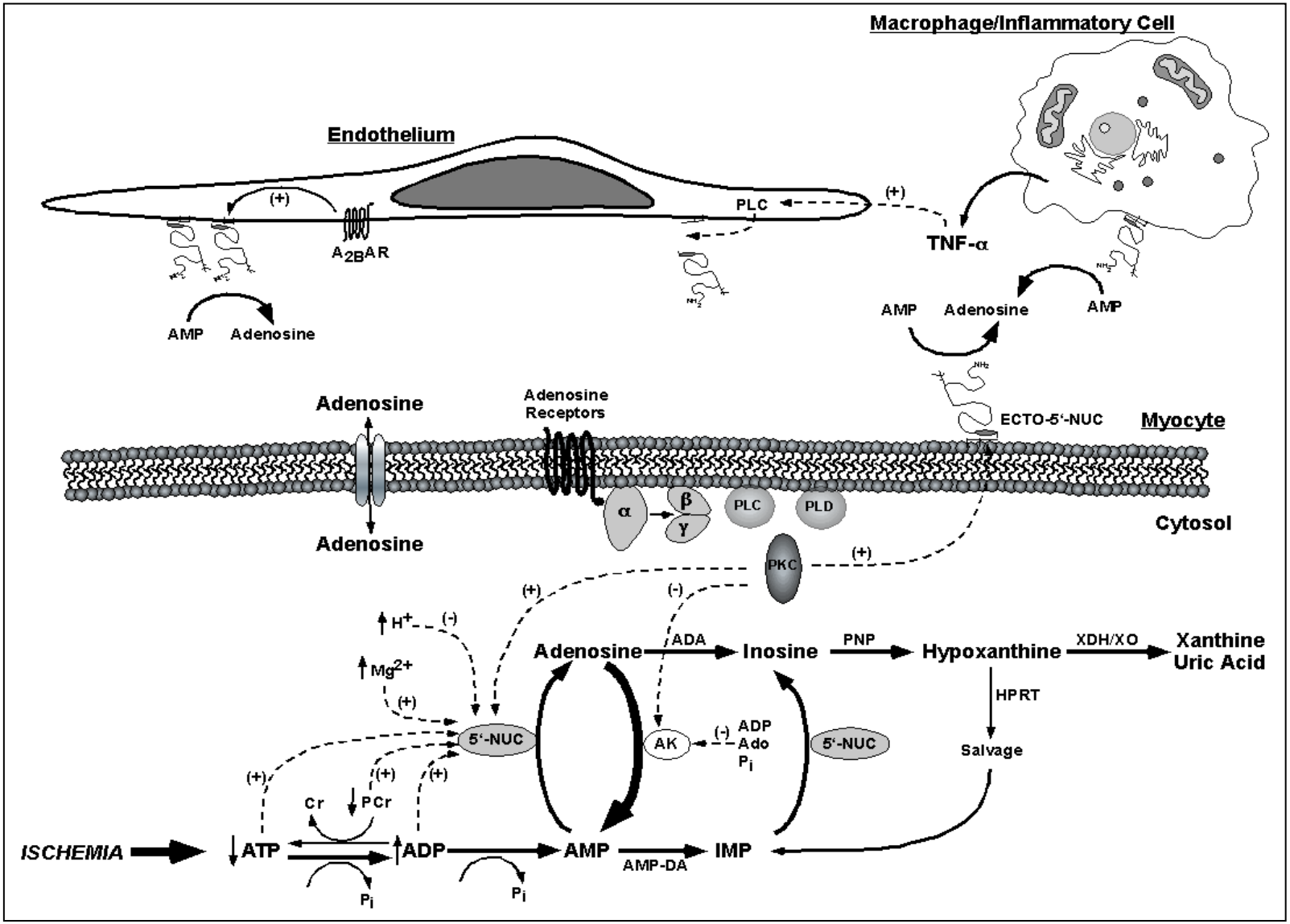

Figure 1.1 Pathways of adenosine formation and catabolism and their potential modulation by ischaemia-reperfusion modified from Headrick *et al*. [10].

Adenosine is formed via enzymatic dephosphorylation of 5'-AMP by intra- and extracellular 5'-NT (cytosolic 5'-NT or ecto 5'-NT, respectively), and from intracellular degradation of s-adenosylhomocysteine (SAH) catalysed by SAH-hydrolase [33,34]. Thus both AMP and SAH are the immediate precursors of adenosine. SAH-hydrolase converts adenosine and homocysteine to SAH in a reversible reaction, although the equilibrium constant favours formation of SAH under prevailing intracellular conditions. In fact, 90% of cytosolic adenosine is tightly bound to SAH, thus preventing its deamination, and ensuring that intracellular concentrations of adenosine are low during basal conditions [35,36]. Under normoxic conditions this "oxygen insensitive" pathway accounts for only 10-25% of total adenosine formation [37-39], and this relatively small proportion declines with stress [40]. In contrast, flux through cytosolic 5'-NT contributes 70% to total adenosine formation [22] (Fig. 1.1).

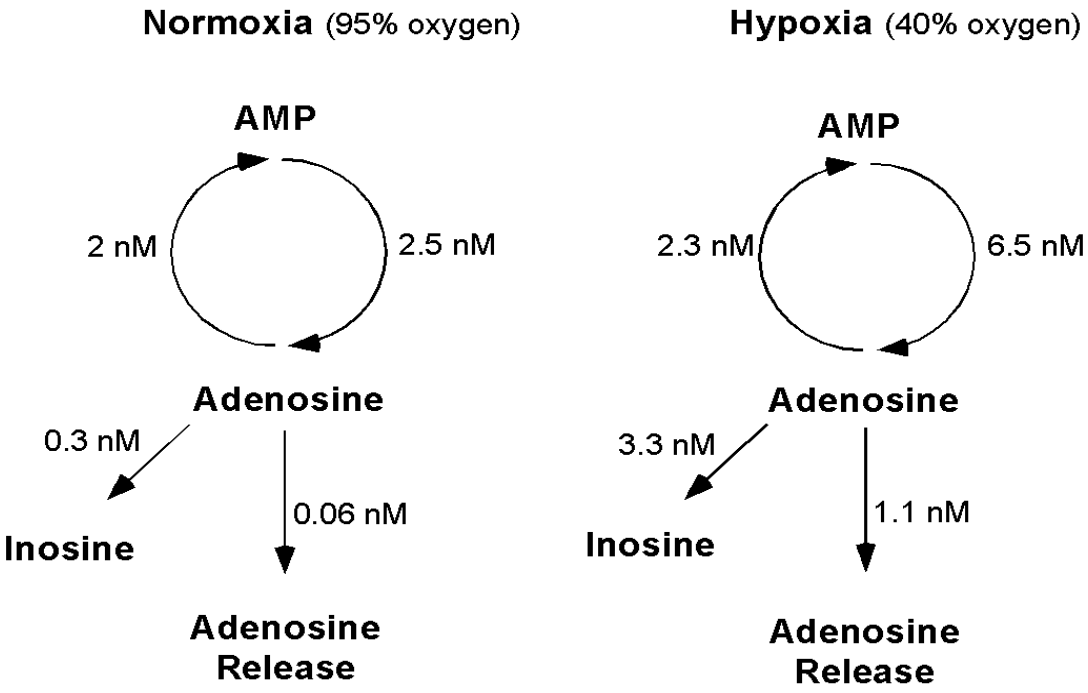

Figure 1.2 Fluxes and cytosolic adenosine concentrations under normoxic and hypoxic conditions modified from Decking *et al.* [22].

While adenosine is predominantly formed in cardiomyocytes [33,41], early work by Schrader and Gerlach [42] revealed that the endothelium could also generate

adenosine. Subsequent studies determined that the endothelium contributes 15-25% to vascular adenosine levels under basal conditions [43,44], whereas the proportion declines during stress (ischaemia, hypoxia, catecholamine stimulation, acidosis) [37,45]. This emphasises the importance of myocardial release during ischaemic insult [10].

Although most studies support the idea that adenosine is largely formed intracellularly [38,46], extracellular formation via ecto-nucleotidases on myocardial and vascular cells represents an alternate source of adenosine formation [47,48] (Fig. 1.1). During hypoxia or ischaemia adenosine formation is at least partially dependent on ecto 5'-NT. A study by Headrick *et al.* found that inhibition of ecto 5'-NT results in an increase in AMP while adenosine levels in venous and epicardial contents are reduced [49]. This suggests that there is a significant contribution from extracellular nucleotides. More recent evidence suggests that 8% of the total production of adenosine is formed in the extracellular compartment and that 70% of this is taken up into cellular regions [50]. Importantly, extracellular production of adenosine may be of particular significance, especially in modifying vascular injury (eg. modification of inflammatory processes). This is consistent with the findings of Vinten-Johansen and colleagues regarding post-ischaemic adenosine mediated protection within the vascular compartment [51-53].

Very recent evidence indicates that in addition to endothelial cells, inflammatory cells can also produce highly localised adenosine, which may be particularly relevant to cardioprotection. Specifically, adenosine is formed via ecto 5'-NT on the surface of neutrophils, mast cells, and activated endothelial cells during inflammatory responses [54-56] (Fig. 1.1). Moreover, Narravula *et al.* found that release and subsequent A_{2B}AR agonism upregulates endothelial ecto-5'-NT expression and activity [55]. Thus, adenosine modulates inflammatory processes via a paracrine feedback loop [54-56], which potentially limits resultant cardiovascular injury and cell death [9,57,58].

Metabolism and Transport

Cytosolic adenosine is either flushed out of the heart or catabolised by two alternate enzymatic processes: i) phosphorylation by adenosine kinase (AK) to AMP followed ultimately by reincorporation into the ATP pool (salvage pathway); or ii) deamination to inosine by adenosine deaminase (AD) [35] (Fig. 1.1). Since AD and AK are primarily cytosolic enzymes, adenosine must be taken up by cells before it can be deaminated or phosphorylated [35]. Intracellularly formed adenosine may be more likely to be phosphorylated than deaminated [22,59], based on K_M and activities for the two enzymes [60]. Recent studies in rat [61] and mouse [62] verify the majority of adenosine is normally cycled back to 5'-AMP, and that during ischaemia this salvage is inhibited by >90% [22]. Moreover, reduced flux through AK has been reported in de-energized myocytes [63]. Under normoxic conditions, the high turnover of this AMP-adenosine cycle limits purine loss from the heart and therefore adenosine formation in cells is much greater than coronary release [59,63]. However under hypoxic conditions when AK is inhibited, an amplification system is established whereby small changes in 5'-AMP and adenosine formation generate large changes in intracellular and extracellular adenosine levels [22,30,31]. Moreover, inhibition of AK by P_i may increase adenosine formation independently of any change in AMP [31]. Modulation of AK activity may account for disproportionate elevations in adenosine relative to [5'-AMP] during ischaemia or hypoxia [10]. Of greater interest, Sinclair *et al.* recently demonstrated that A_1AR activation by elevated adenosine levels may initiate protein kinase C (PKC) dependent inhibition of AK [64]. A_1AR activation may therefore be capable of enhancing adenosine generation and subsequent protection via a positive feedback loop. In contrast, there is evidence for adenosine-mediated inhibition of 5'-NT activity [65], which would counter this effect. Further studies are required to decipher

the precise role of adenosine receptor activation in modulation of adenosine formation. However it is possible that A_1AR mediated inhibition of AK activity and enhanced 5'-NT activity with preconditioning may share common signalling pathways [66].

Once adenosine is formed it is transported across the cell membrane by simple or facilitated diffusion by a nucleoside transporter (nt) where it accumulates in interstitial fluid. The necessity of an effective adenosine transport system is borne out of the need for intracellularly formed adenosine to rapidly activate cell membrane surface receptors and for an equally rapid removal and termination of its action at these sites [35]. From the interstitial fluid it slowly escapes to the intravascular compartment by paracellular washout or is transported into endothelial cells where it is further metabolised to inosine or AMP [67].

Receptors

Locally released adenosine produces a majority of its physiological effects by interacting with specific purinergic receptors (there is also evidence of non-receptor effects). Currently 4 adenosine receptor sub-types encoded by distinct genes have been characterised – A_1, A_{2A}, A_{2B}, and A_3ARs [15,68,69] (Fig. 1.3).

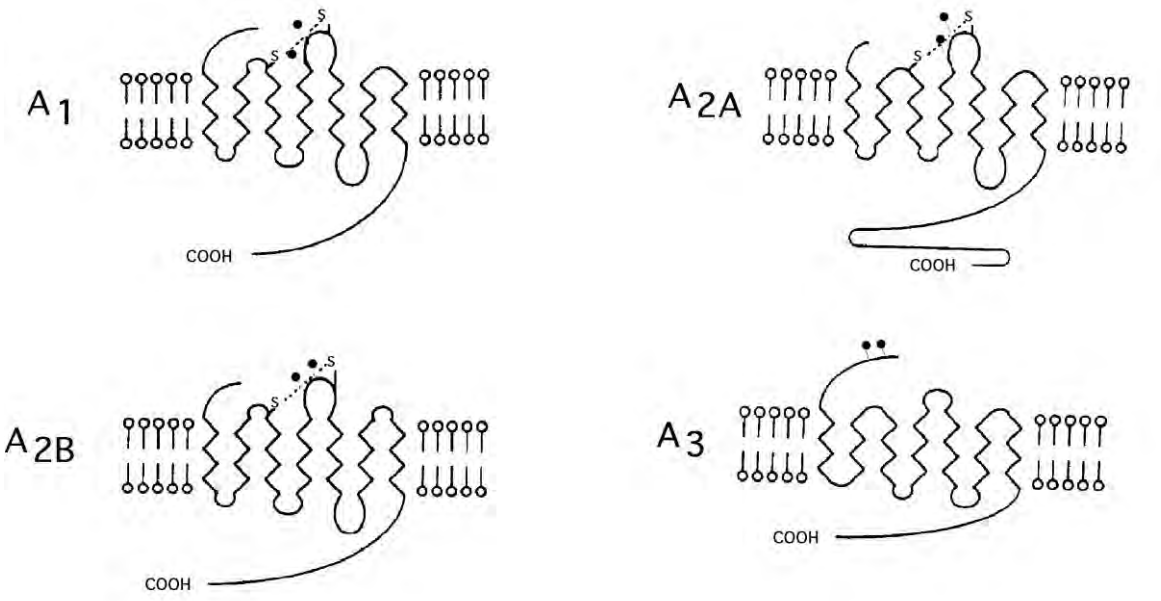

Figure 1.3 Structure of A_1, A_{2A}, A_{2B} and A_3 adenosine receptors.

Originally classified according to their effect (inhibition or stimulation) on adenylate cyclase [70] and on their affinities for adenosine analogues and methylxanthine antagonists, they are now distinguished by their primary amino-acid sequence and molecular weights [71,72]. The A_1, A_{2A}, and A_3ARs have high affinities for adenosine whereas the A_{2B} has a relatively low affinity. They appear to be expressed within cells of the cardiovascular system, although direct and unequivocal evidence of expression of some sub-types is lacking [10]. All are G-coupled receptors, which transfer signals by activating heterotrimeric G proteins and subsequently intiate an effector response [73]. It is also appreciated that additional proteins (other than G proteins) impinge on the signalling reactions by interacting directly with receptors and G protein subunits, including GRKs (G-protein-coupled receptor kinases) and arrestins [73].

Effectors

The A_1 and A_3ARs couple to pertussis-sensitive G_i and G_o family proteins. A_1ARs have been located on myocytes and neutrophils and inhibit the activation of adenylyl cyclase (AC) caused by catecholamines and histamine. Physiological effects of A_1AR stimulation include negative chronotropy and dromotropy, antiadrenergic effects, stimulation of glycolysis and stimulation of neutrophil adherence. Specifically, in atrial tissue, guanine nucleotide binding inhibitory protein ($G_{i/o}$) mediated stimulation of acetylcholine (Ach)/adenosine regulated outward potassium current ($I_{KACh/Ado}$) leads to cAMP-independent action potential shortening and membrane hyperpolarisation in the (sinoatrial) SA and (atrioventricular) AV nodes, which ultimately results in negative chronotropy and dromotropy [74,75]. In atrial myocytes these effects are translated into direct negative inotropic responses [74,75]. In ventricular and atrial myocytes, cAMP-dependent effects of adenosine are mediated by depression of catecholamine stimulated L-type Ca^{2+} channel (I_{Ca}) and transient inward current (I_{TI}) via inhibition of cAMP. Thus, in ventricular tissue adenosine A_1-mediated effects are anti-adrenergic, opposing the positive chronotropic, dromotropic, and inotropic effects of β-adrenergic receptor stimulation [74,75]. In addition, adenosine inhibits norepinephrine release from stimulated sympathetic nerve fibers in the heart [75]. The A_1AR is also coupled to phospholipase activation and membrane-associated PKC in ventricular myocytes [76], activates sarcolemmal ATP sensitive K^+ channels (sarc K_{ATP}) [77], and can modify the activity of putative mitochondrial ATP sensitive K^+ channels (mito K_{ATP}) [78].

The A_3AR has been shown to couple to a number of G proteins, including $G_i\alpha 2$, $G_i\alpha 3$ and $G_q\alpha$ [79]. Like the A_1AR, the A_3AR also inhibits adenylate cyclase [80,81], and apparently modifies mito K_{ATP} function [82,83]. A_3ARs have been localised in heart tissue and may be on endothelium and myocytes, although firm data are not available

[9]. Data from Liang and colleagues supports differential coupling of A_3ARs to phospholipase D (PLD) while A_1ARs couple to phospholipase C (PLC). These effects may be important in generating different levels of cardioprotection [84].

A_{2A}ARs are localised on endothelial cells, vascular smooth muscle, neutrophils and platelets [11,72]. The, A_{2B}ARs are found on endothelial and vascular smooth muscle cells and are also localised on fibroblasts and mast cells [72,85]. In addition, A_{2A}AR mRNA has been found in cardiomyocytes, however their functional significance remains controversial [72]. Preliminary functional data supports the presence of A_{2B}ARs in avian cardiomyocytes [72], and A_{2A} and A_{2B}ARs in mammalian myocytes [86]. A_{2A} and A_{2B}ARs are thought to activate AC via G_s protein, with the A_{2B}AR additionally stimulating PLC via G_q protein [87]. These transduction mechanisms result in vasodilation, renin release, and inhibition of neutrophil superoxide generation and adherence to endothelium. Through these and related "effector" interactions the adenosine receptor family modifies a broad range of signalling pathways [73].

II. CORONARY REGULATION BY ADENOSINE

In this section the role of adenosine in mediating CBF is discussed. Particular emphasis is placed on adenosine's role in modulating coronary vascular resistance under normoxic conditions, highlighting current controversies on receptor subtypes and mechanisms involved. Adenosine's potential vasoprotective role will be assessed in later sections.

Coupling of Contractility to Flow

CBF must be able to match rapid shifts in myocardial metabolic demand. Therefore, it is not surprising that a variety of integrated mechanisms regulate the resistance of blood vessels. These include metabolic control, myogenic autoregulation, flow- or shear stress-mediated vasodilation, neurohumoral influences and endothelium-dependent vasodilation (Fig. 1.4) [88]. Metabolic regulation of CBF via the release of local metabolites such as adenosine is considered one of the most important individual mechanisms, despite the fact that controversy still surrounds the identity of the signals linking metabolism and resistance. Although an increase in metabolic activity undoubtedly produces an increase in vasoactive metabolites at all levels of the coronary vasculature, metabolic control mechanisms appear to be most prominent in small coronary arterioles. Indeed, several studies have demonstrated that small coronary microvessels are the primary target for the effects of the metabolic vasodilator adenosine [89,90]. Adenosine is thought to play a central role as a "metabolic" vasodilator.

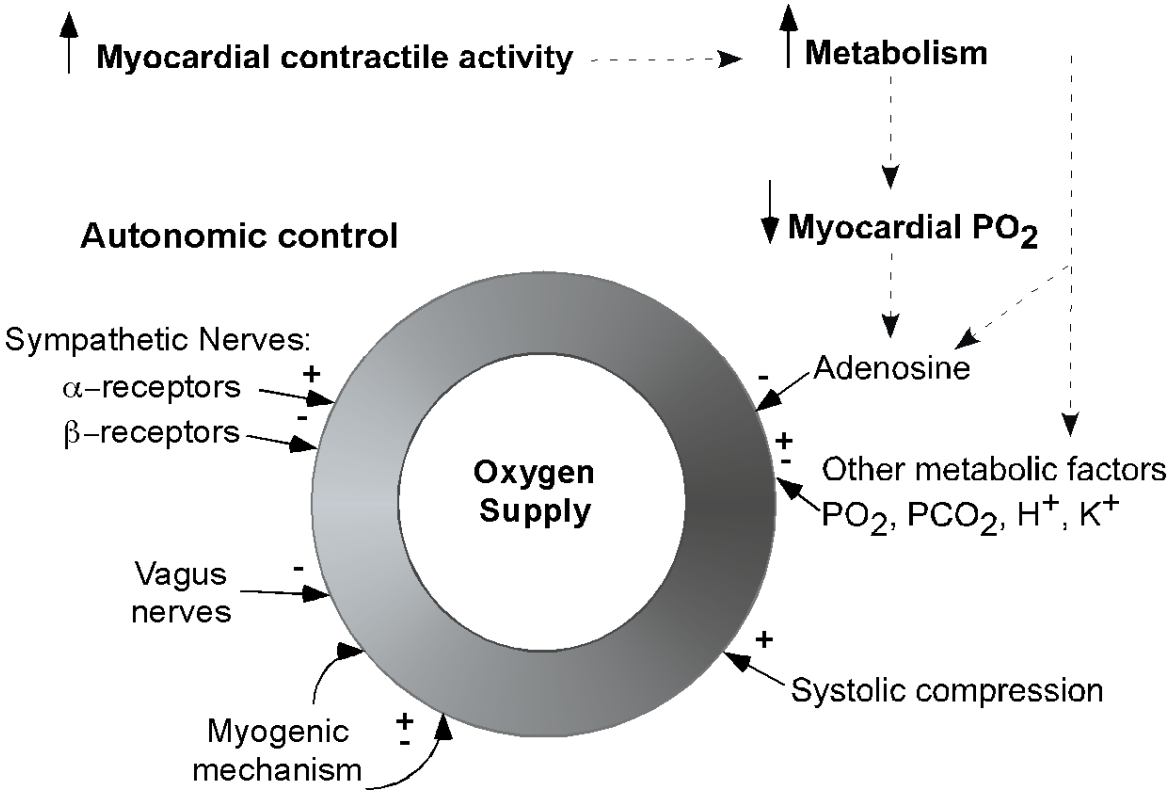

Figure 1.4 Mediators involved in coupling contractility to flow modified from Berne and Levy [88].

"The Adenosine Hypothesis"

In 1963 Berne postulated that an increase in myocardial oxygen consumption produces an incipient fall in myocardial oxygen tension that augments adenosine release from cardiac cells to give rise to vasodilation [12]. Over a decade later, Berne and colleagues linked elevations in adenosine formation with reduced O_2 delivery [13,91]. However, it was subsequently determined that myocardial hypoxia is not the only pre-requisite for stimulated adenosine formation [92], but that elevations in O_2 consumption (ie. during exercise) under normoxic conditions can also markedly enhance adenosine formation [26,93]. This led to the concept that formation is triggered by an imbalance between myocardial energy "supply" and "demand" (reflected by the so-called O_2 supply:demand ratio) [26,93].

Two theories emerged from Berne's original hypothesis. The first model was developed by Schrader and colleagues [93,94] which suggested that adenosine is sensitive to small reductions in oxygen partial pressure (PO_2) levels, sensing any imbalance between O_2 supply/demand and adjusting CBF accordingly in a feedback system. The exact trigger leading to coronary flow metabolite production is not known but this model does assume some degree of hypoxia [35,92,94,95]. Olsson and Bünger [95] revealed an alternative model, which could explain a role for adenosine under normoxic conditions. Based on turnover of ATP during oxygen consumption leading to accumulation of AMP, this model proposes a feed-forward regulation of CBF where oxygen usage parallels cardiac work if there is no borderline hypoxia. This model has not only been supported by studies in isolated heart models [96,97] but has recently been demonstrated *in vivo* [98]. Bian *et al.* found that increases in myocardial O_2 demand can modulate adenosine formation without the requirement for O_2 supply:demand imbalance resulting in myocardial hypoxia [98]. Both theories involve degradation of ATP and describe potential mechanisms by which CBF regulation could be coupled to myocardial energy metabolism.

Physiological Role for Adenosine in the Regulation of CBF?

As mentioned above, close coupling of CBF and myocardial O_2 demand has been proposed to depend primarily on messengers released from the myocardium, such as adenosine. Adenosine released from cardiomyocytes crosses the interstitial space to activate adenosine receptors on coronary vasculature to produce vasodilation and an increase in CBF [95]. Though endogenous adenosine may play an important role in modulating coronary vascular resistance during and following pathophysiological stimuli [13,99], its role in regulation of coronary tone under more physiological conditions *in vivo* remains questionable [100]. While earlier studies in the *in vivo* dog

heart demonstrate that adenosine release is enhanced during conditions of increased myocardial O_2 demand [101-104], more recent studies have failed to find an obligatory role for adenosine in the increase of CBF during conditions of increased myocardial O_2 demand (ie. exercise or pacing tachycardia) [100,105,106]. Furthermore, while adenosine was found to exert a vasodilator influence on the coronary resistance vessels in swine at rest and during exercise, adenosine was not mandatory for exercise-induced coronary vasodilation [107]. In contrast to findings in intact animal models there is ample evidence that adenosine contributes to regulation of coronary vasomotor tone in human heart [108-110]. Thus there may well exist species-related differences in adenosine physiology.

Estimations of interstitial adenosine concentrations have also fueled the debate on the role of adenosine in regulation of CBF. Early speculation was raised on whether adenosine levels are indeed high enough in normoxic hearts to sufficiently activate receptors to induce cellular responses [111]. As measured by microdialysis, interstitial adenosine concentrations under basal conditions in mammalian hearts have been shown to range between 0.1-1.0 μM, [27,49,112,113], and to rise considerably under stress conditions such as ischaemia (to 10-100 μM), hypoxia, or catecholamine stimulation [27,49,112,113]. At these levels the purine may serve a role in regulating both CBF [13,35,95] and cardiac workload [114-117]. In contrast, recent estimates using a mathematical model indicate that the basal concentration of interstitial adenosine *in vivo* is 0.04 μM [106], a level well below the 0.1 μM threshold for coronary vasodilation found by Stepp *et al.* [118]. Moreover, despite a transient increase in interstitial adenosine after pacing or exercise [106], the level was still found to be below the threshold for coronary vasodilation [100,106]. Despite the fact that a pivotal role for adenosine in "physiological" regulation of CBF is yet to be more clearly defined in the

in situ organ, adenosine has been shown to contribute to coronary vasodilation during "pathophysiological" hypoxia and ischaemia *in vivo*, and under a variety of conditions in isolated hearts.

Adenosine Receptor Modulation of Coronary Vascular Resistance

In addition to the controversies discussed above, the adenosine receptor subtypes mediating vasodilation appear to differ between species. Furthermore, conflicting evidence exists regarding the mechanisms involved in adenosine-mediated dilation in different (and also within) vascular beds.

Receptor Sub-Types Involved in Adenosine-Mediated Vasodilation

Vasodilation caused by adenosine is due to activation of A_2ARs, and the A_2AR subtype/s (A_{2A} and/or A_{2B}) that elicit this vasodilation have been characterised in an array of species and tissues with mixed results. Previous studies indicate the existence of functional A_{2A}ARs, which play a major role as mediator of coronary dilation in dog [119], pig [120-122] and guinea pig [123]. However, A_{2B} receptors appear to be of considerable importance in mediating coronary dilation in humans [124] and in the rat [125,126]. With the advent of transgenic models much work is being undertaken in murine heart. However, on commencing this doctoral research the adenosine receptor subtype involved in mediating vasodilation in mouse heart was not known. Preliminary evidence by Morrison *et al.* (2001) indicates that the A_{2A}AR are involved since gene deletion of the A_{2A}AR eliminates 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680) -mediated (specific A_{2A} agonist) coronary dilation in the mouse [127]. However, due to potential problems involved in gene knock-out studies, verification using a pharmacological approach is necessary to confirm these results. Furthermore, findings by Prentice *et al.* [128] denote an absence

of relaxant adenosine A_1, A_{2A}, A_{2B} or A_3ARs in isolated mouse aorta, and have proposed the presence of another as yet undefined mechanism(s). This is a valuable observation, but as indicated by the authors, does not rule out the presence and involvement of A_{2A}ARs in smaller vascular branches in mouse heart.

Mechanisms Involved in Adenosine-Mediated Vasodilation

Early studies supported the idea that adenosine directly stimulated A_2 receptors on vascular smooth muscle to produce vasodilation [13], either through activation of adenylate cyclase [129,130] or via direct changes in Ca^{2+} [131] and/or K^+ fluxes. However, there is now evidence to support the presence of A_2ARs on both endothelial and smooth muscle cells within the same vessel [132-136]. Activation of endothelial receptors elicits vasodilation by endothelial release of endothelial-dependent relaxing factor (EDRF), identified as NO [137,138]. Activation of vascular smooth muscle receptors induces relaxation via the opening of K_{ATP} channels (endothelial-independent processes) [137,138].

Importantly, there is support for mixed NO or endothelial-dependent and independent coronary responses to adenosine in guinea-pig [139-141], dog [142,143], and pig [136,144-147]. Endothelial-independent adenosine responses have been demonstrated in human and porcine coronary vessels [122,124,148,149], and in rat heart [125]. These observations from different species again supports pronounced species differences in mechanisms of adenosine-mediated coronary dilation. Furthermore, there is no information on the mechanisms involved in A_2AR-mediated dilation in mouse heart, therefore, this was an aim of this thesis.

Interestingly, there is a greater weight of seemingly unequivocal data supporting NO-dependence of adenosine A_{2A} responses [139-141], whereas there is controversy regarding NO-dependence of coronary A_{2B} responses [122,124,125,149]. It has been

documented that a considerable amount of dilation mediated by adenosine remains after endothelial denudation or inhibition of the NO pathway, suggesting that vasodilation to adenosine A_{2A} receptor activation is not solely NO dependent [120,121,146,150]. In distinct contrast, A_{2B} receptors may mediate coronary dilation in human vessels via NO and K_{ATP} independent mechanisms [124]. Nevertheless, a role for K_{ATP} channels in adenosine mediated coronary dilation is accumulating [120,146,150,151], despite data arguing against involvement [152,153].

In general, NO released from the endothelium mediates relaxation of the vascular smooth muscle by stimulating soluble guanylate cyclase (sGC) to increase intracellular concentrations of cyclic guanosine monophosphate (cGMP) [154,155]. The later activates cyclic GMP-dependent protein kinase, which leads to an increased extrusion of Ca^{2+} from the cytosol and inhibition of the contractile machinery [156,157]. More recent evidence suggests that adenosine-mediated vasodilation involves activation of endothelial PTX-sensitive G-proteins, which are coupled to the opening of endothelial K_{ATP} channels [136]. This results in membrane hyperpolarisation, accompanying Ca^{2+} influx and vasodilation through subsequent activation of NO synthase [136] (Fig. 1.5). Indeed, previous studies have shown that membrane hyperpolarisation triggers an influx of Ca^{2+}, which is necessary for NO production from NO synthase [158]. Furthermore, earlier evidence supports the existence of K_{ATP} channels on endothelium [159] and suggests that adenosine is capable of producing sustained hyperpolarisation in coronary endothelial cells [160].

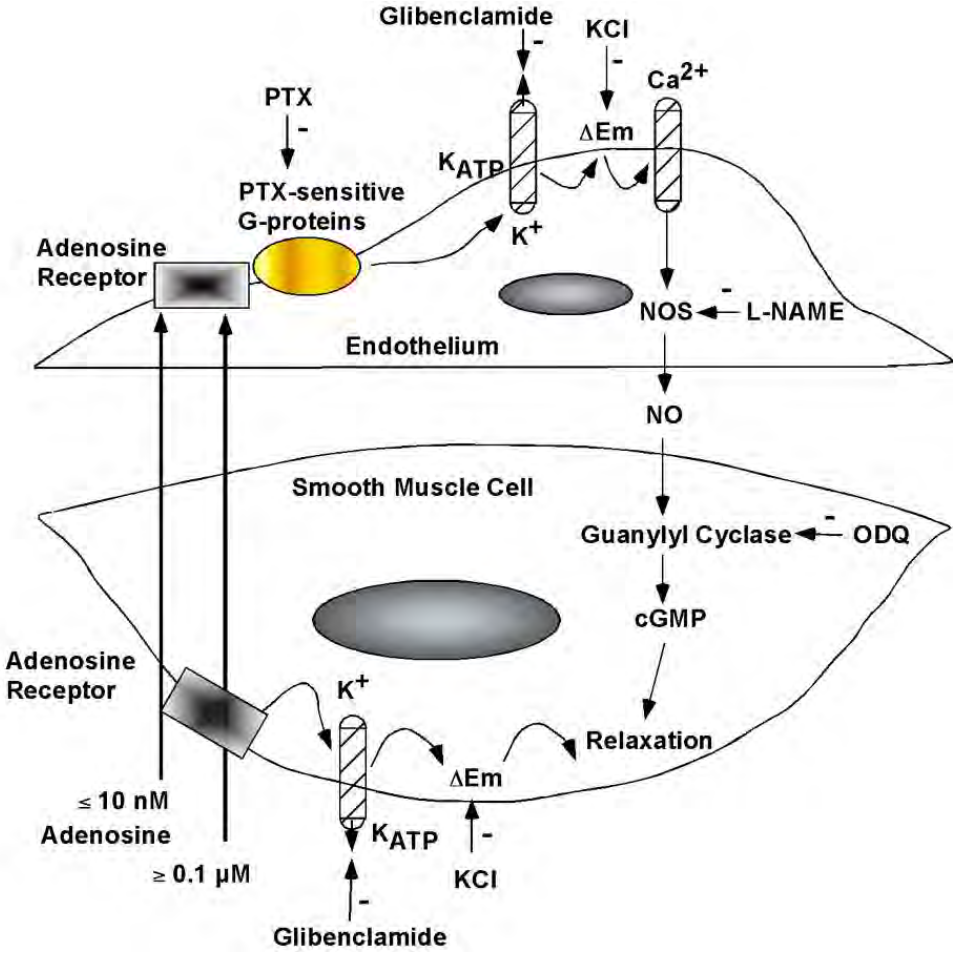

Figure 1.5 Proposed endothelial and vascular smooth muscle signal transduction pathways involved in vasodilation to adenosine modified from Hein and Kuo [136].

As mentioned above, vasodilation to adenosine may also involve direct activation of smooth muscle K_{ATP} channels (Fig. 1.5). However, despite earlier contention that activation of K_{ATP} channels by adenosine involves cAMP/cGMP pathways, more recent evidence from Hein and Kuo does not support this possibility, since dilation was unaffected by cAMP antagonism, and endothelial-independent dilation with sodium nitroprusside (SNP) was not inhibited by glibenclamide [136]. Of greater interest, Hein and Kuo propose that lower concentrations of adenosine (eg. nM range) may selectively open endothelial K_{ATP} channels while higher concentrations (eg μM range) may cause opening of smooth muscle K_{ATP} channels [136]. They postulate that G proteins in endothelium may serve as an amplifier for the cellular signalling

linked to K_{ATP} channels, and therefore may contribute to high sensitivity of endothelium to adenosine [136]. This could explain conflicting evidence on mechanisms of adenosine-mediated dilation, since studies use widely varying concentrations of adenosine agonists to elicit dilation.

III. IMPORTANCE OF THE ENDOTHELIUM IN THE MODULATION OF VASCULAR RESISTANCE

As indicated above, the endothelium may be pivotal in mediating adenosine's vasodilatory responses, particularly at low concentrations. Furthermore, endothelial cells have surface receptors for vasoactive compounds, in addition to adenosine, reflecting the integral involvement of the endothelium in regulating vascular smooth muscle tone [161]. The endothelium is metabolically very active, producing many of the vasoactive substances involved in regulation of coronary vascular resistance, including, adenosine, NO, endothelium derived hyperpolarizing factor (EDHF), prostacyclin (PGI_2), and ET (among others) [162-165].

Since this thesis will focus on coronary function and vascular injury with ischaemia-reperfusion and since the endothelium is of integral importance in both regulation of injury, a more detailed discussion of the endothelium in vascular control is warranted.

Endothelium-Derived Vasoactive Substances

i. EDHF

In addition to EDRF (NO), discussed in the prior section, endothelium releases other relaxing factors. In 1988, Taylor and Weston [166] proposed that a factor exists which produces relaxation by increasing the membrane potential of muscle cells. In general, hyperpolarising of smooth muscle produces relaxation by reducing both the open probability of voltage-dependent Ca^{2+} channels and the turnover of intracellular phosphatidylinositides, thus decreasing the intracellular Ca^{2+} concentration $\{[Ca^{2+}]_i\}$ [167]. This additional factor was termed endothelial-dependent hyperpolarising factor or EDHF [168]. Recent evidence indicates that EDHF-mediated responses in resistance vessels are at least as important as endothelium-derived NO in mediating agonist-induced, endothelium-dependent vasodilatation since neither deletion of the gene encoding endothelial NO synthase nor inhibition of NO synthase blocks agonist-induced vasodilator responses *in vivo* and *in vitro* [169,170]. Interestingly, recent studies also describe enhanced EDHF-mediated responses following ischaemia-reperfusion [171,172], which is thought to involve augmented Ca^{2+} responses downstream of dilatory receptors [173]. Collectively, these studies support EDHF-dependent "compensation" when vasoregulation via NO is impaired.

The identity of EDHF still remains controversial. However, similar to many other endothelial functions, an increase in endothelial Ca^{2+} is a crucial step in mediating EDHF responses [174]. G-protein-coupled receptor agonists including ACh and bradykinin (BKn) [175,176], as well as receptor-independent substances, such as Ca^{2+} ionophores and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase inhibitors thapsigargin and cyclopiazonic acid [177,178], are capable of inducing EDHF-mediated responses. Furthermore, accumulating evidence suggests K^+ plays a pivotal role in mediating

EDHF responses. Not only is there evidence EDHF responses are blocked by high K^+ concentrations, but agonists stimulating endothelium-dependent hyperpolarizations also stimulate efflux of ^{42}K from pre-loaded arteries [179]. Moreover, EDHF is associated with a decrease in membrane resistance of vascular smooth muscle cells [179]. Collectively these findings suggest that hyperpolarization involves an increase in K^+ conductance and that EDHF may be an endothelium-derived K^+ channel opener.

To date, there are several lines of evidence that indicate that K^+ channels involved in the EDHF response are Ca^{2+} -sensitive K^+ channels (K_{Ca}). The first observation was made by Garland's group, who determined that the combination of apamin (a specific inhibitor of small conductance K_{Ca} channels [SK_{Ca}]) plus charybdotoxin (a non-selective inhibitor of large-conductance [BK_{Ca}] and intermediate-conductance channels [IK_{Ca}]) abolishes EDHF-mediated responses [180]. However, a central role for BK_{Ca} channels has since been excluded, as iberiotoxin (a specific inhibitor of BK_{Ca} channels) is unable to prevent NO and prostacyclin independent vasodilatation [181,182]. These data indicate IK_{Ca} and SK_{Ca} channels are predominantly (if not solely) responsible for EDHF responses. What remains unexplained is what cell type expresses the K_{Ca} channels. Recent research has determined that both IK_{Ca} and SK_{Ca} channels are expressed in endothelial cells [183-185], and are activated by stimuli (ACh or BKn) that increase $[Ca^{2+}]_i$. However, there is no evidence that IK_{Ca} channels exist on smooth muscle cells. Furthermore, when charybdotoxin and apamin are selectively applied to the endothelium itself they prevent EDHF–mediated smooth muscle relaxation [186]. Collectively, current evidence suggests that co-administration of charybdotoxin and apamin targets both IK_{Ca} and SK_{Ca} channels expressed on endothelial cells, rather than K^+ channels activated by an EDHF and located on smooth muscle cells.

Despite recent progress into the understanding of EDHF-mediated responses, a variety of hypotheses exist regarding mechanisms involved. The reason for much of the controversey is that "increasing $[Ca^{2+}]_i$ in endothelial cells opens not only SK_{Ca} and IK_{Ca} channels, which results in the efflux of K^+ in the myo-endothelial space, but also leads to the activation of various enzymes including phospholipases and the subsequent metabolism of arachidonic acid by cytochrome P450 epoxygenases" [179]. Currently, 3 hypotheses are extensively supported (reviewed by [179]): i) increased endothelial $[Ca^{2+}]_i$ triggers synthesis of a cytochrome P450 metabolite essential for the subsequent EDHF-mediated responses; ii) endothelial hyperpolarisation is transmitted to vascular smooth muscle via gap junctions; and/or iii) K^+ released from endothelial cells via K_{Ca} channels induces smooth muscle hyperpolarisation by activating smooth muscle K^+ channels and/or Na^+-K^+-ATPase. These mechanisms are not necessarily mutually exclusive and may occur simultaneously or might act synergistically. In short, hyperpolarisations of endothelial cells might be regulated by activation of cytochrome P450 and resultant generation of epoxyeicosatrienoic acids (EETs) [179]. The endothelial hyperpolarisation could then spread to adjacent smooth muscle through myo-endothelial gap junctions (which might be modulated by EETs), and/or efflux of K^+ through endothelial SK_{Ca} and IK_{Ca} channels could elicit hyperpolarisation of surrounding myocytes by activating K_{IR} channels and/or Na^+-K^+-ATPase [179] (Fig. 1.6).

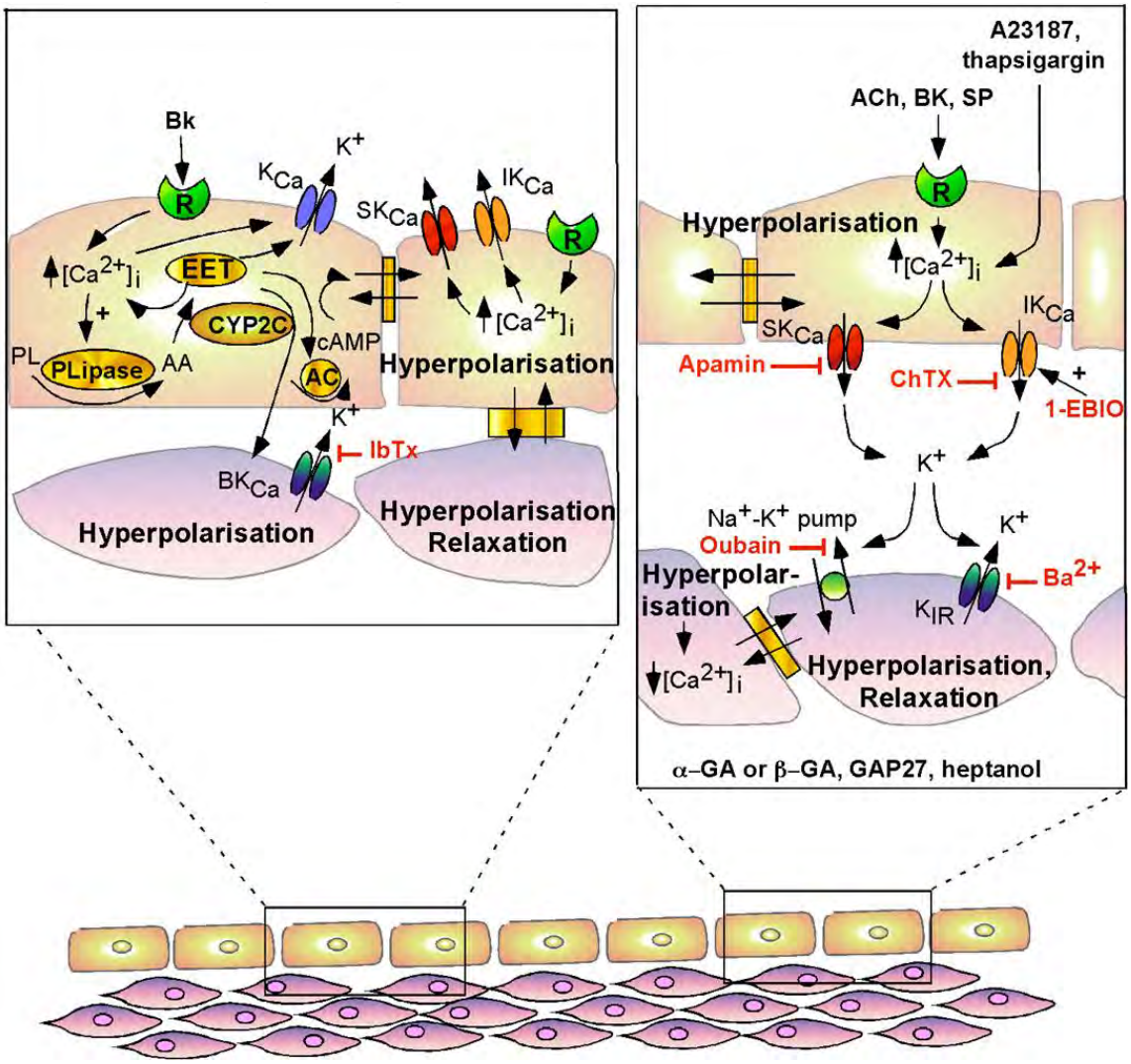

Figure 1.6 Schematic representation of potential pathways involved in EDHF mediated dilation modified from Busse *et al.* (a) Epoxyeicosatrienoic acids (EETs), (b) The role of K^+ ions [179].

ii. Endothelin

ET-1 is produced by endothelial cells and is currently the most powerful vasoconstrictor known [187]. ET-1 regulates cell function, including vascular resistance, by interacting with G-protein coupled receptors (subtypes ET_A and ET_B), stimulating formation of inositol 1,4,5-triphosphate (IP_3) and subsequent release of intracellular Ca^{2+} [188]. The ET_A receptor is expressed in vascular smooth muscle whereas ET_B receptors are predominantly localised to endothelial cells with only a

small population on vascular smooth muscle cells [189,190]. The ET_A receptor is the main contributor to vasoconstriction, while ET_B receptors are thought to primarily result in vasodilation by enhancing NO synthesis [191,192]. Secretion of ET-1 can be induced by thrombin, interleukin-1, growth factor-β1, platelet products and neurohormones such as vasopressin and catecholamines [193].

Conventionally, regulation of CBF is thought of in terms of vasodilation, via production of vasodilators in response to increased myocardial work. However, accumulating evidence indicates that vasoconstrictors, such as ET, may be important in the regulation of coronary vascular tone. In support of this, ET is tightly controlled by the level of circulating NO [194]. Moreover, 'crosstalk' between ET and NO in the control of vascular tone has recently been established [195]. Nevertheless, the most convincing evidence has come from recent studies that show that the contribution of ET-1 mediated constriction decreases during exercise, indicating that dilation may be a combination of increased production of vasodilators and decreased production of ET [196,197]. Importantly, Merkus *et al.* [197] hypothesises that cardiac myocytes, acting as O_2 sensors, produce a vasodilator when PO_2 decreases and a vasoconstrictor when PO_2 increases. Despite evidence implicating involvement of ET, the mechanism for ET production remains unresolved. The fact that myocytes are unlikely to produce ET [198,199] suggests myocyte production of an independent factor(s) to induce the release of ET from vascular cells. The advantage of utilising both dilators and constrictors to rapidly "fine tune" coronary vascular tone becomes obvious when metabolism decreases. Rather than waiting for vasodilator influences to dwindle, myocytes may actively increase vascular tone and thereby actively decrease CBF as MVO_2 falls [197].

iii. Prostanoids

Another potentially important vasodilator is PGI_2. PGI_2 is produced during the metabolism of arachidonic acid by cyclooxygenase and evokes vasodilation via the activation of adenylate cyclase, leading to an increase of intracellular cAMP. Importantly, while some studies support the involvement of PGI_2 in the regulation of coronary vascular tone in humans at rest and during exercise [200,201], there is also evidence that dispute its involvement [202,203]. Interestingly, the involvement of PGI_2 appears species dependent since a role for prostanoids has been identified in humans and swine [200,201,204] but not in dog [205]. Furthermore, prostanoid compensatory effects are observed after NO inhibition in systemic circulation [206], but not in coronary circulation [204].

Vasoprotection in Addition to Vasoregulation?

Since the endothelium is highly susceptible to the injurious effects of ischaemia-reperfusion [2], endothelial-dependent regulatory mechanisms may become disrupted with insult, reducing the capacity to match changes in myocardial energy supply:demand. This raises the question of the importance of vascular injury on the function and/or survival of the organ following ischaemia-reperfusion. To date studies of the effects of ischaemia-reperfusion have almost entirely concentrated on the cardiac myocyte. However, many of the general mechanisms of ischaemia-reperfusion injury pertain to all cardiovascular cells. What follows is an outline of the effects of ischaemia-reperfusion on the heart, ultimately focusing on vascular effects.

IV. MYOCARDIAL ISCHAEMIA-REPERFUSION

The term myocardial ischaemia describes a condition that exists when fractional uptake of O_2 in the heart is insufficient to maintain the rate of cellular oxidation [207]. The ability to limit tissue damage during ischaemia is time-dependent on reperfusion. Paradoxically, although essential, it is the reperfusion phase where the extent of damage is ultimately realised and/or incurred. It has been suggested that ischaemia essentially sets the stage for the damage induced by the reperfusion [208]. Controversy exists due to the technical difficulty in determining whether cell death is caused entirely by the ischaemic history or by reperfusion [208].

Following total or partial occlusion of a coronary artery, metabolic and functional changes are initiated within seconds. These changes, which become progressively more severe with time, are initially of a reversible nature. However, with increasing durations of ischaemia, they become more severe and eventually irreversible injury occurs [209]. As highlighted by Hearse, the major factors influencing the evolution of injury are: i) the extent of residual collateral flow; ii) diseases that may coexist with ischaemia, such as hypertrophy, diabetes, hypertension; iii) heart rate, metabolic rate and tissue temperature; iv) variations in the metabolic response to ischaemia (particularly in relation to patterns of substrate metabolism; v) nutritional and hormone status; vi) the presence of a variety of cardioprotective drugs; vii) previous ischaemia and various adaptive processes; and viii) the age, sex and species of the tissue. Thus, there is no universal time at which tissue becomes irreversibly injured [209].

Mechanisms involved in both reversible and irreversible injury are outlined in subsequent sections. As injury is largely determined by metabolic derangements, these are first discussed.

Metabolic Derangements of Ischaemia-Reperfusion Injury

Metabolic Changes

Myocardial substrate metabolism during ischaemia is highly dependent upon the severity of ischaemia. Metabolic changes appear within seconds of the cessation of coronary flow and energy metabolism shifts from aerobic or mitochondrial metabolism to anaerobic glycolysis almost instantaneously. Under severe ischaemic conditions the sole source of glycolytic substrate becomes glycogen, since there is no blood flow to deliver glucose to the tissue. Moreover, there is no washout of lactate, intracellular pH decreases, and eventually a reduction in the rate of glycolysis occurs due to H^+ inhibition of phosphofructokinase (PFK). Intracellular creatine phosphate (PCr) content, a major reserve source of high-energy phosphate (HEP), is rapidly depleted and P_i levels are elevated. Interestingly, 90% of HEPs are exhausted after 30 seconds of ischaemia, whereas ATP levels decline more slowly. In addition, since the rephosphorylation of ADP to ATP during anaerobic glycolysis occurs at a much slower rate, the adenine nucleotide pool is degraded as the energy bond of ADP is captured via the action of adenylate kinase. In the process, AMP is formed and accumulates intracellularly, where it can be degraded to adenosine. The adenosine diffuses to the extracellular fluid and is ultimately lost from the adenine nucleotide pool after catabolism to inosine and hypoxanthine. Both of these metabolites accumulate and potentially contribute to the production of free radicals on reperfusion (see below).

Continued production of glycolytic ATP undoubtedly limits the degree of injury and dictates the potential viability of the myocardium. Specifically, glycolytic ATP maintains a low level of membrane pump and channel activity, which accounts for its important role in maintaining ion homeostasis during the ischaemic period. Glycolytic ATP ultimately maintains Ca^{2+} homeostasis by not only maintaining the activity of the

Na^+/K^+ ATPase pumps in the sarcolemma, but also by the SR Ca^{2+} ATPase pumps [210]. Furthermore, it has a role in maintaining membrane integrity and limiting ischaemic contracture, which is characterised by the formation of rigor complexes between actin-myosin cross bridges due to a decrease in glycolytic ATP flux rate [210]. The detrimental effects of ischaemic contracture are associated with severe microscopic damage [211] and mechanical compression of the vasculature resulting in compromised coronary flows upon reperfusion [212].

Ionic Homeostasis During Ischaemia

During ischaemia there is not only a rise in intracellular H^+ but also a substantial accumulation of Na^+, which can be markedly attenuated by NHE inhibitors [213,214]. The fact that NHE inhibitors reduce intracellular Na^+ and Ca^{2+} during ischaemia, in conjunction with preventing post-ischaemic contractile dysfunction [214-218], provides support for a pivotal role of NHE in loss of ionic homeostasis during ischaemia. As intracellular pH drops the NHE becomes progressively more active due to stimulation of the H^+ sensor site of the exchanger protein [219]. This initiates the rise in intracellular Na^+ since the primary Na^+ extrusion pathway (Na^+/K^+ ATPase pump) becomes inactive due to low or zero levels of ATP during ischaemia [218,220] (Fig. 1.7).

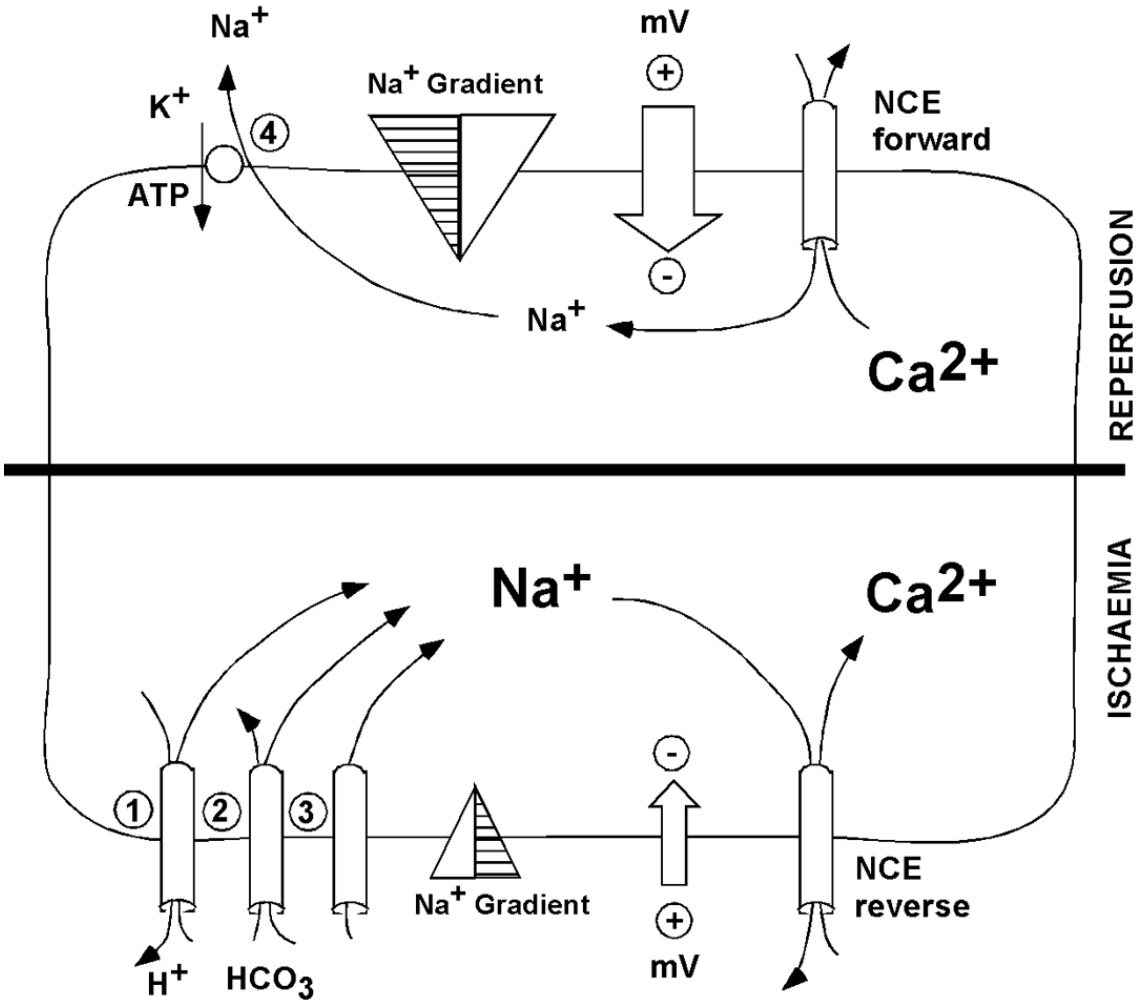

Figure 1.7 Schematic diagram of cation control in ischaemia-reperfusion modified from Piper *et al.* [221].

The resultant rise in intracellular Na^+ promotes the Na^+/Ca^{2+} exchanger (NCE) to work in reverse mode, enhancing Ca^{2+} influx and producing Ca^{2+} overload [208] (Fig. 1.7). An increase in intracellular Ca^{2+} occurs within 15 minutes of ischaemia [222,223]. However, this rise in Ca^{2+} is small in comparison with the large quantity of Ca^{2+} taken up during early reperfusion [213]. Mechanisms of increased Ca^{2+} include increased influx through the voltage dependent Ca^{2+} channels as well as increased flux though exchange pumps [224]. Therefore, while Ca^{2+} plays an important role, it is Na^+ accumulation that appears to be the critical factor which leads to further ionic imbalances all of which can by modulated by NHE inhibition [225]. Interestingly,

several endogenous stimuli that have been shown to be involved in detrimental effects of ischaemia-reperfusion have also been shown to readily activate the NHE by enhancing the sensitivity of the exchanger to intracellular H^+. Recently reviewed by Avkiran [218], such stimuli include thrombin [226], ET [227], angiotensin II [228], and importantly ∝-adrenergic agonists [229].

Osmotic Load/Tissue Osmolarity

As reported by Braasch *et al.* [230], aerobic respiration ceases within 15-30 sec of total occlusion of a coronary artery and results in the by-products of anaerobic metabolism quadrupling in the first min. The lactate concentration as measured by Jennings *et al.* [231] shifts from 1 mM in control tissue to 48 mM in the reversible phase of ischaemia (15 min after onset) to 96 mM in the irreversible phase of ischaemia (45-60 min after onset). While lactate is a primary contributor to osmotic load, the accumulation of P_i due to the metabolism of PCr and nucleotides also participates in elevating the osmotic load [231]. Indeed, the combined contribution of P_i and lactate results in 43% of tissue osmolarity [231], which over time equilibrates either side of the membrane and reduces the force of the gradient while temporarily preventing cell swelling.

The degree of cell swelling during the ischaemic phase is dependent on the degree of perfusion flow present. In no-flow ischaemia there is no metabolite washout in the extracellular space, therefore the transmembrane osmotic gradient is small resulting in only a modest increase in cell volume [232]. During low-flow ischaemia extracellular osmolytes are gradually washed away promoting a larger transmembrane gradient and pronounced influx of water into the cells [233]. Importantly, in both cases reperfusion can result in a further imbalance in lactate concentration, while the sudden washout of extracellular osmolytes promotes explosive swelling of osmotically loaded

cardiac cells [234]. As observed by a number of laboratories under different experimental conditions a hyperosmotic reperfusate containing mannitol can alleviate rapid normalisation of extracellular osmolarity and subsequently prevent cell swelling during a period of mechanical fragility [235-237].

Despite cell swelling being an important feature of ischaemia-reperfusion, it does not necessarily result in sarcolemmal disruption and loss of membrane integrity. Indeed under normoxic conditions myocytes are able to withstand a strong osmotic stress and severe edema without losing sarcolemmel integrity [238]. However if energy depleted cells are osmotically swollen they develop subsarcolemmal blebs and may rupture membranes [239]. This observation has been further characterised by Ruiz-Meana *et al.* [240] who demonstrated the necessary interaction between cell swelling and hypercontracture during reoxygenation to cause sarcolemmal disruption. Therefore, as emphasised by Piper *et al.,* [208] a combination of factors appears necessary in the induction of membrane injury by osmotic stress.

Oxidant Generation

Upon reperfusion, molecular oxygen undergoes sequential reduction to form reactive oxygen species (ROS), including superoxide anion (O_2^{-*}) and hydroxyl radical (OH^*), in addition to hydrogen peroxide (H_2O_2). As will be seen, a predisposition to oxidant generation due to the metabolic and ionic perturbation may be central to several forms of injury.

Oxygen Free Radicals

Free radicals contain one or more unpaired electrons which makes them highly reactive in biological systems. In the myocardium, the reduction of oxygen to water proceeds by two pathways. The mitochondrial cytochrome oxidase reduces 95% of oxygen to water by tetravalent reduction without the production of any intermediates. The remaining 5% of oxygen proceeds by the univalent pathway, in which several intermediates are produced:

1) $O_2 + e^- \rightarrow O_2^{-*}$

2) $2O_2^{-*} + 2H \rightarrow H_2O_2 + O_2$

3) $O_2^{-*} + H_2O_2 \rightarrow O_2 + OH^- + OH^*$

These intermediates include O_2^{-*}, protonated superoxide anion (HO_2^*), H_2O_2, OH^* and singlet oxygen ($^1O_2$). They are all activated oxygen species and are collectively known as partially reduced forms of oxygen (PRFO) [241].

Interestingly, O_2^{-*} is comparatively unreactive and is readily broken down by superoxide dismutase (SOD) to form the even less reactive yet long-lived lipophilc substance, H_2O_2. However, when H_2O_2 tissue concentrations become high the major danger is the production of OH^*. This extremely reactive radical can be produced by three pathways: in the presence of catalytic iron, O_2^{-*} and H_2O_2 interact in a Haber-Weiss reaction to generate OH^*; in the absence of O_2^{-*}, H_2O_2 can generate OH^* through a Fenton reaction where H_2O_2 accepts an electron from a reduced metal ion such as Fe(II) [242,243]; and OH^* is generated from an iron-independent reaction involving the interaction of O_2^{-*} and NO, ultimately producing nitrites and nitrates as shown in the following equations [244]:

$NO + O_2^{-*} \rightarrow ONOO^-$ (peroxynitrite anion)

$ONOO^- + H \leftrightarrow ONOOH$ (peroxynitrous acid)

$ONOOH \leftrightarrow OH^{*} + NO_2$ (nitrogen dioxide)

During reperfusion a large burst of NO is produced [245], and during the same time frame large amounts of O_2^{-*} are also generated [245,246]. The overwhelming affinity of NO and O_2^{-*} ensures their rapid reactivity during the initial moments of reperfusion to form $ONOO^-$ [245].

Sources of Free Radicals

Oxygen free radicals are not only formed during reperfusion but can also be generated during the ischaemic period. In fact, the metabolic alterations that occur during ischaemia facilitate the formation of free radicals from the remaining low levels of oxygen [241]. Moreover, transient reversible ischaemia followed by reperfusion can result in increased production of O_2^{-*} through several potential mechanisms, including; i) increased activity of xanthine oxidases [247], ii) activation of neutrophils [248], iii) activation of the arachidonate cascade [249], iv) accumulation of reducing equivalents during oxygen deprivation [250], v) derangements of the mitochondrial electron transport system resulting in increased univalent reduction of oxygen [251], and vi) auto-oxidation of catecholamines [252] and other substances [243,253].

i. Neutrophils

Neutrophils, when activated, generate several types of free radicals, which are relevant to inflammatory reactions, including acute myocardial infarction [241]. The initial step involves chemotaxis of neutrophils brought about by various mediators released at the site of injury. Within neutrophils an electron transport chain is activated in phagolysosomes which results in a process called “respiratory burst” [248,254]. While neutrophils do contribute to the production of free radicals, a primary role for neutrophils is undermined by studies showing oxidative stress in the absence of blood.

Moreover, neutrophils do not accumulate immediately upon reperfusion [255], when free radical generation peaks [256], but appear when damage has already occurred.

ii. Mitochondrial and other cytoplasmic sources

During ischaemia the components of the mitochondrial electron transport chain become reduced [257,258], allowing an increase of electron leakage from the respiratory chain which, in turn, will react with residual molecular oxygen, leading to formation of O_2^{-*}. Subsequently, on reperfusion the electron egress through cytochrome oxidase will be reduced because of the lack of ADP, again causing formation of oxygen free radicals [241]. Ubisemiquinone and ubiquinol are the main sources of mitochondrial O_2^{-*} [250]. Nicotinamide adenine dinucleotide dehydrogenase (NADH) and dehydroorate dehydrogenase also produce O_2^{-*}[259], whereas amine oxidase in the outer mitochondrial membrane produces H_2O_2.

Xanthine oxidase (XO) is conventionally known as a generator of ROS that contributes to ischaemia-reperfusion injury. Xanthine oxidoreductase is a molybdoenzyme capable of catalyzing the oxidation of hypoxanthine and xanthine in the process of purine metabolism. Xanthine oxidoreductase can exist in two interconvertible forms, either as xanthine dehydrogenase (XD) or XO. The former reduces NAD^+, whereas the latter prefers molecular oxygen, leading to the production of both O_2^{-*} and H_2O_2. Although XO was thought to play a crucial role in generating oxidant stress, some evidence disputes a role for human XO due to its distinctive lack of activity towards xanthine [260]. Moreover, levels of XO have been found to be negible in human myocardium [261,262], though it may be present in vascular cells [263]. Nonetheless, several studies have shown that xanthine oxidase may exist in a molybdenum-deficient form. In this state, the enzyme is unable to use xanthine as a substrate and is not inhibited by allopurinol but can use NADH as an electron donor to

form O_2^{-*} [264,265]. It was therefore concluded that both human XD and XO can oxidise NADH to generate ROS and that the conversion of XD to XO was not necessary for post-ischaemic ROS generation [265]. In addition, recent data indicate that increased circulating plasma XO levels, found after ischaemia-reperfusion [266], can bind to vascular endothelial cells and impair cell function via oxidative mechanisms [263]. Thus, circulating XO can bind in the vascular compartment of humans, utilise endogenous purine substrates to generate reactive species and impair vascular function. In support of this, it was recently observed in an electron spin resonance study that increased activities of both enzymes contribute to increased vascular oxidant stress in patients with coronary artery disease and directly contributed to accompanying endothelial dysfunction [267].

During ischaemia arachidonic acid is released and subsequently metabolised to prostaglandins and leukotrienes via the cyclooxygenase and lipoxygenase pathways, which involves the intermediate generation of oxidising species. Moreover elevated Ca^{2+} can lead to the activation of phospholipases and degradation of cell membrane phospholipids, which has been shown to promote the release of arachidonic acid [268]. As demonstrated by Basu and Karmazyn, accumulation of intracellular arachidonic acid increases tissue injury caused by exogenous free radicals [269].

iii. Auto-oxidation of catecholamines

Increased plasma levels of catecholamines during stressful events such as ischaemia, can lead to oxidation products consisting of quinone derivatives as well as electrons leading to the formation of ROS and potentially myocardial damage [270]. Furthermore, auto-oxidation of catecholamines, which are abundantly released from the ischaemic myocardium, could provide ROS through the formation of adrenochromes [241]. Evidence demonstrating that non-physiological concentrations of catecholamines

can induce functional damage to the heart [271,272] further support the hypothesis that catecholamines can induce free radical related injuries.

Lipid Peroxidation (or Targets for Disruption)

One of the main targets of OH^* and $ONOO^-$ are polyunsaturated fatty acids of membrane phospholipids leading to a chain reaction involving the formation and propagation of lipid radicals [273,274]. The process is initiated when free radicals abstract hydrogen from polyunsaturated fatty acids to form fatty acid radicals with a carbon-center [242,244,254,275]:

$$\text{Lipid-H} + OH^* \rightarrow H_2O + \text{lipid}^*$$

Molecular rearrangement results in the formation of a conjugated diene, with uptake of an O_2 at its center, to yield an oxygen-centered lipid peroxyl radical (lipid-OO^*):

$$\text{Lipid}^* + O_2 \rightarrow \text{Lipid-}OO^*$$

The biologically active lipid peroxy radicals can react with other lipids (extracting H^+), proteins, or nucleic acids and thereby propagate the transfer of electrons and subsequent oxidation of substrate [243,254]:

$$\text{Lipid-}OO^* + \text{lipid-H} \rightarrow \text{lipid-OOH} + \text{lipid}^*$$

Peroxidation of membrane lipids (lipid-OOH) alters membrane fluidity, impairs the function of membrane proteins and semipermeable characteristics [276]. Fragmentation of the membrane and an increased permeability to Ca^{2+} and other ions leads to irreversible cell destruction [242].

V. MECHANISMS OF 'REVERSIBLE' ISCHAEMIA-REPERFUSION INJURY

Ischaemia-reperfusion in the myocardium generates both irreversible injury, marked by necrotic or apoptotic tissue, and also reversible injury, manifested by contractile dysfunction (known as "myocardial stunning"). Furthermore, despite early studies suggesting vascular injury occurs only after prolonged ischaemia [277-279], accummulating evidence demonstrates vascular dysfunction after brief occlusion and supports the existence of "vascular stunning" [280-285]. The following section reviews current information pertaining to the evolution of myocardial and vascular "stunning".

Myocardial Stunning – "Contractile Dysfunction"

First described by Heyndrickx *et al.* in 1975 [286], brief coronary occlusion can produce prolonged contractile abnormalities in the absence of necrosis. Initially thought to be an artefact of the experimental conditions, it wasn't until 1982 that Braunwald and Kloner [287] first coined the term myocardial "stunning". It has since been observed in a variety of experimental settings and shown to be species independent. Eloquently described by Bolli [288] as a "mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage", this syndrome is defined by a mild or sublethal injury. However, it is still not known if stunning and necrosis share the same mechanistic pathway. What is known is that the extent of post-ischaemic dysfunction is determined primarily by the severity and duration of the preceeding ischaemic insult. Therefore, despite being referred to as a type of reperfusion injury, the injury must be initiated to some extent during ischaemia.

Several different hypotheses have been proposed to explain the sequence of events that lead to stunning. This review will focus on the current and most likely instigators of stunning: Ca^{2+} overload [289] and oxidant injury [290,291] (Fig. 1.8).

Oxidant Injury

Initially, evidence that ROS were involved in stunning came from studies of antioxidant therapies to limit contractile dysfunction in open chest dogs [292-294]. Interestingly, it was determined that co-treatment with SOD and catalase was more effective than SOD or catalase alone, indicating that the breakdown of both H_2O_2 and O_2^{-*} are necessary steps in limiting injury. Subsequently, two independent laboratories reported on the importance of OH^* radicals in the genesis of stunning via use of OH^* radical scavenging agents including dimethylthiourea, *N*-2-mercaptopropionyl glycine (MPG), and desferrioxamine [295,296]. More direct evidence for involvement of oxygen free radicals in stunning came from electron spin resonance studies. Bolli and colleagues demonstrated that free radicals were generated and released in the first 5 minutes of reperfusion and that this could be largely prevented (although, not completely) by scavengers if present immediately on reperfusion [256,290].

As extensively reviewed by Bolli and Marban [297], the role of free radical injury in the progression of stunning is well documented and appears to be a rare example of concordance among different laboratories and investigators. What isn't so widely agreed upon is the extent of oxygen-derived free radical involvement. Findings that free radical generation is greatly exaggerated in open-chest dogs as compared with closed-chest conscious dogs [298] sparked substantial controversy and rendered previous findings in open-chest models questionable. No doubt this has contributed to an over-estimation of the role of free radicals in stunning [299]. As highlighted by Opie [300], Bolli's laboratory found that Ca^{2+} antagonism limited the effects of stunning by

approximately 50%, while antioxidant therapy limited injury by the same amount (in different experiments) in closed chest dogs [298]. These studies suggest that ROS injury is not the sole, or even most predominant, mediator of stunning. Moreover, the mechanism by which ROS produce a decrease in contractile dysfunction remains elusive.

By nature, free radicals can attack virtually all cellular targets and can lead to protein denaturation and enzyme inactivation as well as membrane peroxidation [288,301]. Perhaps not surprising, ROS have been shown to disrupt sarcolemma and more specifically ATPase Ca^{2+} transport activity [302]. In addition, it has been demonstrated that free radicals can interact with NCE [297,301,303] and Na^{+}-K^{+} exchange [297,304,305]. Inhibition or impairment of Na^{+}-K^{+} -ATPase activity promotes Na^{+} overload, which subsequently leads to activation of NCE and Ca^{2+} overload [208,213,225]. Another theory to explain free radical mediated contractile dysfunction is borne out of their ability to damage contractile proteins [306]. Evidence exists showing that exposure of myofilaments to O_2^{-*} results in a dose-dependent reduction in maximal Ca^{2+} activated force despite no reduction in Ca^{2+} sensitivity [307]. Finally, it was demonstrated two decades ago that ROS can impair SR function [308], and more recent studies highlight a role for SR modulation in the genesis of stunning [309,310].

Ca^{2+} Hypothesis

As suggested by Shattock [299], it appears that the principle *initiator* of stunning is oxidant stress while the principle *mediator* is decreased Ca^{2+}-induced activation of myofilaments. Original evidence for a central role of Ca^{2+} in stunning came from Kusuoka *et al.* [311], who discovered that "low Ca^{2+}" reperfusion limits the degree of stunning in isolated heart. Today there are three postulated mechanisms that involve disturbances in Ca^{2+} homeostasis in stunning: i) decreased responsiveness of contractile

protein machinery to Ca^{2+}; ii) Ca^{2+} overload; and iii) excitation-contraction uncoupling due to SR dysfunction [297].

Bolli and Marban [297] recently dissected and presented an overview of cardiac excitation-contraction coupling. They highlighted that cardiac contractile force can be effected by: i) modulation of intracellular free Ca^{2+} concentration; ii) modulation of the contractile protein response to Ca^{2+}; and iii) loading. However, while loading undoubtedly contributes to stunning *in vivo*, only factors i) and ii) will be considered for simplicity. Breaking this down even further, if there is a lesion between excitation and Ca^{2+} availability then pathways of Ca^{2+} influx and efflux from the cytosol, in particular ion channels and transporters, would be suspected. However if Ca^{2+} cycling is not the limiting factor then, attention will turn to the response of contractile proteins to Ca^{2+}. The latter consists of three components: the maximal force-generating capacity of the myofilaments; the sensitivity of the myofilaments to Ca^{2+}; and cross bridge cycling kinetics.

Marban and colleagues [312] and Carrozza *et al.* [313] were the first to provide evidence that Ca^{2+} transients are not decreased in the stunned heart, despite a decrease in contractile force. Furthermore, Gao *et al.* [314] determined that stunning was not only associated with a depression in maximal Ca^{2+}-activated force but also decreased sensitivity. These studies suggest Ca^{2+} availability is not limited, but that myofilament responsiveness is disrupted. While the mechanism underlying decreased Ca^{2+} responsiveness is not conclusively established, much evidence points to structural modifications of one or more myofibrillar proteins. Matsumura *et al.* [315] found that α-actinin, a myofilament scaffolding protein, is partially degraded in stunned rat hearts. Moreover, Gao *et al.* [316,317] demonstrated that changes in myofilament sensitivity in stunned heart may be mediated by activation of Ca^{2+}-dependent proteases (calpain I)

and degradation of troponin I (TnI). Calpains cleave proteins when cell Ca^{2+} is elevated [318,319], and have been shown to digest TnI and TnT *in vitro* [320]. Gao and colleagues [316] demonstrated a decrease in myofilament sensitivity after only 5 minutes exposure to calpain I, which could be prevented with a specific calpain I inhibitor. Moreover, degradation of TnI in stunned myocardium could be prevented by modifications of the reperfusate designed to mitigate Ca^{2+} overload [317]. Despite Van Eyk and associates further verifying involvement of calpain I and TnI in manifestation of ischaemic injury [321], and several studies reporting benefical effects of calpain I inhibitors in blunting stunning [322,323], more recent evidence suggests functional recovery of stunned myocardium is independent of TnI fragmentation [324,325]. Treatments that either improved or worsened functional recovery in post-ischaemic hearts were not associated with representative changes in TnI expression [324,325]. Importantly, it is likely that several impaired protein systems may be involved in decreased myofilament responsiveness in stunned myocardium. In support of this, Jeremy and Colleagues [326] demonstrated a role for damage to myosin-regulatory light chain (MCL-2) in stunned myocardium, which, via changes in its phosphorylation state, modulates contractile force generation.

A role for SR dysfunction is supported by several studies [309,310]. Since Ca^{2+} is sequestered by SR Ca^{2+}-ATPase and released from the SR via ryanodine receptor activation, a reduction in stored Ca^{2+} due to abnormal pump function could diminish contractile protein activation. However, since Ca^{2+} availability is not a limiting factor in stunning (previously discussed), and the premise of this theory implies a decreased Ca^{2+} transient, the likelihood that SR dysfunction is a central mechanism appears doubtful.

Fig. 1.8 summarises the converged theory proposed by Bolli and Marban [297]. As described by Hearse *et al.* in 1978 [327], generation of ROS results in damage that is

very similar to that caused by Ca^{2+} overload. Moreover, conditions of oxidant stress in the absence of ischaemia reflect many of the characterisitics of stunned myocardium, including decreased in myofilament responsiveness, and cellular Ca^{2+} overload [328,329]. While many of the effects of free radicals may reflect activation of Ca^{2+}-dependent processes, free radicals can directly impair ATPase pump activity via oxidation and decrease Ca^{2+}-activated force [306,307], as previously noted. Therefore, it is probable that ROS generation, Ca^{2+} overload and decreased myofilament Ca^{2+} responsiveness all have individual roles in pathogenesis of stunning, as proposed by Bolli and Marban [297].

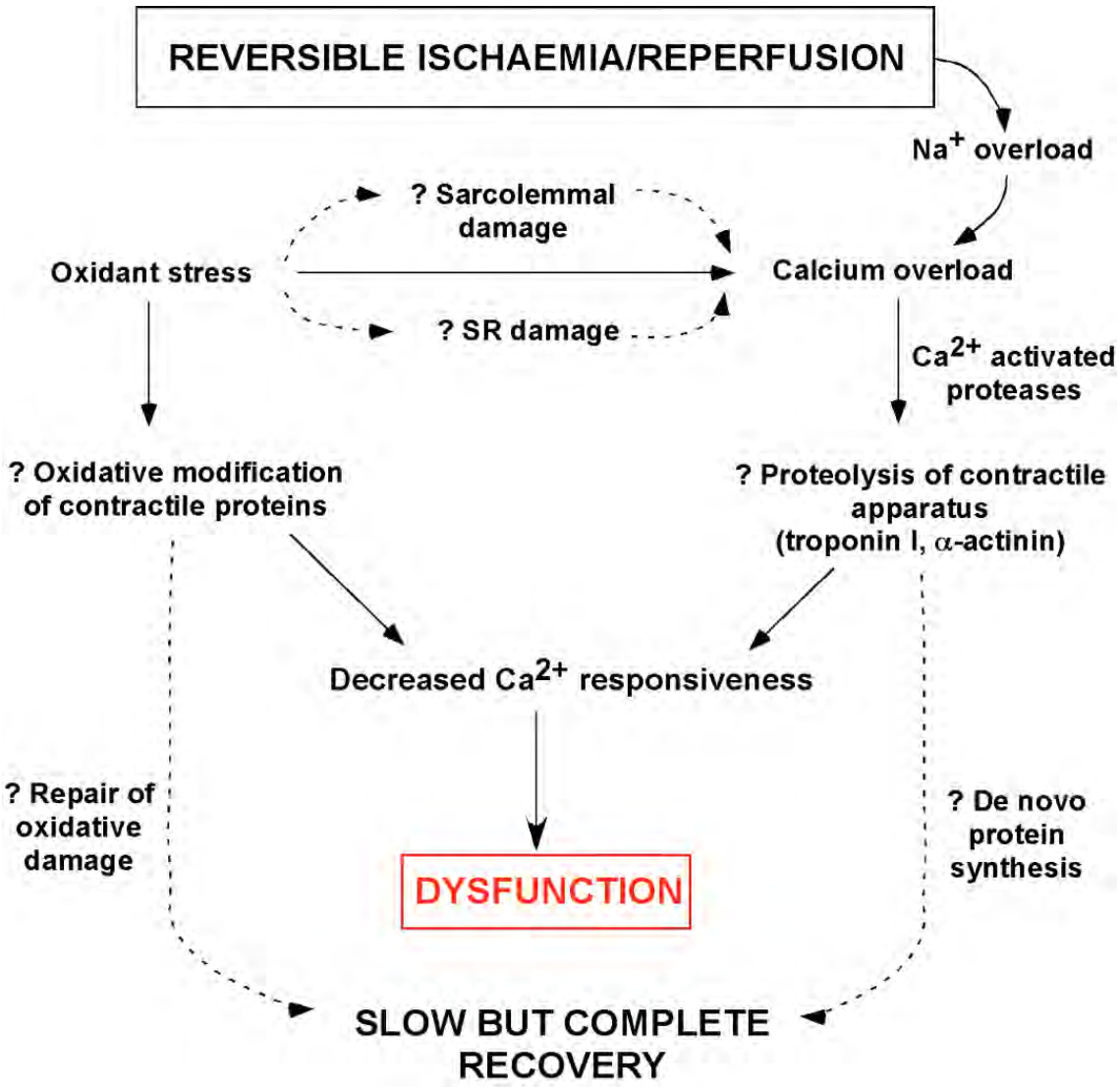

Figure 1.8 Proposed mechanistic basis of myocardial stunning modified from Bolli and Marban [297].

Vascular Stunning – (Endothelial Dysfunction)

In comparison to myocardial stunning, there is very little definitive information regarding manifestation of vascular stunning. Vascular injury it is characterised by endothelial dysfunction, which is defined as a marked reduction in endothelium-dependent relaxation due to reduced release or action (bioavailability) of EDRF [330]. Importantly, responses to endothelium-dependent agonists are used as an index of the functional status of the endothelium and thus existance of endothelial dysfunction

[330,331]. Apart from possible changes to endothelial cell glycocalyx [332], this dysfunction can occur in morphologically normal myocardium. Furthermore, despite meeting the criteria as a reversible form of injury, endothelial dysfunction is not transient and has been determined to persist for at least 2 to 4 weeks [333,334]. Assessing the point of irreversible vascular injury remains a challenge.

Impaired vascular reactivity has been observed in a variety of species and preparations (*in vitro*, *in vivo* and *in situ*) after varying degrees of ischaemic injury, and findings are summarised in Table 1.1 In general, it appears there is a selective impairment of endothelium-dependent relaxation in isolated coronary arteries (*in vitro*). However, in the intact coronary circulation (*in vivo*), vascular reactivity to both endothelium-dependent and –independent agonists is attenuated. This further reduction in vasodilator reserve may reflect involvement of factors such as diastolic compression or capillary plugging by leukocytes (the latter plugging being void in *in vitro* preparations). Importantly, a number of studies demonstrate vasodilatory responses to adenosine are at least partially endothelial-dependent [120,133], endothelial dysfunction due to ischaemia-reperfusion may also limit the capacity of adenosine to increase flow to match increased metabolic demands. Thus, vascular abnormalities (even after very brief episodes of ischaemia) may disrupt normal coronary vascular regulation and matching of energy supply and demand.

Table 1.1 Effects of ischaemia-reperfusion on endothelium dependent and independent agonists in *in vitro*, *in vivo* and *in situ*

Preparation/ Species	Endo-dep Agonist type	Endo-effect Dep	Indep	Refs
In vitro				
Isolated arteries				
canine	ACh (abolished)	+	-	[335]
canine	ACh	-	-	[336]
canine	ADP, 5-HT, thrombin	+	n/a	[336]
canine	ACh, A23187	+	-	[282]
cat	ACh, A23187	+	-	[337]
canine	ACh, ADP, A23187	-	-	[331]
canine	ACh, Bk (partial)	+	-	[284]
pig	ADP, Bk	+	-	[133]
canine	ACh	+	-	[338]
canine	ACh, ADP	+	-	[339]
canine	ADP, ACh SF	+	n/a	[340]
canine	A23187, P-C	-	n/a	[340]
pig	5-HT, A23187, substance P	+	-	[341]
canine	ACh	-	n/a	[172]
Microvascular				
canine	ACh, ADP, A23187	+	-	[331]
In vivo				
CBF				
canine	ACh, Bk,	+	+	[7]
canine	ACh, Bk, TEA	+	-	[342]
canine	ACh	+	n/a	[284]
canine	ACh	+	+	[343]
Human	ACh	+	-	[344]
canine	ACh	+	+	[345]
In situ				
Isolated heart				
rat	Ach	+	-	[346]
rabbit	5-HT	+	-	[347]
rat	ACh, CCh	+	-	[348]
rat	Bk, histamine	-	n/a	[348]

+, impaired dilation; -, dilation unaffected. Endothelium-independent dilators include SNP, $NaNO_2$, NO, glyceryl trinitrate and papaverine.

Note: Receptor-mediated endothelium-dependent dilators are as follows: ACh, ADP
Nonreceptor-mediated endothelium-dependent dilators are as follows: A23187, Phospholipase C

Importantly, it is not known what effect vascular stunning has on myocytes in viable (reversibly injured) myocardium. Although the implications appear to differ from those following occlusions that are sufficiently long to also produce irreversible damage [281,349]. Nevertheless, vascular stunning has important ramifications to humans since the heart is often exposed to transient, reversible ischaemia in numerous clinical settings, including interventional procedures such as angioplasty and coronary bypass operations. Indeed, in these situations, the existence of prolonged post-ischaemic vascular stunning could impair the ability of the coronary vasculature to respond appropriately to recurrent ischaemic episodes and to compensate for the obstruction caused by a critical stenosis [281,350].

The mechanisms that result in loss of endothelial function/NO bioavailability and/or bioactivity remain unclear. Various degrees and forms of endothelial dysfunction exist, including: i) impairment of $G_{\alpha i}$ proteins; ii) reduced release of NO, prostacyclin and/or EDHF; iii) increased release of endoperoxides; iv) increased production of ROS; v) increased generation of ET-1; and vi) decreased sensitivity of vascular smooth muscle to NO, PGI_2 and/or EDHF. Some of the mechanisms and issues relevant to the molecular pathogenesis of post-ischaemic vascular injury are outline below.

Oxidant Stress in Vascular Dysfunction

Ischaemia impairs anti-oxidant defences while subsequent reperfusion enhances levels of ROS [246,351] (Fig. 1.9). Resultant oxidative stress is implicated in mediating endothelial dysfunction in the absence or presence of blood [283,337,352-355]. Radicals generated on reperfusion can potently inactivate NO [356,357], reduce NO-synthase activity [358], and attenuate agonist-stimulated NO release [359]. Proximity of endothelial cells to sites of radical generation may render them particularly susceptible. Compounding these factors, endothelial cells themselves generate radicals [360].

In mammalian cells, potential sources of ROS include the mitochondrial respiratory chain, arachidonic acid pathway enzymes (lipoxygenase and cyclooxygenase), cytochrome p450s, XO, NAH/NADPH oxidases, NO synthase, peroxidases, and other hemoproteins [361]. Some studies argue for roles for specific radicals (eg. OH^{*}) in endothelial dysfunction and vascular injury [355], while others demonstrate no role for specific oxidants, such as H_2O_2, since individual levels achieved are insufficient to mediate injury alone [362]. Furthermore, several studies indicate that native or endogenous antioxidants including SOD, catalase and glutathione peroxidase are not impaired by ischaemia-reperfusion in isolated hearts (regardless of ischaemic severity) [363,364]. This suggests use of exogenously applied antioxidants may be futile in protecting against post-ischaemic vascular injury.

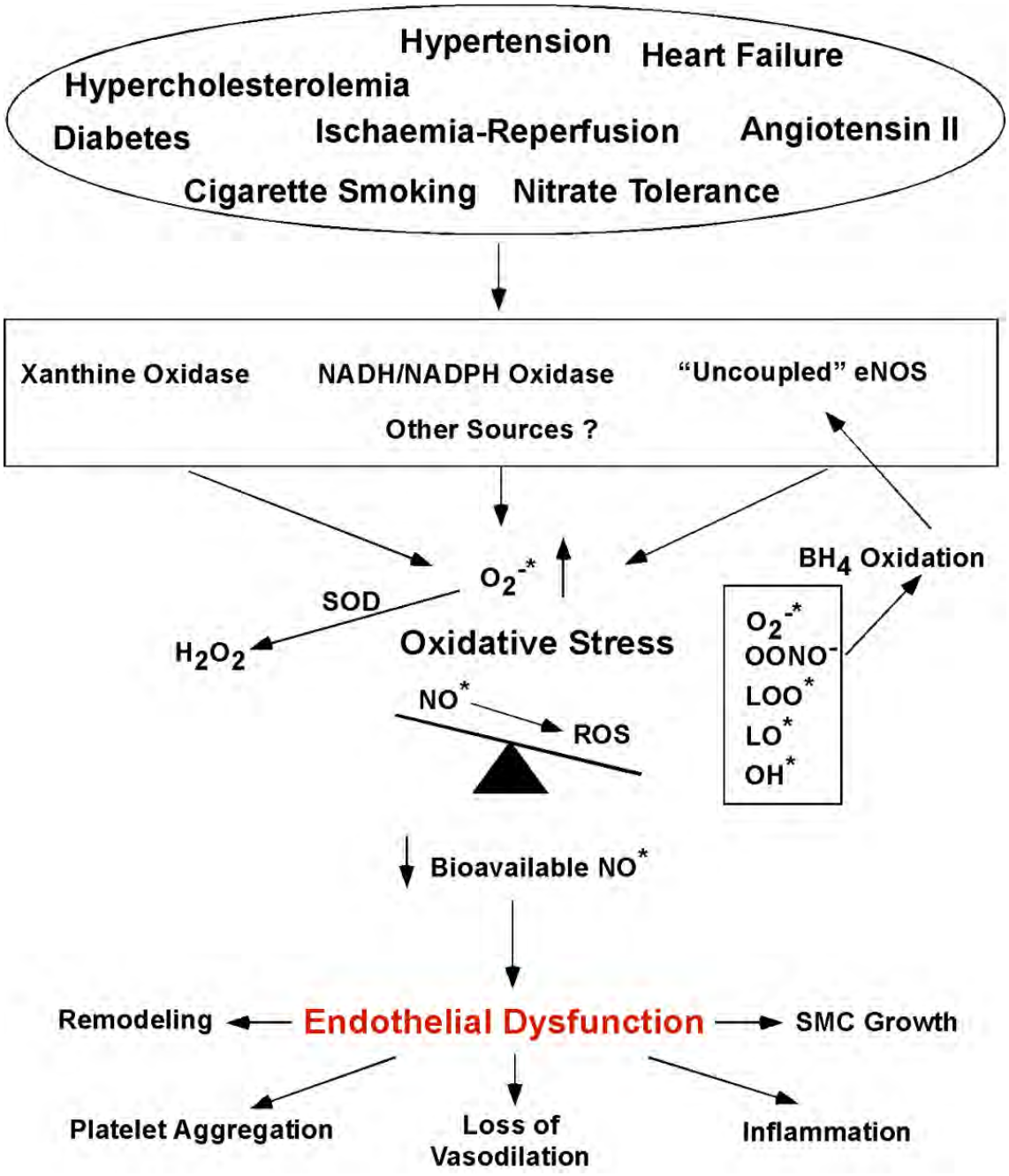

Figure 1.9 Role of oxidant stress in endothelial dysfunction modified from Cai and Harrison [361].

The Central Role of NO

As already noted, reduced NO "bioavailability" appears to be a chief cause of endothelial dysfunction. A decline in NO bioavailability may be caused by decreased expression of endothelial cell NO synthase (eNOS) [365], a lack of substrate or cofactors for eNOS [366], alterations of cellular signalling such that eNOS is not appropriately activated [367], and/or, accelerated NO degradation by ROS [368]. However, there is evidence both for [369] and against [370] impaired NO-synthase activity, for [371] and against [282,331,346] impairment of receptor *vs.* non-receptor mediated responses, and for [348] and against [336,372] inhibition of specific muscarinic responses. Thus, the genesis of dysfunction is extremely controversial.

In addition to representing an end-point of injury, NO-itself may be a "double-edged sword", exerting injurious and beneficial actions during ischaemia-reperfusion. During reperfusion NO may have direct toxic effects, facilitate injurious $ONOO^-$ formation [245], and contribute to apoptosis [373]. On the other hand, NO may exert beneficial effects on endothelial permeability, microvascular function, and platelet/leukocyte aggregation and adhesion [374]. Whether endogenous NO is involved in protecting vessels also remains controversial, with evidence for [375,376] and against [377,378] a role in early preconditioning responses. There is also evidence NO worsens ischaemic tolerance [377], that NO blockade is protective [245], and that NO plays no obligatory role in ischaemic preconditioning [376]. Moreover, recent findings from our laboratory indicate NO plays no role in triggering or mediating protection due to adenosine-mediated preconditioning [379]. Despite varied and confounding data on the role of endogenous NO in protection *vs.* injury, there is a tendency for studies to identify beneficial effects of NO donors and *L*-arginine in myocardial ischaemia-reperfusion injury.

Endothelin

As discussed in Section III, ET is a potent vasoconstrictor and instigates deleterious actions in the myocardium in the form of elevated post-ischaemic diastolic contracture [380-382], and also promotes vascular dysfunction [355,382-385]. Plasma ET levels are increased during ischaemia-reperfusion [386], resulting in activation of NHE [227], and a rise in intracellular Ca^{2+} levels on reperfusion. Maczewski and Beresewicz argue for ET-dependent activation of PKC with resultant generation of OH^{*} radicals and subsequent development of endothelial dysfunction [355]. More recent findings from Beresewicz's group [385] also support an additional role for ET antagonism in relation to "anti-neutrophil" effects.

Guanylate Cyclase Activity

cGMP is an important intracellular messenger which mediates most actions of NO and atrial natriuretic peptide (ANP). NO activates cGMP synthesis by binding to sGC, and subsequently elicits a myriad of NO implicated actions including vascular permeability [387], cell adhesion [388], and NO synthesis. Very recent work indicates endothelial GC activity is impaired by ischaemic conditions [389]. Furthermore, a reduction in myocardial cGMP content has been described in *in situ* rat [390] and rabbit [391] hearts after 30 min ischaemia. Thus reduced generation of cGMP may be an important element in manifestation of post-ischaemic vascular dysfunction. Preliminary results of Agulló *et al.* suggest that acidosis and ATP depletion are sufficient to profoundly inhibit cGMP synthesis [389]. Not only does the time course of cGMP inhibition correlate with the time course of cellular acidosis, but there was a rapid recovery of cGMP synthesis upon normalisation of pH. A similar relation was observed between ATP concentration and cGMP synthesis. Although altered cGMP synthesis was not associated with cell death *in vitro*, endothelial cGMP is known to modulate

events that contribute to myocardial injury. Therefore, reduced cGMP may be of importance in the pathophysiology of ischaemia-reperfusion injury. Alternatively, reductions in post-ischaemic cGMP synthesis could also explain reduced susceptibility to cGMP-mediated apoptosis in post-ischaemic myocardium [389,392].

Vascular Plugging and the Importance of Blood Cells?

For many years leukocytes were thought to be major players in dysfunction, releasing radicals and obstructing capillaries [3]. However despite evidence of neutrophils directly injuring coronary vascular endothelium [393,394], the time course of endothelial dysfunction occurs prior to neutrophil accumulation *in vivo* [395]. Specifically, endothelial dysfunction occurs within 2.5 min of reperfusion [337], whereas neutrophil adherence progressively occurs following 10 min of repefusion [395,396]. In addition, endothelial dysfunction is well documented in blood free models [346-348,397], implicating non-leukocyte dependent processes. The observation that smooth muscle injury occurs in addition to endothelial dysfunction in *in vivo* experimental models supports the idea that blood cells are involved in progression of vascular injury (discussed below; Section VII). Therefore, by the very nature of NO to inhibit platelet adherence and activation, modulate microvascular permeability, and decrease expression of adhesion molecules on activated endothelium [398,399], leukocytes [400] and myocytes [401], it seems probable that endothelial dysfunction augments neutrophil adhesion and subsequently enhances vascular injury. These forms of reversible injury may ultimately be exaggerated or contribute to irreversible injury with more severe insult. Mechanisms of irreversible injury are discussed below.

VI. MECHANISMS OF 'IRREVERSIBLE' INJURY IN MYOCARDIUM AND VASCULATURE

Prolonged occlusion of a major coronary artery will usually lead to transmural infarction, which extends relatively slowly from the subendocardial zone in a "wavefront" [402]. Ischaemia is a dynamic, time-dependent process that begins with transition from reversible to irreversible ischaemic injury, and culminating in replacement of necrotic myocardium by fibrous scar tissue. Although early restoration of blood flow to ischaemic myocardium is necessary to salvage myocytes, abundant evidence indicates reperfusion itself, even after a brief period of ischaemia, has additional deleterious effects that are not expressed during ischaemia, and that can be modified by interventions given only at the onset of reperfusion [403,404]. Thus cell death occurs via both ischaemic and reperfusion dependent processes.

Experimental studies have shown that myocytes undergo cell death during ischaemia and reperfusion by two mechanisms: necrosis (or oncosis) and apoptosis. Necrosis is often described as "accidental collapse of cellular homeostasis, compartmentalisation, and cell membrane integrity with release of cytosolic material and with random nuclear deoxyribonucleic acid (DNA) fragmentation" [405]. In contrast, apoptotic cell death is defined by the "occurrence of cytosolic proteolysis and with enzymatic cleavage of nuclear DNA into oligonucleosome sized fragments in the presence of a functionally intact cell membrane" [405]. Although the process of necrosis and apoptosis may differ in a number of ways, and may seemingly proceed down separate paths, apoptosis and necrosis are unlikely to be two separate, mutually exclusive forms of cell death [405,406]. Indeed, it is becoming evident that there is overlap or cross-over (i.e. switch from apoptosis to necrosis) between these two types of cell death [407]. Importantly, the existence of both types of cell death simultaneously in

myocardium may co-determine the extent of lethal post-ischaemic injury in the myocardium [208,405,406,408].

While there is now considerable evidence for both forms of cell death in myocytes following ischaemia-reperfusion, very little is known about vascular cell death. In particular, there is little to no information on the occurrence of necrosis in post-ischaemic vasculature, although it is suspected that neutrophil adherence to the endothelium may result in vascular necrosis. Vascular apoptosis in the absence of overt necrosis has been observed in atherosclerosis patients [409] and during the process of normal aging [410]. More importantly, preliminary evidence indicates that ischaemia-reperfusion induced apoptotic cell death in endothelial cells precedes that of cardiomyocytes [411]. In addition, different signalling pathways appear to be involved in the induction of apoptosis in myocytes versus endothelial cells [412]. While the contribution of necrosis and apoptosis to cardiac cell loss remains controversial, apoptosis of ischaemic endothelial cells is likely to be of pathophysiologic significance (especially in light of the fact that necrosis may trigger a significant inflammatory response). Injury to these non-contractile components may be a determinant of functional recovery of the heart [2]. The following sections of this thesis will review current knowledge on the mechanisms involved in the induction of necrosis and apoptosis following ischaemia-reperfusion. Although the majority of research has concentrated on irreversible injury in myocardium, evidence for irreversible post-ischaemic vascular injury will be discussed.

Necrosis

Immediate Lethal Reperfusion Injury

Immediate lethal reperfusion injury is manifest as development of the acute form of cell death, necrosis, occurring within the first minutes to first few hours of reperfusion. Recent evidence shows necrotic cell death reaches a peak after 24 hours reperfusion (following 1 hour occlusion of the left anterior descending artery in dogs) [413]. Myocyte death in permanently ischaemic myocardium is traditionally considered as necrosis and is marked by the release of intracardiac enzymes (such as creatine kinase and troponin-T) [414-416]. The end-point of immediate lethal reperfusion injury is mechanical disruption of the sarcolemma, which is probably initiated by severe hypercontracture of the myofibrils. There are 4 potential mechanisms involved in initiating cell death on reperfusion injury: i) re-energisation (paradoxically); ii) rapid normalisation of tissue pH; iii) rapid normalisation of tissue osmolality; and iv) the mitochondrial permeability transition (MPT). In addition, a significant inflammatory response during the first 24 hours has been associated with delayed lethal reperfusion injury [417,418]. The potential causes of immediate lethal reperfusion injury are not entirely independent but probably work in cohesion to generate necrosis (Fig.1.10).

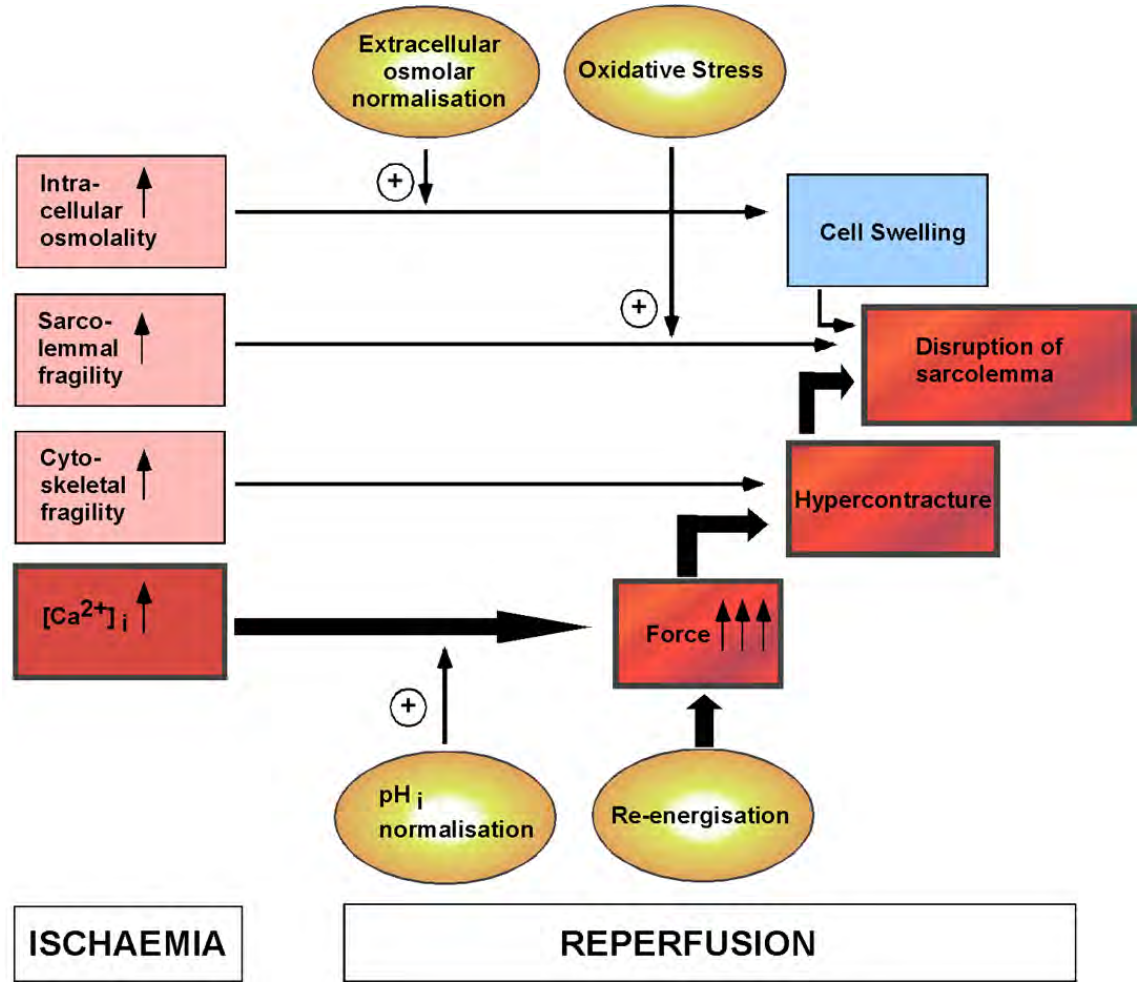

Figure 1.10 Potential mediators of immediate lethal ischaemia-reperfusion injury. Enhancing influences are marked with a plus sign, inhibitory influences with a minus sign modified from Piper *et al.* [208].

i. Re-energisation

As previously discussed, the ischaemic period promotes overload of Na^+ and Ca^{2+} in the cytosol, which leads to a sustained contracture of the cell (Fig. 1.7). The re-energerisation process can promote hypercontracture via one of two pathways, which are activated simultaneously. The first promotes cell recovery by reactivating the two major cation pumps; the SR Ca^{2+}-ATPase pump and the sarcolemma Na^+/K^+-ATPase

pump. This leads to a continuous cyclic effect whereby excess cytosolic Ca^{2+} release and reuptake by the SR occurs. The NCE (forward mode) located in the sarcolemma can control cessation of this event by removing sufficient amounts of Ca^{2+} from the cytosol. Ironically, the prerequisite for the activation of this exchanger is a large Na^+ gradient across the sarcolemma. As Na^+ has also built up in the cytosol during ischaemia it is essential that the Na^+-ATPase be rapidly reactivated, aiding in extrusion of Na^+ [208]. Importantly, studies from Piper's laboratory [419-421] have shown that even if a cardiomyocyte has been extensively depleted of its energy stores and suffered from severe Ca^{2+} and Na^+ overload before re-energisation, there is usually still sufficient metabolic competence to rapidly reactivate SR Ca^{2+} and sarcolemmal Na^+ pumps during early reoxygenation. However, if crucial pump damage has occurred and a diminished Na^+ gradient persists, Ca^{2+} entry will be favoured through a NCE operating in reverse mode [208].

While the first pathway provides the means (Ca^{2+} overload), it is the second pathway which leads to the generation of sustained hypercontracture. During the initial phase of reoxygenation, cytosolic Ca^{2+} is still largely elevated and myofibrillar activation therefore leads to uncontrolled, excessive force generation [208] (Fig. 1.11). This sustained force generation causes hypercontracture, whereby the actin-myosin cross bridges become deformed structurally and cell shortening becomes irreversible [208].

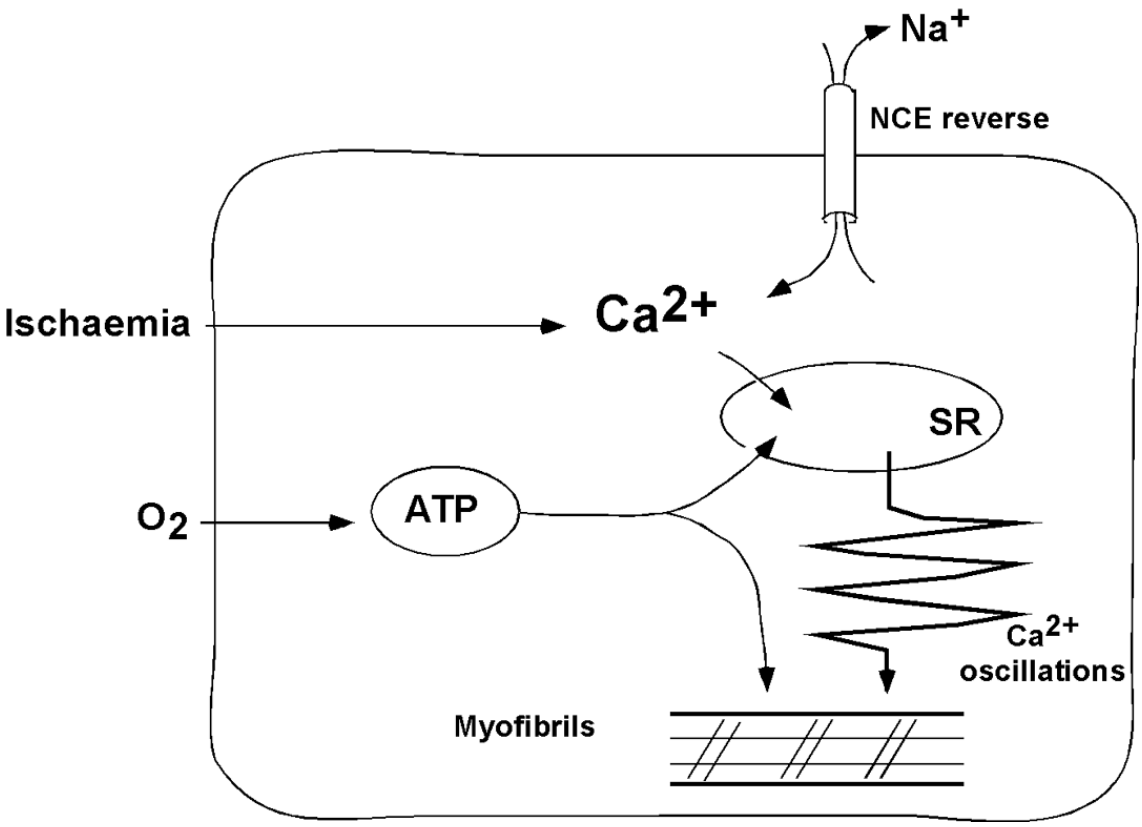

Figure 1.11 Schematic diagram of events leading to hypercontracture upon reperfusion modified from Piper *et.al.* [221].

ii. Rapid normalisation of tissue pH

Rapid normalisation of tissue pH is the second potential cause of immediate lethal reperfusion injury (Fig. 1.12). Ischaemia initiates anaerobic metabolism and the breakdown of ATP, resulting in excess H^+ and acidification of both intracellular and interstitial spaces. Reperfusion promotes activation of NHE and the Na^+/HCO_3^- symporter, which reduces intracellular acidosis and increases influx of Na^+. Intracellular acidosis may be beneficial during ischaemia as it impairs the contractile machinery and aids in preventing occurrence of hypercontracture if maintained during early reperfusion. However, high intracellular acidosis leads to excess Na^+ which can be detrimental via a secondary NCE which enhances the pre-existing levels of Ca^{2+} in

the cell [208]. This rapid removal of H^+ and secondary uptake of Ca^{2+} thus favours the development of hypercontracture if ischaemic-reperfused myocardial cells are allowed to restore a normal intracellular acid-base balance.

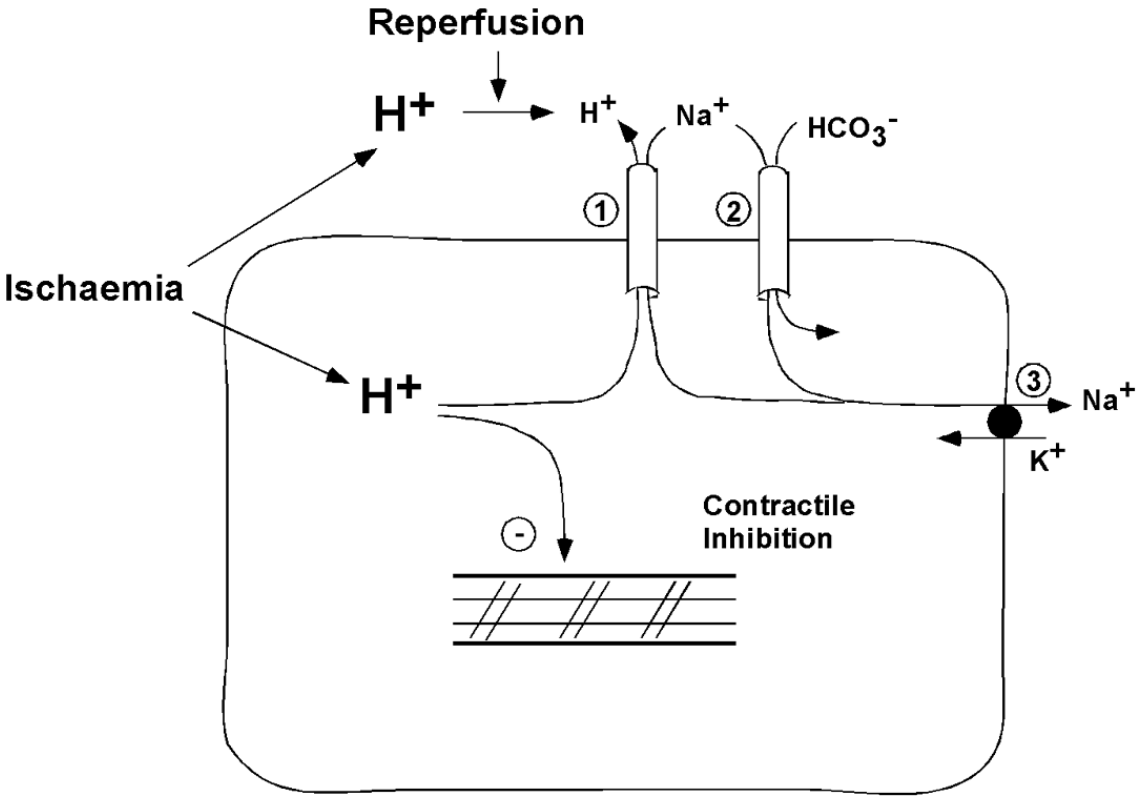

Figure 1.12 Schematic diagram displaying the effects of H^+ accumulation modified from Piper *et al.* [208].

iii. Rapid normalisation of tissue osmolality

Rapid normalisation of tissue osmolality is the third potential cause of immediate lethal reperfusion injury (Fig. 1.13). An intra- and extracellular increase in osmolality is created under the preceding ischaemic conditions. Reperfusion allows the rapid normalisation of extracellular osmolality, which propagates a transsarcolemmal osmotic gradient. The inevitable influx of water induces cell swelling which, in conjunction with other factors, contribute to cell fragility and leads to the cells demise [208].

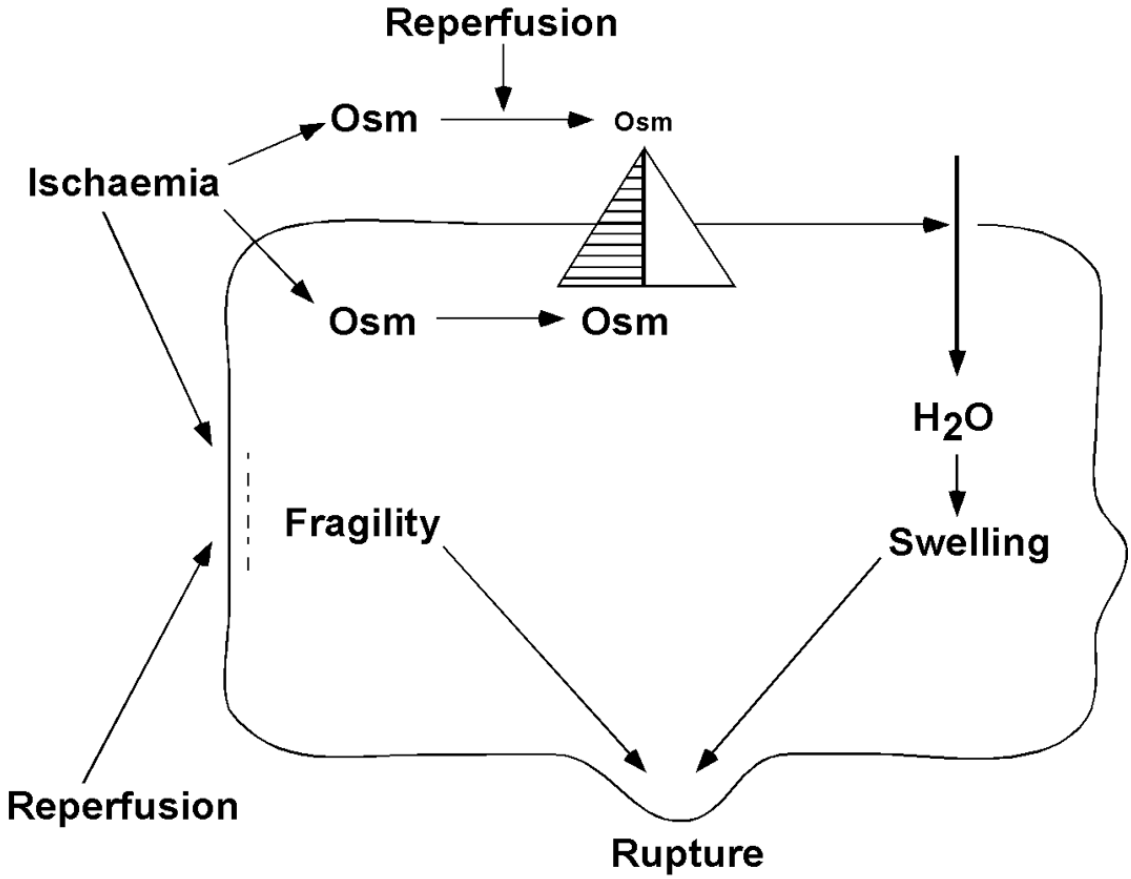

Figure 1.13 Schematic diagram displaying cell swelling as a result of an osmotic gradient modified from Piper *et al.* [208].

iv. Mitochondrial Permeability Transition

The phenomenon of the MPT was first described several decades ago [422], and refers to the massive swelling and uncoupling of mitochondria that occurs when mitochondria are exposed to supraphysiological matrix concentrations of Ca^{2+}. Indeed, conditions for MPT are ideal when associated with adenine nucleotide depletion, elevated phosphate concentration, membrane depolarisation, and oxidative stress [423]. The MPT is now known to be the result of a conformational change in the membrane protein adenine nucleotide translocase (ANT), whose normal function is to catalyse the transport of ADP into and ATP out of the mitochondria [424,425]. Accumulating work

from Halestrap's laboratory indicates that under conditions favouring MPT, the enzyme CyP-D binds to ANT, and when triggered by Ca^{2+}, causes a conformational change, converting the specific transporter into a nonspecific pore [426,427] (Fig. 1.14). The selective permeability of the mitochondrial inner membrane is essential for the maintenance of the membrane potential and pH gradient that drive ATP synthesis during oxidative phosphorylation. Therefore, when conditions are optimal for MPT pore opening, mitochondria become uncoupled and the proton-translocating ATPase actively hydrolyses rather than synthesises ATP [423].

Not surprisingly, it has been proposed that MPT plays a major role in reperfusion injury and subsequent cell death, since conditions necessary for MPT pore opening are exactly those created during ischaemia-reperfusion [428]. During ischaemia, Ca^{2+} starts accumulating while low pH (induced by high concentrations of lactic acid) inhibits the MPT. Upon reperfusion the rapid normalisation of tissue pH and the combination of oxidative stress and elevated Ca^{2+} concentrations make ideal conditions for MPT pore opening [423,428] (Fig. 1.14). If MPT pore opening is extensive and prolonged, mitochondria remain uncoupled and are unable to generate the ATP required for maintaining ionic homeostasis and repairing tissue damage [423,428]. In addition, uncoupled mitochondria may actively hydrolyse ATP derived from both glycolysis and residual oxidative phosphorylation. In time the permeability barrier of the plasma membrane will also be compromised through phospholipase A_2 action, and leakage of cell contents and disruption of ion gradients will then ensure cell death [423,428]. As identified by Kerr *et al.* [429], there is a strong correlation between the amount of pore opening and the degree to which the heart recovers after ischaemia-reperfusion injury. Even if some pore opening occurs during early reperfusion, a full recovery will still be made provided pores close in the subsequent minutes [429]. These

data indicate that MPT is a critical factor in immediate lethal reperfusion injury, which is not only associated with contractile dysfunction and ionic homeostatic imbalance due to an inability to synthesise sufficient ATP but also ensuing cell death. Importantly, recent studies show MPT inhibition does increase ischaemic tolerance [430-432].

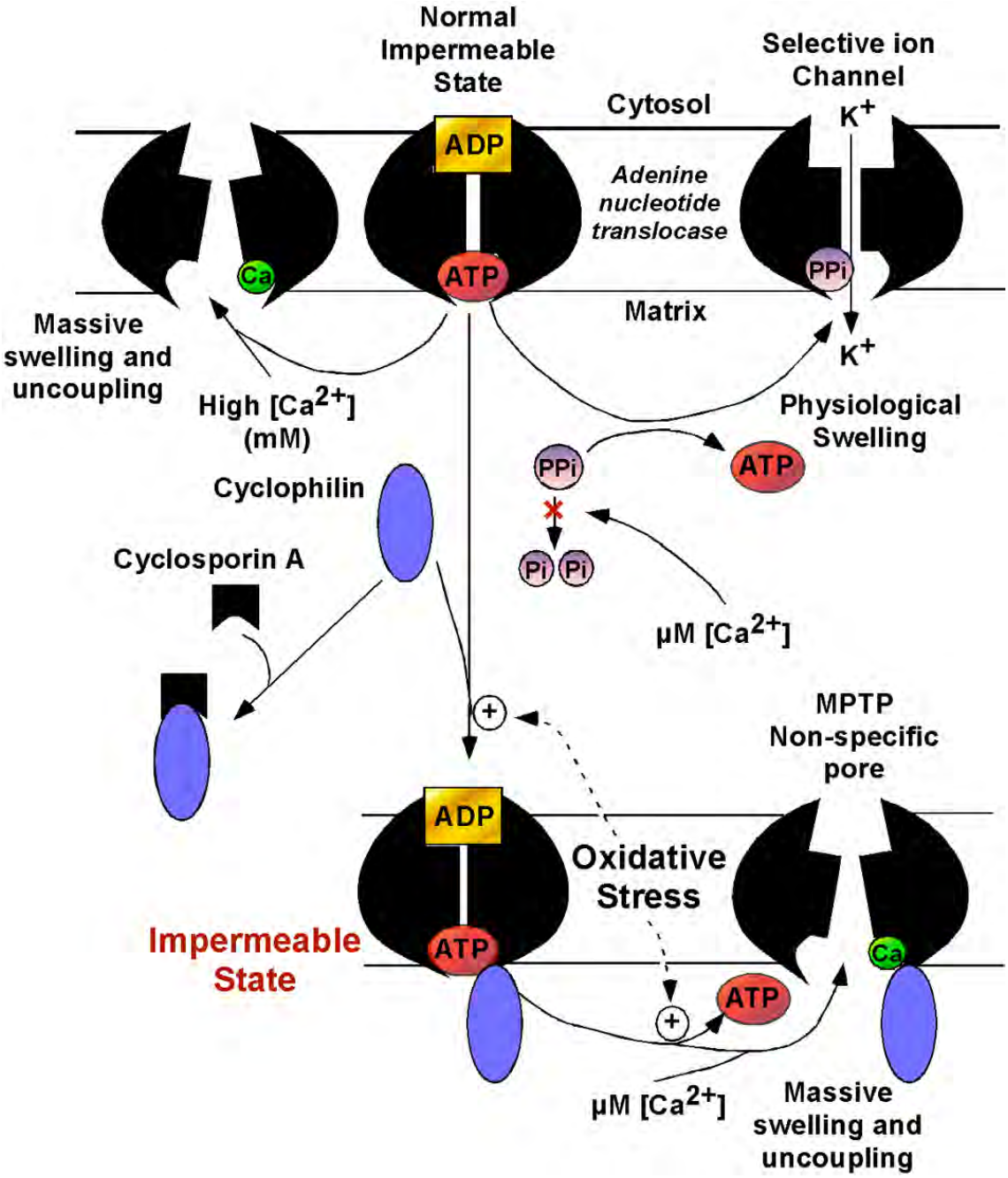

Figure 1.14 Proposed mechanism of the MPTP modified from Halestrap *et al.* [433].

v. Neutrophil Activation and the Inflammatory Response

The inflammatory component of reperfusion injury is initiated by the interaction between polymorphonuclear neutrophils (PMNs) and the coronary vascular endothelium. This interaction begins immediately upon reperfusion, and may continue for 72 hours or more [434]. During the early moments of reperfusion, PMNs are recruited to the endothelial surface; the pro-adhesive properties are stimulated by ROS, thrombin, histamine, tumour necrosis factor-alpha (TNF-α), platelet activating factor, and C5a [435-438] (Fig. 1.15). The interactions between coronary vascular endothelium and neutrophils are mediated by a highly specific and temporally orchestrated sequence of events involving the early (p-selectin, L-selectin) and late (ICAM-1, VCAM, PECAM) expression of adhesion molecules on both the endothelium and PMNs. P-selectin is stored as preformed granules in the Weibel-Palade bodies, which are rapidly translocated to the endothelial surface in response to oxygen radicals, thrombin, C5a, TNF-α, IL-1, and reperfusion [439,440]. Interaction with P-selectin on endothelium causes the neutrophil to start rolling and attaching loosely on the endothelial surface. This "rolling phenomenon" plays a critical role in the pathogenesis of the early phase of reperfusion injury in the myocardium [337,396,441-448].

After the initial tethering of PMNs to the vascular endothelium, firm adherence is facilitated by interaction between CD11b/CD18 on PMNs and ICAM-1 on the endothelium. ICAM-1 is constitutively expressed at low levels, but *de novo* protein synthesis and surface expression is stimulated by cytokines (eg. TNF-α) beginning at 4-6 hours after reperfusion, and peaking at 24 hours. This later response is in contrast to the early (<30 minutes) expression of P-selectin. Firm adhesion is followed by transendothelial migration of PMNs into the extravascular (myocyte) compartment.

The inflammatory cell component of reperfusion injury, characterised by PMN-endothelial cell interactions described above, initiates a broad range of tissue injuries. The early PMN adherence to endothelium is prerequisite to a constellation of pathophysiological processes that ultimately lead to infarction, vascular injury and apoptosis (Fig. 1.15). Several studies have shown that inhibition of the early phase P-selectin-dependent interaction between neutrophils and endothelium reduces vascular injury, infarction, apoptosis and neutrophil accumulation, suggesting that intervention in the early phases of reperfusion will attenuate down-stream physiological outcomes [337,394,396,448]. The physiological consequences of reperfusion injury can also be attenuated by inhibiting the later, ICAM-1 and PECAM-1 [449] mediated stages of neutrophil-endothelial cell interactions [442,450-453].

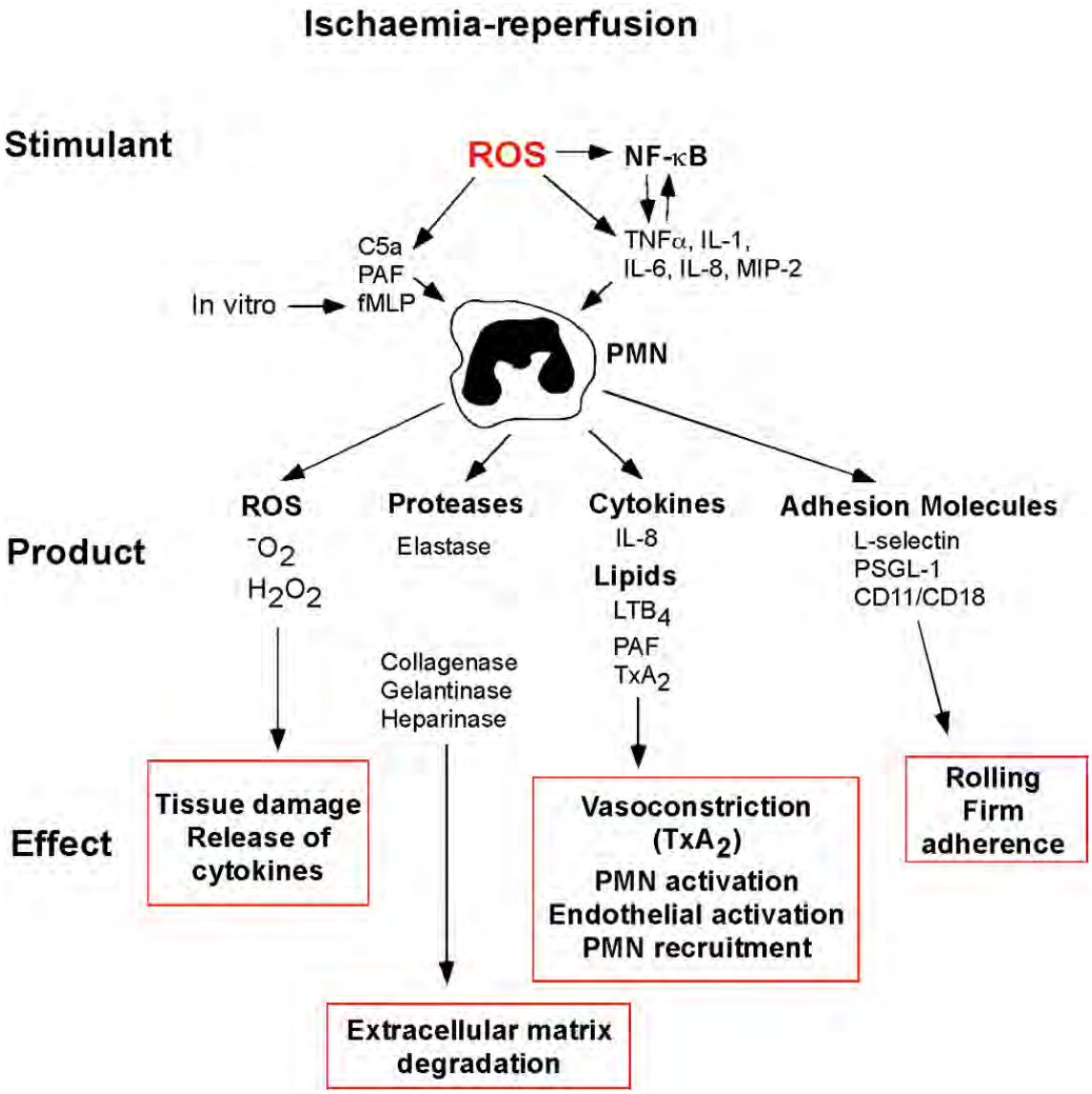

Figure 1.15 Schematic diagram of activation of neutrophils and the end-effectors involved in lethal ischaemia-reperfusion injury modified from Vinten-Johansen [454].

Apoptosis

In the 1970s Kerr and colleagues first described a novel form of cell death distinct from necrosis which they designated 'apoptosis' (from the Greek word for falling) [455]. It wasn't until the early 1990s that apoptosis was first reported following reperfusion in cardiomyocytes [456]. Since then many reports have focused on confirming the appearance of apoptosis in ischaemic cardiomyocytes and on its distribution *in vivo* in animals [413,457-461] and humans [462-466]. Although some

studies have demonstrated myocardial apoptosis during ischaemia, a growing body of evidence indicates that apoptosis is reperfusion-dependent [407,456,459,461,467]. A study by Vinten-Johansen and colleagues found that while a prolonged ischaemic period caused significant infarction, very few cells were in fact apoptotic [461]. A milder ischaemic period followed by reperfusion resulted in a potentiated number of apoptotic cells in the peri-necrotic area, in association with DNA laddering [461]. Indeed, the depletion of intracellular ATP levels during ischaemia blocks the activation of the down-stream pro-apoptotic genes, which prevents the typical apoptotic changes from taking place. Reperfusion rapidly restores the intracellular ATP levels, providing the energy necessary to allow execution of the apoptotic pathway [468]. This would contribute to observed mixes of apoptotic and necrotic phenotypes post-ischaemia [468-470], and association of apoptosis with reperfusion (when energy is available) rather than ischaemia itself [456,467]. An energy threshold below which necrosis is favoured and above which apoptosis is favoured, is supported by work in neonatal cardiomyocytes [471]. This dependence may contribute to development of "secondary necrosis" in apoptotic cells [469].

It is still not clear whether apoptosis precedes or is followed by necrosis, or whether both mechanisms of cell death occur simultaneously by separate pathways. However, recent evidence supports the latter [461]. Interestingly, during early reperfusion necrotic cell death rapidly develops which is followed by a slower appearance of apoptosis during late reperfusion. As mentioned previously, the extent of necrosis peaks at 24 hours of reperfusion, whereas, apoptotic cells increase slowly up to 72 hours of reperfusion [413]. This gives strength to the idea that progressively developed apoptotic cell death may participate in extension of myocardial infarction in later phase of reperfusion. Moreover, Zhao *et al.* used the endonuclease inhibitor

aurintricarboxylic acid (ATA) at the onset of reperfusion to examine whether ATA reduces extension of infarction by inhibiting apoptosis [472]. They determined that ATA significantly reduced the area of necrosis, in the absence of a direct effect of ATA on pathogenesis of necrosis. Their observation suggests a link between apoptosis and necrosis and involvement of cross-talk between these processes.

Evidence of Vascular Apoptosis

In addition to myocardial apoptosis, there is also evidence of vascular endothelial apoptosis in post-ischaemic hearts [411]. Moreover, this may occur via pathways distinct from those mediating cardiac apoptosis [412]. Although mechanisms involved in the induction of vascular apoptosis remain unclear, a very recent report indicates oxidative stress in human endothelial cells increases caspase activity [473]. Capases are specialised cysteine-dependent proteases that cleave major structural proteins of the cytoplasm and nucleus (discussed below). Importantly in normal healthy cells caspases are present as poorly active or inactive zymogens that can be activated by autocleavage or processing by other proteases [474]. However during ischaemia mitochondrial cytochrome c is released [475,476] and leads to the activation of the caspase cascade [477,478] and the induction of apoptosis. Moreover, Zhang *et al.* demonstrated that ox-LDL upregulates apoptosis inducing factor (AIF) expression in endothelial cells in a concentration and time dependent manner, and was associated with translocation of AIF from cytoplasm to the nuclei [479]. Collectively these observations hint at a role for apoptosis in vascular dysfunction and injury during oxidative stress (eg. Ischaemia-reperfusion). Interestingly, apoptosis of ischaemic coronary endothelial cells may actually help to quickly reseal the intima of the coronary vasculature by new endothelium, since apoptosis shortens the time required for elimination of injured cells [480]. Furthermore, the mode of execution of inflammatory cells that accumulate in the

post-ischaemic vasculature may also be meaningful. A necrotic death would lead to the release of toxic components and may cause excessive inflammation, whereas apoptosis may ultimately prevent expansion of injury [480]. Nonetheless, the fact that endothelial cell apoptosis precedes myocyte cell apoptosis suggests that strategies which allow endothelial cell rescue may potentially protect the myocardium or enhance its capacity for salvage [411].

Methodological Considerations

Following the morphological definitions of apoptosis (as discussed above), its biochemical characteristics were elucidated. However, the simple use of TUNEL-positivity and DNA ladder detection as a determination of apoptosis has resulted in misunderstandings of the modes of cardiomyocyte death. TUNEL detects single-strand DNA breaks as well as double-strand DNA breaks with free 3'-OH termini [480]. Therefore, TUNEL is not as specific for apoptosis as originally thought, and necrotic cells may be being detected as TUNEL-positive. Since this methodological flaw was realised, it has become imperative to additionally assess other markers such as plasma membrane integrity by electron microscopy [481]. As determined by Ohno *et al.,* electron microscopy combined with TUNEL revealed that TUNEL-positive cardiomyocytes exhibited membrane damage [481]. They speculated that TUNEL-positivity may be secondary to irreversible membrane damage.

To add to the confusion regarding the distinction between necrosis and apoptosis, a recent report documented the occurence of apoptosis without DNA fragmentation [482]. Interestingly, a capase-activated deoxyribonuclease (CAD), which breaks at internucleosomal DNA sites, was identified and cloned and an inhibitor of CAD (ICAD) was also identified. By binding to CAD, ICAD inhibits DNA fragmentation induced by caspase activation. However instead of escaping from suicide

those cells were still killed as a result of caspase activation [482]. This implies that caspase activation is able to kill cells without DNA fragmentation, that DNA fragmentation may not be a "hallmark" of apoptotic cell death, and that backward signalling from activated caspases to determinants of cell death, such as MPT, may have a role in caspase activation-induced cell death [480]. Thus, it is difficult to draw a clear line between apoptotic cell death and non-apoptotic cell death by the appearance of fragmented DNA in post-ischaemic cardiomyocytes [480]. This emphasises that a combination of techniques should be utilised when assessing for the presence of apoptosis, including microscopic methods of evaluation, analysis of pro- and anti-apoptotic proteins and down-stream caspase activation, and detection of mitochondrial function in addition to DNA fragmentation [406].

Possible Mechanisms Triggering Apoptosis

Induction of apoptosis is controlled by intrinsic and extrinsic signals. Every cell contains all components of the suicide machinery and is ready to engage in self-destruction unless it is actively signalled not to do so [483]. Extrinsic signals, including cytokines, hormones, oxidised lipids, chemotherapeutic, ionising or viral agents, can initiate apoptosis. Interestingly, while intrinsic-mediated apoptosis usually leads to a slow progressive loss of cells (chronic), extrinsic signals trigger acute and massive loss. Ultimately, it is the relative abundance of pro-apoptotic and anti-apoptotic molecules within the cell at a given time that will determine the cell's fate. The apoptosis cascade can be divided into four stages: i) initiation; ii) effector activation; iii) structural alterations and DNA degradation; and iv) recognition and removal of apoptotic bodies [483].

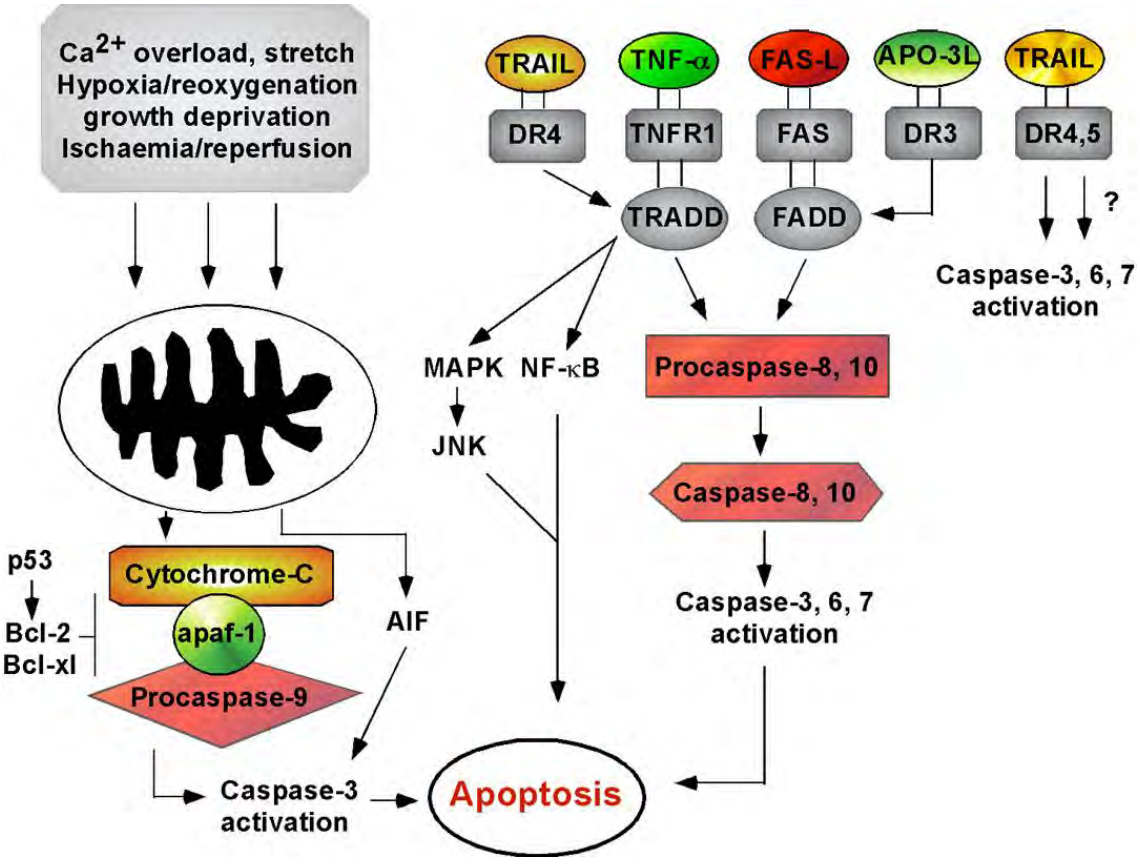

Figure 1.16 Mechanisms of receptor-dependent –independent caspase activation modified from Zhao and Vinten-Johansen [406].

It is generally accepted that the process of apoptosis involves activation of death receptor-dependent and –independent signal transduction pathways [460,484] (Fig. 1.16). During initiation phase death-promoting molecules such as TNF-α or Fas ligand bind to their respective death receptors on the cell surface with subsequent recruitment of death domain proteins (FADD, TRADD, RIP) required for caspase-8 activation, and the induction of the lethal proteolytic cascade. The receptor-independent pathway involves the release of cytochrome C and AIF into the cytoplasm from the intemembrane space of mitochondria in response to cell stimulation. Upon release from the mitochondria, cytochrome C directly forms a trimeric complex with Apaf-1 in an ATP-dependent manner. This complex activates procaspase 9 resulting in cleavage into

caspase 9, the most upstream caspase in the mitochondrial apoptotic pathway. The release of activated caspase 9 subsequently cleaves procaspase 3 into its activated form (caspase 3). AIF may independently exert its apoptotic action by directly stimulating caspases [406]. Furthermore, AIF is also known to mediate caspase-independent DNA fragmentation [485]. Thus, there may be 3 paths to apoptosis: i) receptor-dependent activation; ii) mitochondrial caspase-dependent; and iii) mitochondrial caspase-independent.

The effector phase is characterised by the loss of mitochondrial membrane potential and mitochondrial damage, which leads to activation of the initiator protease, caspase-9, and propagates the cascade of downstream caspases (located at pivotal junctions in apoptotic pathways). However, ligation of death receptors, such as Fas, activates caspase-8, can also then processes downstream effector enzymes. Enzymatically active caspase-8 cleaves BID, and the truncated protein tBID, relocates to the mitochondria where it induces caspase-9 processing. Caspases are present as inactive pro-enzymes, most of which are activated by proteolytic cleavage. They are responsible for the deliberate disassembly of a cell into apoptotic bodies.

The execution phase is controlled by the Bcl-2 family members [486], acting upstream from the caspases, through inhibition of cytochrome c and/or AIF. Generally, apoptosis is believed to be a result of activation of caspases. However AIF is a newly described pathway, which leads to cell injury independent of caspase activation [487]. AIF is normally confined to the mitochondrial inter-membrane space, but translocates to the nucleus when apoptosis is induced. AIF plays a complementary or cooperative role along with caspases that lead to nuclear apoptosis. The Bcl-2 family of proteins contains both inhibitors (Bcl-2, Bcl-xL) and inducers (Bcl-xS, BAX, Bid, Bad, Bak) of apoptosis acting as homodimers and heterodimers. The balance between anti-apoptotic and pro-

apoptotic Bcl-2 family members is critical to determining if a cell undergoes apoptosis. After an appropriate signal, Bax or Bak, for example, undergo a conformational change and move to the mitochondrial membrane where they cause release of cytochrome c into the cytosol [488,489].

The DNA fragmentation phase (DFF) results in irreversible loss of cell viability and is due to production of two subunits, DFF45 (ICAD) and DFF40 (CAD). Cleaveage of DFF45 by caspase-3 activates the nuclease activity of DFF40, which ultimately induces structural alterations and nuclear fragmentation. After completion of the apoptotic process, dead cells are removed from the tissue as a result of specific recognition and phagocytosis by adjacent professional and/or non-professional cells through a variety of mechanisms that implicate phosphatidylserine (PS), thrombospondin/CD36 binding site, and the vitronectin receptor or CD14. Indeed, one of the earliest hallmarks of cells undergoing apoptosis is the loss of phospholipid membrane asymmetry and exposure of PS in the exoplasmic leaflet of the plasma membrane. In addition, cells undergoing apoptosis generate ROS that may induce membrane peroxidation. This results in changes to the cells surface and the development of oxidative-specific epitopes, which can serve as ligands for recognition and phagocytosis by macrophages [483].

Strategies that limit apoptosis reduce myocardial and potentially vascular damage as a result of ischaemia and enhance salvage ability. Therefore, understanding the mechanisms that lead to apoptosis is crucial in the development of therapeutic strategies against this injury. Moreover, evidence indicates that the MPT with subsequent release of apoptotic signals is also a characteristic feature of apoptotic cell death and plays a central role in the apoptotic process. Very recent research supports the idea that the MPT is in fact the first indicator of activation of the apoptotic cascade

[490]. Therefore, not only does mPTP opening result in necrosis, but also in induction of apoptosis [423,432,491-494]. Whether mPTP leads to cell death predominantly by induction of apoptosis or necrosis is still a matter of debate. It has been argued that extensive mPTP opening results in rapid ATP depletion and leads to necrotic cell death, whereas less extensive mPTP opening may result in apoptotic cell death [493]. There is strong evidence that the induction of apoptosis by mPTP opening involves cytochrome c mediated caspase activation. The most common mode for cytrochrome c release from mitochondria is via opening of the mPTP. As recently shown by Martinou *et al.* MPT causes mitochondrial swelling which results in rupture of outer mitochondrial membrane in conditions of reduced Bcl-2 expression [495].

VII. "NO-REFLOW" PHENOMENON

As highlighted and discussed above ischaemic injury is not limited to the myocardium but may also impact upon the vasculature. It may range from vascular stunning (reversible) to cell death. Since the main aim in the treatment of ischaemia is to restore blood flow or to 'reperfuse' the ischaemic vascular bed, impaired vascular function may prevent that goal being attained [496]. Therefore, the manifestation of severe vascular injury may unfavourably impact upon the overall recovery of the myocardium. The extreme form of vascular abnormality is the no-reflow phenomenon. The concept of the "no-reflow" phenomenon was initially observed in a canine model of myocardial infarction by Krug *et al.,* in 1966 [497] and later by Kloner *et al.* in 1974 [349]. They detected persistent subendocardial perfusion defects or regions of no-reflow using myocardial tracers, such as carbon black or thioflavin S (a fluorescent stain for endothelium). Kloner *et al.* eloquently described the observation of no-reflow as the inability to adequately perfuse myocardium after temporary occlusion of an epicardial

coronary artery without evidence of persistent mechanical obstruction [349]. Using electron microscopic examination of the cardiac microvasculature within the anatomic no-reflow zones they revealed significant capillary damage in the form of swollen endothelium and intraluminal endothelial protrusions and, less commonly, intraluminal platelets and fibrin thrombi. These changes were always confined to areas of myocyte necrosis and temporarily lagged behind myocyte cell death with various duration of ischaemia [498].

The no-reflow phenomenon initiates more pronounced injury in the subendocardium, reflecting the wavefront phenomenon of ischaemic myocyte cell death [402]. It is also more pronounced with longer periods of coronary occlusion [349]. Moreover, no-reflow appears to be a process rather than an immediate event that occurs at the moment of reperfusion. Experimental studies showed that the no-reflow area increases with time after reperfusion [499-502]. Initial studies documented a significant rise in the zone of no-reflow between 2 min and 3.5 hr of reperfusion [499]. However, after further characterisation Kloner and colleagues demonstrated that expansion of no-reflow predominantly occurs within 1 hour of reperfusion, with markedly less progression within the second hour, and only a minimal (if any) increase between 2 and 8 hours of reperfusion [501].

A multitude of mechanisms have been proposed to explain the occurrence of the no-reflow phenomenon. However, the significance and mutual interrelations are not yet clarified. The potential mechanisms include endothelial dysfunction and mechanical dysfunction which comprises several hypotheses including: extravascular compression of the coronary arteries caused by interstitial and/or intracellular edema [349,498,503-505]; diastolic compression (contracture) [212,506]; and microvascular “plugging” by leukocytes and/or platelets [417,507,508]. A final explanation is “appropriate”

autoregulation resulting from the decreased metabolic demands of the myocardium after arrest [347], leading to an apparent decrease in vascular function. Preliminary results from Hashimoto *et al.* indicate that this mechanism remains unlikely since metabolic regulation appeared to be maintained after global ischaemia [347]. Therefore, only endothelial and mechanical dysfunction will be evaluated as mechanisms of the no-reflow phenomenon.

Endothelial Dysfunction

As previously discussed in detail (Section V; vascular stunning), endothelial dysfunction or vascular stunning can occur after brief episodes of ischaemia in the absence of irreversible myocardial injury. This might suggest that endothelial dysfunction is not a primary mediator of no-reflow. However, endothelial dysfunction results in increased vascular resistance and is associated with biochemical changes that alter normal vascular function. Indeed, post-ischaemic attenuation of EDRF reduces the intrinsic protection of the endothelium against inflammatory elements such as neutrophil and platelet adhesion and aggregation (Fig. 1.17). Therefore, endothelial dysfunction may lead to the progression of vascular injury and the manifestation of the no-reflow phenomonen.

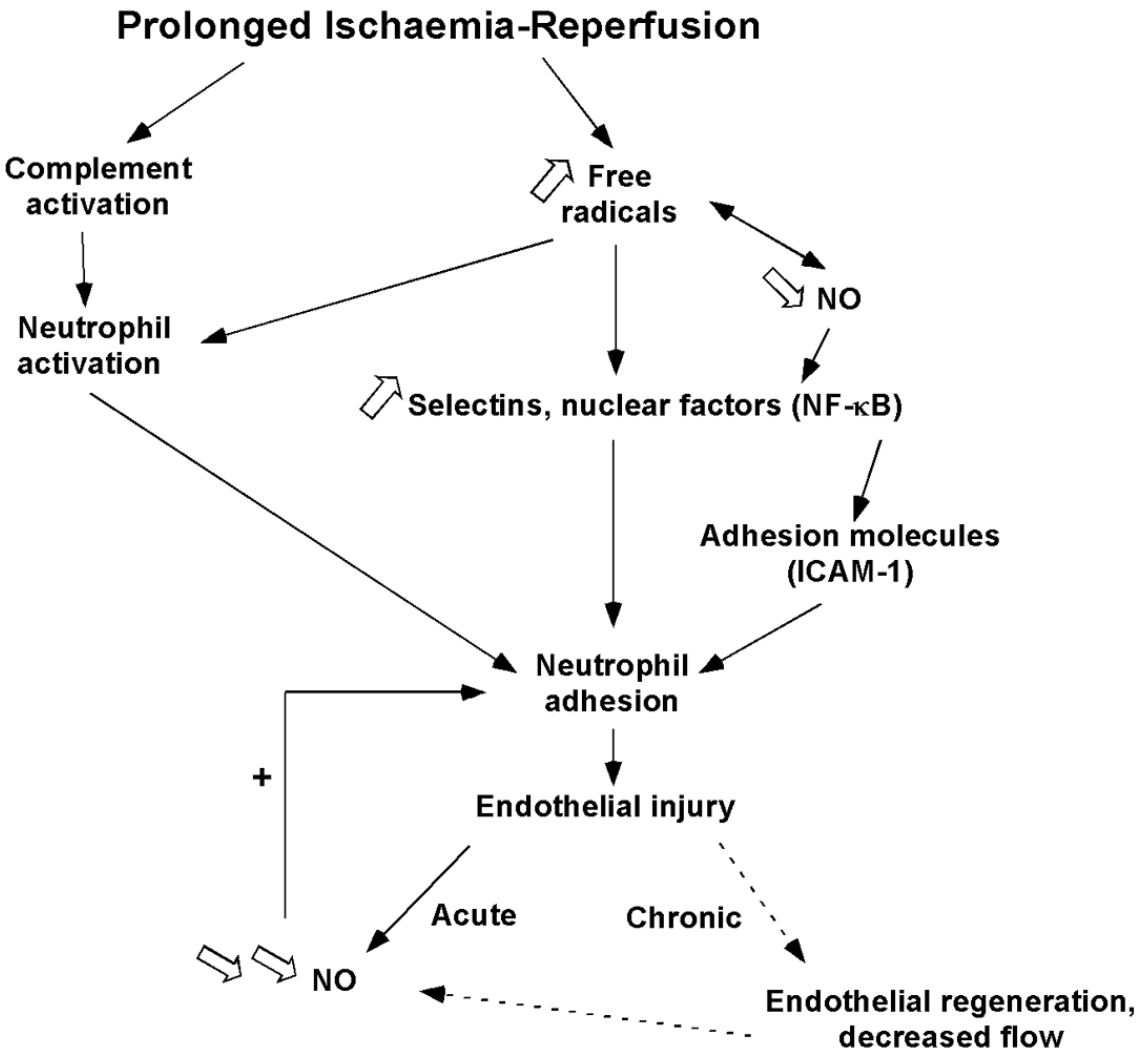

Figure 1.17 Potential mechanisms of endothelial injury after severe ischaemia-reperfusion modified from Laude *et al.* [509].

Mechanical Vascular Dysfunction

Oedema and Cell Swelling

It has been suggested that tissue oedema caused by reperfusion injury could produce physical resistance to blood flow. Myocardial oedema and cell swelling is produce in response to the rapid changes in tissue osmolality which arise upon reperfusion. It is postulated that cellular and interstitial swelling compresses the microcirculation leading to increases in vascular resistance and reduction in blood flow

[235,349,498,503-505]. However, this probably cannot explain the worsening of myocardial blood flow within the first 2 hr of reperfusion, because tissue oedema in general develops very rapidly after reflow. In support of this, Reffelmann *et al.* found that regional myocardial blood flow, which serves as an indicator of the development of oedema, did not decrease during the period of rapid expansion of no reflow, nor did it correlate with the area of no-reflow [501]. Furthermore, there are mixed results on a reduction of oedema using a hyperosmolar buffer (such as mannitol) resulting in improvement in CBF [347,510].

Myocardial Contracture

Extravascular compression of microvessels due to ischaemic contracture and diastolic dysfunction of the myocardium has also been implicated as a cause for coronary microvascular flow deficits after an ischaemic insult [1,212,511]. Ischaemic contracture is described as in an increase in diastolic tension or a reduction in myocyte length [1,2,212]. Humphrey *et al.* [212] demonstrated that the onset of the 'no-reflow' phenomenon was associated with the onset of ischaemic contracture, although they found that the vascular defect continued to deteriorate while the intensity of the contracture declined. These observations led to the hypothesis that the pathogenesis of the no-reflow phenomenon is biphasic. It was postulated that ischaemic contracture compresses and closes the vessels. A secondary mechanism, probably rigor mortis, then supervenes to prevent the affected vessels from reopening when reperfusion is attempted [212]. The diastolic dysfunction that is present upon reperfusion occurs as a result of a reduced dissociation of thick and thin filaments, an increased intracellular Ca^{2+} and the failure to remove Ca^{2+} from contractile proteins, all of which are associated with insufficient ATP levels [512]. In addition to myocyte contraction, recent work supports a potential role for endothelial cell contraction contributing to the no-

reflow phenomenon [513]. Indeed the use of phalloidin to stabilise endothelial actin filaments resulted in a reduction in capillary dimensions that was consistent with constriction that occur post-ischaemia [513]. However, a direct role for endothelial cell contraction in no-reflow is yet to be established.

Leukocyte Adhesion and Red Blood Plugging

The progression of vascular damage and the no-reflow phenomenon during reperfusion is considered to be primarily due to leukocyte plugging [255,396,514] or leukocyte interactions with endothelial cells, platelets, and myocytes as a result of an inflammatory response [515]. Engler *et al.* determined that capillary plugging by neutrophils occurred in regions of no-reflow in previously ischaemic tissue [514]. In addition, the gradual development of no-reflow was associated with increasing numbers of neutrophils [499]. Nonetheless, it is clear that factors other than neutrophil plugging and neutrophil-mediated damage must play a role in the no-reflow phenomenon since isolated asanguinously (blood-free) perfused hearts are still subject to incomplete perfusion [516,517]. Therefore, while neutrophil interactions may play a substantial role in the development of no-reflow, they appear to only be a secondary mechanism of action. The no-reflow phenomenon is likely multifactorial. As highlighted by Rezkalla *et al.*, during the ischaemic phase, endothelial damage, including endothelial swelling and myocyte edema, may lead to initial no-reflow zones [518]. Reperfusion, additional oedema, myocyte contraction, platelets, fibrin, and leukocyte plugging all result in expansion of the no-reflow zones over the first hours of reperfusion.

VIII. RELATIONSHIP BETWEEN VASCULAR AND MYOCARDIAL INJURY

The relationship of vascular dysfunction to the concomitant delay in recovery of myocardial function has yet to be fully elucidated. A number of experimental studies verify that vascular dysfunction does contribute substantially to myocardial depression [498,519,520], and reveal that no-reflow appears tightly coupled to infarct size [4,5,501]. A very recent study by Reffelmann *et al.* assessed the long-term significance of no-reflow on myocardial infarct size [521]. They determined that no-reflow persists for 1 month after reperfusion and is a significant predictor of infarct expansion [521]. In the clinical setting, angiographic no-reflow phenomenon predicts short- and long-term cardiac complications after acute myocardial infarction (AMI), including vascular remodelling that ultimately leads to progressive heart failure and cardiac death [522-524]. Moreover, endothelial dysfunction has proven to be a successful predictor of long-term atherosclerotic disease progression and cardiovascular conditions such as unstable angina, myocardial infarction and stroke [344,525,526]. Thus both experimental and clinical studies have drawn the identical conclusion that vascular injury is closely related to myocardial salvage. However, they are yet to clarify whether the correlation between no-reflow and myocardial recovery is due to microvascular damage impeding infarct salvage or vice versa.

In support of vascular injury impacting upon myocardial recovery, reduced coronary vasodilator function has been demonstrated in infarcted and normal myocardium following myocardial infarction [527]. Furthermore, patients with chronic stable angina due to single vessel coronary artery disease have been shown to have reduced maximal myocardial blood flow, not only in areas perfused by the stenosed artery but also in regions of angiographically “normal” coronaries [528,529].

Interestingly, endothelial dysfunction has also been reported in asymptomatic children and young adults with risk factors for atherosclerosis, such as hypercholesterolaemia and smoking [530,531]. Collectively these studies suggest that endothelial dysfunction preceeds acute myocardial events and that myocardial infarction caused by atherogenesis is directly related to the severity of endothelial dysfunction. This indicates that treatment of endothelial dysfunction may well become a cornerstone for effective management of many coronary pathologies, and the subsequent occurrence of myocardial infarction [532,533].

The fact that intracoronary adminstration of adenosine upon reperfusion can reduce vascular injury (in terms of PMN adherence to vascular endothelium and agonist induced endothelium-dependent dilation) and the incidence of the no-reflow phenomenon [507,534-538], indicates that vascular injury can be at least partially reversed. However, very little is known about the adenosine receptor subtypes or mechanisms involved in modulation of post-ischaemic vascular injury. This is an area of investigation in this thesis (see Chapters 5 and 6). The following section reviews current literature on adenosine receptor-mediated myocardial protection, and the signalling pathways involved.

IX. ADENOSINERGIC PROTECTION AGAINST ISCHAEMIA-REPERFUSION

Increased production of adenosine during ischaemia has several potentially cardioprotective effects. In 1985 Ely and co-workers [539] showed enhanced recovery of function and higher post-ischaemic ATP levels in hearts treated with adenosine, and proposed that adenosine served as an intracellular substrate to enhance salvage resynthesis of ATP during reperfusion. They also concluded that adenosine slowed ATP breakdown during ischaemia [539]. In the last decade, many studies report adenosine's cardioprotective effects during both ischaemia [540] and reperfusion [507,541]. Furthermore, it is now known that, via receptor-dependent and independent (metabolic) paths, adenosine can substantially modify the acute response to ischaemic insult, in addition to generating a more sustained ischaemia-tolerant phenotype (preconditioning). While the molecular basis for acute adenosinergic cardioprotection remains incompletely understood, it may involve; protection against SR dysfunction induced by Ca^{2+} paradox [542]; modulation of NHE activity [543]; energy metabolism [544,545] and oxidant injury [546-548]; enhanced NO bioavailability [549]; and inhibition of the MPT and mitochondrial uncoupling [431,550].

Adenosine-Mediated Protection Against Myocardial and Vascular Injuries

A_1AR-Mediated Cardioprotection

Lasley and colleagues provided much of the early evidence supporting a central role for the A_1AR in cardioprotection [540,551-553]. Subsequent investigations using more selective pharmacological agents verify A_1AR agonist mediated protection (for

review see [10]). Moreover, a relatively small number of studies document impaired tolerance with A_1AR antagonists [18,21], supporting protection via endogenous adenosine. In addition to myocardial protection, preliminary evidence indicates that A_1AR activation might afford coronary vascular protection [554]. While there are clinical complications associated with the use of A_1AR agonists in humans, transgenic A_1AR overexpression in animal models has been demonstrated to increased resistance toward ischaemia (increased post-ischaemic contractile function, decreased lactate dehydrogenase release, and decreased infarct size) [555-558]. Additionally, A_1AR overexpression attenuates apoptosis and caspase-3 activity [559]. Indeed, if an increase in cardiac adenosine receptors could be achieved in humans by means of gene transfer or other molecular techniques, it may be possible to chronically enhance the tolerance of the myocardium to ischaemia without systemic side effects and without the need for continuous administration of receptor agonists. Signalling via the A_1AR has been shown to provide protection not only in animal tissues but in human myocardium [560,561].

Confounding findings do exist with respect to A_1AR mediated cardioprotection. For example, protection with A_1AR agonists is not always observed [18,21,555], and as already noted only a limited number of studies demonstrate exacerbation of ischaemic injury with A_1AR antagonism. Lasley and colleagues have put forward an intriguing hypothesis to explain some of the inconsistencies in A_1AR mediated responses. They observed translocation of A_1ARs from caveolae upon agonist stimulation [562], a form of compartmentalization, which may explain lack of direct effects of A_1AR agonism under certain conditions. Based upon effects of ischaemia and endogenous adenosine on caveolae translocation, this process could regulate A_1AR responsiveness prior to and during ischaemia. This possibility awaits further testing.

The mechanisms by which A_1ARs protect against ischaemia-reperfusion injury are under investigation. Work from Liang and colleagues suggest coupling to PLC (whereas A_3 couple to PLD) [84] and activation of PKC [563]. Moreover, mitochondrial K_{ATP} channels and MAPK are also implicated [563,564].

A_{2A}R-Mediated Cardioprotection

The A_2ARs are classified as sub-types A and B (A_{2A}ARs and A_{2B}ARs, respectively). Key reports indicate while A_1AR mediated cardioprotection is principally exerted during ischaemia [18], A_{2A}AR mediated effects predominate during reperfusion [51,53,565-567]. The A_2ARs are thought to primarily exist in vascular (endothelial and smooth muscle) and blood cells (neutrophils and platelets), where they mediate vasodilatory and anti-inflammatory actions, respectively. Extensive research by Vinten-Johansen and colleagues determined that selective A_2 receptor activation reduces infarction by inhibiting neutrophil adherence to endothelium and decreasing the production of O_2^{-*} independent of K_{ATP} channels [9,52,53,57,393,541]. More recently they also revealed that A_{2A}AR mediated protection results in alterations in anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins [58], which were ultimately associated with inhibition of apoptotic death on reperfusion.

While there is strong support for beneficial effects of A_{2A} agonism in irreversibly infarcted tissue, there is only one study showing A_{2A} mediated protection in stunned myocardium [567]. Lasley *et al.* [567] recently demonstrated that intracoronary infusion of A_{2A}AR agonist CGS 21680 during reperfusion reduces infarct size and increases contractility. The latter effect was achieved using a low concentration with miminal direct hemodynamic effects, and was suggested to reflect positive inotropic effects of A_{2A}AR-mediated elevations in [cAMP] (since PDE inhibition and elevations in cAMP exert positive inotropic actions). This hints at the posssibility of A_{2A}AR

effects directly in ventricular myocytes. Indeed, expression of mRNA encoding the A_{2A}AR in rat ventricular myocytes [568], together with A_{2A}AR agonist mediated changes in cAMP and contraction of chick [569] and rat myocytes [92,568,570], support expression of A_{2A}ARs on myocytes. Despite preliminary evidence that porcine myocardial homogenate is devoid of A_{2A}AR transcript [121], immunological evidence indicates a canine A_{2A}AR-like protein exists in human and porcine ventricular tissue [571]. Moreover, A_{2A}AR protein expression in rat ventricular myocytes has been demonstrated using superior Western blot technology [572]. Interestingly, activation of putative myocardial A_{2A}ARs does not result in characteristic inotropic effects (increases in cAMP levels or altered twitch amplitude) [572]. However, these authors argue that the A_{2A}AR is localised in the soluble fraction of the myocyte, precluding receptor interaction with membrane-bound G_s proteins and AC. This challenges a direct role for myocyte A_{2A}ARs in cardioprotection.

Consistent with the idea that A_{2A} mediated effects are restricted to the vasculature, accumulating evidence indicates that little, if any, protection is achieved via A_{2A}AR activation in a blood free model [542,573,574]. Furthermore, A_{2A}AR activation fails to protect isolated cardiomyocytes [575]. Since the primary targets of A_2AR agonists appear to be neutrophils and vascular cells, these findings are not surprising. Nevertheless, two investigations in buffer perfused hearts support direct A_{2A}AR-mediated protection in rat [576] and rabbit [577] myocardium. One explanation for the disparatity amongst these findings is that the latter studies were performed in hearts perfused in constant-flow mode [576,577]. Recently our group determined that this results in reduced coronary perfusion pressure during A_{2A}AR agonism with subsequent modification of myocardial contractility [574]. This renders interpretation of functional changes in the latter studies problematic and questionable.

While a number of studies support A_{2A}AR mediated protection *in vivo*, there is little evidence for acute A_{2B}AR mediated cardioprotection, partially due to the lack of selective and potent A_{2B}AR agonists/antagonists. Given the low affinity of the A_{2B}AR, it is probable the protein is only activated by pathophysiological elevations in adenosine. However, it is interesting to note A_{2B}ARs expressed in CHO cells have an EC_{50} for MAPK activation of only 25 nM, in contrast to low potency for endogenous receptors in HEK293 cells [578]. This suggests potency may be substantially modified by relative levels of receptor and G-protein expression. In any case, evidence the A_{2B}AR is expressed in cardiovascular tissue remains indirect, and signalling involved in A_{2B}AR responses in cardiac tissue remains to be delineated. There is evidence, however, that A_{2B}ARs may regulate post-ischaemic remodeling via inhibition of cardiac fibroblast growth [579], providing a level of "chronic" protection.

A_3AR-Mediated Cardioprotection

In the early 1990s, a new adenosine receptor, the A_3 receptor, was cloned in rats by Meyerhof *et al.* [580] and Zhou *et al.* [80], and subsequently in sheep [581], humans [582,583], rabbits [584], and more recently dogs [585]. Early studies by Armstrong and Ganote [586], and Liu *et al.,* [587] indicated that A_3ARs may play a protective role in the heart during ischaemia/reperfusion. However, key issues relate to cellular location of the receptor, and specificity of pharmacological agents employed. While there are several studies demonstrating A_3AR mediated protection in isolated myocytes [556,560,586,588-590], a cardiac A_3 receptor has yet to be identified. Nevertheless, accumulating pharmacological evidence indicates A_3AR agonism provides cardioprotection in a variety of models and species [82,83,574,590-595]. Furthermore, the A_3AR has been implicated in preconditioning of chick [588,591,596], rabbit [83,589,597,598], and human [560] hearts.

One major challenge encountered is the lack of potent, selective, and species-independent A_3 receptor agonist and antagonists. Questions have arisen regarding the validity of prior findings, and thus the role (if any) of A_3ARs in cardioprotection. In particular the A_3 agonist IB-MECA, which has been employed in many studies, was recently found to be only slightly more potent at binding to rabbit A_3ARs than A_1ARs [599]. Therefore, agents thought to possess high specificity may interact with other sub-types. As discussed by Hill and colleagues, this may stem in part from the greater species diversity of A_3 receptors compared with greater homology for A_1 receptors [584]. Highlighting the issue, Kilpatrick *et al.* recently acquired evidence that cardioprotection via A_3AR agonism was abrogated by selective A_1AR antagonism [600]. These findings, however, are equivocal since effects of A_1AR antagonism alone were not assessed, and this has been shown to impair ischaemic tolerance in some models [18,21]. More recent work verified A_3AR agonist mediated protection is unrelated to A_1AR activation in rabbit [601] and rat [82]. The latter study utilised the chlorinated derivative, Cl-IB-MECA, which is 2,500-fold more selective for the A_3 *vs* A_1 [602], in comparison to IB-MECA (which is only 50-fold selective) [603]. Co-administration of a selective A_1AR antagonist confirmed the cardioprotective effects of Cl-IB-MECA were not the result of a spillover effect mediated via A_1ARs.

Given issues regarding selectivity at the A_3AR, transgenic or knockout approaches might appear promising in identifying roles of this receptor. It has been determined that the level of A_3ARs is low in wild-type hearts from most species (in comparison to the A_1AR) [80,599]. This may present a unique opportunity to increase endogenous protection genetically. Current studies show that transgenic animals overexpressing the A_3AR in myocytes and intact hearts do possess enhanced tolerance to ischaemic/hypoxic stress [544,556,604]. It remains to be determined if adminstration

of a selective A_3AR agonist in this transgenic model will induce further protection. Interestingly there does appear to be a threshold effect regarding A_3 overexpression. While low levels of A_3 overexpression are cardioprotective, high levels (mice carrying 6 vs 1 copies of the transgene) of overexpression may lead to development of dilated cardiomyopathy [604]. Furthermore, low levels of A_3AR activation may reduce injury whereas high levels enhance apoptosis and mast cell degranulation [605,606]. Recent reports from transgenic animals with low level A_3 overexpression indicate that A_3ARs exert significant cardioprotective effects against both reversible (stunning) [544] and irreversible injury (infarction) [604]. In contrast, a paradoxical ischaemia-tolerant phenotype has been observed in A_3AR knockout mice, supporting detrimental rather than protective functions of A_3ARs [607,608]. Unfortunately, knockout models should be considered with even more caution than transgenic models since phenotypic results of life-long absence of a protein do not simply reflect the effect of acute deletion or inhibition of the protein/response. As discussed recently [593], this phenotype may reflect the impact of "compensatory" mechanisms, and/or result from expression of associated background or "hitch-hiker" genes. Indeed, studies of compensatory processes in knockout animals may prove to be a more fruitful avenue of research in knockout models.

The mechanisms by which A_3ARs protect ischaemic myocardium have yet to be determined. On the basis of pharmacological studies, either PKC [597] or K_{ATP}^+ channels [82,83,609] have been implicated in the response to A_3 stimulation (analogous to A_1AR signalling). Liang's group show coupling to PLD and RhoA with more prolonged PKC activation than with A_1AR activation (coupling via PLC) [563,610]. There is also evidence of MAPK signalling via A_3ARs [611].

Earlier work focused on the ability of A_3 receptors to cause mast cell degranulation (which is either ineffective [612] or detrimental [613] in rat heart models of ischaemia-reperfusion). A_3AR activation has been shown to induce hypotension by mast cell degranulation in rats *in vivo* [597,614]. However, in humans and dogs, mast cell activation is probably mediated by the adenosine A_{2B}, not the adenosine A_3 receptor [585,615]. In contrast, the A_3 receptor in these species exerts anti-inflammatory effects via inhibition of eosinophil migration [615,616], neutrophil degranulation [617], and TNF-α release from macrophages [618]. Furthermore, A_3 activation decreases adherence of PMNs to endothelium, attenuating adherence-dependent vascular and contractile injury [619]. Thus, although both adenosine A_1 and A_3 receptor activation protect against ischaemic injury, agonists of the latter may be more promising as cardioprotective agents in the clinical setting due to a lack of hemodynamic side effects, anti-inflammatory effects, and a more sustained duration of protection than adenosine A_1 receptor agonists [591].

Another potential A_3 receptor-mediated effect includes modulation of apoptosis [590,620-623]. Again highlighting the A_3 threshold effect, A_3ARs can either mediate cell protection and cell death, simply depending on the degree of receptor activation. Interestingly, sub-maximal activation of the A_3AR has been shown to decrease apoptotic injury induced by ischaemia [590,620,623], while high concentrations of agonists can trigger cell death by either necrosis or apoptosis [621,622]. Evidence in support of a role for A_3ARs in attenuating apoptosis reveals that A_3AR are capable of coupling to two well-known cell survival-signalling pathways: the phosphatidylinositol 3'-kinase/Akt kinase pathway [624-626]; and the Ras/Raf-1/MEK/ERK 1/2 pathway [627]. Anti-apoptotic effects of A_3ARs are supported by results of Maddock and

colleagues [590], who reported that post-ischaemic Cl-IB-MECA reduced apoptosis is rat cardiac myocytes.

In addition to the above mentioned effect of A_3AR, A_3 signalling preserves ischaemic ATP levels and recovery of cardiac energetics [628], as well as modulating SR Ca^{2+} handling [542]. This results in protection against energetic dysfunction as well as ionic imbalance. Lastly, a recent investigation found preconditioning via transient A_3AR activation preserved hypoxic coronary dilation in post-ischaemic hearts [554]. Since the model used was devoid of blood elements, A_3 anti-inflammatory effects are unlikely, and these data hint that A_3ARs may exist on endothelium where they mediate direct vasoprotection.

The above review highlights several controversies regarding adenosine protection. In particular, the receptor subtypes and mechanisms involved in adenosine-mediated coronary dilation remain questionable. Moreover, no study has assessed the mechanisms involved in adenosine-mediated dilation or reactive hyperaemia in the increasingly studied mouse heart. Since species differences have been acknowledged in the literature, and transgenic/knockout mouse models are being increasily utilised, it is important to elucidate the receptor subtype and mechanisms involved in adenosine mediated dilation in mouse heart.

Of greater interest, there is also limited information on the impact of ischaemia-reperfusion on coronary vascular function in asanguinous models. Moreover, whether post-ischaemic vascular injury modulates adenosine-responses and normal flow-function matching in the heart remains unclear, and whether adenosine receptors modulate vascular injury is not known. Thus, the following aims are addressed in this thesis:

i. To functionally characterise the adenosine receptor sub-type mediating coronary dilation in mouse heart (chapter 3)
ii. To elucidate the mechanisms by which adenosine mediates dilation in mouse heart (chapter 3)
iii. Reveal the molecular mechanisms involved in mediating reactive hyperaemia responses in mouse heart (chapter 4)
iv. To assess the impact of ischaemia/reperfusion on coronary function in the asanguinous mouse heart (chapter 5)
v. To assess the mechanisms involved in endothelial dysfunction in the asanguinous mouse heart and to identify the role of adenosine receptors in ameliorating coronary injury (chaper 6)

CHAPTER 2

METHODS

I. LANGENDORFF PERFUSED MURINE HEART MODEL

Investigations conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publications No. 85-23, revised 1996). Hearts were isolated and prepared as described previously [629]. Specifically, animals (see Table 2.1) were anaesthetised with 50 $mg.kg^{-1}$ sodium pentobarbitone administered intraperitoneally, a thoracotomy was performed and hearts excised into ice-cold perfusion fluid. The aorta was cannulated with a 21g stainless steel cannula constructed from a syringe needle and perfused at 80 mmHg with Krebs bicarbonate buffer containing (in mM): NaCl, 120; $NaHCO_3$, 22; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; MgCl, 1.2; glucose, 11; and EDTA, 0.5. Perfusate was equilibrated with 95% O_2, 5% CO_2 at 37°C, giving a pH of 7.4 and PO_2 ≥600 mmHg at the aortic cannula over a 1-5 $ml.min^{-1}$ flow range. Buffer was initially passed through a 5.0 μ filter on preparation, and all perfusate delivered to hearts was filtered via an in-line 0.45 μ Sterivex-HV filter (Millipore, Bedford, MA, USA) to continuously remove micro-particulates (Fig. 2.1). The left ventricle was vented with a polyethylene drain to prevent Thebesian accumulation and after completion of surgical manipulations (ie. instrumentation with a balloon for functional measurements), hearts were immersed in a 37°C organ bath. Perfusate temperature was continuously assessed by needle thermistors at the entry into the aortic cannula and at close proximity to the heart in the organ bath (Fig. 2.1). Temperatures were monitored using a 3 channel Physitemp TH-8 digital thermometer (Physitemp Instruments Inc, Clifton, NJ, USA). Coronary flow was monitored via ultrasonic flow-probe (1N probe, Transonic Systems, Ithaca, NY, USA). Perfusion pressure was monitored using a P23XL pressure transducer (Viggo-Spectramed, Oxnard, CA, USA) connected to a MacLab (ADInstruments, Castle Hill,

Australia). After 20-min stabilisation hearts were switched to pacing via silver electrodes, using a Grass S9 stimulator (Grass, Quincy, MA, USA). Hearts were paced at 400 beats.min^{-1} via silver left ventricular electrodes (0.5 ms square pulses, 20% above threshold, typically 2-5 V) and stabilised for a further 10 min. Importantly, the heart rate of the mouse *in situ* is 550-600 beats.min^{-1}, however, based on characterisation studies the optimal heart rate for isolated perfused mouse hearts is 400-420 beats.min^{-1} [629].

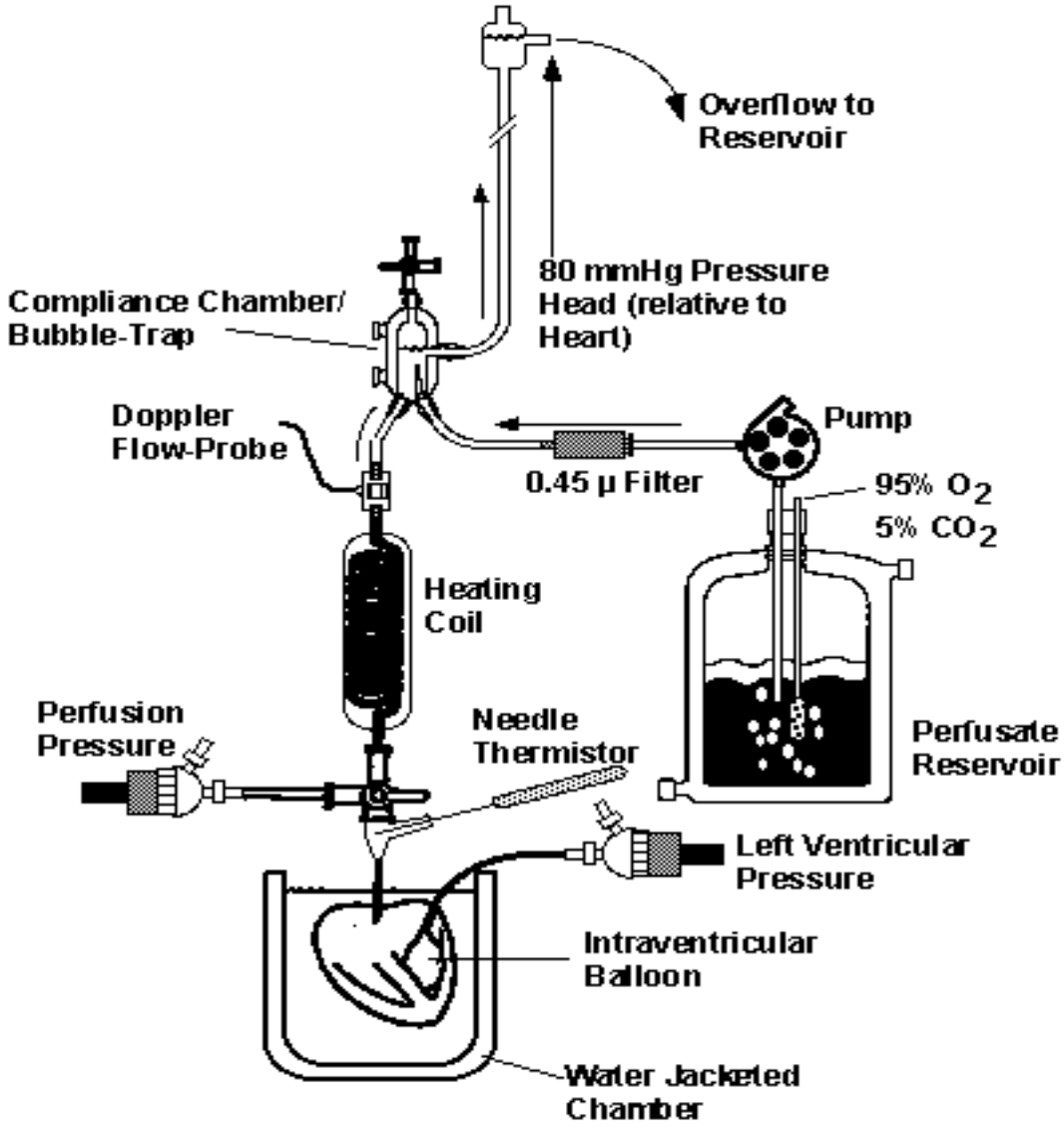

Figure 2.1 Schematic diagram of apparatus utilised to acquire data.

Table 2.1 Animal usage, age, species, average body and blotted heart weights for inidividual studies

Study	Species	Useage	Age (weeks)	Average body weight (g)	Average blotted heart weight (mg)
Chapter 3	Male C57/B16 mice	159	7-12	26.3±0.3	118±8
Chapter 3	Male Wistar rats	44	12-16	337±15	1220±190
Chapter 4	Male C57/B16 mice	56	8-12	20-25	112±2
Chapter 5	Male C57/B16 mice	196	7-12	26.0±0.3	105±2
Chapter 6	Male C57/B16 mice	234	7-12	27.0±0.2	113±1
Chapter 6	A_1AR overexpressed transgenic mice	14	7-12	25.0±0.8	116±1

Measurement of Isovolumic Contraction via Intraventricular Balloon

In hearts in which ventricular contractile function was assessed, fluid-filled balloons were inserted into the left ventricle via the mitral valve [21,629]. Fluid-filled balloons were constructed of pliable polyvinyl chloride plastic film (eg. cling or saran wrap) secured with a 4-0 suture onto a short length of polyethylene tubing, which was initially exposed to a flame to create a small flange. The plastic film used in construction of each balloon was initially stretched over a syringe tip to give a more spherical form to the balloon. Balloons were 6-8 mm in length when deflated. Balloons were connected to a P23XL pressure transducer by fluid-filled polyethylene tube. The balloons have an unstressed volume of ~60 µl as described previously [629]. Balloon volume was controlled by a calibrated Hamilton 500 µl threaded plunger syringe (Hamilton Co., Reno, NV, USA). End-diastolic pressure was initially set at 5 mmHg during the equilibration phase or stabilisation. Functional data were recorded at 1 KHz

on a 4/s MacLab unit connected to an Apple computer. The ventricular pressure signal was digitally processed using Chart software (v.3.6.3; ADInstruments, Castle Hill, Australia) to yield peak systolic and diastolic pressures, ±dP/dt (rate of rise and fall in ventricular pressure), and heart rate. Other parameters were calculated from these data using Excel (v.98, Microsoft) and Kaleidagraph (v.3.0; Abelbeck Software) software and will be described in relevant sections.

Assessment of Coronary Vascular Function

For analysis of coronary function, hearts were switched to constant flow perfusion 10-min prior to acquisition of concentration-response curves. Flow was controlled by a Gilson MiniPuls 2 peristaltic pump (Gilson, Middleton, WI, USA) set to a flow rate yielding 100-mmHg aortic pressure (13.2±0.7 $ml.min^{-1}.g^{-1}$ in mice). This permitted analysis of coronary dilation without the complication of changes in coronary flow rate and drug delivery rate. The dilatory agents were then infused in incremental concentrations for 1-3 min at each concentration (during which vascular responses stabilised). Changes in aortic pressure were measured. Only one concentration-response curve was acquired per heart.

Measurement of Reactive Hyperaemic Responses

To assess reactive hyperaemic vasodilation, hearts were randomly subjected to 5, 10, 20, 40 or 60 sec periods of total vascular occlusion separated by 5-15 min periods of reperfusion (during which flow recovered to pre-occlusion levels). Flows were determined from continuous flow-meter recordings made in the Chart data acquisition program (ADInstruments, Castle Hill, Australia). Peak hyperaemic responses were determined (in absolute units and as % of baseline resistance), together with % flow-debt repayment.

Flow-debt repayment was calculated as:

100% x excess flow during hyperaemia ($ml.g^{-1}$) / flow-debt ($ml.g^{-1}$),

where flow-debt is the product of baseline flow ($ml.min^{-1}.g^{-1}$) x period of occlusion (min), and excess flow during hyperaemia was calculated as the volume of flow during the period of the hyperaemic response minus the baseline (pre-occlusion) flow. The period of the hyperaemic response was defined as the time from onset of reperfusion to the point where flow fell to the pre-occlusion level.

In chapter 4 resting coronary flow was reduced by different inhibitory agents studied, therefore calculation of % flow debt repayment (commonly assessed in studies of reactive hyperaemia) was not meaningful. Rather, we examined the overall hyperaemic response (Figs 4.1, 4.3-4.9), together with changes in peak hyperaemic flow and total flow repayment during reperfusion (Fig. 4.2).

II. EXPERIMENTAL PROTOCOLS

Refer to individual study Chapters for details.

III. DATA ANALYSIS

Responses to agonists were compared between treatment groups using a multi-way analysis of variance for repeated measures, followed by Tukeys HSD post-hoc test for multiple comparisons when differences were detected. pEC_{50} values were obtained from concentration-response data (expressed as absolute units) by fitting the following four-parameter logistic equation to data for individual experiments:

$$\text{Response} = A \pm B - \frac{B}{1 + ([\text{agonist}]/EC_{50})^{\text{slope factor}}}$$

where *A* is the response at zero dose (ie. the pre-infusion value), and *B* is the response at infinite dose. The equation was fit to data using the non-linear regression module of the Statistica program (Statsoft, Tulsa, OK, U.S.A.), and individual pEC_{50} values derived from each fit. pEC_{50}s were compared between treatment groups by one-way analysis of variance with Tukeys HSD post-hoc test. In all statistical tests $P<0.05$ was considered indicative of statistical significance. Dilatory responses to agonists were calculated as a % of resting resistance, or were scaled (as a %) to the maximal dilatory response observed. All values are reported as mean ± s.e.mean.

Peak hyperaemic flows and repayment flows over 1 min reperfusion in the different treatment groups were compared by analysis of variance with Tukeys H.S.D. post-hoc test when significant effects were detected.

Functional responses to ischaemia-reperfusion were compared via multi-way analysis of variance with repeated measures, followed by Tukeys post-hoc test. In all tests $P<0.05$ was accepted as indicative of statistical significance. All values are reported as mean±S.E.M.

IV. CHEMICALS

Table 2.2 List of compounds utilised

Compound Abrev.	Function	EC_{50}	[Conc] utilised	Initial Solvent
2-CAD	Non-selective AR agonist	8.4		Buffer
8-CSC	A_{2A}AR antagonist		1 μM	DMSO
Adenosine	All AR agonist	6.8	50 μM	Buffer
ADP	Endothelial dependent dilator	7.4		Buffer
Alloxazine	A_{2B}AR antagonist		1 μM	0.1 M NaOH
BDM	Activate protein phosphatase		5 mM	Milli Q water
BIIB-513 MS	NHE-1 inhibitor		1 μM or 10 μM	Milli Q water
Catalase	Converts H_2O_2 to H_20		600 U/ml	Milli Q water
CGS-21680	A_{2A}AR agonist		1 nM	Buffer
CHA	A_1AR agonist		50 nM	Buffer
Chloro-IB-MECA	A_3AR agonist		100 nM	Buffer
CPA	A_1AR agonist		100 nM	Buffer
DPCPX	A_1AR antagonist		200 nM	0.1 M NaOH
Glibenclamide	K_{ATP} channel inhibitor		5 μM	100 % DMSO
Indomethacin	Cyclooxygenase inhibitor		100 μM	0.1 M NaOH
L-NAME	Irreversible inhibitor of NO synthetase		50 μM	Buffer
Minoxidil	K^+ channel activator		0.1 – 3.0 μM	Milli Q water
MPG	Antioxidant		300 μM	Buffer
MRS 1220	A_3AR antagonist		100 nM	70% DMSO
NECA	A_2AR agonist	9.3		Buffer
PD 142893	ET_A & ET_B antagonist		200 nM	Milli Q water
R-PIA	A_{2B}AR agonist	7.7		Buffer
SCH 58261	A_{2A}AR antagonist		100 nM	60% DMSO
SNP	Releases NO	7.1	3 μM	Buffer
SOD	Converts O_2^{-*} to H_2O_2 and O_2		150 U/ml	Milli Q water

All compounds were infused into the coronary circulation through a 0.22 μM filter at no more than 1 % of coronary flow to achieve final concentrations indicated. NaOH or DMSO concentrations did not exceed 0.4 mM or 0.01% respectively in any group. Preliminary studies (data not shown) verified these low solvent levels fail to modify ventricular contractile function, coronary tone, and responses to vasodilators. BIIB-513 MS and SCH 58261 were kindly donated by Boehringer Ingleheim and Schering-Plough Research Institute, respectively. All other listed compounds were purchased from Sigma. All chemicals used were of analytical grade or better.

CHAPTER 3

FUNCTIONAL CHARACTERISATION OF CORONARY VASCULAR ADENOSINE RECEPTORS IN THE MOUSE

I. ABSTRACT

Coronary responses to adenosine agonists were assessed in perfused mouse and rat hearts. The roles of nitric oxide (NO) and ATP-dependent K^+ channels (K_{ATP}) were studied in the mouse. Resting coronary resistance was lower in mouse *vs.* rat, as was minimal resistance (2.2±0.1 *vs* 3.8±0.2 $mmHg.ml^{-1}.min^{-1}.g^{-1}$). Peak hyperaemic flow after 20-60 s occlusion was greater in mouse. Adenosine agonists induced coronary dilation in mouse, with pEC_{50}s of 9.4±0.1 for 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethyl carboxamidoadenosine (CGS21680, A_{2A}-selective agonist), 9.3±0.1 for 5'-N-ethylcarboxamidoadenosine (NECA, A_1/A_2 agonist), 8.4±0.1 for 2-chloroadenosine (A_1/A_2 agonist), 7.7±0.1 for N^6-(R)-(phenylisopropyl)adenosine (R-PIA, A_1/A_{2B} selective), and 6.8±0.2 for adenosine. The potency order (CGS21680=NECA>2-chloroadenosine> R-PIA>adenosine) supports A_{2A}AR-mediated dilation in mouse coronary vessels. 0.2-2 μM of the A_{2B}-selective antagonist alloxazine failed to alter CGS21680 or 2-chloroadenosine responses. pEC_{50}s in rat were 6.7±0.2 for CGS21680, 7.3±0.1 for NECA, 7.6±0.1 for 2-chloroadenosine, 7.2±0.1 for R-PIA, and 6.2±0.1 for adenosine (2-chloroadenosine>NECA=R-PIA>CGS21680> adenosine), supporting an A_{2B}AR response. NO-synthase antagonism with 50 μM N^G-nitro *L*-arginine (*L*-NOARG) increased resistance by ~25%, and inhibited responses to CGS21680 (pEC_{50}=9.0±0.1), 2-chloroadenosine (pEC_{50}=7.3± 0.2) and endothelial-dependent ADP, but not sodium nitroprusside (SNP). K_{ATP} channel blockade with 5 μM glibenclamide increased resistance by ~80% and inhibited responses to CGS21680 in control (pEC_{50}=8.3±0.1) and *L*-NOARG-treated hearts (pEC_{50}=7.3±0.1), and to 2-chloroadenosine in control (pEC_{50}=6.7±0.1) and *L*-

NOARG-treated hearts (pEC_{50}=5.9±0.2). In summary, mouse coronary vessels are more sensitive to adenosine than rat vessels. A_{2A}ARs mediate dilation in mouse coronary vessels versus A_{2B} receptors in rat. Responses in the mouse involve a sensitive NO-dependent response and K_{ATP}-dependent dilation.

II. INTRODUCTION

Adenosine receptor sub-types mediating coronary vasodilation appear to differ between species, and controversy exists regarding mechanisms contributing to adenosine-mediated dilation in different (and also within) vascular beds. For example, while there is evidence of NO-dependent components to adenosine responses in coronary vessels [120,121,139-141,143,144,146], this is not universally observed [122,124,125,148,630]. There are only preliminary data available regarding the receptor sub-type mediating coronary dilation in the mouse [127], and there are no data regarding mechanisms involved. Therefore, the primary goal of the present study was to functionally characterise the adenosine receptor sub-type mediating coronary dilation in mouse heart. To identify the receptor, coronary responses to adenosine (the endogenous and non-selective agonist), CGS 21680 (A_{2A} selective agonist), NECA (A_1/A_2 agonist), 2-chloroadenosine (A_1/A_2 agonist), and R-PIA (A_1/A_{2B} agonist) were examined, together with the effects of A_{2B}-selective antagonism with alloxazine. The potential contributions of NO and K_{ATP} channel activation to adenosine receptor responses were assessed via competitive inhibition of NO-synthase with *L*-NOARG and inhibition of K_{ATP} channels with glibenclamide. Coronary vascular function in mouse was also compared to that in the more thoroughly characterised rat heart.

III. MATERIALS AND METHODS

Langendorff perfused murine heart model

Hearts were perfused as described in Chapter 2.

Experimental protocol

To identify the adenosine receptor sub-type mediating coronary dilation in mouse, stabilised hearts were treated with a dilatory agonist (2-chloroadenosine, R-PIA, CGS21680, NECA, adenosine, SNP, or ADP) infused in incremental concentrations. As mentioned previously, agonists were infused at each concentration for 1-3 min period during which vascular responses stabilised. Only one concentration-response curve was acquired per heart. Changes in aortic pressure were measured, and coronary flow was constantly monitored to verify a constant flow rate.

To examine the effects of A_{2B} antagonism in mouse hearts, responses to CGS21680 and 2-chloroadenosine were acquired in the presence of 200 nM alloxazine (n=7 for both groups). Responses to 2-chloroadenosine were also acquired in a sub-set of hearts treated with a higher 2 μM concentration of alloxazine (n=4). Alloxazine infusion was initiated 10 min prior to acquisition of agonist concentration-response curves.

For NO-synthase inhibition in mouse hearts, infusion of 50 μM *L*-NAME (final concentration) was initiated after stabilisation. After a further 10 min period concentration-response curves for CGS21680 (n=10), 2-chloroadenosine (n=8), ADP (n=6), or SNP (n=7) were acquired, as described above. To further examine mechanisms of adenosine-mediated dilation, K_{ATP} channel inhibition was studied in mouse hearts. Infusion of 5 μM glibenclamide was initiated alone or in conjunction with 50 μM *L*-

NAME. After a further 10 min period concentration-response curves for CGS21680 and 2-chloroadenosine were acquired (n=6 in all groups). In preliminary experiments 5 μM glibenclamide was shown to fully block dilatory responses to 0.1-3.0 μM of the K_{ATP} opener minoxidil (data not shown).

Reactive hyperaemic responses were assessed in a separate group of mouse (n=7) and rat hearts (n=8) which were perfused at a constant aortic pressure of 100 mmHg after pacing was initiated. After a further 10 min stabilisation hearts were subjected to 20 s of total coronary occlusion followed by reperfusion for 15 min, before studying the response to 60 s of coronary occlusion and reperfusion.

IV. RESULTS

Coronary vascular function in mouse versus rat heart

Baseline coronary tone, peak reactive hyperaemic responses, and minimal vascular tone observed during infusion of the relatively non-selective A_2AR agonist 2-chloroadenosine are shown in Table 3.1 Resting coronary resistance was lower in mouse versus rat hearts (~7.5 *vs.* 9.5 $mmHg.ml^{-1}.min^{-1}.g^{-1}$). The minimal resistance achieved during dilation with 2-chloroadenosine was also lower in mouse (2.2 $mmHg.ml^{-1}.min^{-1}.g^{-1}$) versus rat heart (3.8 $ml^{-1}.min^{-1}.g^{-1}$) (Table 3.1). The peak reactive hyperaemic response and extent of flow repayment following 60 s occlusion was significantly greater in mouse versus rat (Table 3.1). Similarly, the peak response following a shorter 20 s occlusion was greater in mouse (35.8±2.3 $ml^{-1}.min^{-1}.g^{-1}$ at ~5 s of reperfusion) versus rat (24.3±1.2 $ml^{-1}.min^{-1}.g^{-1}$ at ~15 s), as was repayment (136±9% in mouse versus 98±6% in rat). Collectively, data show that resting and

minimum coronary resistances are lower in mouse. However, the dynamic range over which coronary vessels dilate is similar in both species (~5.5 mmHg.ml^{-1}.min^{-1}.g^{-1}).

Table 3.1 Coronary vascular functional parameters in mouse and rat hearts

	Resting Resistance (mmHg.ml ^{1}.min ^{1}.g ^{1})	**Min. Resistance** (mmHg.ml ^{1}.min^{-1}.g^{-1})	**Peak Hyperaemia** (ml.min^{-1}.g^{-1})	**% Repayment**
Mouse	7.6±0.4	2.2±0.1	38.6±1.8	137±9
Rat	9.4±0.7*	3.8±0.2	25.4±1.1	105±7*

Resting resistance was determined at the end of stabilization immediately prior to adenosine agonist infusions. The minimum resistance was the resistance achieved during maximal stimulation with 2-chloroadenosine. Peak hyperaemia was the peak reactive hyperaemic conductance achieved following 60 s of coronary occlusion, and the % repayment is the flow-debt repayment during this hyperaemic response. Values are means ± s.e.mean of individual experiments (n≥6). * P<0.05 *vs.* values in mouse hearts.

Effects of adenosine receptor agonists in mouse versus rat heart

All adenosine receptor agonists concentration-dependently dilated the coronary circulation in mouse and rat hearts (Fig. 3.1). Response magnitude, expressed as % change in resistance, was similar in both species. CGS21680 was the most potent agonist in mouse, whereas it was one of the less potent in rat. The rank order of potencies was CGS21680= NECA>2-chloroadenosine> R-PIA>adenosine for mouse. Statistical analysis of pEC_{50}s yielded *P* values of 0.879 for NECA *vs.* CGS21680, and less than 0.001 for 2-chloroadenosine *vs.* NECA and CGS21680, R-PIA *vs.* 2-chloroadenosine, and adenosine *vs.* R-PIA. This rank order of potencies supports A_{2A}-mediated dilation, and contrasted with that in the rat: 2-chloroadenosine>NECA=R-

PIA>CGS21680>adenosine (Table 3.2). Statistical analysis of pEC_{50}s yielded *P* values of 0.001 for NECA *vs.* 2-chloroadenosine, 0.835 for R-PIA *vs.* NECA, and less than 0.005 for CGS21680 *vs.* R-PIA and adenosine *vs.* CGS21680. This rank order of potencies supports an A_{2B}-mediated response in rat. In addition, sensitivity to all adenosine agonists was higher in mouse versus rat. Furthermore, as shown in Table 3.2 (and apparent in curves depicted in Fig. 3.1), the steepness or slope of concentration-response curves is greater in mouse *vs.* rat. Slope factors for all adenosine agonists were significantly higher in mouse (Table. 3.2).

A_{2B}-selective antagonist alloxazine, when infused at 200 nM concentration, failed to alter responses to either A_{2A}-selective CGS21680 (pEC_{50}=9.2±0.1) or non-selective 2-chloroadenosine (pEC_{50}=8.2±0.1) (Fig. 3.2). A 10-fold higher concentration of alloxazine also failed to alter responses to 2-chloroadenosine in mouse heart (Fig. 3.2B).

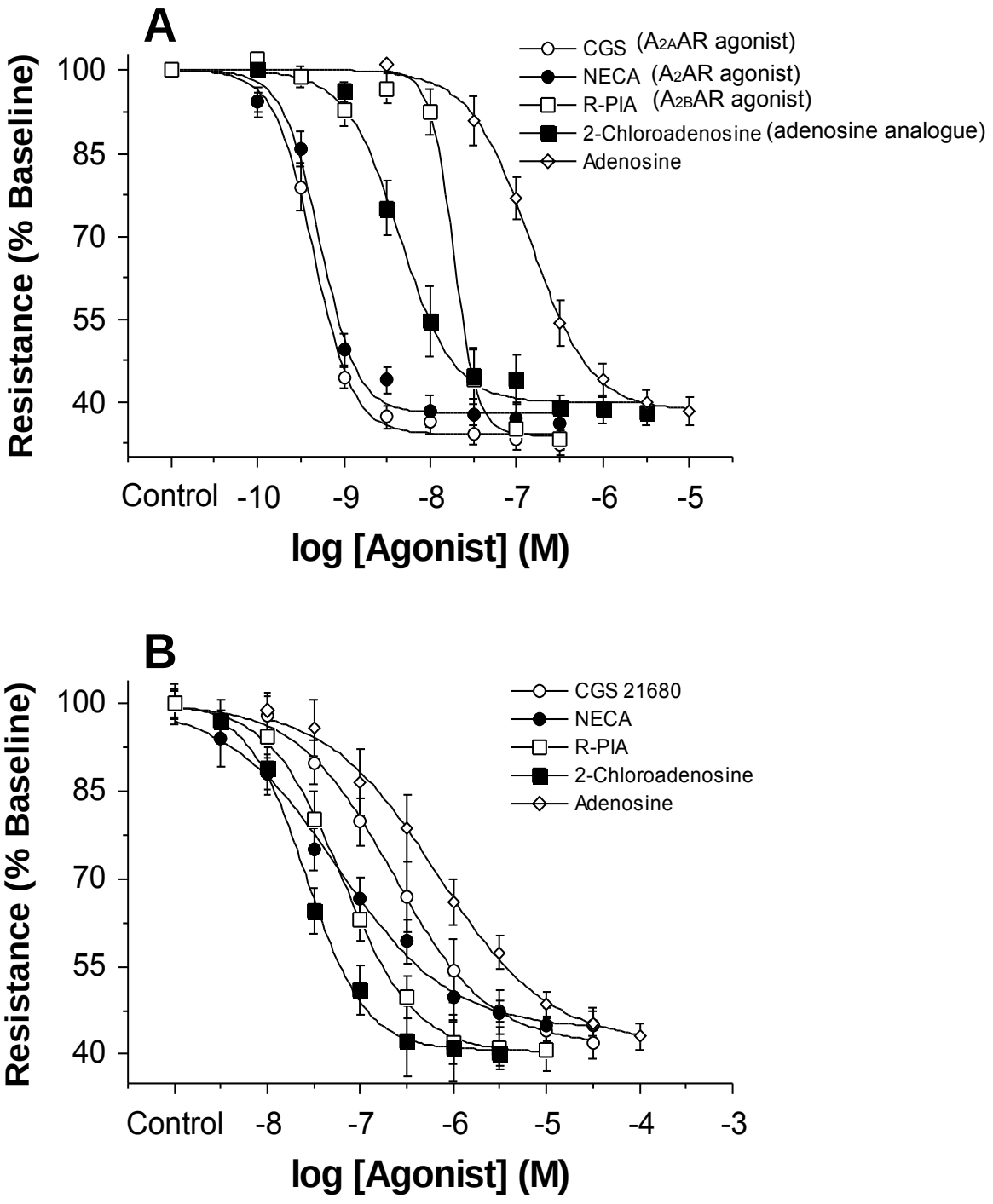

Figure 3.1 Concentration-response curves for adenosine agonist mediated vasodilatation in A) mouse, and B) rat hearts. Responses to adenosine (n=8 for mouse, n=7 for rat), 2-chloroadenosine (n=7 for mouse, n=7 for rat), R-PIA (n=6 for mouse, n=8 for rat), NECA (n=7 for mouse, n=7 for rat), and CGS 21680 (n=8 for mouse, n=7 for rat) were studied. Responses are shown as % of baseline coronary resistance. All values are means ± s.e.mean.

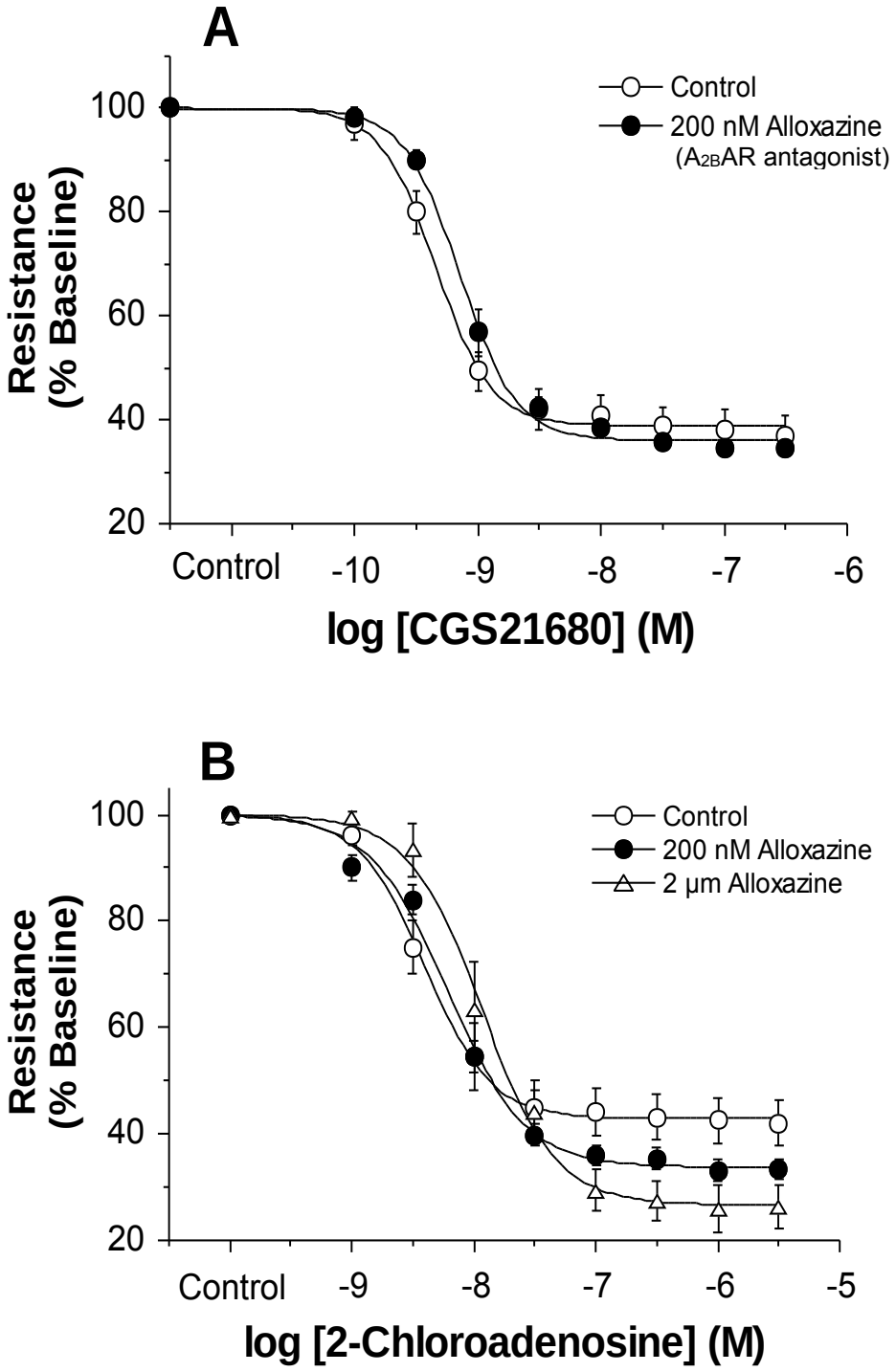

Figure 3.2 Concentration-response curves for A) CGS21680 and B) 2-chloroadenosine in mouse hearts in the presence and absence of alloxazine. Responses were obtained for CGS21680 alone (n=8) or in the presence of 200 nM alloxazine (n=7), and for 2-chloroadenosine alone (n=7) or in the presence of 200 nM (n=7) or 2 µm (n=4) alloxazine. Control data for CGS21680 and 2-chloroadenosine are taken from Fig. 1. Responses are shown as % coronary resistance. All values are means ± s.e.mean. * $P<0.05$ *vs.* values in untreated hearts ($P<0.05$).

Table 3.2 Concentration-response data for adenosine agonists in mouse and rat hearts

	pEC_{50}	Response ($mmHg.ml^{-1}.min^{-1}.g^{-1}$)	Response (% Resistance)	Slope
MOUSE				
CGS21680	9.4±0.1	5.2±0.4	68±2	2.4±0.4
CGS21680+L-NOARG	9.0±0.1†	6.1±0.8	64±2	2.2±0.5
CGS21680+Glibenclamide	8.3±0.1†‡	5.9±0.3	64±6	1.5±0.3
CGS21680+L-NOARG+Glib	7.3±0.1†‡	7.2±0.7	56±3	3.2±0.5
2-Chloroadenosine	8.4±0.1	4.0±0.4	62±2	2.1±0.4
2-Chloro+L-NOARG	7.3±0.2†	5.4±0.8	68±2	2.6±0.5
2-Chloro+Glibenclamide	6.7±0.1†‡	6.1±0.7	63±3	1.2±0.3
2-Chloro+L-NOARG+Glib	5.9±0.2†‡	8.2±0.7†	68±2	3.3±1.1
NECA	9.3±0.1	4.5±0.6	63±3	3.4±1.6
R-PIA	7.7±0.1	6.1±0.7	67±2	3.9±0.7
Adenosine	6.8±0.1	5.1±0.4	63±2	2.2±0.4
SNP	7.1±0.3	5.7±0.5	64±1	1.2±0.2
SNP+L-NOARG	7.2±0.2	7.4±0.4†	72±1†	1.1±0.2
ADP	7.4±0.1	5.0±0.8	64±3	4.8±1.1
ADP+L-NOARG	6.7±0.1†	7.0±0.9	69±2	7.8±1.6
RAT				
CGS21680	6.7±0.2*	5.5±0.3	59±2	0.8±0.2*
2-Chloroadenosine	7.6±0.1*	5.6±0.5	61±4	1.4±0.2*
NECA	7.3±0.1*	5.2±0.6	57±4	0.7±0.3*
R-PIA	7.2±0.1*	5.4±0.4	60±3	1.1±0.3*
Adenosine	6.2±0.1*	5.3±0.3	58±3	0.7±0.2*

The negative log of half-maximal effective concentrations (pEC_{50}) was calculated from individual EC_{50} values obtained for each agonist using the logistic equation:

$$\text{Response} = A \pm B - \frac{B}{1 + ([\text{agonist}]/EC_{50})^{\text{slope factor}}}$$

Response amplitude (*B*) was calculated in absolute resistance units and % of resistance. Slope is the slope factor in this equation. Responses were acquired in the absence and presence of 50 μM *L*-NOARG, 5 μM glibenclamide or 50 μM *L*-NOARG + 5μM glibenclamide (*L*-NOARG+Glib). Values are means ± s.e.mean of individual experiments (n≥6). * P<0.05 rat *vs* mouse; † P<0.05 *vs.* untreated hearts; ‡ P<0.05 *vs.* *L*-NOARG treated hearts.

Effects of NO-synthase inhibition in mouse heart

Treatment of mouse hearts with 50 μM *L*-NOARG significantly increased baseline coronary resistance by ~25% to 9.5±0.6 $mmHg.ml^{-1}.min^{-1}.g^{-1}$ ($P<0.05$). Responses to the adenosine receptor agonists CGS21680 and 2-chloroadenosine were significantly reduced by *L*-NOARG (Figs 3.3 and 3.4). Inhibitory effects were present whether responses were expressed relative to resting resistance (Fig. 3.3) or scaled to maximal dilation (data not shown). Effects of *L*-NOARG were primarily observed at low to moderate agonist concentrations, with no depression of responses to high agonist levels (ie. above 10^{-8} M CGS21680 and 10^{-7} M 2-chloroadenosine) (Fig. 3.3). Coronary sensitivities to both CGS21680 and 2-chloroadenosine (reflected by pEC_{50}s) were significantly depressed by *L*-NOARG treatment, with a greater shift in 2-chloroadenosine sensitivity (13-fold) versus CGS21680 sensitivity (2.5-fold) (Table 3.2). Representative traces from hearts treated with 2-chloroadenosine and CGS21680 ± L-NOARG, are shown in Fig. 3.4.

L-NOARG failed to reduce responses or alter sensitivity to the endothelial-independent dilator SNP (Fig. 3.3C, Table 3.2). There were no significant inhibitory effects when responses were expressed either as % of resting coronary resistance (Fig. 3.3C), or scaled to the maximal dilation observed (data not shown). Indeed, due to slightly increased resting resistance with *L*-NOARG, there was an insignificant trend towards slightly enhanced responses to higher levels of nitroprusside (when expressed relative to the initial resting resistance). Dilatory responses to the endothelial-dependent dilator ADP were significantly inhibited by *L*-NOARG (Fig. 3.3D, Table 3.2).

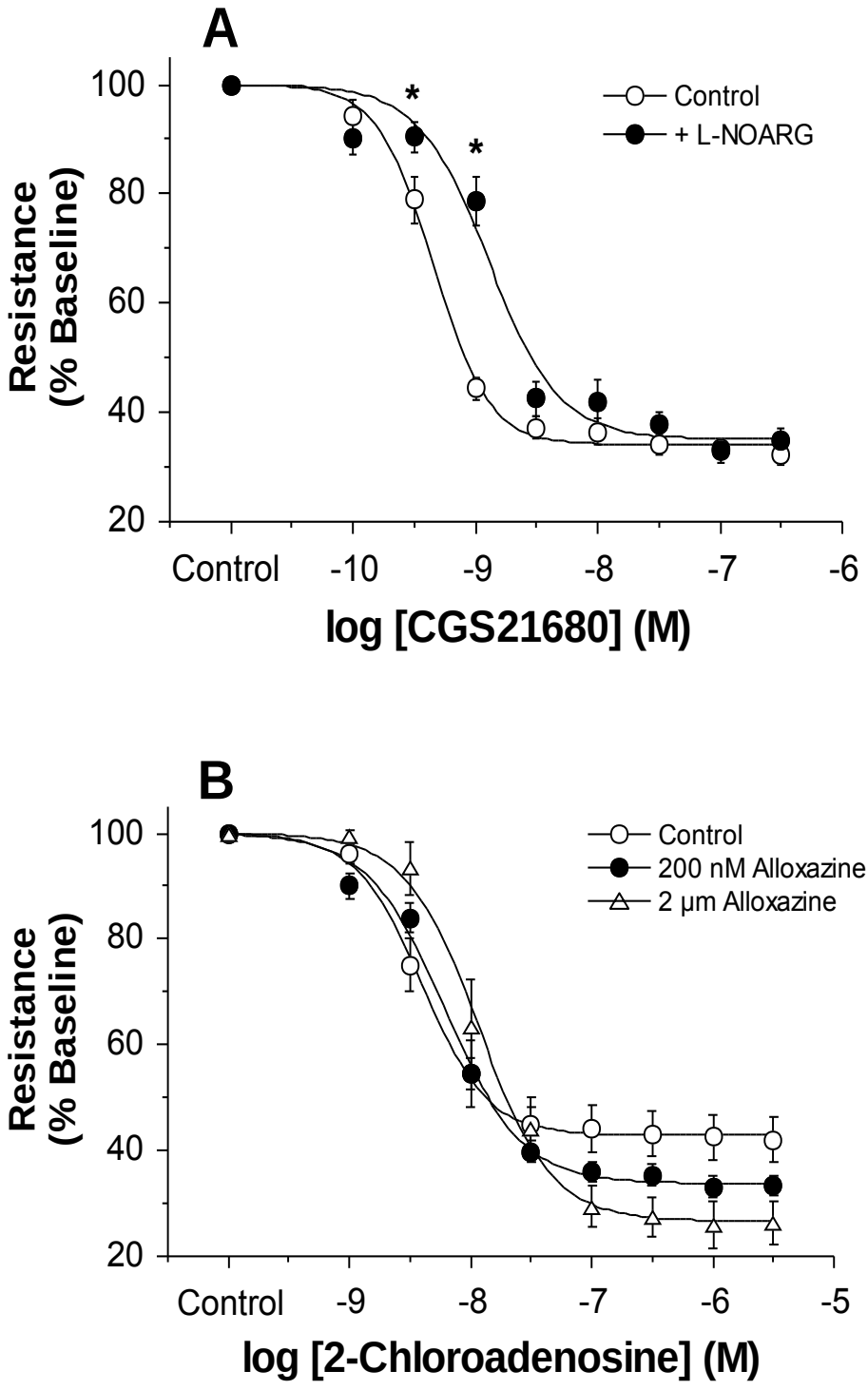

Figure 3.3 Concentration-response curves from mouse hearts in the presence and absence of 50 µM *L*-NOARG. Responses were obtained for A) CGS21680 (n=10) and B) 2-chloroadenosine (n=8). Control responses for CGS21680 and 2-chloroadenosine are taken from the data shown in Fig. 1. Responses are shown as % of baseline coronary resistance. All values are means ± s.e.mean. * $P<0.05$ *vs.* values in untreated hearts ($P<0.05$).

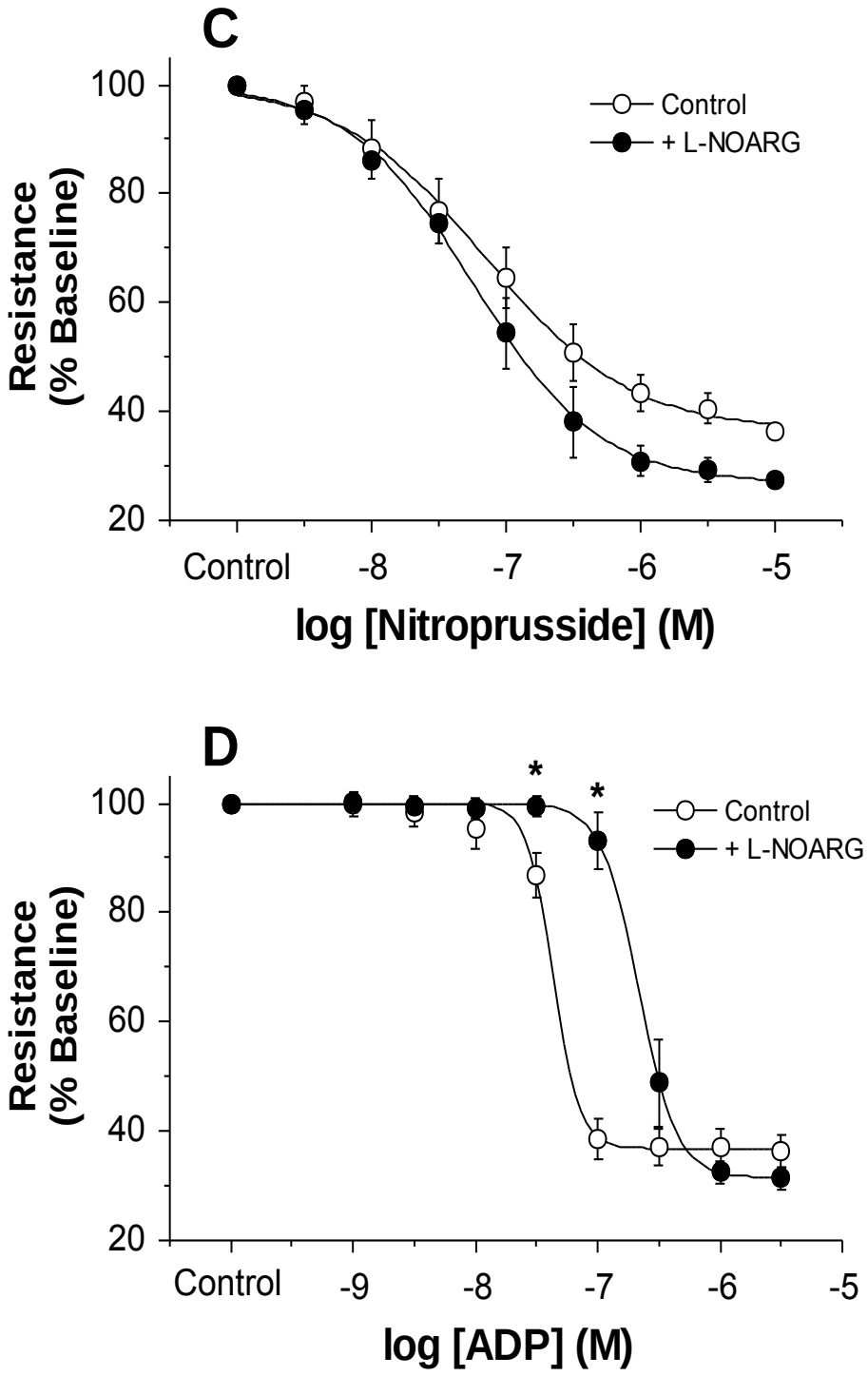

Figure 3.3 Concentration-response curves from mouse hearts in the presence and absence of 50 μM *L*-NOARG. Responses were obtained for C) SNP (n=6 in both groups) and D) ADP (n=7 in both groups). Responses are shown as % of baseline coronary resistance. All values are means ± s.e.mean. * P<0.05 *vs.* values in untreated hearts (P<0.05).

Curiously, a significant number of hearts displayed phasic oscillations in coronary tone during treatment with *L*-NOARG, rendering it difficult to acquire concentration-response data in those hearts. Representative traces from various experiments, including the latter hearts displaying oscillations in tone, are shown in Fig. 3.4.

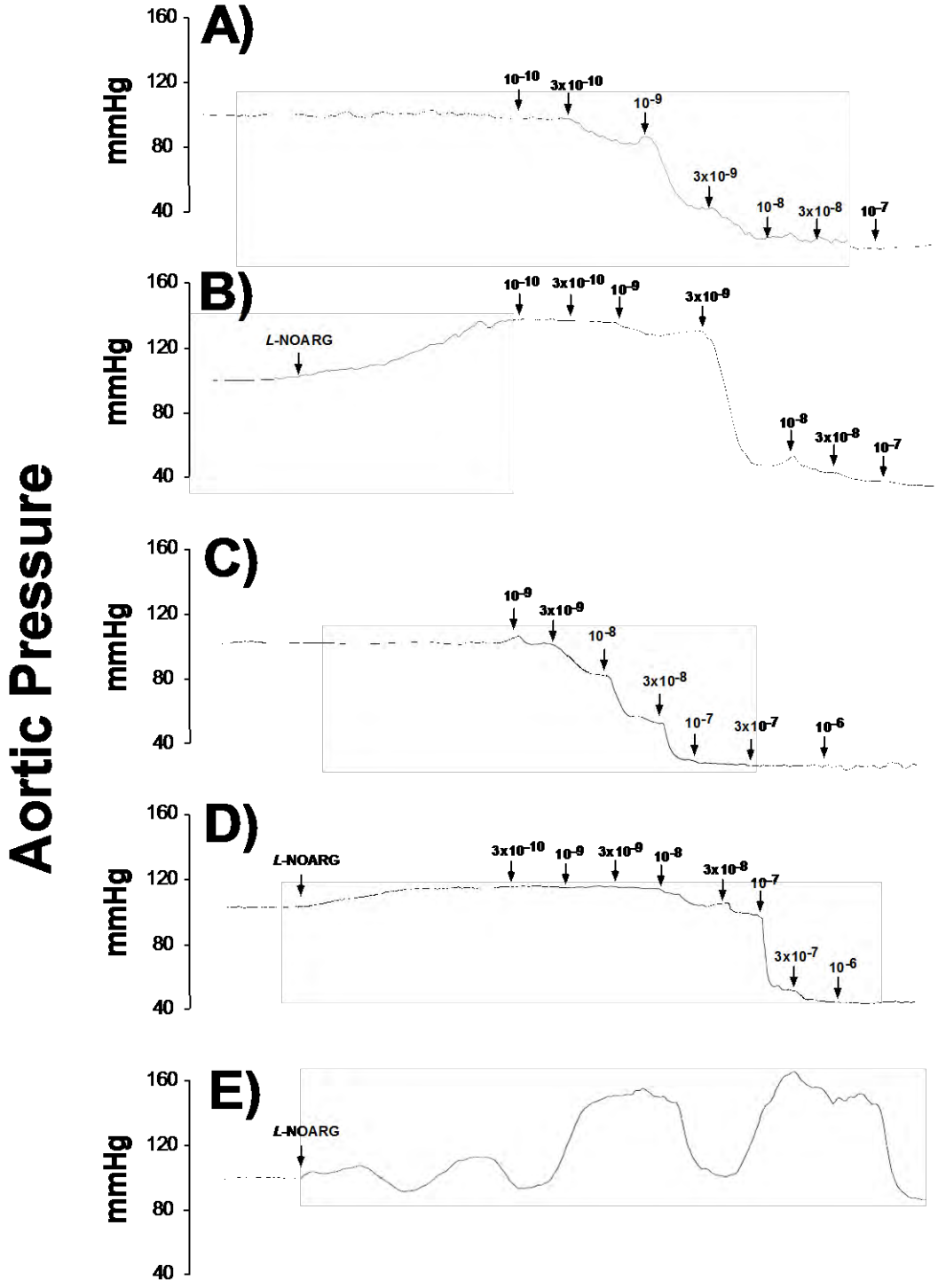

Figure 3.4 Representative tracings from mouse hearts treated with A) CGS21680, B) CGS21680 + 50 µM *L*-NOARG, C) 2-chloroadenosine, and D) 2-chloroadenosine + 50 µM *L*-NOARG. The trace shown in E) is from a heart displaying phasic oscillations in coronary tone during infusion of 50 µm *L*-NOARG.

Effects of K_{ATP} channel inhibition in mouse heart

Treatment of mouse hearts with 5 μM glibenclamide significantly increased baseline coronary resistance by ~35% to 9.8±0.5 mmHg.ml^{-1}.min^{-1}.g^{-1} ($P<0.05$). Treatment with 5 μM glibenclamide + 50 μM L-NOARG further increased resting resistance to 12.6±0.7 mmHg.ml^{-1}.min^{-1}.g^{-1} ($P<0.05$). Responses to CGS21680 and 2-chloroadenosine were significantly inhibited by glibenclamide, and were further inhibited by co-treatment with glibenclamide + *L*-NOARG (Fig. 3.5). The degree of inhibition observed with glibenclamide was considerably greater than that with *L*-NOARG alone. The pEC_{50}s for CGS21680 and 2-chloroadenosine were shifted to higher concentrations by an order of magnitude in the presence of glibenclamide (Table 3.2). The substantial inhibitory effects of glibenclamide were additive to those for *L*-NOARG. The pEC_{50}s for CGS21680 and 2-chloroadenosine were further shifted by at least an order of magnitude in hearts treated with glibenclamide + *L*-NOARG together, relative to pEC_{50}s for hearts treated with either glibenclamide or *L*-NOARG alone (Table 3.2).

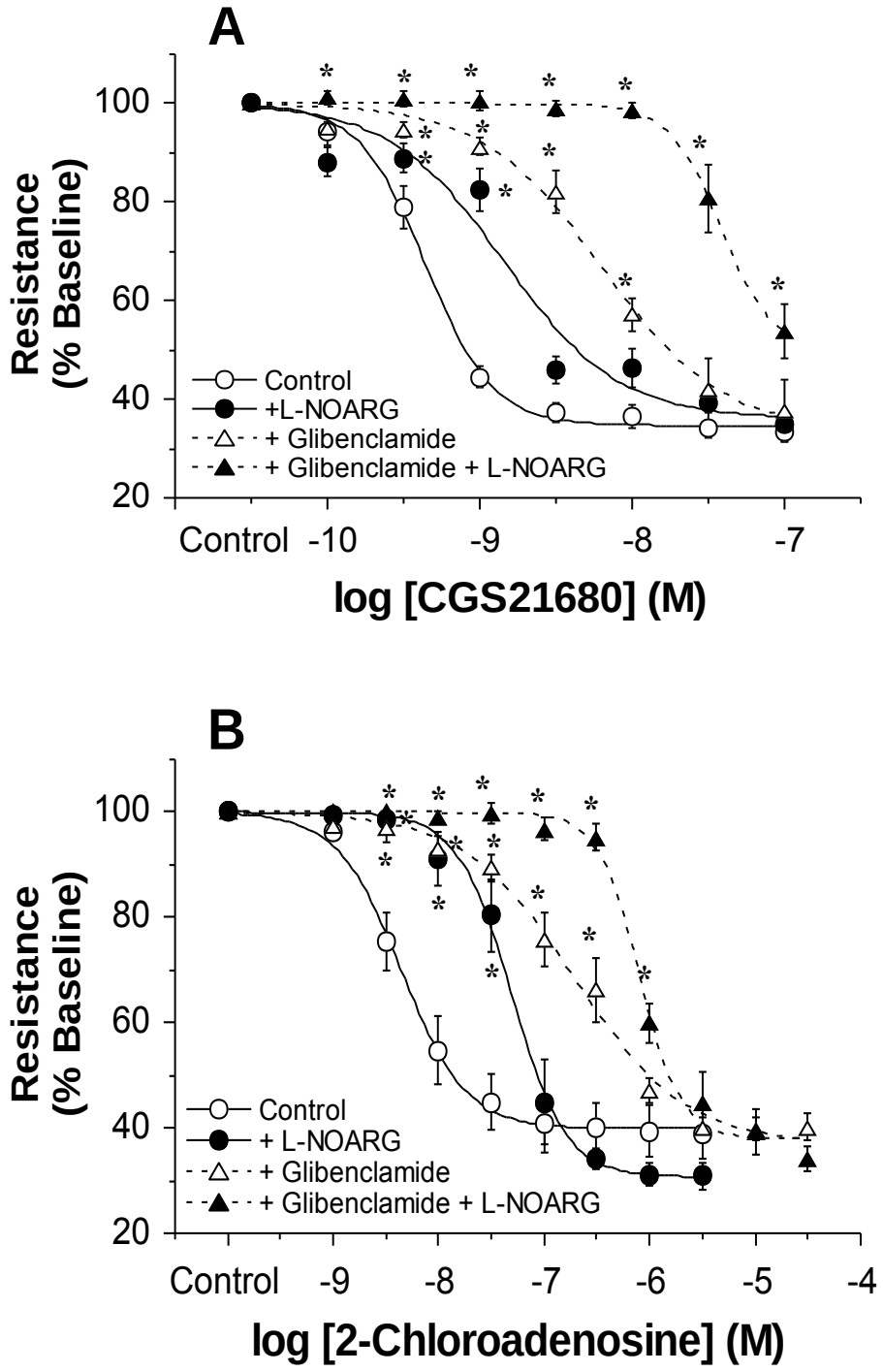

Figure 3.5 Concentration-response curves from mouse hearts in the presence and absence of 5 μM glibenclamide or 5 μM glibenclamide + 50 μM *L*-NOARG. Responses were obtained for A) CGS21680 (n=10), and B) 2-chloroadenosine (n=8). Control responses and responses in the presence of *L*-NOARG alone are also shown (from Fig. 3.3). Responses are shown as % of baseline coronary resistance. All values are means ± s.e.mean. * P<0.05 *vs.* values in untreated hearts (P<0.05). † P<0.05 *vs.* values in *L*-NOARG treated hearts (P<0.05).

V. DISCUSSION

Little information exists regarding adenosine receptor sub-types mediating coronary dilation in the mouse, or mechanisms of adenosine-mediated coronary dilation in this species. The present data indicate that mouse heart is more sensitive to adenosine agonists than rat heart, and show that different receptor sub-types mediate dilation in these species. Adenosine-mediated coronary dilation in the mouse involves NO-dependent and K_{ATP}-dependent components.

Coronary vascular function in mouse versus rat heart

Baseline and maximally dilated coronary resistances were lower in mouse versus rat heart (Table 3.1). Additionally, reactive hyperaemic responses were higher in the mouse (Table 3.1). The mouse circulation therefore appears to operate at lower resting and dilated resistances than in the rat, likely reflecting correlations between vascular density, mitochondrial capacity, metabolic rate and body mass [631,632]. Owing to greater metabolic rate in mouse (ie. mouse heart rate is 550-600 beats.min^{-1} versus ~350 beats.min^{-1} in the rat), the murine heart requires a much greater O_2 delivery. This can be accomplished by limited adaptations in O_2 carrying capacity of blood and O_2 transport from blood to mitochondria, and/or it can be met by increased coronary vascularization and coronary flow, as shown here. Higher hyperaemic responses and lower coronary resistance in mouse heart permits greater resting and maximal O_2 delivery.

The adenosine receptor mediating coronary dilation in mouse and rat hearts

Endogenous adenosine may play an important role in modulating coronary vascular resistance during and following pathophysiological stimuli [13,99], although

its role in regulation of coronary tone under more physiological conditions *in vivo* is questionable [100]. We show that murine coronary vessels are highly sensitive to adenosine agonists. Sensitivities to all agonists, including adenosine, are higher in mouse than in rat (Fig 3.1., Table 3.2). Importantly, the rank order of agonist potencies differs between mouse and rat (Table 3.2). As noted initially by Gurden *et al.* (1993), two differing orders of agonist potencies are observed for A_2 receptor mediated vascular responses [633]. They observed an order of potency of CGS21680≥NECA>R-PIA in canine coronary vessels, reflecting an A_{2A} receptor-mediated response, and NECA>R-PIA>CGS21680 in guinea pig aorta, reflecting an A_{2B} receptor-mediated response. Kull and colleagues (1999) documented a functional potency profile of NECA≥CGS21680>R-PIA≥2-chloroadenosine>adenosine for rat and human A_{2A} receptors [634]. We obtained an order of CGS21680=NECA>2-chloroadenosine>R-PIA>adenosine in the mouse which is comparable to that for canine [633], human and rat A_{2A} receptors [634]. In contrast we obtained a profile of 2-chloroadenosine>NECA= R-PIA>CGS21680>adenosine in rat, supporting a primarily A_{2B} response.

To test for A_{2B} receptors the A_{2B} selective antagonist alloxazine was infused (Fig. 3.2). While there is a lack of potent and highly selective A_{2B} antagonists, alloxazine exhibits ~10-fold selectivity for murine A_{2B} receptors versus A_{2A} receptors [635] and completely inhibits A_{2B} receptors at 2 μM. We chose a lower concentration in order to minimise antagonism at the A_{2A} receptors which appear to be present. At 200 nM alloxazine failed to alter responses to A_{2A} selective CGS21680 and non-selective 2-chloroadenosine (Fig. 3.2). Hearts receiving the maximally effective 2 μM concentration also displayed unaltered responses to 2-chloroadenosine (Fig. 3.2B).

These data verify A_{2A} mediated responses to both receptor agonists, and indicate an absence of functional A_{2B} receptor in mouse coronary vessels.

Previous studies indicate that A_{2A} receptors mediate coronary dilation in dog [119], pig [120,122], and guinea pig [123]. Functional identification of A_{2A} receptors in the pig [120,122] has recently been supported by molecular analysis [121]. In contrast, A_{2B} receptors are of primary importance in mediating coronary dilation in humans [124] and in the rat [125,126]. A preliminary study by Morrison *et al.* (2001) indicates that gene deletion of the A_{2A} receptor eliminates CGS21680-mediated coronary dilation in the mouse [127]. While findings from genetically modified knock-out animals are not directly applicable to wild-type tissues, and although we have only assessed receptor identity in a single strain of mice, and rodent strains can show variability in cardiovascular responses, our data together with the findings of Morrison *et al.* (2001) support A_{2A}-mediated coronary dilation in the murine heart [127].

It is worth noting that the slope of concentration-response curves is greater for all agonists in mouse versus rat (Table 3.2). This suggests that the efficacy of adenosine agonists at murine A_{2A} receptors may be greater than at rat A_{2B} receptors, and/or supports heterogeneity in adenosine responses in rat. Lewis and Hourani provide evidence that both A_{2A} and A_{2B} receptors may mediate dilation in rat heart [125], and there is support for an unidentified, potentially intracellular receptor in rat vessels [636,637] in addition to mouse aorta [128]. This multiplicity of effector mechanisms may contribute to a broader concentration range over which agonists act in rat. The narrow range and steep slope in mouse is consistent with a single coronary receptor sub-type.

In assessing the identity of the adenosine receptor we chose to assess the rank order of agonist potencies, and study effects of a selective antagonist. This conventional pharmacological approach remains a key method for identification of adenosine receptors [120,122-124,126,633-637], and was adopted despite alternate methodologies, including epigenetic approaches (targeted gene knock-out and anti-sense oligonucleotides) [638]. Despite selectivity of epigenetic techniques, these methods possess limitations. Anti-sense studies in intact organs are largely restricted to brain and lungs due to difficulties in selectively introducing oligonucleotides, and these studies are limited by poor penetration into cells and nuclei. With respect to gene knock-out, an A_{2A} knockout mouse has been assessed in terms of neurophysiological responses [639], and there are preliminary data regarding adenosine and CGS21680 mediated coronary dilation in this model [127]. While these findings support an important A_{2A} response in murine vessels, interpretation of gene knock-out data are limited since they assess effects of a gene's long-term absence rather than the normal role of the gene product itself. Absence of the gene may lead to unknown developmental, morphological and functional changes, together with unpredicted compensatory responses. Receptor identification remains best achieved via complimentary pharmacological and molecular approaches.

Role of NO in adenosine receptor-mediated coronary dilation in mouse

There is evidence that adenosine is a mixed endothelial-dependent/independent dilator, activating both endothelial and smooth muscle receptors within the same vessel [120,133-135,141,640]. To test the potential involvement of NO in adenosine responses, we studied effects of NO-synthase inhibition with *L*-NOARG. *L*-NOARG significantly increased baseline resistance and inhibited responses to endothelial-dependent ADP but not SNP, verifying selective inhibition of NO-dependent responses, and supporting a

role for endogenous NO in control of resting tone in murine coronary vessels (Table 3.1, Fig. 3.3). Curiously, a number of hearts displayed phasic oscillations in coronary resistance during *L*-NOARG treatment (Fig. 3.4). Though the mechanism of these oscillations is unclear, it may reflect competition between constriction due to reduced NO release and relaxation due to locally released dilators. Importantly, while *L*-NOARG did not alter responses to SNP, it significantly attenuated responses to 2-chloroadenosine and CGS 21680, substantially increasing EC_{50}s and threshold concentrations at which agonists induced dilation (Fig. 3.3, Table 3.2).

While our data agree with studies supporting partial NO- or endothelial-dependent coronary responses to adenosine in guinea pig [139-141], dog [142,143], and pig [120,144,146,147], they contrast with studies in human and porcine coronary vessels [122,124,148,149], and in rat heart [125]. Varying observations from different species supports pronounced species differences in the mechanisms of adenosine-mediated coronary dilation. Contradictory observations made within a single species (eg. [120,122,144,146,149]) demonstrates a need for further research. In this respect, both A_{2A} and A_{2B} receptors may mediate coronary dilation in the same species and there appear to be differences in transduction mechanisms for these sub-types. There is a greater weight of unequivocal data supporting NO-dependence of adenosine A_{2A} responses [139-141] whereas there is controversy regarding NO-dependence of coronary A_{2B} responses [122,124,125,149].

Functional antagonism versus NO-synthase inhibition

There may be two effects of *L*-NOARG in coronary vessels - enhanced functional antagonism increasing the resistance over which dilatory agonists can induce relaxation, and direct antagonism of NO-synthase dependent responses. Lewis &

Hourani (1997) recently concluded that apparent antagonism of adenosine via NO-synthase inhibitors may reflect functional antagonism (ie. vasoconstriction) [125]. They found that *L*-NOARG reduced adenosine responses in rat, but also reduced responses to NO-synthase independent SNP. However, responses to SNP were quite low in their study and *L*-NOARG only modestly reduced these in contrast with 2- to 10-fold differences in adenosine-response magnitude [125]. We show that *L*-NOARG increases resting coronary resistance (Table 3.1), and this should alter dilatory response amplitude. If "pre-constrictors" do not directly inhibit mechanisms of action of a dilator, enhanced functional antagonism and resting tone simply increases the amplitude (but not sensitivity) of dilatory responses, as noted by Lew (1995) [641]. Functional antagonism is useful in increasing response amplitude for dilators, removing constraints imposed by degree of pre-constriction, and permitting analysis of agonist efficacies [641,642]. These factors are exemplified by responses to SNP: *L*-NOARG tends to enhance response amplitude to high levels of SNP when responses are expressed relative to the higher initial tone (Fig. 3.3C). It is necessary to normalize such responses to make meaningful comparisons, and to remove differences which are not relevant [641]. These minor differences are absent when SNP responses are scaled to maximal dilation (data not shown). Importantly, in direct contrast to SNP, responses to low levels of adenosine agonists are substantially reduced by *L*-NOARG while maximal responses are unaltered (Fig. 3.3), the threshold effective concentration of 2-chloroadenosine is increased ~10-fold (Fig. 3.3), and coronary sensitivity to 2-chloroadenosine and CGS21680 is significantly reduced (Table 3.2). Selective effects of *L*-NOARG on A_2-mediated responses (and on ADP responses) supports significant NO-dependence of adenosine-mediated dilation in mouse heart.

Assuming that responses to low levels of 2-chloroadenosine in the presence of *L*-NOARG can be considered largely NO-synthase independent, we can estimate NO-synthase dependent dilation by subtraction of NO-synthase independent responses from control (mixed) responses [133,640]. As shown in Fig. 3.6 the NO-synthase dependent *L*-NOARG sensitive response is important at low levels of A_2 receptor activation. Dilation with 1-30 nM 2-chloroadenosine is almost entirely NO-dependent (Fig. 3.6). At higher levels of activation NO-synthase independent dilation predominates. This is consistent with previous observations indicating that NO-dependent adenosine responses are sensitive yet small in amplitude [133-135,141,640]. While small amplitude might be considered evidence for a minimal functional role [141], high sensitivity (Fig. 3.6) ensures that it is quantitatively important at low, physiologically relevant levels of receptor activation.

Role of K_{ATP} channels in adenosine receptor-mediated coronary dilation in mouse

In addition to release of NO, activation of K_{ATP} channels is implicated in A_{2A}AR-mediated coronary dilation [120,121,146,150]. In contrast, A_{2B} receptors may mediate coronary dilation in human vessels via NO and K_{ATP}-independent mechanisms [124]. Our data reveal that K_{ATP} blockade with glibenclamide, at a level which fully antagonises responses to minoxidil, markedly inhibits responses to CGS21680 and 2-chloroadenosine (Fig. 3.5). Importantly, effects of K_{ATP} channel inhibition are additive to (and greater than) those for NO-synthase inhibition via *L*-NOARG. Considering the 120- to 300-fold reduction in sensitivity to CGS21680 and 2-chloroadenosine with combined NO-synthase and K_{ATP} inhibition, these two pathways appear to be the primary mechanisms contributing to A_{2A}-mediated dilation. The more pronounced inhibitory effects of K_{ATP} channel blockade and additivity with the effects

of *L*-NOARG suggest that A_{2A} receptors trigger NO-mediated dilation via mechanisms distinct from activation of smooth muscle and/or endothelial K_{ATP} channels.

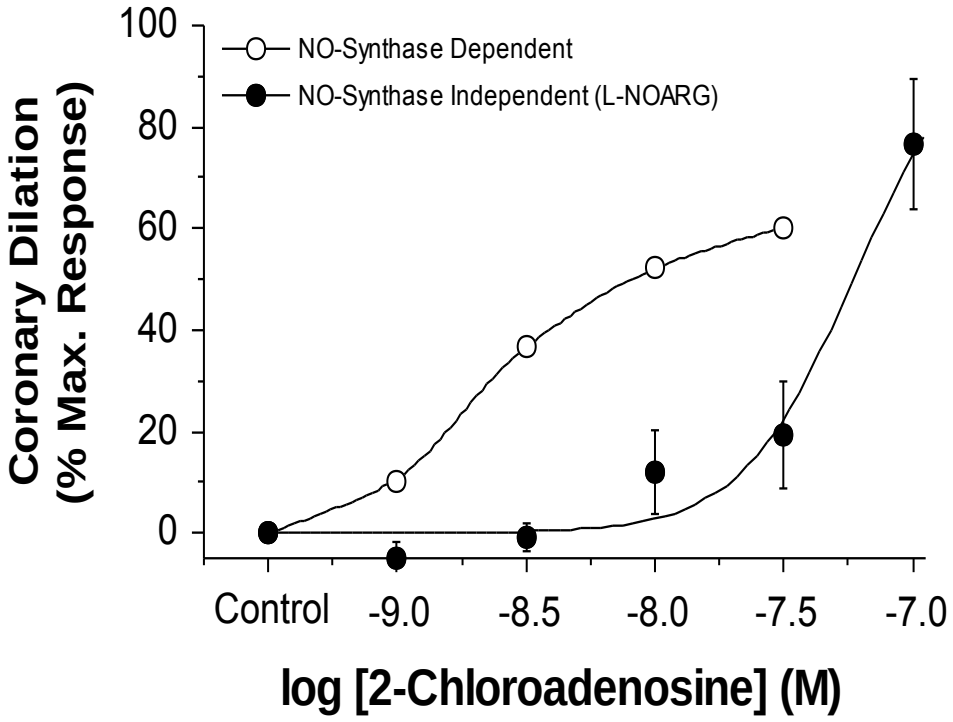

Figure 3.6 NO-synthase dependent and NO-synthase independent responses to 2-chloroadenosine in perfused mouse heart. The raw data is derived from the results shown in Fig. 3.3. The NO-synthase independent response is represented by the dilatory response in the presence of 50 μM *L*-NOARG. The NO-synthase dependent response is calculated as the difference between the control response (in absence of *L*-NOARG) and the NO-synthase independent response (in presence of *L*-NOARG). Responses are scaled to % of maximal dilation observed.

Concluding remarks

In conclusion, the present study demonstrates that peak coronary flows, reactive hyperaemia and adenosine sensitivity are all substantially greater in mouse versus rat heart. Adenosine agonist potency profiles and effects of alloxazine support A_{2A}AR-mediated coronary dilation in mouse versus A_{2B}AR-mediated dilation in rat. Our data demonstrate a significant NO-dependent and K_{ATP} channel-dependent components to adenosine mediated coronary dilation in the mouse. A_{2A}-mediated dilation appears more strongly dependent on K_{ATP} channels than NO formation. However, although NO-independent dilation predominates over NO-dependent dilation at moderate to high agonist levels, the high-sensitivity NO-dependent response may play an important role under physiological conditions when adenosine concentrations and the level of A_{2A} receptor activation are relatively low.

CHAPTER 4

MEDIATORS OF CORONARY REACTIVE HYPERAEMIA IN ISOLATED MOUSE HEART

I. ABSTRACT

Having identified the functional adenosine receptors present in murine coronary vessels, the mechanisms regulating coronary tone under basal conditions and during reactive hyperaemic responses were assessed in isolated perfused mouse hearts. Blockade of NO-synthase (50 μM *L*-NAME), K_{ATP} channels (5 μM glibenclamide), adenosine A_{2A} receptors (100 nM SCH58261), and prostanoid synthesis (100 μM indomethacin) all reduced basal coronary flow ~50%. Effects of *L*-NAME and glibenclamide were partly additive, while co-administration of SCH58261 and indomethacin with these inhibitors failed to further limit flow. Substantial hyperaemia was observed after 5, 10, 20, or 40 s occlusions, with a flow increasing to a peak of 51±2 $ml.min^{-1}.g^{-1}$. Glibenclamide most effectively inhibited hyperaemia after 5-10 s occlusions (by 50-60%) followed by *L*-NAME (~25%). There was no evidence for compensatory changes in NO and/or K_{ATP} responses when one response was blocked during brief occlusions. With longer (20-40 s) occlusions, glibenclamide and *L*-NAME were increasingly ineffective, reducing peak flows ~10-20%. Treatment with *L*-NAME + glibenclamide inhibited peak hyperaemia by ~70% following 20 s occlusion, and ~35% following 40 s occlusion. Greater effects of combined treatment (*vs.* the sum of individual effects) support compensatory changes in NO or K_{ATP} dependent dilation when the other is inhibited. In contrast to initial flow changes, sustained dilation over the first 60 s post-occlusion was almost entirely K_{ATP} and NO dependent, irrespective of occlusion duration (both contributing equally to this dilation). Antagonism of A_{2A}ARs modestly reduced hyperaemia (by 20-30%) whereas indomethacin failed to substantially modify hyperaemic responses. Inhibitory effects of SCH58621 were not additive with those of *L*-NAME and glibenclamide, supporting NO and K_{ATP}-channel dependent actions of the A_{2A} receptor. These data support a primary role for K_{ATP} channels and

NO in mediating sustained elevations in flow, irrespective of occlusion duration (5-40 s). Whereas K_{ATP} channels are of primary importance in mediating initial flow adjustments after brief (5-10 s) occlusions, K_{ATP} (and NO) independent processes are increasingly important with longer (20-40 s) occlusion. Evidence is also presented for compensatory changes in K_{ATP} and/or NO mediated dilation when one path is blocked, and for a modest role for A_{2A}ARs in hyperaemia.

II. INTRODUCTION

Reactive hyperaemia is a temporary increase in flow in response to brief periods of occlusion. This graded response provides repayment of so-called "flow debt" (or O_2 debt) incurred during occlusion, potentially hastening metabolic and functional recovery of post-ischaemic tissue. Despite considerable research into mechanisms of reactive hyperaemia, controversy still remains regarding relative contributions of different mediators. Both nitric oxide (NO) and ATP-sensitive K^+ (K_{ATP}) channel dependent mechanisms are implicated [643-652], with mixed support for some role for adenosine as mediator [630,646,652-654]. In contrast, most studies find little evidence for a role for prostanoids [649,652,654-657], despite some support for a role in mediating coronary basal tone [201]. Importantly, conventional analysis of hyperaemic responses using inhibitors of individual pathways is complicated by potential redundancy and compensation by other dilatory mechanisms. Moreover, it is possible differing processes contribute to initial and later flow adjustments during hyperaemia (the latter potentially flow-dependent). The aim of the current study was to examine individual roles of K_{ATP} channels, NO, A_{2A}ARs, and prostanoids in mediating peak and sustained flow changes during reactive hyperaemic response in intact mouse heart.

III. MATERIALS AND METHODS

Langendorff perfused murine heart model

Hearts were prepared for vascular studies as outlined in Chapter 2.

Experimental protocol

To assess reactive hyperaemic vasodilation hearts were randomly subjected to 5, 10, 20 or 40 sec periods of total vascular occlusion separated by 5-min periods of reperfusion (during which flow recovered to pre-occlusion levels). Peak hyperaemic flow and total repayment flow during the initial 1 min reperfusion were determined for each occlusion period. Flows were determined from continuous flow-meter recordings in the Chart data acquisition program (ADInstruments, Castle Hill, Australia). Reactive hyperaemic responses were assessed in the absence (control; n=8) or presence of 50 μM *L*-NAME (NO synthase inhibitor, n=10), 5 μM glibenclamide (K_{ATP} channel antagonist; n=10), 100 nM SCH58261 (selective A_{2A}AR antagonist; n=8), 100 μM indomethacin (cyclooxygenase inhibitor; n=6), 5 μM glibenclamide + 50 μM *L*-NAME (n=6), or 5 μM glibenclamide + 50 μM *L*-NAME + 100 nM SCH58261 + 100 μM indomethacin (n=8). Treatment with antagonists was commenced 15 min prior to inducing reactive hyperaemic responses. As previously mentioned, resting coronary flow was reduced by different inhibitory agents studied, therefore calculation of % flow debt repayment (commonly assessed in studies of reactive hyperaemia), was not meaningful. Rather, we examined the overall hyperaemic response (Figs 4.1, 4.3-4.9), together with changes in peak hyperaemic flow and total flow repayment during reperfusion (Fig. 4.2).

IV. RESULTS

Effects of inhibitors on basal coronary tone

Baseline coronary flow was 14 $ml.min^{-1}.g^{-1}$ in untreated hearts. Treatment with *L*-NAME, glibenclamide, SCH58261, and indomethacin led to 40-50% reductions in resting coronary flow (to 7-8 $ml.min^{-1}.g^{-1}$). Combined treatment with *L*-NAME and glibenclamide exerted an even greater effect on basal flow, which was reduced by almost 75% (Table 4.1). Addition of SCH58261 and indomethacin to these inhibitors failed to further limit basal flow.

Table 4.1 Effects of different treatments on resting coronary tone

	Coronary Flow ($ml.min^{-1}.g^{-1}$)	**Resistance** ($mmHg.ml^{-1}.min^{-1}.g^{-1}$)
Untreated (*n*=8)	14.2±2.7	5.6±1.1
L-NAME (*n*=10)	6.8±0.8*	11.8±1.4*
Glibenclamide (*n*=10)	7.2±0.7*	11.1±1.1*
SCH58261 (*n*=8)	6.9±1.1*	11.6±1.8*
Indomethacin (*n*=6)	7.6±1.0*	10.5±1.4*
L-NAME+Glibenclamide (*n*=6)	3.5±0.3*	22.8±1.9*
L-NAME+Glibenclamide+ SCH58261+Indomethacin (*n*=8)	4.4±0.6*	18.0±2.5*

The pre-occlusion flows were assessed immediately prior to the reactive hyperaemia protocol. Values are means ± s.e.mean. * P<0.05 *vs.* Untreated hearts.

Reactive hyperaemic responses in control hearts

All hearts showed a substantial hyperaemic response following transient coronary occlusions. Coronary reactive hyperaemia was graded, increasing with the period of occlusion (Fig. 4.1). Peak hyperaemic flows increased from ~30 $ml.min^{-1}.g^{-1}$ after 5 s occlusion to 52 $ml.min^{-1}.g^{-1}$ after 40 s occlusion (Fig. 4.2A). Total flow repayment over 1 min reperfusion was also graded, increasing from 13 to 33 $ml.g^{-1}$ after 5 and 40 s occlusions, respectively (Fig. 4.2B).

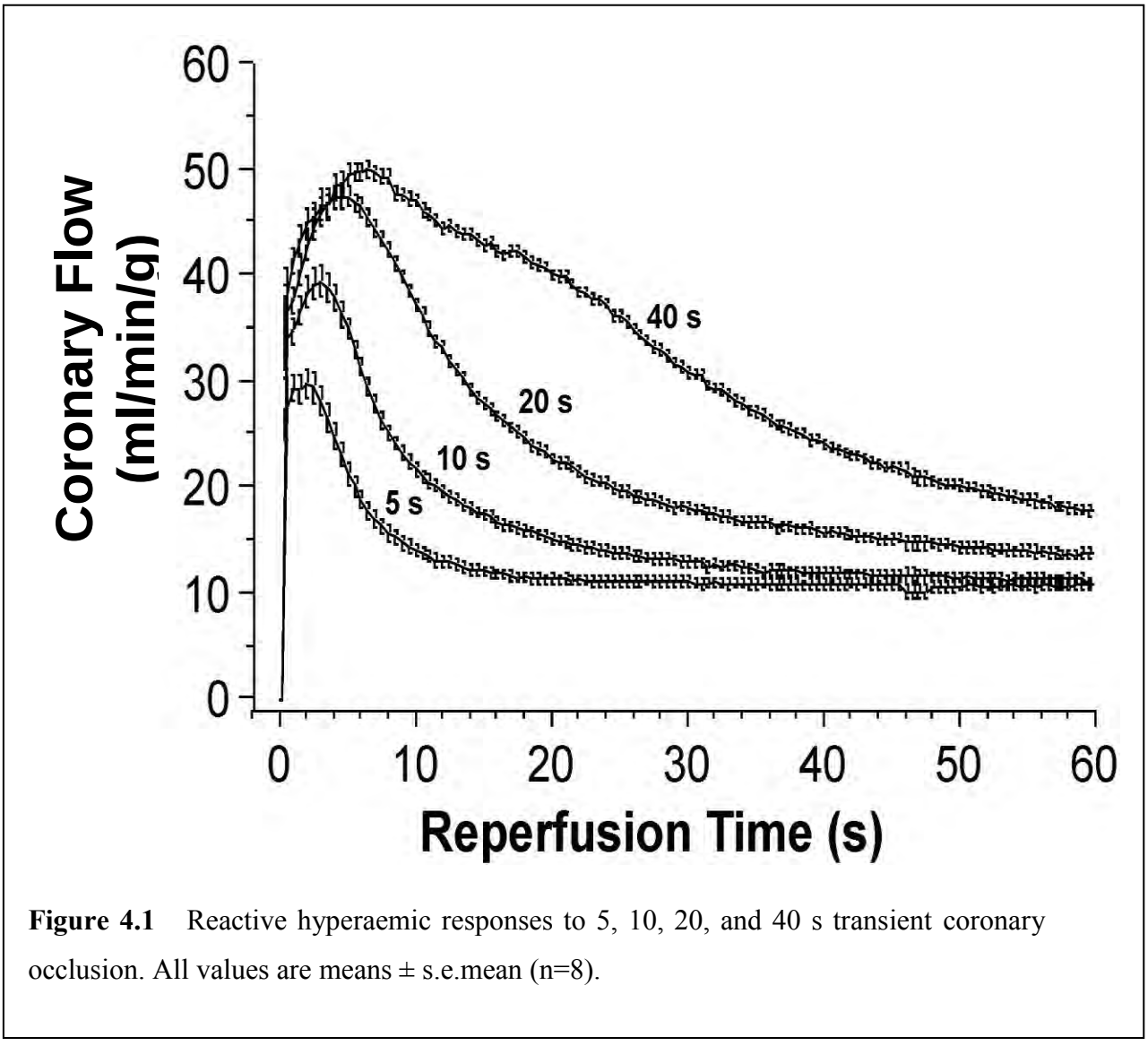

Figure 4.1 Reactive hyperaemic responses to 5, 10, 20, and 40 s transient coronary occlusion. All values are means ± s.e.mean (n=8).

Effects of NO synthase and K_{ATP} channel inhibition on reactive hyperaemia responses

The NO-synthase inhibitor *L*-NAME exerted minor inhibitory effects on peak hyperaemic flows after all occlusion periods (Figs 4.2 and 4.3). However, *L*-NAME did significantly lower flow during the period following the maximal dilation (this was evident with all periods of occlusion). The K_{ATP} channel blocker glibenclamide was the most effective inhibitor studied, substantially limiting peak hyperaemic flows (Fig. 4.2) and the more prolonged dilation following the initial hyperaemia (Fig. 4.4). Inhibitory effects of glibenclamide were evident with all periods of occlusion, although effects on peak flow were gradually lessened as period of occlusion increased. Combined treatment with *L*-NAME + glibenclamide was more effective than *L*-NAME and glibenclamide individually in limiting hyperaemic responses to all occlusion periods (Fig. 4.5), although effects of glibenclamide and *L*-NAME + glibenclamide were similar with the brief 5 and 10 s occlusion (Figs 4.4 and 4.5).

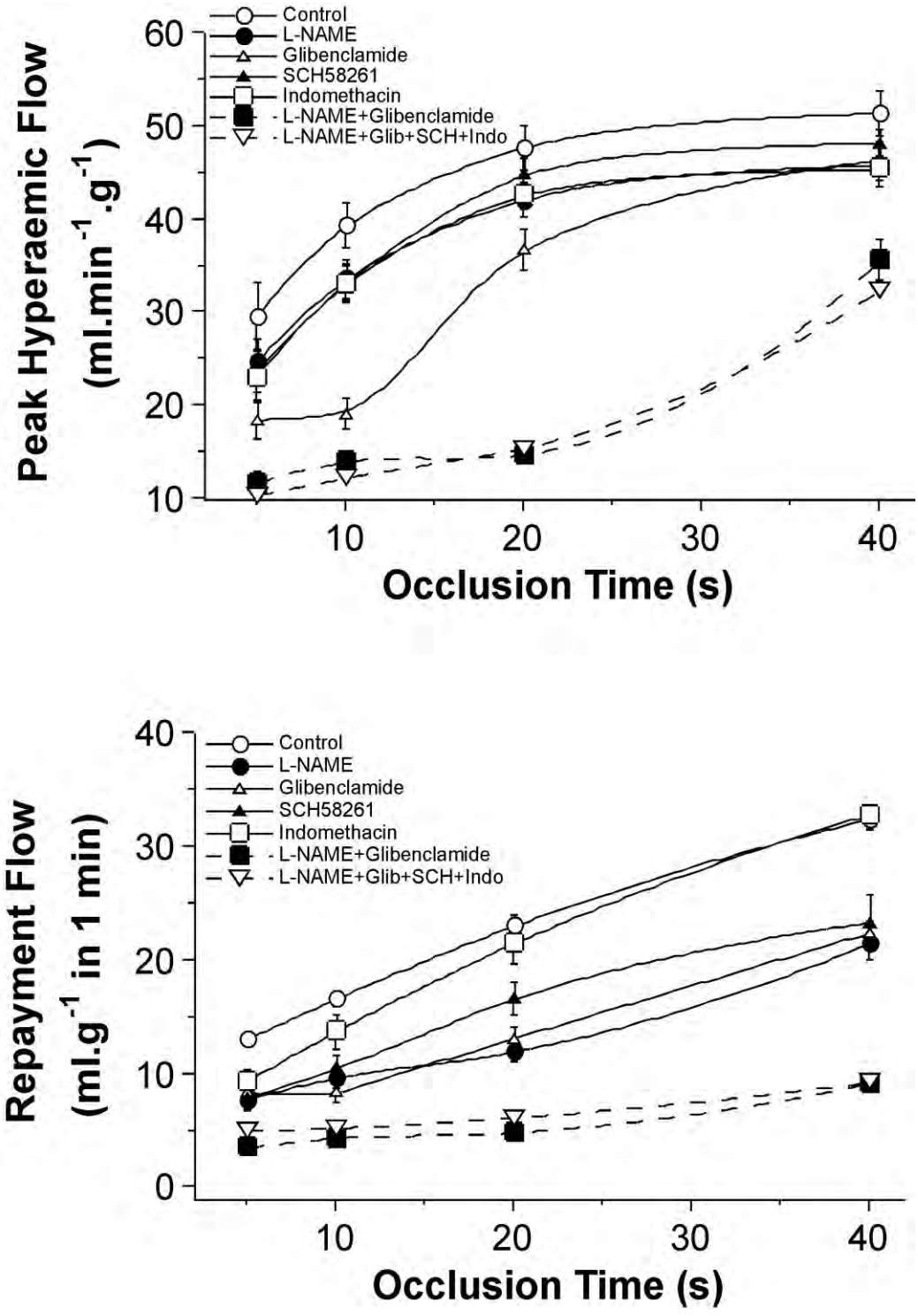

Figure 4.2 Effects of inhibitors on A) peak hyperaemic flow and B) repayment flow over the initial 1 min of reperfusion following 5, 10, 20, and 40 s occlusions. Data were acquired in the absence (n=8) or presence of *L*-NAME (n=10), glibenclamide (n=10), SCH58261 (n=8), indomethacin (n=6), *L*-NAME + glibenclamide (n=6), or *L*-NAME + glibenclamide + SCH58261 + indomethacin (n=8). All values are means ± s.e.mean. * P<0.05 *vs.* values in untreated hearts.

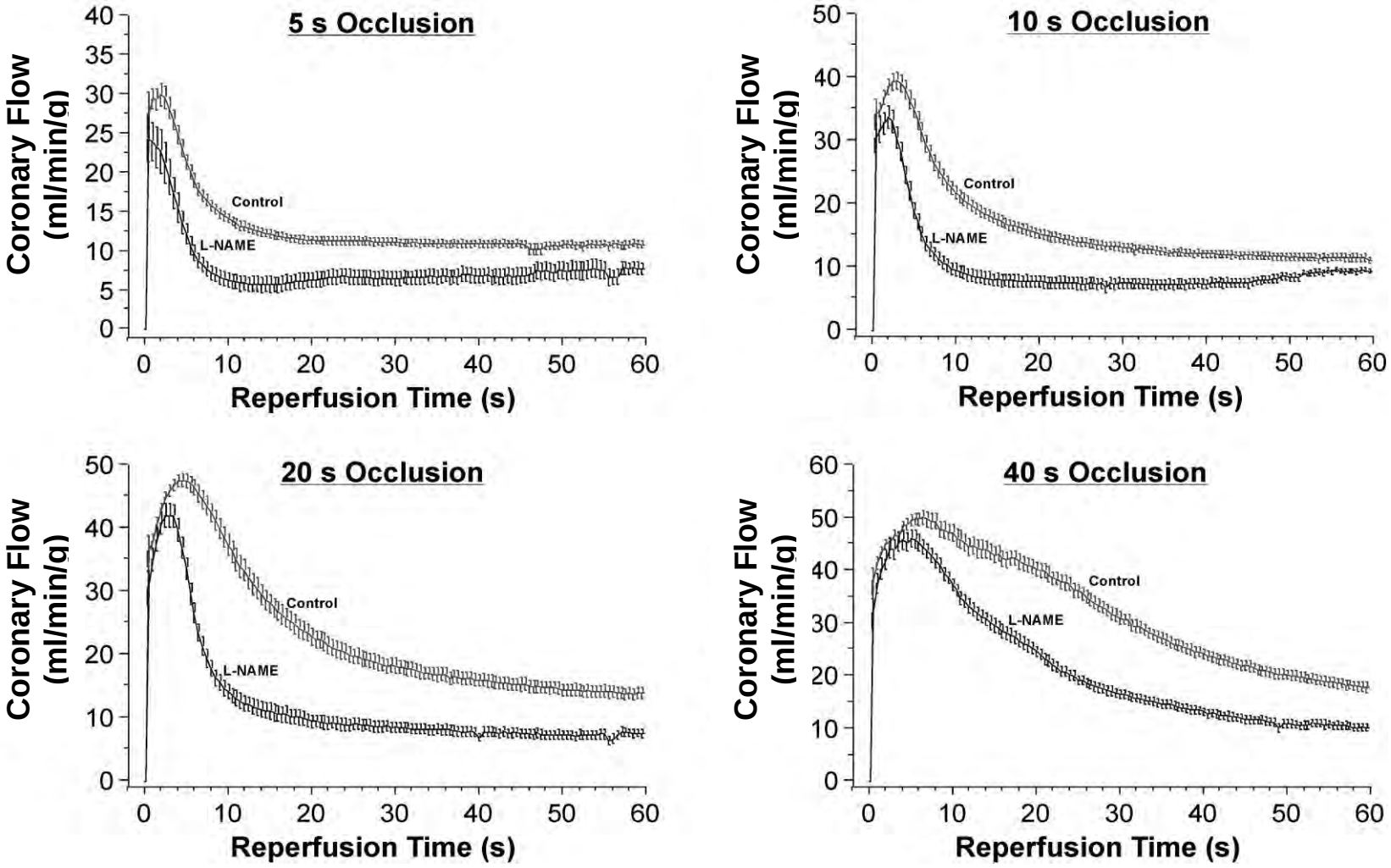

Figure 4.3 Effects of NO synthase inhibition with *L*-NAME on hyperaemic response to A) 5 s, B) 10 s, C) 20 s, and D) 40 s occlusions. All values are means ± s.e.mean (n=10).

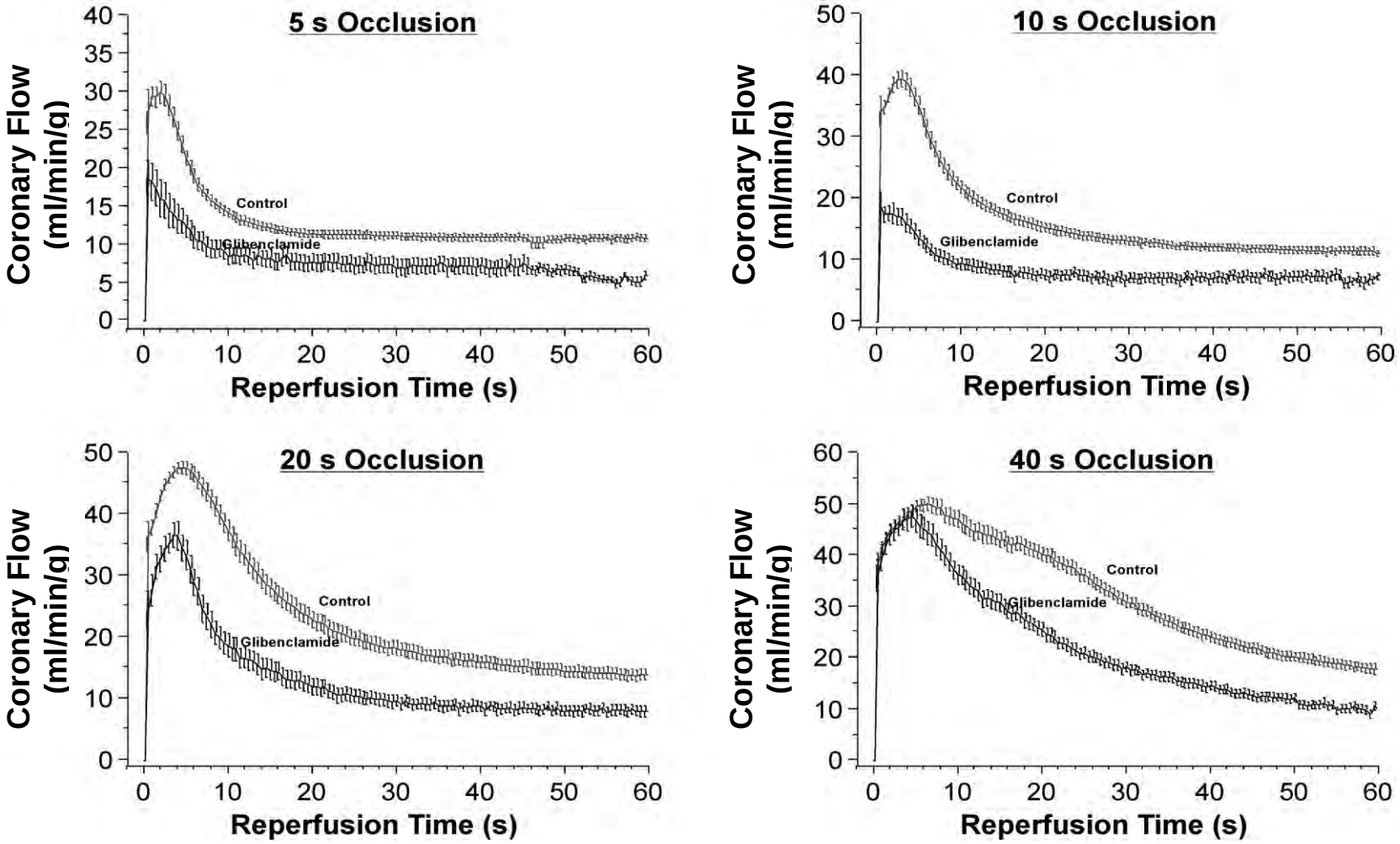

Figure 4.4 Effects of K_{ATP} channel inhibition with glibenclamide on hyperaemic response to A) 5 s, B) 10 s, C) 20 s, and D) 40 s occlusions. All values are means ± s.e.mean (n=10).

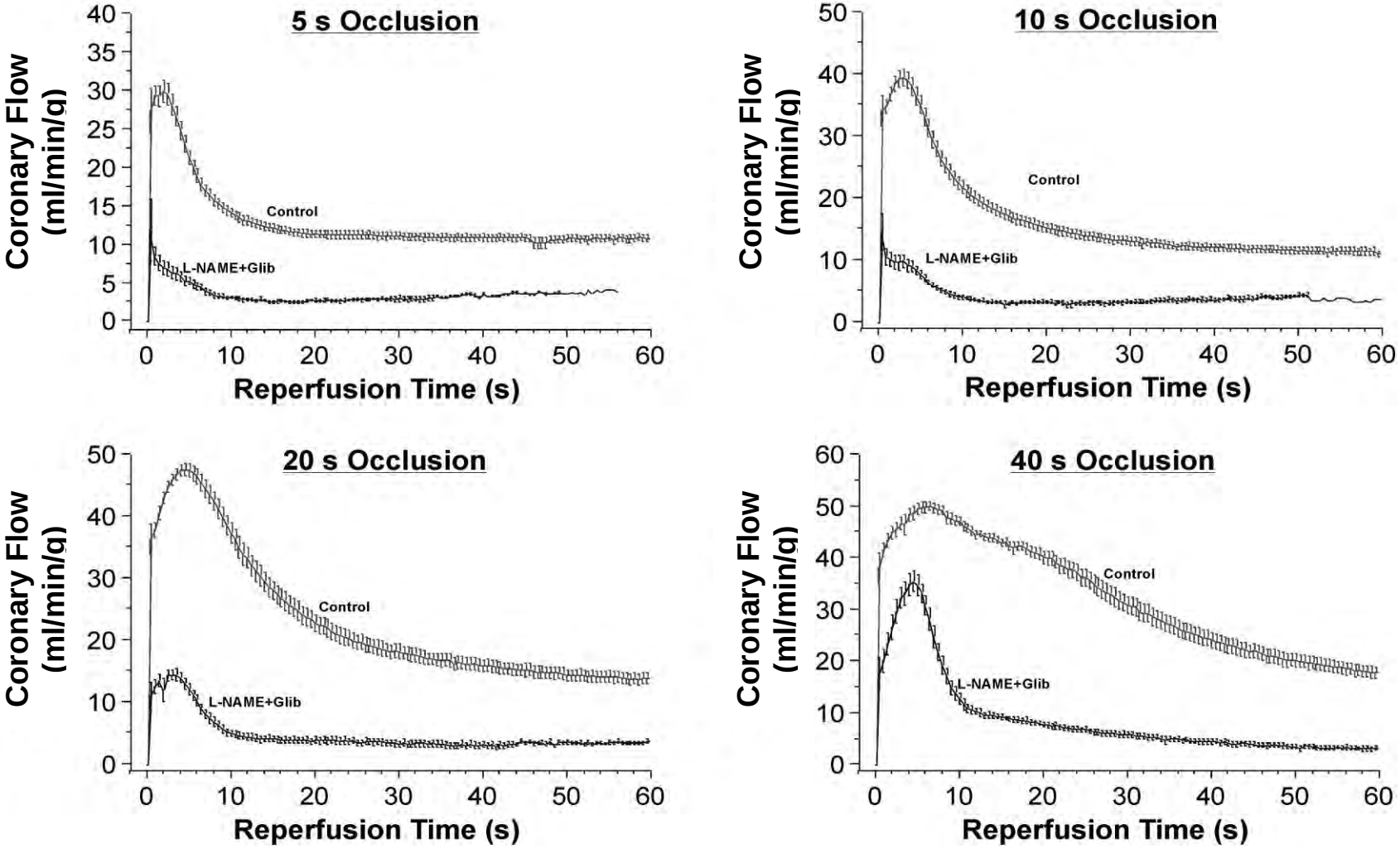

Figure 4.5 Effects of simultaneous NO synthase and K_{ATP} channel inhibition with *L*-NAME + glibenclamide (*L*-NAME + Glib) on hyperaemic response to A) 5 s, B) 10 s, C) 20 s, and D) 40 s occlusions. All values are means ± s.e.mean (n=6).

Effects of A_{2A} adenosine receptor antagonism and cyclooxygenase inhibition on reactive hyperaemia responses

The A_{2A} selective antagonist SCH58261 modestly attenuated peak hyperaemic flow following 5 and 10 s occlusions, but was largely ineffective following 20 and 40 s occlusions (Figs 4.2 and 4.6). However, repayment flow over 1 min of reperfusion was significantly reduced by SCH58261 after all periods of occlusion (Fig. 4.2B). The cyclooxygenase inhibitor indomethacin modestly limited peak hyperaemic flow responses to 5 and 10 s occlusion but was ineffective after 20 and 40 s occlusions (Figs 4.2 and 4.7). Indomethacin generally failed to modify the more prolonged dilation following the initial peak response (Fig. 4.7). Co-infusion of SCH58261 and indomethacin with *L*-NAME and glibenclamide (Fig. 4.8) was no more effective in limiting hyperaemic responses than *L*-NAME + glibenclamide (Fig. 4.5).

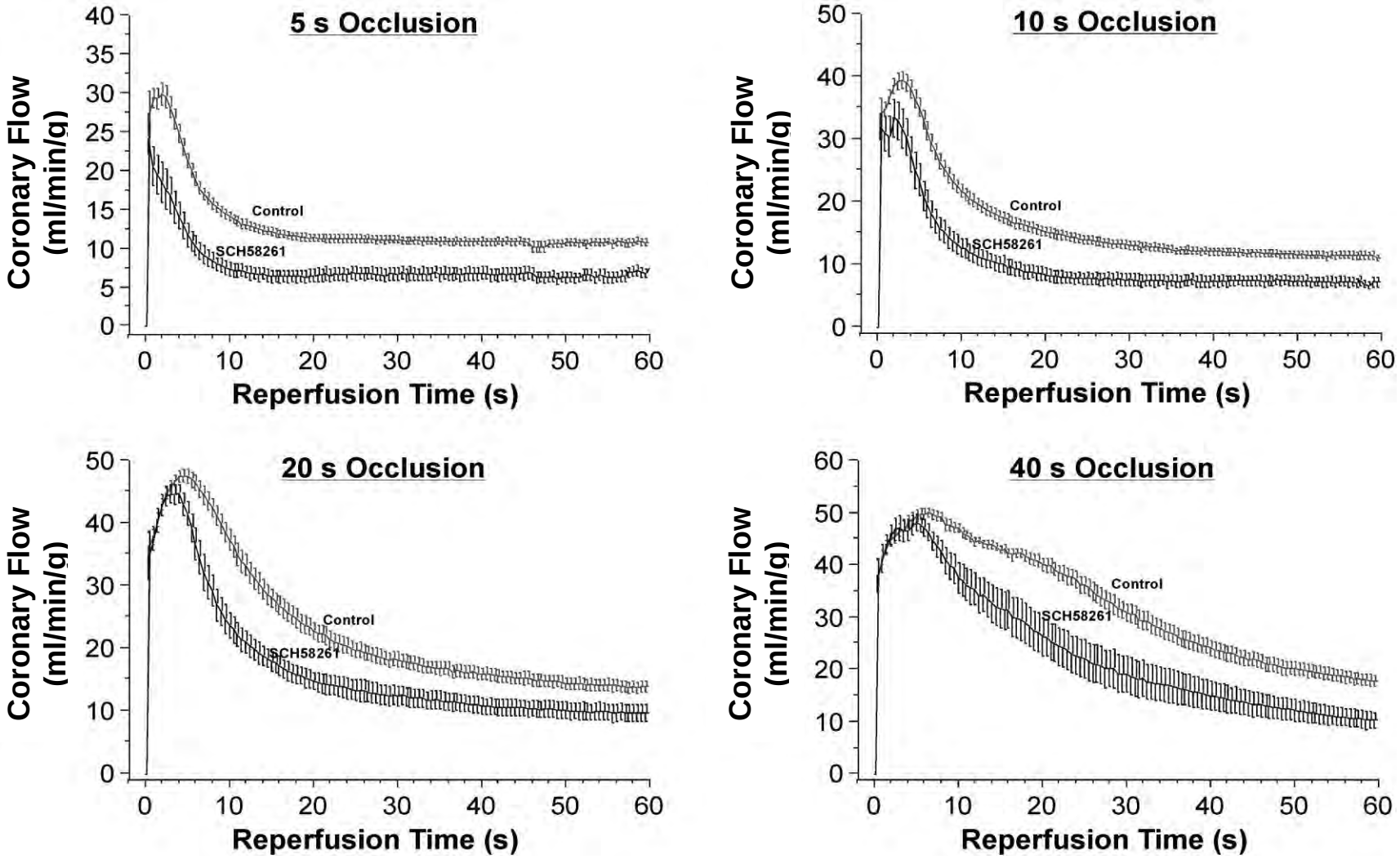

Figure 4.6 Effects of A_{2A} adenosine receptor antagonism with SCH58261 on hyperaemic response to A) 5 s, B) 10 s, C) 20 s, and D) 40 s occlusions. All values are means ± s.e.mean (n=8).

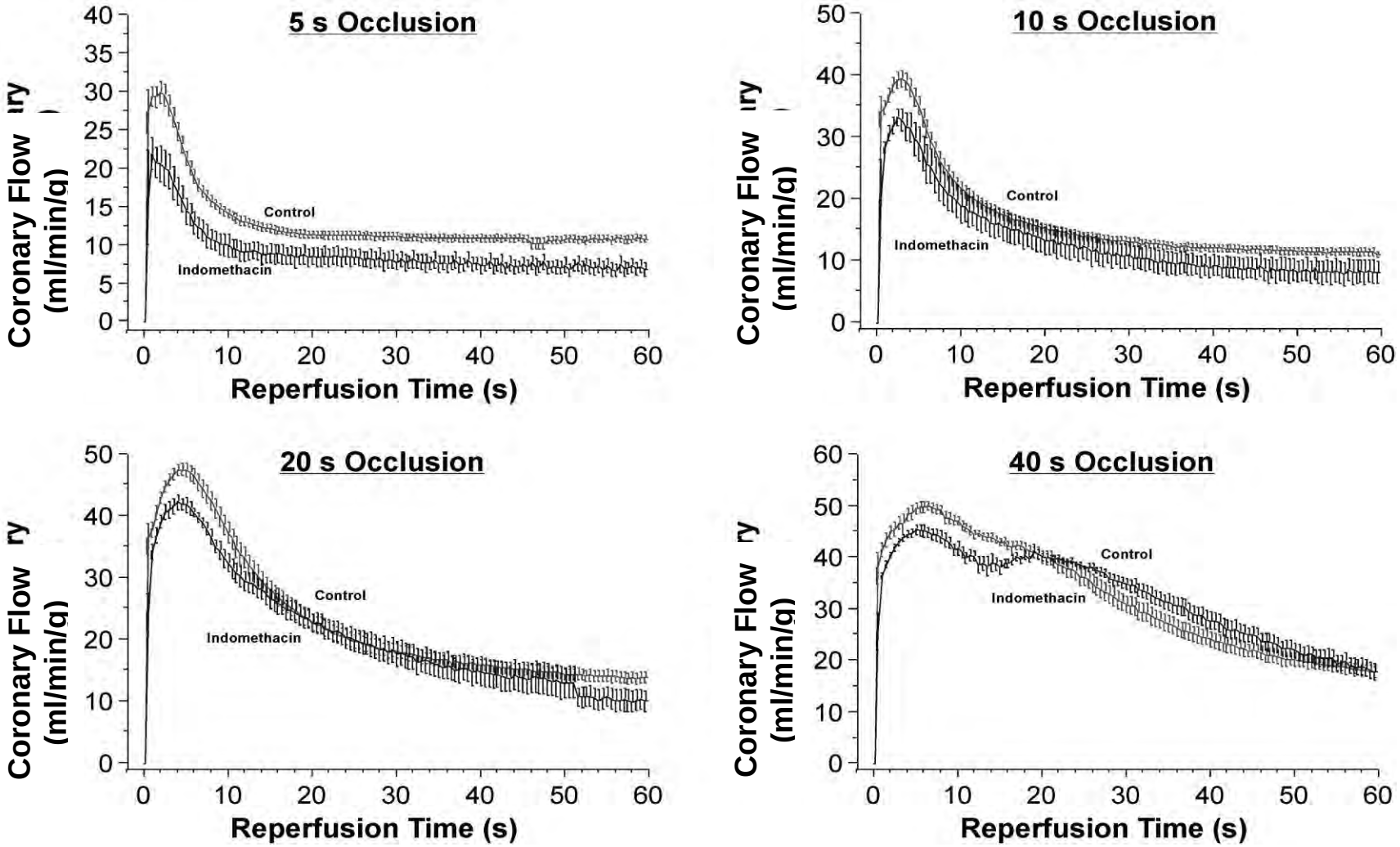

Figure 4.7 Effects of cyclooxygenase inhibition with indomethacin on hyperaemic response to A) 5 s, B) 10 s, C) 20 s, and D) 40 s occlusions. All values are means ± s.e.mean (n=6).

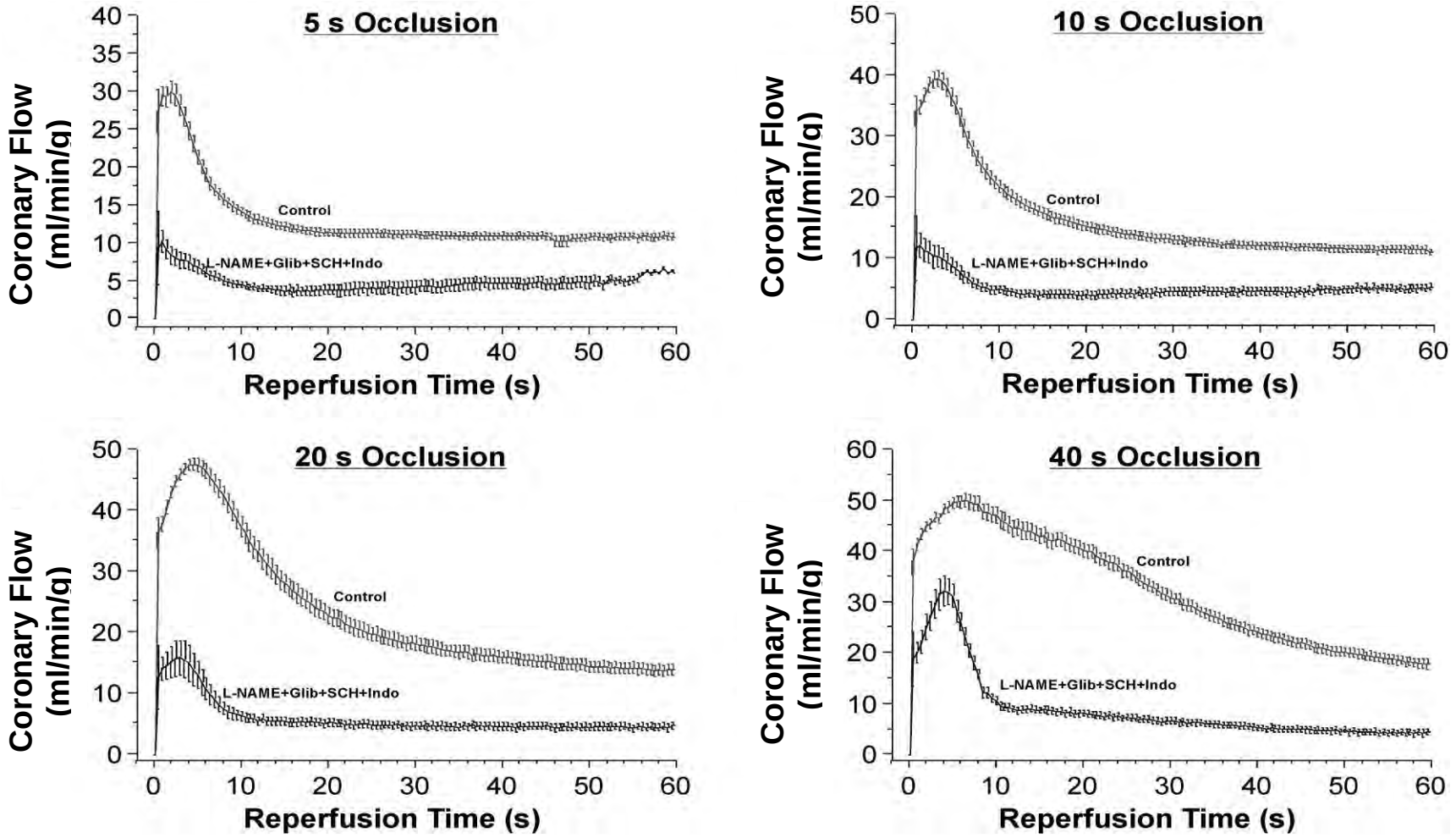

Figure 4.8 Effects of simultaneous NO synthase, K_{ATP} channel, A_{2A} adenosine receptor, and cyclooxygenase inhibition with *L*-NAME + glibenclamide + SCH58261 + indomethacin (*L*-NAME + Glib + SCH + Indo) on hyperaemic response to A) 5 s, B) 10 s, C) 20 s, and D) 40 s occlusions. All values are means ± s.e.mean (n=8).

Relative roles of NO and K_{ATP} channels in hyperaemic responses to brief (5 s) and prolonged (40 s) occlusions

By assuming that hyperaemic flows in the presence of inhibitors of NO synthase or K_{ATP} channels reflect NO and K_{ATP} independent responses, it is possible to calculate the dependent and independent components of hyperaemic responses to different periods of occlusion (Fig. 4.9). The data shown in Fig. 4.9A demonstrate that K_{ATP}-dependent mechanisms are of chief importance in mediating the initial hyperaemic response to brief coronary occlusion. The role of NO-dependent processes in initiating initial dilation is negligible. However, the role of NO-dependent dilation increases during the post-occlusion period such that it is of chief importance from ~10-20 s post-occlusion. Both K_{ATP} and NO dependent processes then contribute equally to coronary flow control at these later times. The situation changes with longer (40 s) occlusion (Fig. 4.9B), with the initial hyperaemic response being almost entirely independent of NO and K_{ATP} channels. The K_{ATP} and NO dependent mechanisms become increasingly important after the initial 5-10 s post-occlusion after which they become predominant control mechanisms (Fig. 4.9B).

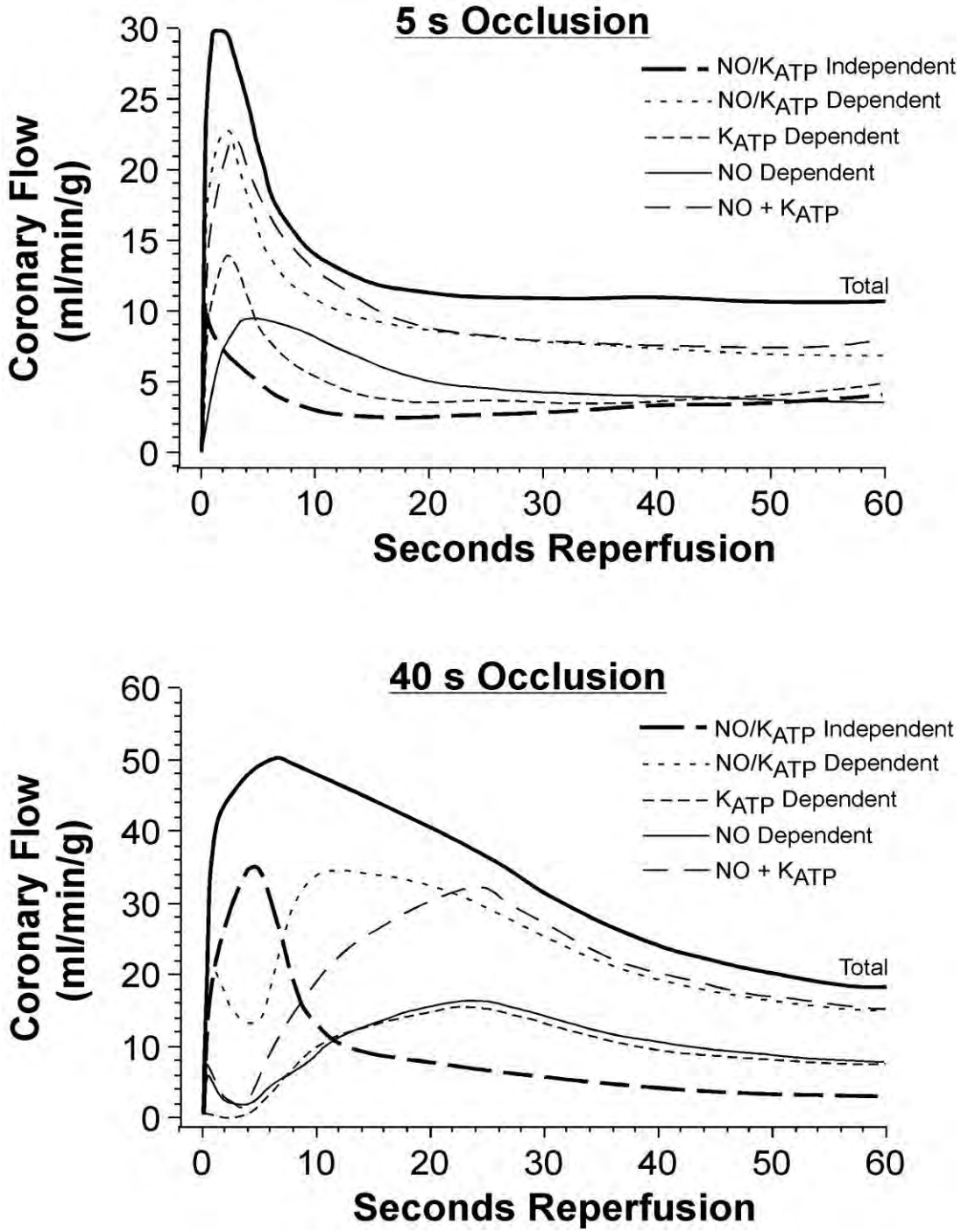

Figure 4.9 Relative contributions of NO and K_{ATP} channel-dependent and independent mechanisms to control of coronary flow during reactive hyperaemic responses to A) brief 5 s occlusion, and B) prolonged 40 s occlusion. Data were calculated from hyperaemic responses in untreated (Total), *L*-NAME treated (NO independent flow), glibenclamide treated (K_{ATP} channel independent flow), and *L*-NAME + glibenclamide treated hearts (NO and K_{ATP} channel independent flow). The NO and K_{ATP} channel dependent responses were calculated by subtraction of independent flow from total flow responses. Also shown is the summated NO dependent and K_{ATP} channel dependent responses (from the individual effects of *L*-NAME and glibenclamide, respectively), demonstrating agreement between individual and combined treatments throughout hyperaemia following brief occlusion, and for the later stages of hyperaemia following prolonged occlusion.

V. DISCUSSION

The present data demonstrate that mechanisms contributing to coronary reactive hyperaemic responses differ dependent upon the period of occlusion. Specifically, our findings indicate that K_{ATP} channels mediate the majority of the initial hyperaemic response to brief coronary occlusions (5-10 s), with both K_{ATP} channels and NO contributing equally to later dilation. In contrast, with more prolonged occlusion (eg. 40 s), the initial peak flow response is mediated primarily by K_{ATP} channel and NO independent processes, whereas these two mechanisms do contribute equally to later dilation. There is evidence for compensatory changes in K_{ATP} and/or NO dependent dilation during the early stages of the hyperaemic response, and for a modest role for A_{2A} adenosine receptors in hyperaemia.

Regulation of basal coronary tone

There is little information regarding control of resting coronary tone in the increasingly studied mouse heart. Our data (Table 4.1) indicate that NO and K_{ATP} channels contribute equally (and in an additive manner) to basal coronary tone, and that adenosine and prostanoids play a role in control of resting flow via NO and/or K_{ATP} channel dependent processes (since effects of A_{2A} and cyclooxygenase inhibition were not additive with NO and K_{ATP} channel inhibition). Conflicting data have been acquired regarding contributions of K_{ATP} channels, NO, adenosine receptors and prostanoids to maintenance of resting coronary tone. A majority of studies verify K_{ATP} channel inhibition reduces basal coronary flow [153,648,652,658-665], with a small number finding no effects of K_{ATP} inhibition on basal coronary tone [100,666]. Similarly, inhibition of NO synthase has been found to limit basal coronary flow in different models [201,647,650-653,666-677], with a small number of studies finding no effect of NO synthase blockade on resting coronary tone in animal models and humans [100,678-

680]. Reasons for these discrepancies are unclear. Species differences do not appear to be key since evidence for and against these mediators have been acquired in the same species and between species. Generally the same inhibitors (eg. glibenclamide, *L*-NAME) are employed. One key issue relates to "redundancy" and compensation by other dilatory control mechanisms when one process is pharmacologically blocked. Thus, it is feasible lack of support stems from compensatory changes in other processes. Nonetheless, this does not explain why individual inhibitors have been found to reduce resting flow in multiple models.

Inhibition of prostanoid synthesis has also been shown to limit basal coronary tone in some models [201], and specifically in hearts from subjects with coronary artery disease [108,200,202] and animal models of coronary artery disease [681,682]. However, a majority of studies in non-diseased models reveal no effects of cyclooxygenase inhibitors on resting coronary flow [205,650,670,672]. Reasons for these discrepancies are also unclear, but tend to suggest a minimal role for prostanoids in healthy hearts. In addition, it is possible that indomethacin exerts direct coronary effects, as suggested by Edlund *et al.* [108]. This might explain the lack of effect of other cyclooxygenase inhibitors in mouse heart [650] *vs.* reduced flow with indomethacin observed here (Table 4.1).

In terms of adenosine A_2 receptors, there are also varied reports on their role in regulating basal coronary tone, with a preponderance of studies failing to identify a key role for endogenous adenosine [100,105,683,684]. Nonetheless, there is some support for such a role in humans and also various animal models [109,685-688]. The data of Tayama and colleagues [689] also suggest that compensatory changes may cloud effects of adenosine antagonism, which does limit basal flow when NO control is inhibited.

An issue that should not be overlooked when studying small rodent hearts is the difference in mass-specific metabolic rate, which may impact on control of basal tone. As body mass declines mass-specific metabolic rate (and heart rate) rises in a regular fashion. Thus, in mouse the myocardial VO_2 is1.6-fold higher than in rat, 2.4-fold higher than in guinea pig [629]. The greater apparent contribution of adenosine receptors to maintenance of resting coronary tone in the mouse may stem in part from a higher resting metabolic rate.

Roles of K_{ATP} channels and NO in reactive hyperaemia

Prior studies present evidence for NO [647,649-651,690] and K_{ATP} channel involvement [643-646,648,652] in coronary reactive hyperaemia in different species. The present findings reveal that with brief occlusion (5-10 s), K_{ATP} channel dependent dilation (blocked by glibenclamide) accounts for 60-70% of initial hyperaemia (Figs 4.4 and 4.9). Most prior studies documenting effects of K_{ATP} channel blockade observe substantial reductions in both peak flows and flow repayment (eg. [643,646,652]), consistent with our observations. In contrast, NO (blocked by *L*-NAME) plays little role in initial hyperaemia, contributing primarily to the subsequent dilation over the remaining min of reperfusion (Figs 4.3 and 4.9). Blockade of both processes almost abolishes hyperaemic responses to 5-20 s. Nonetheless, a significant initial hyperaemic dilation is observed in the presence of the inhibitors following all periods of occlusion. As the period of transient occlusion is increased to 20 and 40 s, the efficacy of *L*-NAME and glibenclamide declines such that each individually reduces dilation by only 10-15% (with 40 s occlusion), and collectively by ~25%. The changes with occlusion period are diagrammatically presented in Fig 4.9. These changes might be argued as evidence for an enhanced role for K_{ATP} and NO independent mechanisms in responses to more prolonged occlusion. This is consistent with prior work indicating that K_{ATP} channels

contribute to hyperaemia after short (30 s) but not long (300 s) occlusions [654]. However, an alternate explanation is that this shift may simply reflect an impaired ability of inhibitory agents employed (glibenclamide and *L*-NAME) to overcome dilatory responses mediated with longer occlusion.

It is interesting to note that effects of *L*-NAME and glibenclamide together are more effective than the sum of individual effects of these inhibitors. For example, while each reduces hyperaemic flow by ~20% after 20 s occlusion, the two agents combined reduce flow by almost 70% (Fig. 4.5). This is most readily explained by compensatory changes in either NO-dependent or K_{ATP} channel dependent flow when the other process is inhibited. There is support for such compensatory changes in dilatory pathways in other models (eg. [647,691]). An alternate possibility is incomplete inhibition of a dilatory mechanism which is both K_{ATP} and NO dependent. Infusion of the two agents together is therefore more inhibitory than each individually, resulting in an apparent synergism in inhibiting dilation. There is some support for this from recent studies. Prior studies [120,136] present evidence coronary endothelial K_{ATP} channels trigger NO release in response to adenosine. Similarly, Deka *et al.* [692] found endogenous NO contributes to coronary dilatory responses to K_{ATP} openers. Conversely, Miyoshi *et al.* [693] present evidence NO activates vascular K_{ATP} channels to mediate dilation. Thus, synergistic actions of *L*-NAME and glibenclamide may stem from enhanced antagonism of a NO and K_{ATP} dependent dilatory mechanism in the presence of both inhibitors. If this was the case, one would predict this synergism would be observed after all periods of occlusion or would become increasingly evident with longer occlusions (when each individual inhibitor may become less capable of blocking effects of enhanced levels of dilation). Indeed, we find more evidence for this effect after the longer 40 s occlusion compared with shorter occlusion periods (Figs 4.3-4.5,

and 4.9). Thus, we cannot exclude this explanation. However, while almost identical effects of K_{ATP} channel and NO synthase blockade after 20 and 40 s occlusions are consistent with this possibility, this cannot explain selective effects of K_{ATP} channel inhibition on initial peak flows following 5-10 s occlusions.

In contrast to studies supporting a key role for NO, Shinoda *et al.* [654] found no effect of *L*-NAME on reactive hyperaemia whereas K_{ATP} channels did appear to play a central role. Similarly, other studies find no effect of NO antagonism on reactive hyperaemic flows [667]. Kingsbury *et al.* [652] found K_{ATP} channels are major mediators of reactive hyperaemia in guinea pig hearts, with little role for either NO or prostaglandins. Similarly, Gattullo *et al.* [690] found no evidence for a role for NO, though it is again possible that lack of effect of NO inhibition may result from compensatory changes in other dilatory mechanisms.

Roles for adenosine and prostanoids in mediating hyperaemic responses

Adenosine has received considerable attention as an endogenous mediator of hyperaemic responses [105,630,646,649,652,653,694-697]. Early studies with relatively poor antagonist treatments showed reactive hyperaemic responses were mediated in part by adenosine [105,694,695], with flow repayment and duration of reactive hyperaemia reduced up to 30%, and with minor (if any) changes in peak flows. More recent studies support a role for adenosine in mediating flow repayment but not peak dilation [646,654], or support a minor role in mediating peak dilation [653]. Our data are largely consistent with these observations, supporting a modest effect of the A_{2A} antagonist SCH58261 on peak flow with brief (but not prolonged) occlusion, and significant reductions in flow repayment after all periods of occlusion (Figs 4.2 and 4.6). Most studies support a 30-35% reduction in hyperaemic flow repayment with adenosine antagonism in different models [105,649,652,653,656,657,694,695,697], in good

agreement with the current reductions in flow repayment after 10-40 s occlusions (Fig. 4.2B). However, these estimates generally do not take into account potential compensation during adenosine blockade.

Gryglewski and colleagues noted an apparent shift in the importance of adenosine *vs.* NO in mediating hyperaemia with short *vs.* long occlusions [657]. Specifically, hyperaemia after brief (1-10 s) occlusion was NO-dependent whereas both adenosine and NO played a role after longer (≥20 s) occlusions. Otomo *et al.* have presented evidence that adenosine and NO are both involved in reactive hyperaemia, but that adenosine only plays a substantial role in the presence of significant ischaemia [653]. The data of Kanatsuka *et al.* indicates adenosine receptors are particularly important in smaller coronary vessels [646]. Others have acquired evidence implicating adenosine in at least a fraction of the reactive hyperaemic response *in vivo* [630]. Yamabe *et al.* acquired support for roles for both adenosine and NO, and found the two compounds apparently exert additive effects [698].

We and others have shown adenosine mediated coronary dilation involves endothelial (NO-dependent) and direct smooth muscle (K_{ATP} dependent) responses [120,136,675]. Since effects of K_{ATP} and NO synthase inhibition far exceed those of A_{2A}AR antagonism, processes additional to A_{2A} receptor activation must trigger K_{ATP} and NO-mediated hyperaemia. Since indomethacin failed to substantially modify hyperaemia, these other mediators appear unrelated to prostanoids. One possibility is flow-dependent activation of NO (and possibly K_{ATP} channel) dependent dilation. this may indeed account for the NO and K_{ATP} dependent elevations in flow following the initial peak dilation.

A number of studies also support our observation (Figs 4.2 and 4.7) that prostanoids play a negligible role in reactive hyperaemia [649,652,654-657], in

agreement with the current findings in mouse heart (Figs 4.2 and 4.7). While inconclusive, the pattern of hyperaemia in the presence of indomethacin does support some minor role for endogenous prostanoids during the early (initial 10 s) component of dilation after all periods of occlusion (Fig. 4.7). However, this pattern change is not reflected in significant changes in peak flow or overall flow repayment.

Conclusions

In summary, the present data indicate that mechanisms contributing to coronary reactive hyperaemia vary with the period of prior occlusion and also during the hyperaemic response itself. Vascular K_{ATP} channels mediate the major fraction of initial peak hyperaemic responses to brief (5-10 s) occlusions whereas with longer (20-40 s) occlusions other K_{ATP} (and NO) independent mechanism play a primary role. In terms of the more prolonged elevation in flow following the peak hyperaemic responses, both K_{ATP} channel and NO dependent mechanisms contribute equally, and are matched by unidentified mechanisms distinct from these signalling elements. We also acquire evidence for compensatory changes in either K_{ATP} channel or NO dependent dilation when only one of these is inhibited following prolonged coronary occlusion, which can complicate interpretation of findings using inhibitors of individual dilatory pathways. Finally, our data reveal that adenosine A_{2A} receptors may play a modest role in mediating hyperaemic responses whereas prostanoids appear to be of little (if any) importance.

Limitations

It is apparent that other mechanisms are involved in mediating reactive hyperaemic responses, since we were unable to completely abolish coronary flow reserve using a co-treatment approach with K_{ATP} channel, NO, prostanoid and A_2AR inhibition. One potential mechanism that has not been assessed in this study or previously is the role of EDHF in reactive hyperaemic responses. EDHF-mediated dilation appears to play an important role in the physiological regulation of coronary blood flow in resistance vessels both *in vitro* and *in vivo* [699,700]. Moreover, accumulating evidence indicates that NO and EDHF are intimately connected, in that normal NO production exerts negative feedback inhibition on EDHF-induced vasodilation [701,702]. As suggested by Nishikawa *et al.* EDHF may be a "second line of defence" when the NO pathway is compromised. Therefore EDHF-mediated responses may be augmented in the presence of impaired endothelial NO production. Collectively, these findings hint that EDHF may play a part in reactive hyperaemic responses in our murine model, adding to the complexity of observed compensatory effects already seen between K_{ATP} channel and NO inhibition. It is acknowledge that these additional studies are of great importance and are therefore a priority in future studies.

CHAPTER 5

CORONARY FUNCTION AND ADENOSINE RECEPTOR-MEDIATED RESPONSES IN ISCHAEMIC-REPERFUSED MOUSE HEART

I. ABSTRACT

This study assessed the impact of ischaemia-reperfusion (I/R) on coronary function, and the role of endogenous adenosine in modifying post-ischaemic vascular function in asanguinous hearts. Vascular function was studied in Langendorff perfused mouse hearts subjected to 20-25 min global normothermic ischaemia and 30 min reperfusion. Ischaemia altered the dependence of flow on work-rate observed in normoxic hearts, and inhibited reflow by mechanisms additional to diastolic compression. Coronary responses were selectively reduced: 2-chloroadenosine and ADP dilated with pEC_{50}s of 8.4±0.1 and 7.4±0.1 in non-ischaemic hearts *vs.* 7.7±0.1 and 7.1±0.1 after 20 min ischaemia ($P<0.05$). Sensitivity was further reduced after longer (25 min) ischaemia. Responses to nitroprusside were unaltered. Competitive NO-synthase antagonism (50 μM *L*-NAME) reduced sensitivities to 2-chloroadenosine and ADP up to 10-fold in normoxic hearts, and thus eliminated the inhibitory effects of I/R. K_{ATP} blockade with 5 μM glibenclamide impaired sensitivity pre- and post-ischaemia, and did not eliminate the inhibitory effects of I/R. A_1AR antagonism with 100 nM 8-cyclopentyl-1,3-dipropylxanthine worsened effects of ischaemia on coronary sensitivity to 2-chloroadenosine. A_{2A}AR antagonism with 100 nM 8-(3-chlorostyryl)caffeine reduced post-ischaemic flow by 50%, yet paradoxically enhanced post-ischaemic contractile recovery. These data show that ischaemia modifies vascular control and impairs NO- *vs.* K_{ATP}-dependent coronary dilation in an asanguinous model. Endogenous adenosine protects against vascular dysfunction via A_1 receptors, and determines coronary reflow via A_{2A} receptors. However, intrinsic A_{2A} activation apparently worsens contractile dysfunction.

II. INTRODUCTION

While research into the pathogenesis of ischaemic injury has focussed on cardiac myocytes, increasing evidence supports a role for coronary injury [2,3,703]. Ku first documented impaired endothelium-dependent responses in post-ischaemic coronaries [704]. Subsequent studies verify vascular dysfunction in a variety of models and species [132,282,331,336,337,340,348], including humans [705]. Dysfunction is an early event, evident after brief ischaemia [132,285], and endothelial cells appear the primary site of injury.

Although the pathogenesis of vascular injury remains incompletely understood, oxidant-mediated NO inactivation is implicated [356,706]. Radical generation reduces NO-synthase activity [358] and basal and agonist-stimulated NO release [359]. Proximity of endothelial cells to sites of radical generation may render them susceptible. Moreover, they appear less tolerant of oxidant stress compared with other cells, likely due to low anti-oxidant content [707,708]. Compounding this, endothelial cells generate radicals [360]. Leukocytes are also thought to play a major role through radical release and capillary obstruction [3,396,709,710]. However, impaired reflow and coronary dysfunction occurs in asanguinous hearts [337,340,347,397], implicating non-leukocyte dependent mechanisms. The impact of ischaemia-reperfusion on coronary function in the absence of blood has been the focus of few studies [347]. The primary goal of the present study was to examine the impact of ischaemia-reperfusion on coronary function in an asanguinous model.

A secondary goal was to examine the role of adenosine in modifying post-ischaemic coronary function. There is evidence for reduced vascular injury through adenosine-mediated inhibition of neutrophil activation and radical formation [537,711,712] and also for impaired vascular injury with adenosinergic therapy in

humans [713,714]. While it is generally thought these responses are dependent on blood elements and A_2 and A_3ARs [537,711,712], there is some evidence for A_1-mediated vascular protection [715] and reduced vascular injury with interventions involving A_1 receptors (eg. preconditioning) [716]. Thus, the roles of endogenous adenosine in controlling post-ischaemic coronary function were assessed.

III. MATERIALS AND METHODS

Langendorff perfused murine heart model

Hearts were prepared for functional and vascular studies as outlined in Chapter 2.

Experimental protocol

Functional responses to ischaemia

To assess functional responses to ischaemia, hearts were subjected to 20 (n=8) or 25 min (n=11) global normothermic ischaemia followed by 30 min reperfusion. To identify effects of coronary dilation, and receptor-mediated effects of endogenous adenosine, hearts subjected to 20 min ischaemia were treated with 5 nM of the dilatory A_{2A} agonist 2-[p-(2-carboxyethyl) phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680, n=7) or 150 nM of A_1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, n=7) prior to and for 15 min following ischaemia. Hearts subjected to 25 min ischaemia were treated with 5 nM of A_{2A} agonist CGS21680 (n=7) or 100 nM of A_{2A} antagonist 8-(3-chlorostyryl)caffeine (n=7), prior to and following ischaemia.

Relationships between diastolic pressure, work-rate and coronary flow

To assess the impact of diastolic vascular compression on coronary flow, normoxic hearts were perfused with an intra-ventricular balloon in place (n=6). Balloon volume was increased using a calibrated Hamilton 500 µl threaded syringe (Hamilton Co., Reno, NV, USA) to give end-diastolic pressures of 5-60 mmHg [629]. After 2 min stabilization at each volume, diastolic pressure and coronary flow were measured. This was first performed under control conditions, after which balloon volume was reduced to baseline levels and hearts stabilised for 15 min prior to maximal coronary dilation with 5 nM CGS21680. After 10 min the protocol was repeated. The relationship between

diastolic pressure and coronary flow was then determined. Data for flow and diastolic pressure during initial 5 min reperfusion for the ischaemic hearts described above were also plotted with these data. Early reflow occurs in maximally or near maximally dilated vessels and is therefore largely unrelated to metabolic rate. There are also no differences in systolic compression between groups at this time. Thus, early reflow is determined primarily by mechanical effects of diastolic compression.

To examine dependence of flow on myocardial work-rate, coronary flow rate and cardiac work (indicated by the rate-pressure product), were determined in a group of normoxic hearts (n=19) beating at varying rates. The rate-pressure product (heart rate x ventricular developed pressure) was employed as this is linearly related to oxygen consumption under varying conditions [717], and accounts for contributions of heart rate and pressure work on work-rate. Hearts were stabilised at intrinsic rate (381±11 beats.min^{-1}), then subjected to 5 min periods of pacing at 420, 480, and 540 beats.min^{-1}. Flow and function were measured at the end of each period. A similar experiment was undertaken in hearts subjected to 20 min ischaemia and 30 min reperfusion (n=8). After ischaemia-reperfusion function and flow were assessed at 400, 420, 480 and 540 beats.min^{-1}. Data for flow and function (between 10 and 30 min reperfusion) for the experimental groups were also plotted to contrast relationships in the different groups. We examined later reperfusion times since post-ischaemic hyperaemia has abated and hearts recovered substantial cardiac function at these times. While the latter hearts were not subjected to changes in pacing rate, the data permit assessment of the qualitative nature of the relationships between flow and work-rate (if any) in the varied experimental groups.

Vascular responses to 2-chloroadenosine, ADP and nitroprusside

To assess coronary sensitivity, hearts were treated with dilatory agonist (2-chloroadenosine, ADP or sodium nitroprusside), infused incrementally for 1-3 min at each concentration (during which vascular responses stabilised). One drug was applied per heart. Changes in aortic pressure were measured. For NO-synthase or K_{ATP} channel inhibition, infusion of 50 μM nitro-*L*-arginine methylester (*L*-NAME) or 5 μM glibenclamide was initiated 10 min prior to concentration-response curves. To assess the role of A_1ARs in modifying dysfunction, responses to 2-chloroadenosine were studied in hearts subjected to 20 min ischaemia in the presence of 150 nM DPCPX (applied prior to ischaemia and for the initial 15 min of reperfusion).

IV. RESULTS

Functional effects of ischaemia-reperfusion

Coronary flow initially recovered to above pre-ischaemic levels during reperfusion following 20 min ischaemia (Fig. 5.1A). This hyperaemia was absent after 25 min ischaemia (Fig. 5.2A). Flow through reperfusion was lower in the 25 *vs.* 20 min ischaemic group (Fig. 5.2A). Recoveries for diastolic (Figs 5.1B and 5.2B) and systolic pressures (Figs 5.1C and 5.2C) were incomplete following ischaemia. Diastolic pressure was significantly higher throughout much of the reperfusion period in the 25 *vs.* 20 min group (Fig. 5.2B) whereas systolic pressure only differed at the final time point (Fig. 5.2C). Coronary dilation with CGS21680 failed to alter early reflow but modestly increased flow during late reperfusion (Figs 5.1A and 5.2A). The dilator failed to modify contractile recovery from 20 (Fig. 5.1) or 25 min ischaemia (Fig. 5.2).

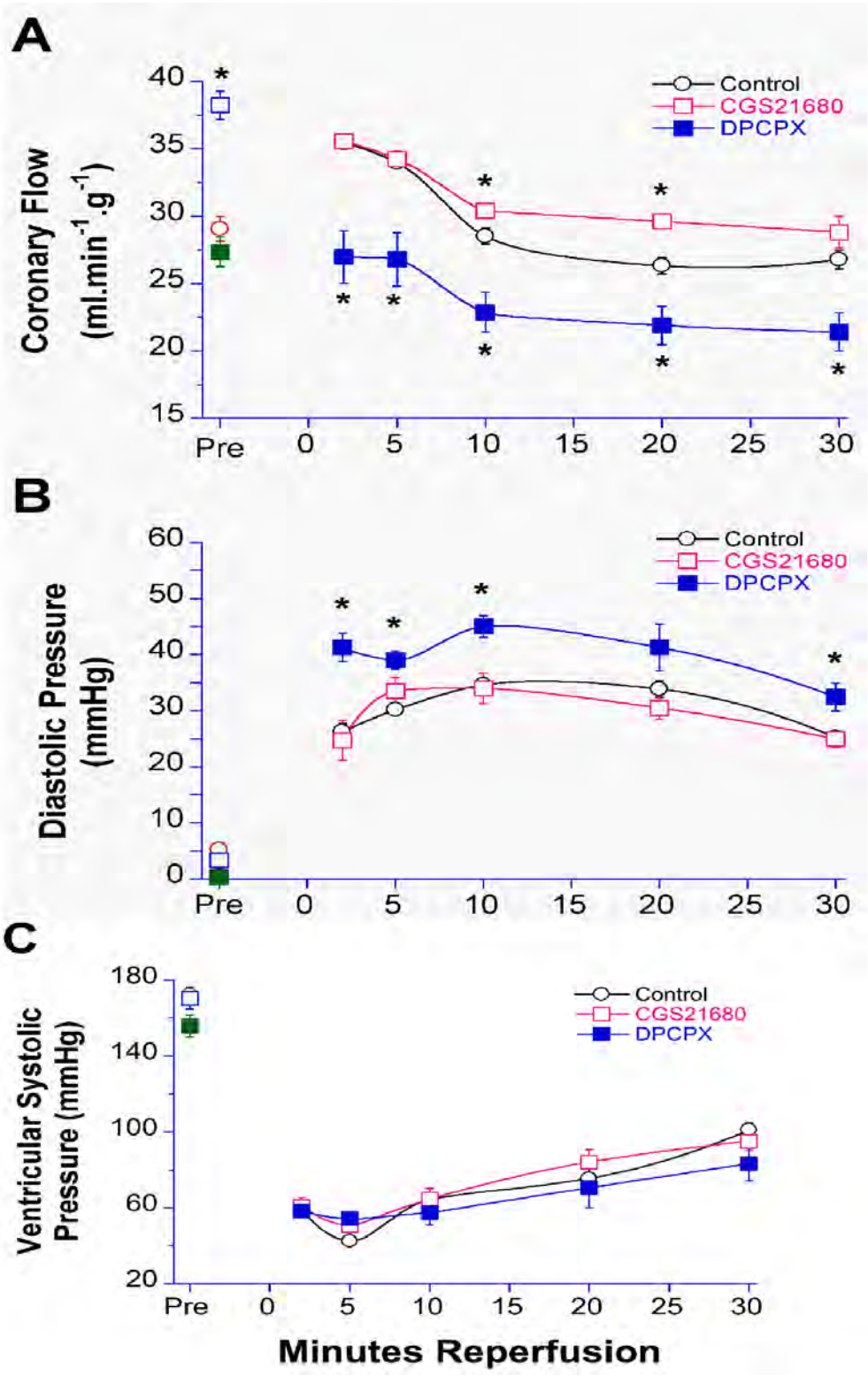

Figure 5.1 Recoveries for A) coronary flow, B) diastolic pressure, and C) systolic pressure after 20 min ischaemia. Data are shown for control hearts (n=8), and hearts treated with dilatory A_{2A} adenosine agonist CGS21680 (Dilated, n=7) or the A_1AR antagonist DPCPX, (n=7). Values are means±SEM. *, P<0.05 *vs.* untreated. Note that pre-ischaemic data points for some groups are obscured by symbols for other groups.

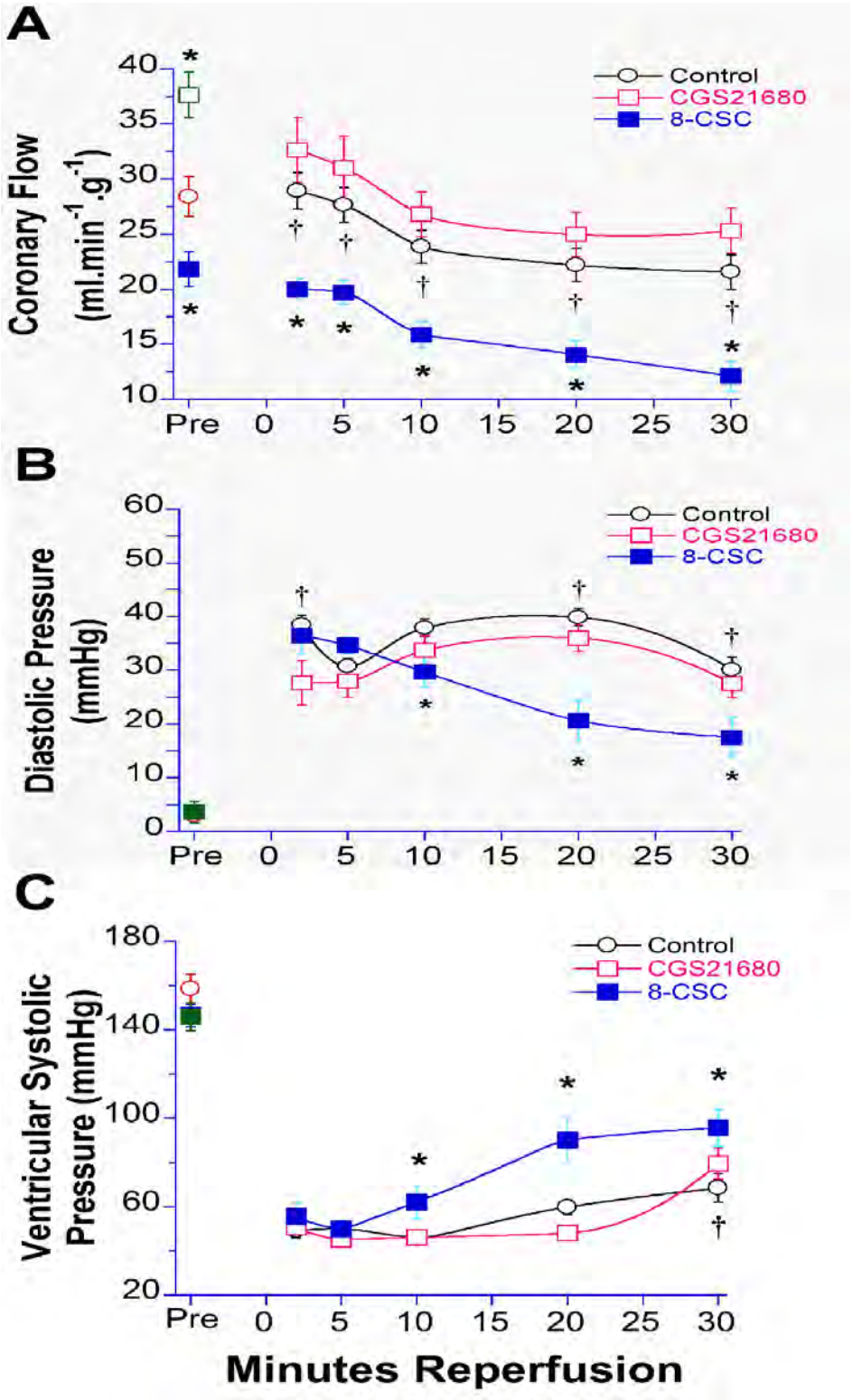

Figure 5.2 Recoveries for A) coronary flow, B) diastolic pressure, and C) systolic pressure after 25 min ischaemia. Data are shown for control hearts (n=11), and hearts treated with dilatory A_{2A} adenosine agonist CGS21680 (Dilated, n=7) or the A_{2A}AR antagonist 8-CSC (n=7). Values are means±SEM. *, P<0.05 *vs.* untreated; †, P<0.05 *vs.* 20 min control. Note that pre-ischaemic data points for some groups are obscured by symbols for other groups.

The dependence of flow on diastolic pressure (vascular compression) is depicted in Fig. 5.3A. Coronary flow displayed an inverse linear dependence on diastolic pressure in non-ischaemic hearts (Fig. 5.3A). The relation predicts a 1.5 $ml.min^{-1}.g^{-1}$ decline in sub-maximally or maximally dilated flow for every 10 mmHg increase in diastolic pressure. Coronary flow during the initial 5 min of reperfusion after 20 min ischaemia falls within the range of flows and pressures for maximally dilated non-ischaemic hearts (Fig. 5.3A). Supporting maximal dilation, CGS21680 increased pre-ischaemic flow (Table 5.1) but failed to increase early reflow relative to diastolic pressure in the 20 min group (Fig. 5.3A). In contrast, early reflow following 25 min reperfusion was much lower than predicted from data for flow and diastolic pressure in non-ischaemic hearts (and hearts subjected to 20 min ischaemia) (Fig. 5.3A).

Dependence of flow on cardiac work is depicted in Fig 5.3B. These data reveal that coronary flow increases in a linear manner with the rate-pressure product when work-rate is increased by varying heart rate. Regression analysis of data for non-ischaemic hearts yielded the relation: flow = -1.71 + 0.54 x rate-pressure product ($r=0.71$). In contrast, in post-ischaemic hearts the pacing protocol elevated work-rate but failed to substantially alter coronary flow (flow = 23.91 + 0.10 x rate-pressure product, $r=0.49$). A similar "flat" relation was observed for post-ischaemic hearts not subjected to the pacing protocol. While data for intrinsic function in these hearts was not acquired during pacing, the findings demonstrate qualitatively different relationships between cardiac function and coronary flow post-ischaemia. Indeed, there was a paradoxical trend towards higher flows at lower work rates post-ischaemia (Fig. 5.3B). Flow relative to work-rate was also higher than predicted from non-ischaemic data, and lower in 25 *vs.* 20 min ischaemic hearts.

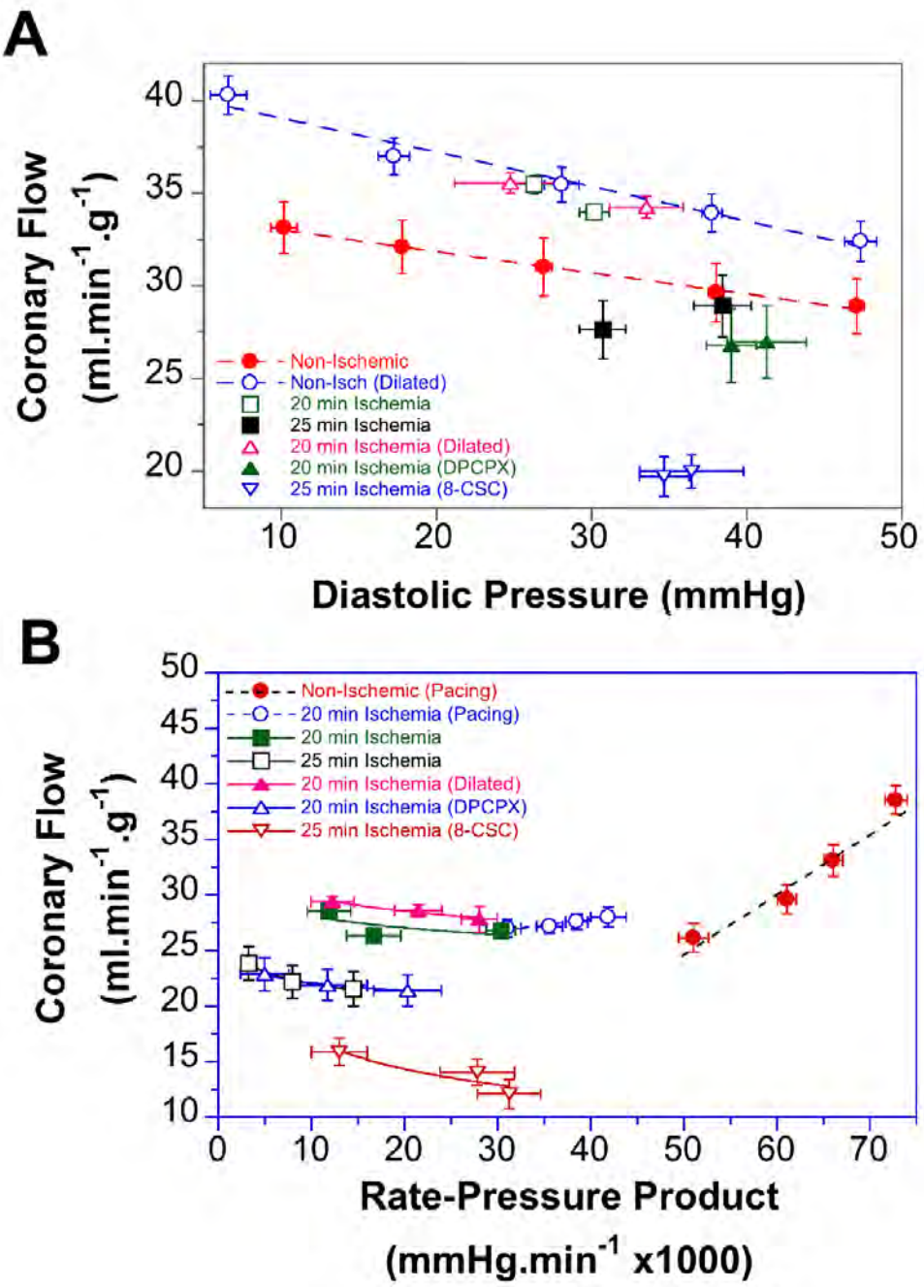

Figure 5.3 A) Dependence of coronary flow on diastolic pressure in normoxic hearts (n=6) untreated or treated with a maximal dilatory concentration of CGS21680 (5 nM), and during reperfusion following 20 or 25 min ischaemia. B) Relationship between coronary flow and work-rate (reflected by the rate-pressure product) in normoxic hearts subjected to electrical pacing (n=19), post-ischaemic hearts subjected to 20 min ischaemia and 30 min reperfusion before assessing effects of pacing (n=8), and in post-ischaemic hearts paced at 400 beats.min^{-1} and subjected to 20 (n=8) or 25 min (n=11) ischaemia, 20 min ischaemia with CGS21680 (Dilated, n=7) or DPCPX (n=7), or 25 min ischaemia with CGS21680 (Dilated, n=7) or 8-(3-chlorostyryl)caffeine (8-CSC, n=7). All values are means±SEM.

Table 5.1 Pre-ischaemic functional parameters

	Control	CGS21680	8-CSC	DPCPX
Coronary Flow ($ml.min^{-1}.g^{-1}$)	29.1±0.9	38.2±1.0	21.8±1.4*	27.4±1.1
Diastolic Pressure (mmHg)	3±2	4±1	2±1	2±1
Rate-Pressure Product ($mmHg.min^{-1}$ x1000)	66.8±1.7	66.8±2.8	60.0±2.6	62.2±2.4
+dP/dt ($mmHg.s^{-1}$)	6155±387	5789±142	5272±278*	5728±453
-dP/dt ($mmHg.s^{-1}$)	3958±172	3717±118	3333±181	3873±157

Normoxic function was measured immediately prior to ischaemia. Hearts were untreated (Control, n=19) or treated with 5 nM CGS21680 (n=14), 100 nM 8-(3-chlorostyryl)caffeine (8-CSC, n=7), or 150 nM antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, n=7). Values are means±SEM. * P<0.05 Control.

Effects of A_1 and A_{2A} adenosine receptor antagonism

Effects of A_1AR antagonism are shown in Fig. 5.1 Antagonism failed to alter pre-ischaemic function (Table 5.1), but modestly reduced post-ischaemic reflow (Fig. 5.1A), elevated post-ischaemic diastolic pressure (Fig. 5.1B), and reduced contractile recovery (Fig. 5.1C). DPCPX shifted relationships between reflow and diastolic pressure (Fig. 5.3A), and post-ischaemic flow and contractile function (Fig. 5.3B). Early reflow was lower than predicted based on diastolic contracture, and the relation between flow and work-rate was almost identical to that for a more severe 25 min ischaemic insult (Fig. 5.3B).

The A_{2A}-selective antagonist 8-(3-chlorostyryl)caffeine reduced pre-ischaemic coronary flow by ~7 $ml.min^{-1}.g^{-1}$ and produced a modest decline in +dP/dt (Table 5.1, Fig. 5.2A). No other functional parameters were altered. Antagonism markedly reduced post-ischaemic coronary flow by up to 10 $ml.min^{-1}.g^{-1}$ (Fig. 5.2A). Reduced reflow occurred despite significantly reduced post-ischaemic diastolic pressure (Fig. 5.2B) and enhanced contractile recovery (Fig. 5.2C).

Effects of ischaemia-reperfusion on coronary vascular responses

Ischaemia-reperfusion impaired sensitivity to 2-chloroadenosine (Fig. 5.4, Table 5.2) and endothelial-dependent ADP (Fig. 5.5, Table 5.2). Longer 25 min ischaemia further reduced sensitivity. Sensitivity to endothelial-independent SNP was unaltered by ischaemia (Fig. 5.5). Responses to 2-chloroadenosine were significantly inhibited by 50 μM *L*-NAME pre-ischaemia (Fig. 5.4, Table 5.2). Similarly, ADP responses were significantly inhibited by *L*-NAME (Fig. 5.5, Table 5.2). In post-ischaemic hearts *L*-NAME was less effective in modifying responses to 2-chloroadenosine and ADP (Figs 5.4 and 5.5, Table 5.2). Moreover, ischaemia-reperfusion failed to alter sensitivity to 2-chloroadenosine and ADP in the presence of *L*-NAME. *L*-NAME failed to modify responses to SNP pre- or post-ischaemia (Figs 5.4 and 5.5).

In contrast to reduced effects of *L*-NAME post-ischaemia, K_{ATP} channel blockade with glibenclamide significantly impaired sensitivity to 2-chloroadenosine post-ischaemia (Fig. 5.4, Table 5.2). Reflecting roles for NO release and K_{ATP} channel activation in control of resting coronary tone, *L*-NAME increased resting coronary vascular resistance by 2.0±0.4 and 2.1±0.5 $mmHg.ml^{-1}.min^{-1}.g^{-1}$ in non-ischaemic and post-ischaemic hearts, respectively, and glibenclamide increased resistance by 2.0±0.4 and 2.0±0.4 $mmHg.ml^{-1}.min^{-1}.g^{-1}$ in non-ischaemic and post-ischaemic hearts, respectively. In the final series of experiments it was found that selective A_1 antagonism

with DPCPX during ischaemia and early reperfusion worsened effects of ischaemia-reperfusion on sensitivity to 2-chloroadenosine (Fig. 5.6, Table 5.2).

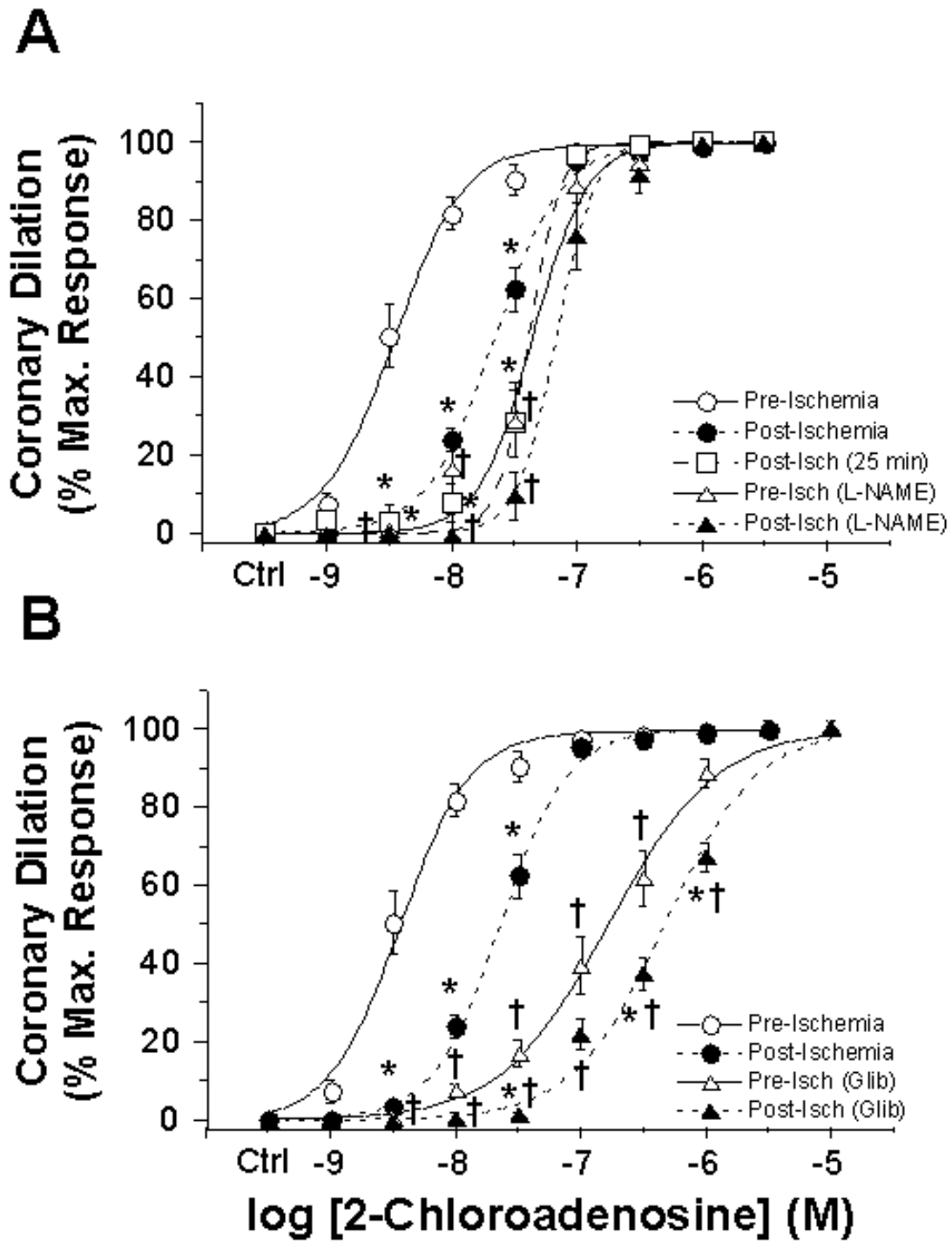

Figure 5.4 Concentration-response curves for 2-chloroadenosine in non-ischaemic and post-ischaemic hearts. A) Effects of NO-synthase inhibition with *L*-NAME, and B) effects of K_{ATP} channel inhibition. Responses were determined for untreated non-ischaemic hearts (n=7), hearts subjected to 20 (n=9) or 25 min of ischaemia (n=7), non-ischaemic (n=8) and post-ischaemic hearts (n=12) treated with 50 μM *L*-NAME, and non-ischaemic (n=9) and post-ischaemic hearts (n=6) treated with 5 μM glibenclamide. Values are means±SEM. *, P<0.05 *vs.* non-ischaemic; †, P<0.05 *vs.* untreated.

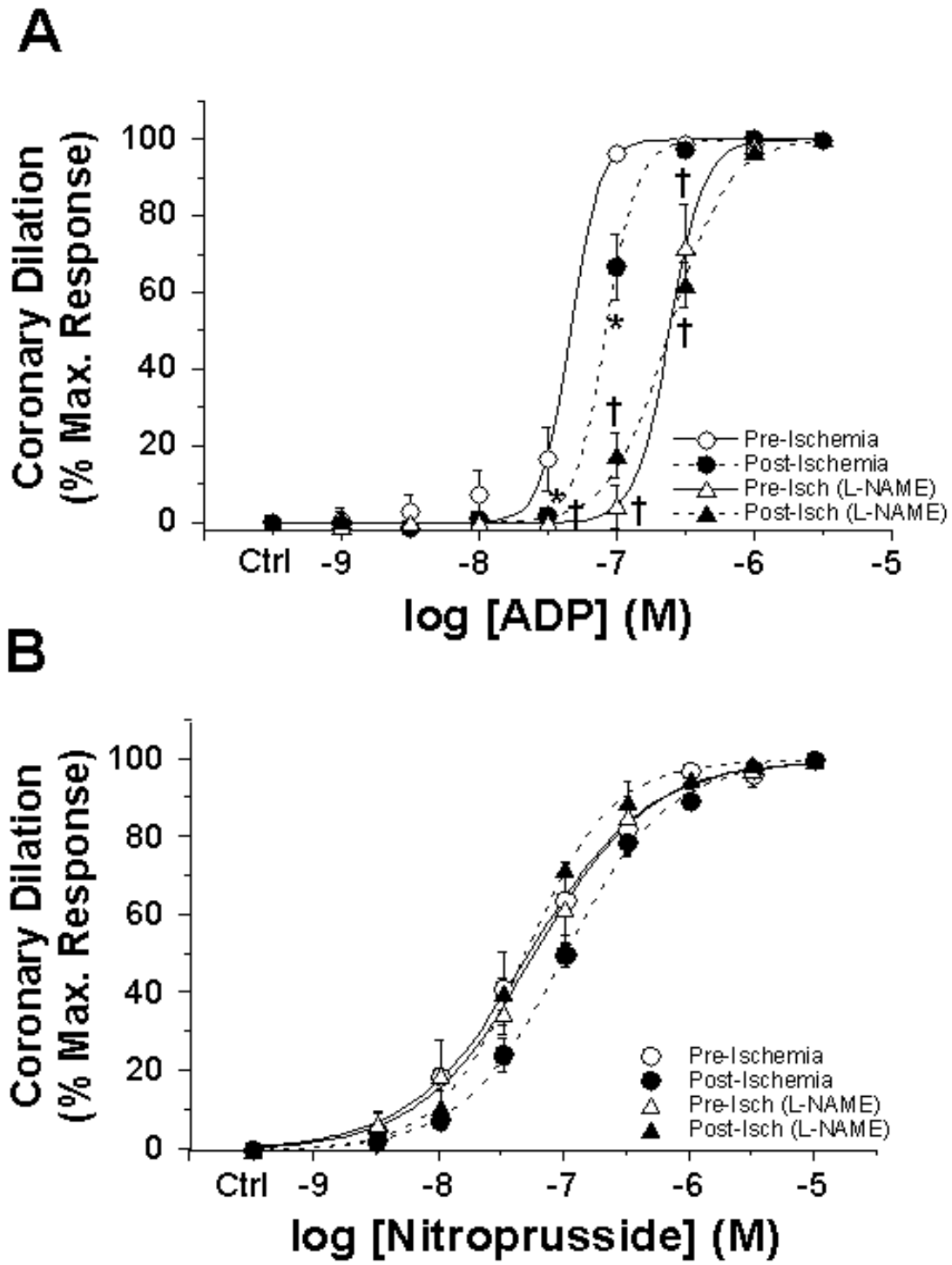

Figure 5.5 Concentration response curves for A) ADP, and B) SNP in non-ischaemic and post-ischaemic hearts. Responses were determined for ADP in the absence (n=7 non-ischaemic, n=8 post-ischaemic) and presence of 50 μM *L*-NAME (n=9 non-ischaemic, n=8 post-ischaemic), and for sodium nitroprusside in the absence (n=6 non-ischaemic, n=6 post-ischaemic) and presence of 50 μM *L*-NAME (n=6 non-ischaemic, n=8 post-ischaemic). Values are means±SEM. *, P<0.05 *vs.* non-ischaemic; †, P<0.05 *vs.* untreated.

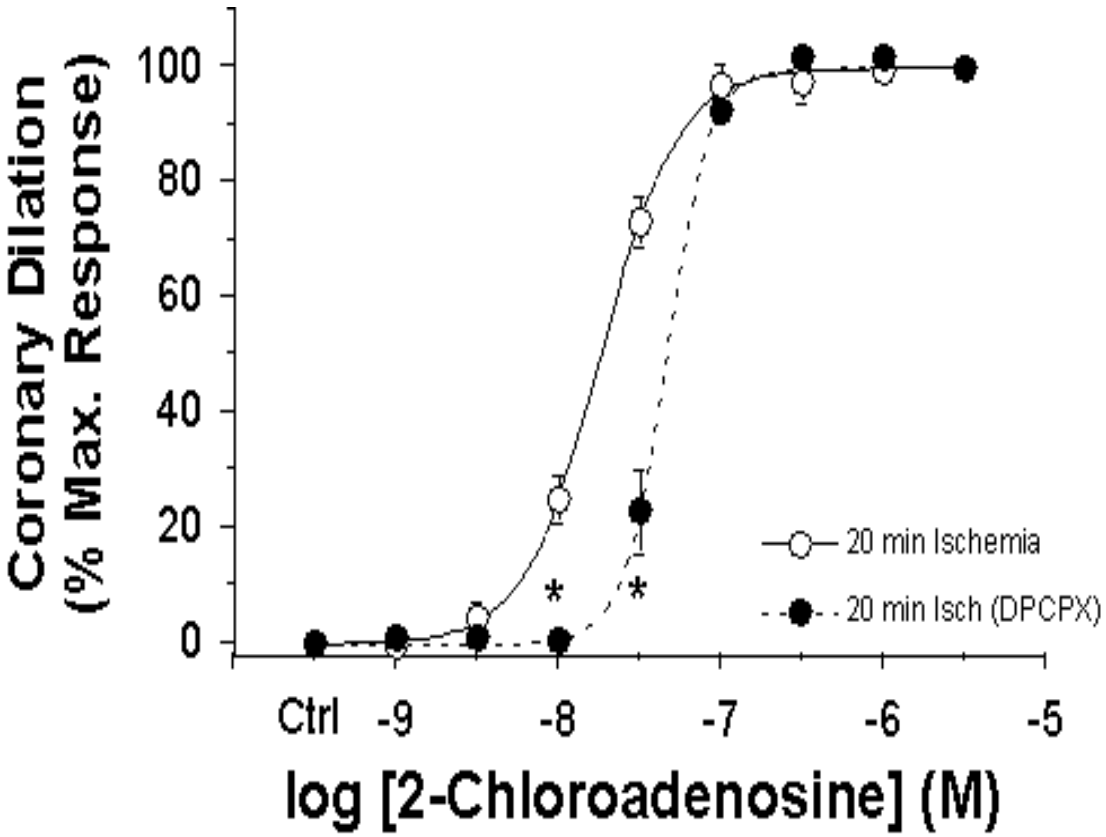

Figure 5.6 Effect of A_1AR antagonism on post-ischaemic sensitivity to 2-chloroadenosine. Data are shown for hearts subjected to 20 min ischaemia and 30 min reperfusion in the absence (n=9) or presence of antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, n=6). Values are means±SEM. *, $P<0.05$ *vs.* untreated hearts.

Table 5.2 Coronary concentration-response data for 2-chloroadenosine, ADP and sodium nitroprusside

	pEC$_{50}$	***Minimum Resistance*** (mmHg/ml/min/g)
2-Chloroadenosine		
Non-Ischaemic (n=7)	8.4±0.1	2.2±0.1
Post-ischaemic (n=9)	7.6±0.1*	2.5±0.1
Post-Ischaemic 25 min (n=7)	7.3±0.1*	3.5±0.2*
Non-Ischaemic+*L*-NAME (n=8)	7.4±0.1*	2.4±0.2
Post-ischaemic+*L*-NAME (n=12)	7.2±0.1*	3.2±0.1*†
Non-Ischaemic+Glibenclamide (n=9)	6.7±0.1*	4.2±0.4*
Post-Ischaemic+Glibenclamide (n=6)	6.3±0.1*†‡	5.0±0.2*‡
Post-Ischaemic+DPCPX (n=6)	7.2±0.1*‡	2.8±0.3
ADP		
Non-Ischaemic (n=7)	7.4±0.1	2.7±0.2
Non-Ischaemic+*L*-NAME (n=9)	6.6±0.1*	3.0±0.2
Post-ischaemic (n=8)	7.0±0.1*	2.9±0.2
Post-ischaemic+*L*-NAME (n=8)	6.6±0.1*†	3.0±0.2
Sodium Nitroprusside		
Non-Ischaemic (n=6)	7.1±0.2	3.2±0.2
Non-Ischaemic+*L*-NAME (n=6)	7.1±0.1	2.8±0.1*
Post-ischaemic (n=6)	7.0±0.1	2.6±0.2*
Post-ischaemic+*L*-NAME (n=8)	7.3±0.1	2.6±0.1

Coronary sensitivity (pEC_{50}) and minimum coronary vascular resistances during dilation were determined in non-ischaemic and post-ischaemic hearts in the absence and presence of *L*-NAME or glibenclamide. Effects of A_1AR antagonism during ischaemic were also assessed (DPCPX). Values are means±SEM of individual experiments. * P<0.05 *vs.* Non-Ischaemic; † P<0.05 Post-Ischaemic treated *vs.* Non-Ischaemic treated; ‡ P<0.05 *vs.* post-ischaemic untreated.

V. DISCUSSION

The aims of this study were to identify effects of ischaemia on coronary function in the absence of blood, and to identify roles of adenosine receptors in modifying post-ischaemic vascular function. Data indicate that 20-25 min ischaemia results in vascular dysfunction in an asanguinous model, implicating non-leukocyte dependent pathways. Furthermore, findings support vascular protection by A_1AR, and control of reflow via A_{2A} receptors.

Post-ischaemic coronary vascular function in asanguinous hearts

As noted by Nevailenen *et al.* [718] and recently by Gavin *et al.* [703], capillary compression by edema and contracture may be primary determinants of reflow, with vascular dysfunction a secondary factor. Supporting this, we recently showed early reflow correlates inversely with diastolic contracture [574]. We verify here that reflow after 20 min ischaemia is consistent with maximally dilated flow for non-ischaemic hearts, when corrected for elevated diastolic pressure and compression. Maximal dilation is additionally verified by lack of dilation with CGS21680 (Figs 5.1A and 5.2A). However, more prolonged ischaemia results in early reflow not predicted from the relation between flow and diastolic compression for normoxic hearts (Fig. 5.3A). Thus, we conclude that peak reflow after 20 min ischaemia is not limited by early vascular dysfunction, while there is evidence for an abnormality after 25 min ischaemia.

In contrast to predicted links between work-rate and flow in normoxic hearts, post-ischaemic coronary flow appeared largely insensitive to work-rate (Fig. 5.3B). Furthermore, coronary flow was lower, relative to work-rate, after more prolonged ischaemia. This further supports vascular dysfunction, although we find no direct evidence of impaired reflow (relative to work rate) after the relatively severe insults, previously shown to induce substantial injury and necrosis [629]. Indeed, post-

ischaemic coronary flow during late reperfusion (10-30 min) appears higher than necessary for the level of work performed (Fig 5.3B). Significantly impaired reflow may therefore require the presence of blood components and related pathological processes.

Post-ischaemic coronary dysfunction

In addition to dissociation of flow from work- rate, coronary dysfunction is directly reflected in impaired sensitivity to 2-chloroadenosine and ADP (Figs 5.4 and 5.5, Table 5.2). 2-Chloroadenosine dilates via mixed NO-synthase and K_{ATP}-dependent paths [675] whereas ADP is endothelial-dependent, acting through release of NO, EDHF and possibly prostaglandins [719,720]. Since ischaemia-reperfusion failed to modify sensitivity to endothelial independent SNP, dysfunction is specific for endothelial responses. A role for impaired NO bio-availability is supported by the fact that ischaemia-reperfusion failed to alter sensitivity to 2-chloroadenosine or ADP when NO-synthase was inhibited with *L*-NAME (Figs 5.4 and 5.5). Moreover, when K_{ATP} channel dependent dilation was inhibited, sensitivity to 2-chloroadenosine was still reduced by ischaemia (Fig. 5.4). Thus, when the contribution of NO synthase dependent dilation is reduced (with *L*-NAME), ischaemia has little additional effect, yet when the contribution of K_{ATP} channel-dependent dilation is reduced (with glibenclamide), ischaemia still impairs vascular function.

Quite opposing data exist regarding the genesis of endothelial dysfunction. There is evidence for [369] and against [370] impaired NO-synthase activity, for [340,369,721] and against [282,331,337] impairment of receptor *vs.* non-receptor mediated responses, and for [348] and against [336] inhibition of muscarinic responses. We exclude post-ischaemic down-regulation of receptors, or impaired receptor-effector coupling, since this would require comparable reductions in receptor number and/or

coupling for two distinct receptor groups with different transduction mechanisms. Irrespective of mechanism, our data indicate that endothelial dysfunction occurs independently of leukocyte-mediated injury. Endothelium itself may be a source of oxidants [360], just as underlying cells may generate radicals during ischaemia-reperfusion. Post-ischaemic dysfunction in the absence of blood observed here (Figs 5.4 and 5.5), and previously [337,347,348,397], indicate that the "classical" path, involving oxidant release and injury via leukocyte activation and adherence, is not the sole source of dysfunction.

Vascular protection via intrinsic activation of A_1 adenosine receptors

Since A_1 receptor activation protects murine hearts from ischaemia [21], we tested whether they might protect against vascular injury. DPCPX worsened diastolic contracture and contractile recovery (Fig. 5.1), consistent with prior observations [21]. Post-ischaemic flow was also impaired modestly, and flow relative to workload was reduced to levels observed after more prolonged ischaemia (Fig. 5.3B), suggesting vascular effects of the antagonist. In support of a vascular effect, DPCPX worsened effects of ischaemia-reperfusion on coronary sensitivity to 2-cloroadenosine (Fig. 5.6). These findings implicate A_1-mediated vascular protection by endogenous adenosine, consistent with the study of Maczewski and Beresewicz [715]. These findings may be of relevance to the clinically beneficial effects of adenosinergic therapies. For example, adenosine has been employed successfully as an adjunctive therapy in myocardial infarction and angioplasty [713,714]. While experimental data indicate vascular effects are due to A_2 receptor-mediated changes in neutrophil/leukocyte function [537,711,712] whereas myocardial effects are A_1 and/or A_3 mediated, our data and the findings of Maczewski and Beresewicz [715] support existence of vascular protection via A_1 receptors independent of blood cells.

Role of A_{2A} adenosine receptors in reflow and injury

Data support a key role for A_{2A}ARs in controlling coronary flow pre- and post-ischaemia (Fig. 5.2A, Table 5.1). There is support for vasoregulation by endogenous adenosine *in vivo* and *in vitro* under pathophysiological conditions [151,722,723]. Although there is evidence for vasoregulation under physiological conditions in animals [107] and humans [109], adenosine's physiological role remains controversial [100]. We targeted A_{2A} receptors since murine coronary responses are A_{2A}-mediated [629]. 8-(3-chlorostyryl)caffeine is selective for A_{2A} receptors, with poor antagonistic properties at A_1 receptors. Lack of effect on A_1 or A_3 receptors is reflected in the fact that antagonism enhanced post-ischaemic contractile and reduced diastolic pressure (Fig. 5.2) whereas A_1 and A_3 antagonists produce the opposite effect [21,574]. Reduced flow is therefore not the result of enhanced vascular compression or reduced workload. Since A_{2A} agonism increases late but not early reflow, A_{2A}-mediated dilatation by endogenous adenosine appears maximal initially, declining as reperfusion continues.

Improved recovery from ischaemia with 8-(3-chlorostyryl)caffeine was unexpected, as there is little evidence for A_{2A} receptor modulation of contractile dysfunction. We recently showed A_{2A} agonism is ineffective in altering contractile recovery in the absence of blood [724], consistent with lack of effect of CGS21680 here (Figs 5.1 and 5.2). However, Strickler *et al.* [596] have observed that A_{2A} antagonism enhances protection with preconditioning in cultured myocytes. Whilst from a different model, the data of Strickler and colleagues together with our observations support a detrimental A_{2A}-mediated effect in ischaemic myocardium [596]. Since A_{2A} agonism did not worsen recovery whereas A_{2A} antagonism enhanced recovery (Figs 5.1 and 5.2), this response appears maximally activated by endogenous adenosine during ischaemia and early reperfusion, consistent with the high sensitivity of A_{2A} receptors.

Conclusions

In summary, graded coronary dysfunction is observed following 20-25 min ischaemia in the absence of blood-borne elements. Dysfunction appears specific for NO-dependent responses. Additionally, we find that post-ischaemic reflow is determined significantly by A_{2A} receptor activation by endogenous adenosine, and that A_1 receptor activation by endogenous adenosine protects against vascular injury. A vascular component may therefore exist in A_1-mediated cardioprotection. Finally, we also observe improved functional recovery with A_{2A} antagonism (despite reduced reflow), supporting a detrimental A_{2A} mediated response to endogenous adenosine. The latter warrants further investigation.

CHAPTER 6

ADENOSINE RECEPTOR-MEDIATED VASOPROTECTION IN POST-ISCHAEMIC MOUSE HEART

I. ABSTRACT

The primary aim of this study was to determine whether adenosine receptor (A_1, A_{2A}, or A_3) activation modulates post-ischaemic coronary dysfunction in "asanguinous" hearts. Coronary vascular and myocardial function were assessed in Langendorff perfused mouse hearts subjected to 20 min ischaemia and 30-45 min reperfusion. Ischaemia-reperfusion (I/R) significantly impaired coronary sensitivity to the mixed endothelial dependent/independent dilator 2-chloroadenosine (pEC_{50} = 7.5±0.1 *vs.* 8.4±0.1 in non-ischaemic), and endothelial dependent dilator ADP (pEC_{50} = 6.8±0.1 *vs.* 7.6±0.1 in non-ischaemic). Responses to nitroprusside (endothelial independent) were unaltered. Selective A_1AR agonism (50 nM CHA) failed to modify coronary dysfunction while A_1AR antagonism (200 nM DPCPX) worsened effects of I/R (2-chloroadenosine pEC_{50}=6.9±0.1). In contrast, A_3AR agonism (100 nM Cl-IB-MECA) reduced effects of I/R (pEC_{50}s=8.0±0.1 and 7.3±0.1 for 2-chloroadenosine and ADP, respectively) whereas antagonism (100 nM MRS1220) was ineffective. Antagonism of A_{2A}ARs (100 nM SCH58261) was also ineffective. Coronary dysfunction was limited by Na^+/H^+ exchange (NHE) inhibition with 10 or 50 μM BIIB-513 (2-chloroadenosine pEC_{50}s = 7.8±0.1 with both doses), an effect not additive with A_3AR agonism. The Ca^{2+} desensitizer and putative protein phosphatase activator BDM (5 mM) also limited dysfunction (2-chloroadenosine pEC_{50} = 7.8±0.1) whereas ET antagonism (200 nM PD142893) and anti-oxidant therapy (300 μM MPG + 150 U/ml superoxide dismutase + 600 U/ml catalase) were ineffective. These data indicate post-ischaemic coronary endothelial dysfunction is reduced by intrinsic activation of A_1ARs, and exogenous activation of A_3ARs. Protection is unrelated to inhibition of ET or oxidant stress. However, A_3AR vasoprotection may share signalling with NHE inhibition.

INTRODUCTION

The adenosine receptor system has been extensively investigated in terms of effects on myocardial injury during and following ischaemia-reperfusion [9,10,69,540]. In contrast, almost nothing is known regarding potential effects of this receptor system on post-ischaemic coronary vascular injury. Available evidence supports an important role for coronary vascular injury in generating the post-ischaemic phenotype and governing ultimate salvage of post-ischaemic hearts [2,3]. Impaired endothelium-dependent control or "endothelial dysfunction" may underlie more cardiovascular diseases than currently known, and can trigger vascular events including vasospasm, thrombosis or re-stenosis, exacerbating the post-ischaemic phenotype. Indeed, endothelial dysfunction predicts risk of cardiovascular events in patients with coronary artery disease, and is linked to worsened clinical outcomes in treatment of cardiovascular disease [526,725]. Moreover, it is emerging that endothelial factors or receptors may directly regulate cardiac contractility [726] and protect against post-ischaemic arrhythmogenesis [727]. Thus, vascular endothelium has an important function not only in determining coronary vascular activity, but also myocardial function and injury. Therapeutic interventions aimed at limiting vascular injury are likely to be of considerable benefit in the setting of ischaemia-reperfusion. In the current study effects of A_1AR, A_{2A}AR, and A_3AR activation by exogenous and endogenous agonists in modulating post-ischaemic coronary dysfunction in murine hearts were examined. A blood-free model was assessed since the commonly accepted notion of neutrophil-mediated damage in generating vascular injury is increasingly questioned [8], with ample support for dysfunction in the absence of neutrophils or other blood-borne elements [346-348,686]. Effects of adenosine receptors were also compared with other “vasoprotective” interventions (anti-oxidant treatment, NHE inhibition, ET

antagonism, treatment with a Ca^{2+} desensitizer and vascular protein phosphatase activator) to identify potential mechanisms of protection.

II. MATERIALS AND METHODS

Langendorff perfused murine heart model

Hearts were prepared for functional and vascular studies as outlined in Chapter 2.

Experimental protocol

Effects of Ischaemia-Reperfusion on Coronary Vascular Function

To assess effects of ischaemia-reperfusion on coronary vascular function, responses to the mixed endothelial dependent and independent adenosine agonist 2-chloroadenosine, the endothelial-dependent dilator ADP, and the endothelium independent dilator sodium nitroprusside (SNP) were examined in non-ischaemic hearts and hearts subjected to 20-min ischaemia and 30-min reperfusion. To examine effects of adenosine receptor activation by exogenous and endogenous agonists on coronary dysfunction, specific dilatory responses were examined in the presence of adenosine receptor agonists or antagonists, infused 10 min prior to ischaemia and for the initial 15 min of reperfusion. A_1ARs and A_3ARs, responses to 2-chloroadenosine were studied in untreated non-ischaemic (n=11) and post-ischaemic hearts (n=18), and in post-ischaemic hearts treated with 50 nM of the A_1AR agonist N^6 -cyclohexyladenosine (CHA; n=8), 200 nM of the A_1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; n=7), 100 nM of the A_3AR agonist 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide (Cl-IB-MECA; n=7), or 100 nM of the A_3AR antagonist 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino][1,2,4]-triazolo[1,5-c]quinazoline (MRS1220;

n=7). Responses to ADP were examined in transgenic hearts overexpressing cardiac A_1ARs under non-ischaemic conditions (n=6) or after ischaemia-reperfusion (n=8). To access the role of endogenously activated A_{2A}ARs, responses to ADP were studied in non-ischaemic (n=9) and post-ischaemic hearts (n=9), and in non-ischaemic and post-ischaemic hearts treated with 100 nM of the A_{2A}AR antagonist 5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-epsilon]-1,2,4-triazolo[1,5-c)pyrimidine (SCH58261; n=7 in both cases). We examined ADP since coronary dilation in response to adenosine analogues is A_{2A}AR-mediated in mouse heart [675].

In addition to adenosine receptor agonists and antagonists, we assessed effects of anti-oxidant therapy with infusion of 300 μM MPG + 150 U/ml SOD + 600 U/ml catalase (n=6), effects of mixed endothelin ($ET_{A/B}$) blockade with 200 nM PD142893 (n=8), effects of NHE-1 inhibition with 10 μM (n=9) or 50 μM (n=7) benzamide,N-(aminoiminomethyl)-4-[4-(2-furanylcrbonyl)-1-piperazinyl]-3-(methylsulfonyl), methanesulfonate] (BIIB-513), and effects of 5 mM of the Ca^{2+} desensitizer and putative protein phosphatase activator BDM (n=7). Furthermore, we examined effects of 10 μM BIIB-513 + 100 nM Cl-IB-MECA (n=6) and 50 μM BIIB-513 + 100 nM Cl-IB-MECA (n=8). BIIB-513 has been shown to be 8 times more potent than HOE-642 or cariporide, and is 37,000 times more selective for NHE-1 versus NHE-3 [216].

To further characterize effects of A_3AR agonism and NHE inhibition, we examined responses to the endothelial-dependent dilator, ADP. Groups studied included non-ischaemic hearts (n=9), and post-ischaemic heart in the absence (n=9) or presence of 10 μM BIIB-513 (n=7), 100 μM Cl-IB-MECA (n=6) or 10 μM BIIB-513 + 100 μM Cl-IB-MECA (n=7).

Ventricular Contractile Responses To Ischaemia

To assess ventricular contractile responses to ischaemia-reperfusion, hearts were subjected to 20-min global normothermic ischaemia followed by 45-min reperfusion (n=9). Effects of A_3AR agonism were examined by treatment with 100 nM Cl-IB-MECA (n=8) prior to and for 15 min following ischaemia. Effects of NHE inhibition were examined by similar treatment with 10 μM (n=9) or 50 μM (n=9) BIIB-513. Effects of simultaneous A_3AR agonism and NHE inhibition were assessed by treatment with 100 nM Cl-IB-MECA + 50 μM BIIB-513 (n=8).

Effects of Ischaemia-Reperfusion±A_3AR Agonism On Reactive Hyperaemic Responses

To assess the functional impact of coronary dysfunction, reactive hyperaemic responses were studied in hearts subjected to 5, 10 and 20 sec of transient occlusion (separated by 5-min periods of reperfusion) in non-ischaemic and post-ischaemic hearts. Peak hyperaemic flow and flow repayment over the initial 2min of reperfusion were determined. Flow repayment was expressed as % of flow debt incurred during occlusion (basal flow x occlusion period), with repayment calculated as the post-occlusion flow above baseline (post-occlusion flow - pre-occlusion flow). After acquiring hyperaemic responses in non-ischaemic hearts, all hearts were then stabilised for an additional 10 min period before being subjected to 20 min global ischaemia and 30 min reperfusion. Hyperaemic responses were again assessed at this time. This protocol, was performed in untreated control hearts (n=6) and in hearts receiving 100 nM Cl-IB-MECA 10 min prior to ischaemia and for the initial 15 min of reperfusion (n=6).

III. RESULTS

Adenosine Receptor-Mediated Protection Against Coronary Dysfunction

Ischaemia-reperfusion significantly impaired coronary sensitivity to 2-chloroadenosine and ADP, while responses to SNP were unaltered (Table 6.1). Ischaemia-reperfusion shifted the EC_{50} values for 2-chloroadenosine and ADP by an order of magnitude (Table 6.1). Exogenous activation of the A_1AR during ischaemia and early reperfusion failed to modify coronary dysfunction (Fig. 6.1A). However, A_1AR antagonism markedly worsened coronary dysfunction (Fig. 6.1A), further shifting the EC_{50} for 2-chloroadenosine (Table 6.1).

In contrast to lack of effect of A_1AR agonism, exogenous A_3AR activation significantly limited post-ischaemic vascular dysfunction (Fig. 6.1C, Table 6.1). Antagonism of A_3ARs did not modify coronary function (Fig. 6.1C, Table 6.1). Similarly, antagonism of A_{2A}ARs was also ineffective in modifying vascular dysfunction (Fig. 6.1B, Table 6.1).

Table 6.1 Concentration-response data for 2-chloroadenosine, ADP, and SNP in non-ischaemic and post-ischaemic hearts

	pEC$_{50}$	***Minimum Resistance*** (mmHg/ml/min/g)
2-Chloroadenosine		
Untreated		
Non-Ischaemic (n=11)	8.4±0.1	2.2±0.1
Post-Ischaemic (n=18)	7.5±0.1*	2.8±0.1*
A_1AR Agonism/Antagonism		
Post-Isch+CHA (n=8)	7.6±0.1*	2.5±0.1
Post-Isch+DPCPX (n=7)	6.9±0.1*†	3.1±0.1*
A_3AR Agonism/Antagonism		
Post-Isch+Cl-IB-MECA (n=7)	8.0±0.1*†	2.7±0.2*
Post-Isch+MRS1220 (n=7)	7.5±0.1*	2.4±0.1
Endothelin Antagonism, Anti-Oxidant Treatment, and BDM		
Post-Isch+PD142893 (n=8)	7.5±0.1*	2.5±0.1
Post-Isch+SOD+Catalase+MPG (n=6)	7.5±0.1*	2.8±0.1*
Post-Isch+BDM (n=7)	7.8±0.1*†‡	2.6±0.1*
NHE Inhibition±A_3AR Agonism		
Post-Isch+10 μM BIIB-513 (n=9)	7.8±0.1*†‡	2.6±0.1*
Post-Isch+50 μM BIIB-513 (n=7)	7.8±0.1*†‡	2.5±0.1
Post-Isch+10 μM BIIB+Cl-IB-MECA (n=6)	7.9±0.1*†	2.0±0.1
Post-Isch+50 μM BIIB+Cl-IB-MECA (n=8)	8.0±0.1*†	2.2±0.1
ADP		
Untreated		
Non-Ischaemic (n=9)	7.6±0.1	2.3±0.1
Post-Ischaemic (n=9)	6.8±0.1*	2.5±0.1
A_{2A}AR Antagonism		
Non-Isch+100 nM SCH58261 (n=7)	7.5±0.1	2.5±0.1
Post-Isch+100 nM SCH58261 (n=7)	6.5±0.1*	2.6±0.1
A_3AR Agonism		
Post-Isch+100 nM Cl-IB-MECA (n=6)	7.3±0.1*†	2.8±0.2*
NHE Inhibition±A_3AR Agonism		
Post-Isch+10 μM BIIB-513 (n=7)	7.3±0.1*†	2.9±0.2*
Post-Isch+10 μM BIIB+Cl-IB-MECA (n=7)	7.3±0.1*†	2.6±0.1
Cardiomyocyte A_1AR Overexpression		
Non-Isch Wild-type (n=6)	7.5±0.1	2.3±0.1
Non-Isch + A_1AR Transgenic (n=6)	7.3±0.1	3.2±0.2*
Post-Isch Wild-type (n=9)	6.8±0.1*	2.5±0.1
Post-Isch A_1AR Transgenic (n=8)	6.8±0.1*	2.6±0.2
SNP		
Non-Ischaemic (n=6)	7.1±0.2	3.2±0.2
Post-Ischaemic (n=6)	7.0±0.1	2.6±0.2

Coronary sensitivity (pEC_{50}) and minimum coronary vascular resistances during dilation were determined in pre-ischaemic and post-ischaemic hearts. Effects of NHE inhibition (BIIB-513), A_3AR activation (Cl-IB-MECA) or combined treatment with BIIB-513 and Cl-IB-MECA during ischaemia were also assessed. Values are means±SEM of individual experiments. * $P<0.05$ versus non-ischaemic; † $P<0.05$ versus post-ischaemic untreated; ‡ $P<0.05$ versus post-ischaemic Cl-IB-MECA treated.

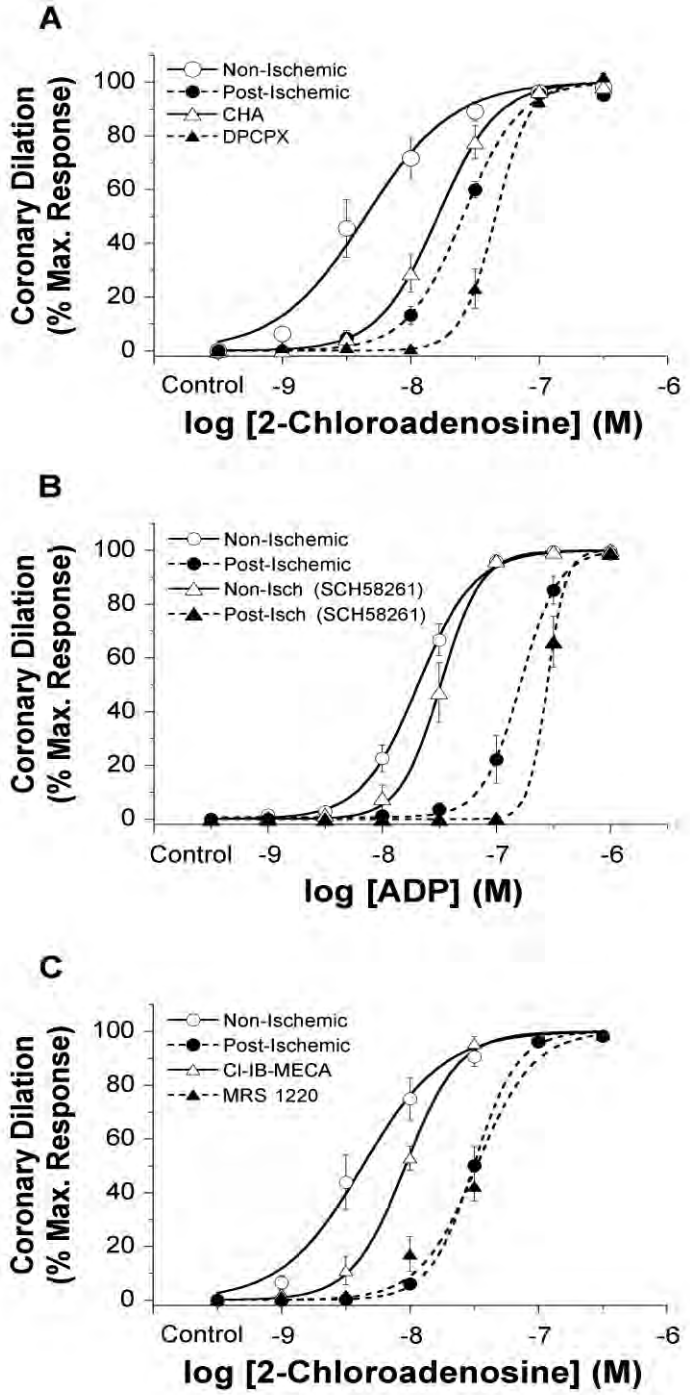

Figure 6.1 Coronary responses to 2-chloroadenosine or ADP in non-ischaemic and post-ischaemic hearts treated with A_1AR agonist or antagonist (A), A_{2A}AR antagonist (B), or A_3AR agonist or antagonist (C). Responses to 2-chloroadenosine were assessed in non-ischaemic hearts (n=11) or hearts subjected to 20-min ischaemia in the absence (n=18) or presence of 50 nM CHA (n=8), 200 nM DPCPX (n=7), 100 nM Cl-IB-MECA (n=7), 100 nM MRS1220 (n=7). To access the role of A_{2A}ARs, responses to ADP were acquired in non-ischaemic and post-ischaemic hearts in the absence (n=9 in both cases) or presence of 100 nM SCH58261 (n=7 in both cases). Values are means±SEM.

Effects of Ischaemia-Reperfusion on Coronary Reactive Hyperaemia

To assess the functional relevance of coronary dysfunction we examined graded reactive hyperaemic responses to 5, 10 and 20 s occlusions in non-ischaemic and post-ischaemic hearts (Fig. 6.2). Ischaemia-reperfusion significantly impaired peak hyperaemic flows and flow repayment for each period of occlusion (Fig. 6.2A and 6.2B). Agonism of A_3ARs during ischaemia and early reperfusion significantly improved peak hyperaemic flow, but insignificantly potentiated flow repayment (Fig. 6.2).

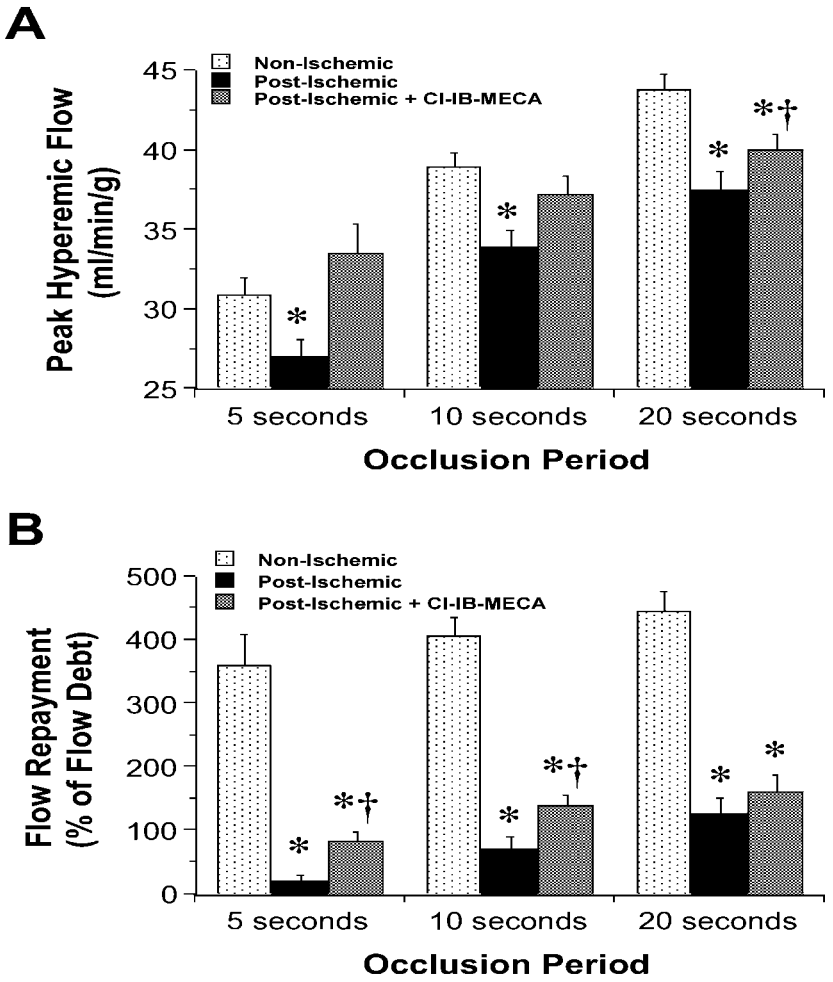

Figure 6.2 Reactive hyperaemic responses assessed from peak hyperaemic flow response (A) and flow debt repayment (B) in non-ischaemic and post-ischaemic hearts in the presence (n=6) or absence (n=6) of Cl-IB-MECA. Values are means±SEM. * P<0.05 versus non-ischaemic; † P<0.05 versus post-ischaemic untreated.

Effects of NHE inhibition, endothelin antagonism, anti-oxidants and BDM on coronary dysfunction

Coronary dysfunction, assessed from the shift in response to 2-chloroadenosine, was significantly reduced by NHE inhibition with BIIB-513 and also by the drug BDM (Fig. 6.3A). Protection with BIIB-513 was identical for both 10 and 50 μM levels of inhibitor (Table 6.1). Coronary dysfunction was unaltered by ET inhibition (Fig. 6.3A) or anti-oxidant therapy (Fig. 6.3B).

Myocardial and vascular protection via exogenous A_3AR activation and NHE inhibition

Further analysis of the protective effects of A_3AR agonism and NHE inhibition showed A_3AR agonism exerted a superior degree of protection against impaired coronary sensitivity to 2-chloroadenosine (Table 6.1, Fig 6.4A), whereas A_3AR agonism and NHE inhibition equally reduced the shift in sensitivity to ADP (Table 6.1, Fig. 6.4B). Interestingly, beneficial vascular effects of A_3AR activation and NHE inhibition were not additive (Fig. 6.4).

We also assessed myocardial protection via A_3AR agonism and NHE inhibition. Pre-ischaemic contractile function was unaltered by Cl-IB-MECA and BIIB-513 (Table 6.2). Both agents improved functional recovery, reducing post-ischaemic diastolic contracture and enhancing pressure development (Fig. 6.5). Inhibition of NHE was superior in limiting diastolic dysfunction (Fig. 6.5B). Despite overall improvement in ventricular function, only treatment with 50 μM BIIB-513 resulted in a modest increase in early coronary reflow (Fig. 6.5A). Myocardial effects of BIIB-513 were concentration dependent, with 10 μM selectively reducing diastolic contracture to enhance pressure development whereas 50 μM improved both diastolic and systolic pressure to further enhance pressure development (Fig. 6.5B-6.5D). Myocardial protective effects of BIIB-513 and Cl-IB-MECA were not additive (Fig. 6.5C-6.5D).

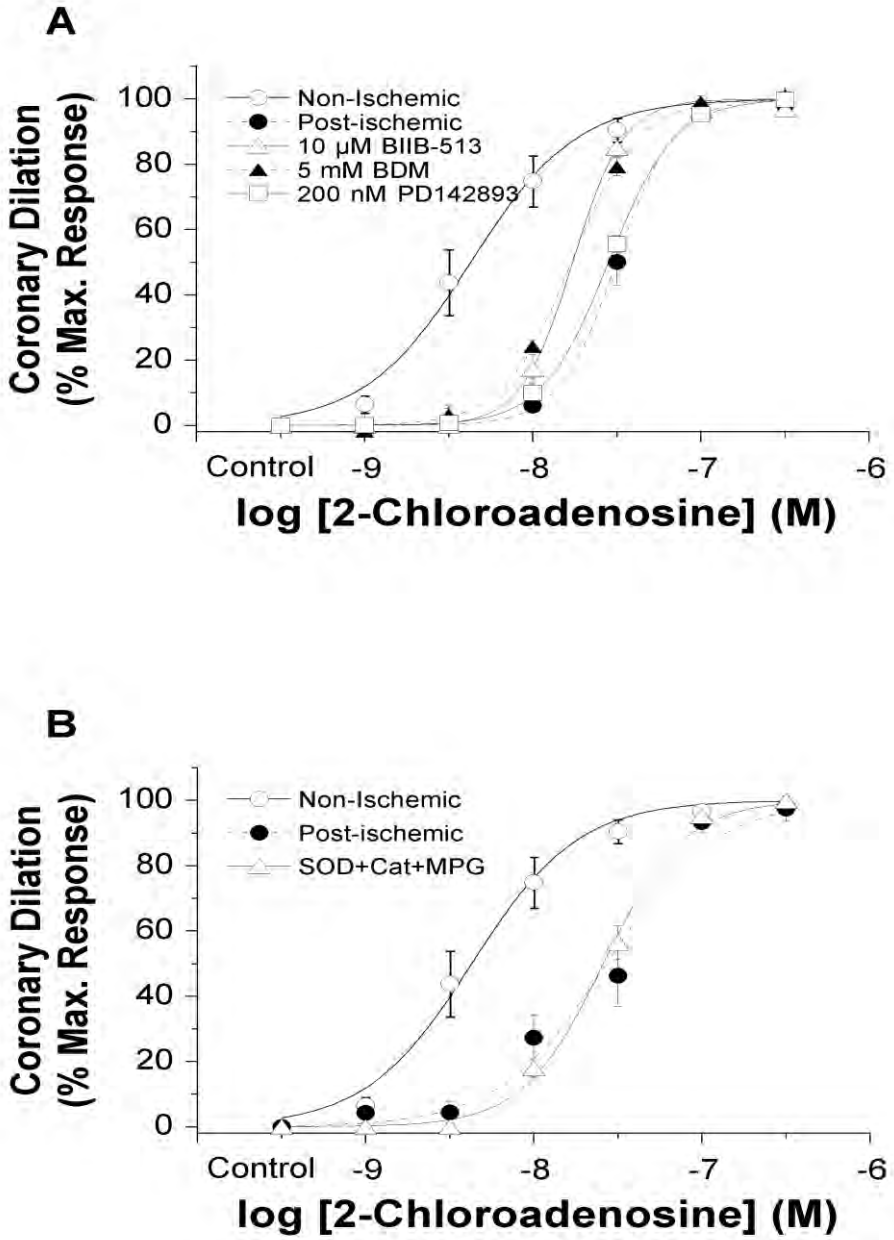

Figure 6.3 Effects of (A) various Ca^{2+} modulators and (B) antioxidant therapy on post-ischaemic coronary vascular sensitivity to 2-chloroadenosine were assessed in the presence of 10 μM BIIB-513 (n=9); 200 nM PD142893 (n=8); 5 mM BDM (n=7); or 300 μM MPG + 150 U/ml SOD + 600 U/ml catalase (n=6). Values are means±SEM.

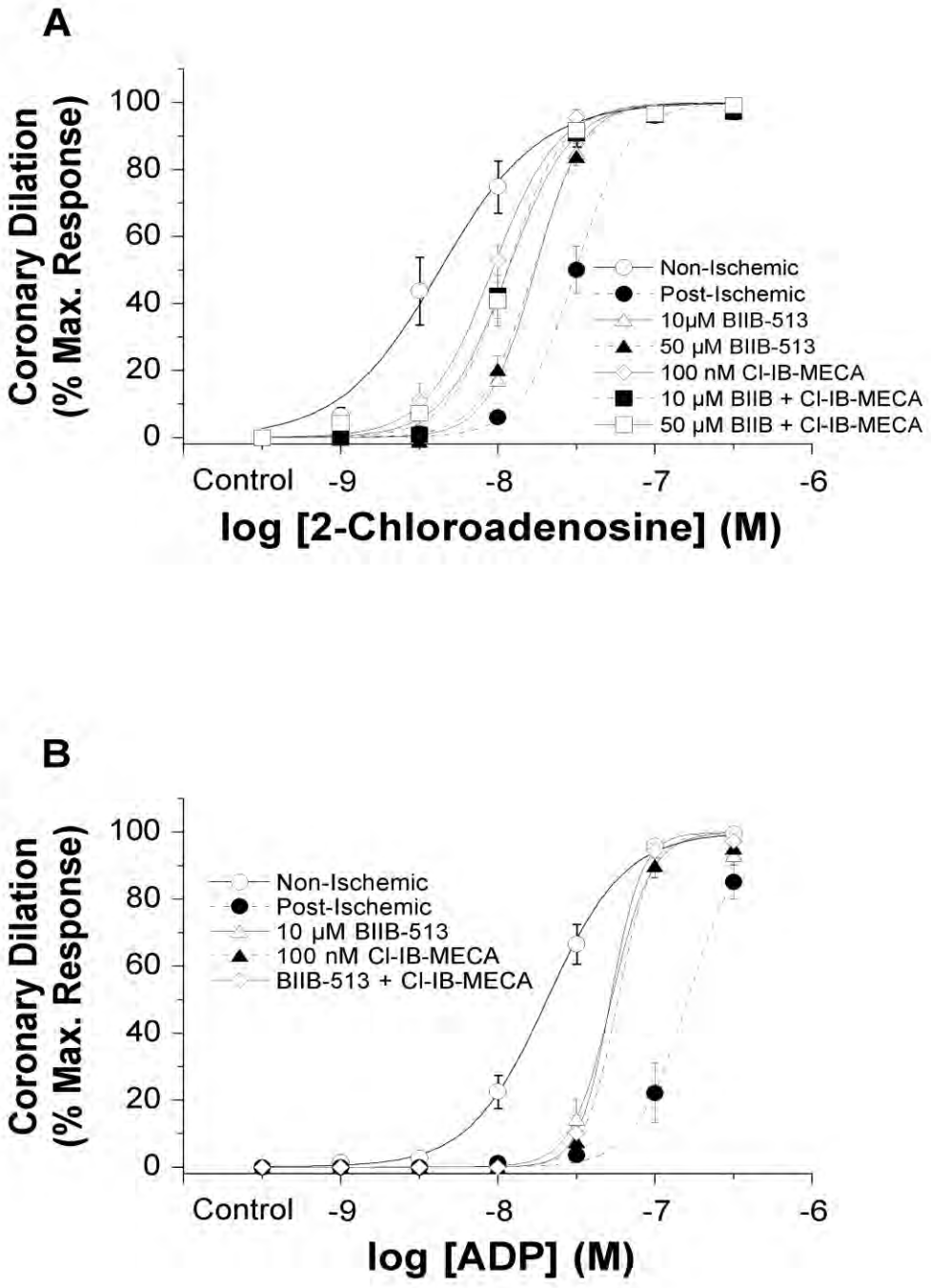

Figure 6.4 Effects of A_3AR activation alone or in conjunction with NHE inhibition on post-ischaemic sensitivity to (A) 2-chloroadenosine and (B) ADP were assessed. Data are shown for non-ischaemic and post-ischaemic hearts treated with 10 or 50 μM BIIB-513, 100 nM Cl-IB-MECA, or 10 or 50 μM BIIB-513 plus Cl-IB-MECA. Values are means±SEM.

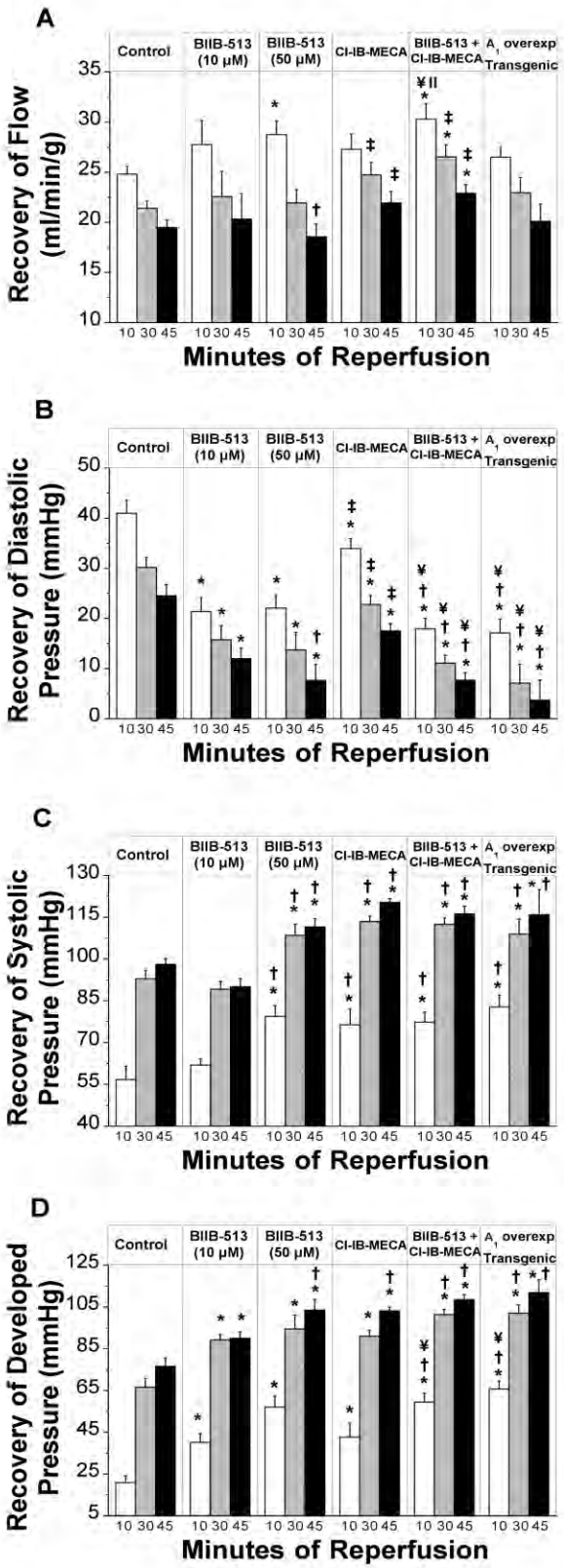

Figure 6.5 Recoveries for (A) coronary flow, (B) diastolic pressure, (C) systolic, and (D) developed pressure after 20 min ischaemia. Recoveries were measured at 10, 30 and 45 min reperfusion. Data are shown for control hearts (n=9), and hearts treated with 50 μM BIIB-513 (n=9), 100 nM Cl-IB-MECA (n=8), BIIB-513 plus Cl-IB-MECA (n=8) or A_1AR overexpressed transgenic hearts (n=10). Values are means±SEM. *P<0.05 versus untreated; † P<0.05 versus 10 μM BIIB-513; ‡ P<0.05 versus 50 μM BIIB-513; ¥ P<0.05 versus Cl-IB-MECA; II P<0.05 versus A_1AR overexpressed.

Table 6.2 Pre-ischaemic contractile function and coronary flow

	Control	BIIB-513 (10 μM)	BIIB -513 (50 μM)	Cl-IB-MECA	BIIB+ Cl-IB-MECA
Coronary flow ($ml.min^{-1}.g^{-1}$)	30.3±1.7	30.1±2.6	28.8±2.3	35.3±1.7	31.8±2.3
Diastolic pressure (mmHg)	1±1	1±1	2±2	2±1	1±1
Systolic pressure (mmHg)	159±2	141±4	154±2	161±4	150±2
Rate-Pressure Product ($mmHg.min^{-1}$ x1000)	62.4±1.8	63.8±1.7	71.0±1.3	66.0±1.5	62.0±1.3

Normoxic function was measured immediately prior to ischaemia. Hearts were untreated (Control, n=9) or treated with 10 or 50 μM BIIB-513 (n=9 in both groups), 100 nM Cl-IB-MECA (n=8) or 50 μM BIIB-513 and 100 nM Cl-IB-MECA (n=8). Values are means±SEM. $^{*}P<0.05$ versus control.

Relation Between Myocardial and Coronary Injuries and Protection

To assess the relation between myocardial and coronary injuries, we studied hearts overexpressing cardiomyocyte A_1ARs, which displayed markedly enhanced recovery of myocardial contractile function (Fig. 6.5). However, A_1AR overexpression failed to modify coronary dysfunction (Fig. 6.6). Plotting cardiac protective effects of A_1AR overexpression, A_3AR agonism, NHE inhibition, and A_3AR agonism + NHE inhibition against vasoprotective effects (EC_{50} shifts for ADP) reveals no correlation between the two forms of injury/protection (Fig. 6.7).

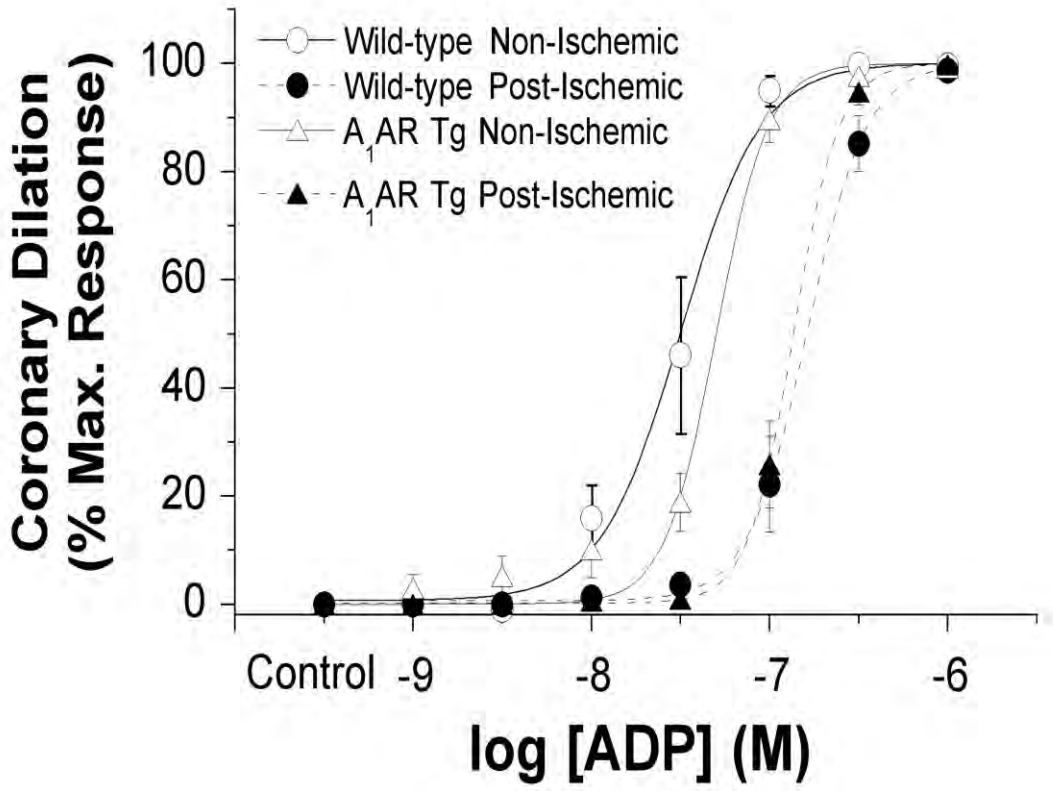

Figure 6.6 Effects of myocardial A_1AR overexpression on coronary vascular sensitivity to ADP. Responses were assessed in transgenic hearts under non-ischaemic and post-ischaemic conditions (n=6 and n=8, respectively), and in wild-type counterparts under non-ischaemic and post-ischaemic conditions (n=5 and n=9, respectively). Values are means±SEM.

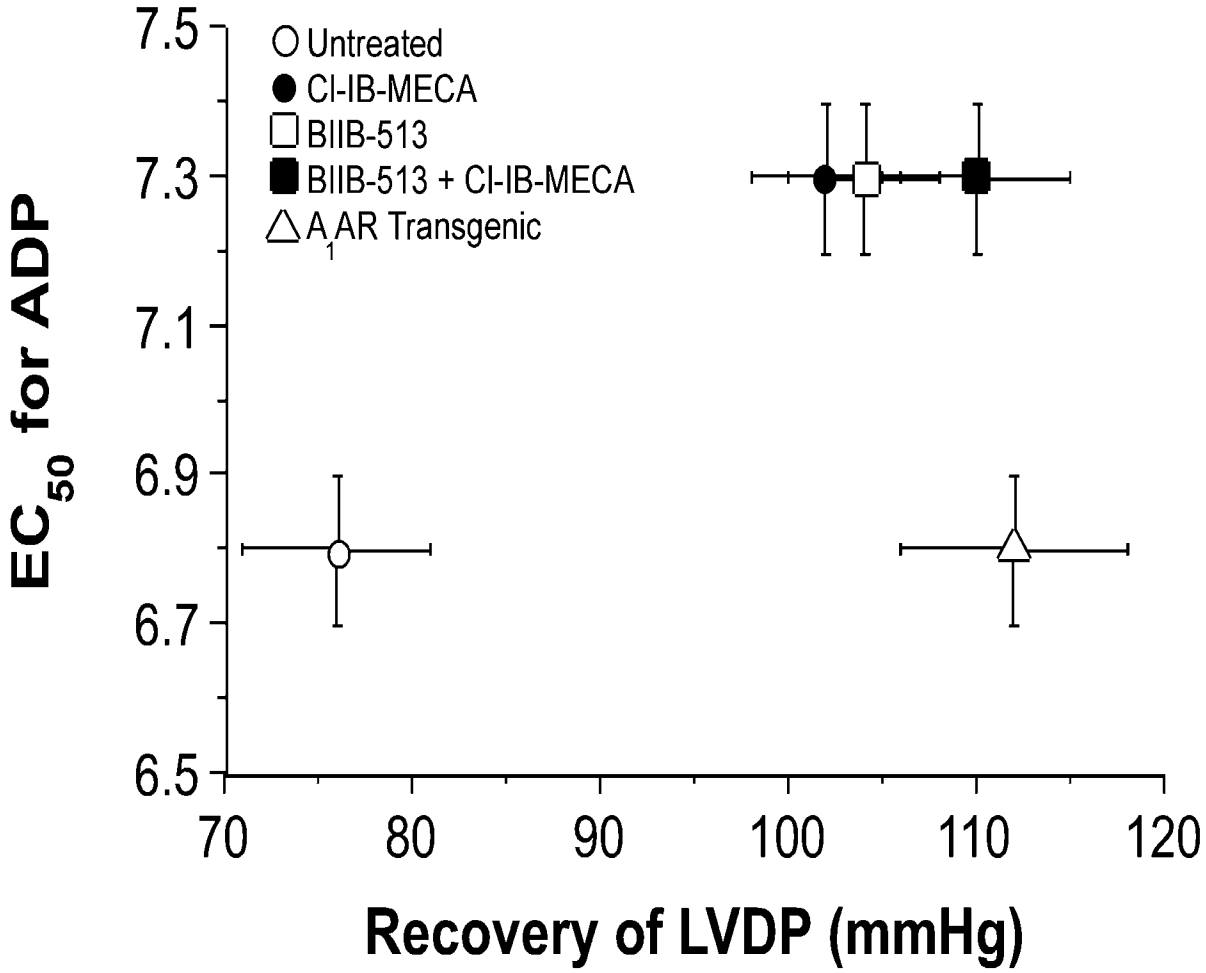

Figure 6.7 Relationship between myocardial and coronary vascular injury/protection in ischaemic-reperfused hearts either untreated, or treated with 100 nM Cl-IB-MECA, 50 μM BIIB-513, or 50 μM BIIB-513 + 100 nM Cl-IB-MECA. Data is also shown for transgenic hearts overexpressing myocardial A_1ARs. Myocardial injury was assessed from recovery of left ventricular developed pressure while relative differences in vascular injury were assessed from pEC_{50} values for ADP mediated coronary dilation. Values are means±SEM.

IV. DISCUSSION

The primary aim of this study was to determine whether specific adenosine receptor activation by endogenous or exogenous agonists might protect against post-ischaemic coronary dysfunction in intact hearts. The data verify selective impairment of endothelial function following ischaemia-reperfusion, and reveal exogenous A_3AR agonism (and endogenous activation of A_1ARs) significantly limits this dysfunction. Coronary vasoprotection does not stem from reduced cardiac injury, and does not appear to involve inhibition of oxidant damage or ET. The level of protection with A_3AR agonism is equal to or superior to that with NHE inhibition, with lack of additivity between these stimuli providing preliminary evidence for common protective signalling.

Adenosine Receptor-Mediated Protection Against Post-Ischaemic Coronary Dysfunction

Cardioprotection via activation of A_1ARs has been well documented [9,10,69,540]. More recently our group [686] and others [355] have acquired evidence A_1ARs may also protect coronary vasculature during ischaemia-reperfusion. Interestingly, exogenous A_1AR agonism was unable to provide vascular protection suggesting either that A_1ARs do not protect coronary vessels, or that the endogenous A_1AR response is maximal. In support of the latter, A_1AR antagonism exacerbates coronary dysfunction. Thus, there appears to be an endogenous A_1AR mediated vasoprotection, consistent with relatively high sensitivity of A_1ARs to adenosine.

Activation of A_{2A}ARs has been shown to limit reperfusion injury *in vivo* [9,51], with mechanisms thought to primarily involve anti-neutrophil actions. We initially attempted to assess A_2AR agonism during ischaemia and early reperfusion. However, profound dilation precluded accurate assessment of potential protection and post-

ischaemic coronary function. We did, however, test effects of A_{2A}AR antagonism, which was ineffective in modifying dysfunction (Fig. 6.1). As noted by Lasley *et al.*, [567] only a small percentage of A_{2A}ARs are required to be activated by locally generated adenosine to induce significant effects, due to large receptor reserve. Accumulation of endogenous adenosine during ischaemia and early reperfusion should be more than sufficient to activate A_{2A}ARs, as supported by reduced early reflow following A_2AR antagonism [686]. Thus, the lack of effect of antagonism argues against intrinsic A_{2A}AR mediated vasoprotection in this model.

Despite some controversy there is considerable support for a role of A_3ARs in myocardial protection [10,69,593,628,728], though cardiomyocyte A_3AR expression is yet to be confirmed. The current data support findings of myocardial protection with A_3AR agonism [593,728], and reveal a substantial vasoprotective effect of this receptor sub-type (Fig. 6.1C). The A_3 agonist Cl-IB-MECA was applied at a concentration above its inhibition constant (K_i) at A_3 receptors (~1 nM), yet well below its K_i at A_1 or A_2 receptors (500-1000 nM) [605]. Prior studies show this level of Cl-IB-MECA exerts little effect on A_2-mediated dilation [729], and reveal protective effects which are unrelated to A_1 receptor activation (at least in rabbit) [601]. The fact that Cl-IB-MECA did not induce dilation indicates lack of activation of A_2 receptors (Table 6.2). Moreover, since A_1 agonism was ineffective, vasoprotection via Cl-IB-MECA cannot be attributed to this receptor, leaving an A_3AR-dependent action. To date vasoprotection via A_3AR activation has only been assessed in one study [554]. These investigators found preconditioning via transient A_3AR activation preserved hypoxic coronary dilation in post-ischaemic hearts.

To assess the functional relevance of vascular injury (and A_3AR mediated vasoprotection), reactive hyperaemic responses to brief occlusion were assessed in non-

ischaemic and post-ischaemic hearts. Ischaemia-reperfusion not only inhibited dilatory responses to exogenous agonists (Fig. 6.1), but significantly limited reactive hyperaemic responses mediated by endogenous stimuli (Fig. 6.2). This effect was significantly countered by A_3AR agonism during the prolonged ischaemic insult. Thus, coronary dysfunction is manifest by impaired responses to both exogenous and endogenous dilators, with both effects countered by A_3AR agonism.

No Roles for Oxidant Stress and Endothelin?

In examining the potential mechanism of action of A_3AR (and A_1AR) activation, we sought to identify factors contributing to the injury. Oxidative stress is considered a key mediator of vascular injury and dysfunction, reducing bio-availability of NO [730,731]. Oxidant-induced endothelial dysfunction occurs in the absence [346,732] and presence [731] of blood. Interestingly adenosine is implicated in protecting against oxidant generation and injury in cardiac tissue *in vitro* [733] and *in vivo* [547]. Recently, Narayan *et al.* showed A_1ARs reduce formation of ROS (in a K_{ATP} channel-dependent manner) in ischaemic-reperfused hearts [548]. There is also evidence A_3AR activation enhances anti-oxidant status in endothelial, smooth muscle, and cardiac cells [17]. Thus, it was hypothesised that vasoprotection via adenosine receptor activation might involve anti-oxidant effects. However, application of a "cocktail" of anti-oxidants (SOD, catalase, MPG) failed to modify vascular dysfunction (Fig. 6.3B, Table 6.1). It is therefore unlikely an anti-oxidant action underlies vasoprotection via adenosine receptors in this model. These findings also indicate oxidant stress may not contribute to coronary dysfunction observed in this model. An alternate possibility is that these anti-oxidants may contribute to an imbalance between NO synthesis and catabolism. As indicated by Visioli [734], enhanced stimulation of NO synthase may lead to generation of reactive nitrogen species. Since superoxide plays a role in disposing of excess NO,

SOD treatment may paradoxically enhance this oxidant path. There is some support for NO-synthase dependent injury during ischaemia-reperfusion [735]. However, inclusion of MPG together with SOD renders exaggerated NO radical generation an unlikely explanation for the lack of effect of the anti-oxidant cocktail.

ET is implicated in vascular dysfunction [355,382,384], and can activate NHE [227], elevating intracellular Ca^{2+} and diastolic tension on reperfusion. Adenosine receptor activation has been shown to counter coronary effects of ET [736]. Thus, dysfunction and adenosinergic vasoprotection could involve modulation of ET responses. Since vascular effects of ET are mediated by both ET_A [381] and ET_B receptors [737], effects of a mixed ($ET_{A/B}$) antagonist, previously shown to exert vasoprotection in guinea-pig and rat hearts [355,382,385] were tested. Although ET antagonism limited post-ischaemic diastolic contracture (data not shown), consistent with prior studies [381,382], it failed to modify coronary dysfunction (Fig. 6.3A, Table 6.1). Since accumulating evidence indicates ET antagonism protects by attenuating radical release [384,385], this observation is consistent with lack of effect of anti-oxidants (Fig. 6.3B). These findings collectively indicate coronary dysfunction does not involve ET or oxidant dependent injury, and therefore that adenosine receptor mediated vasoprotection does not involve modulation of these processes.

Vasoprotection via NHE Inhibition and BDM

Inhibition of NHE is considered by many a new "gold standard" in experimental cardioprotection, even more efficacious than ischaemic preconditioning. The observation of improved myocardial recovery with NHE inhibition (Fig. 6.5) is consistent with prior data in isolated heart models [214,217]. In addition data reveal NHE inhibition limits post-ischaemic vascular dysfunction, albeit to a lesser extent than A_3AR activation (Fig. 6.4, Table 6.1). The role of NHE in modulating vascular injury

has largely been studied *in vivo*. However, the current findings support preliminary evidence NHE inhibition protects against vascular dysfunction in the absence of blood [738]. A drawback to this latter study, however, is that hearts were pre-treated with ACh, rendering it likely they were inadvertently preconditioned and confounding interpretation of data.

The effects of BDM on vascular injury were also studied, since this agent has been shown to protect myocardial tissue [739], can modify vascular actomyosin activity [740], activate vascular protein phosphatases [741], counter vasoconstrictor stimuli [742], and limit vascular Ca^{2+} entry [743]. One prior study shows BDM-mediated protection of endothelial dependent and independent responses in ischaemic hearts [744]. In support of the study of Cartier *et al.* [744] BDM treatment attenuated post-ischaemic vascular dysfunction. The precise mechanism of action of BDM remains to be determined, but potentially involves altered protein phosphatase activity [741], and/or Ca^{2+} handling and contractile control [745-747]. Nonetheless, it may be useful in limiting vascular dysfunction in addition to myocardial injury. The agent has been shown to be well tolerated in humans [748].

Potential Mechanisms of Vasoprotection Via A_3ARs and NHE Inhibition.

Since protection via NHE inhibition was not additive with A_3AR mediated vasoprotection, it is possible these stimuli activate common mechanistic paths or end-effectors. Indeed, adenosine receptor signalling is coupled to NHE modulation in cardiac and non-cardiac tissues [543,750]. Moreover, signalling via A_3ARs involves RhoA and phospholipase D dependent PKC activation [610], and Rho and PKC signalling inhibit NHE activity [751]. Furthermore, A_1ARs, which share signalling elements with A_3ARs, inhibit activation of sarcolemmal NHE activity [543]. The specific role of NHE inhibition in A_3AR mediated protection remains to be more

directly tested.

The present data indicate the vasoprotective effects of A_3AR activation and NHE inhibition do not involve blockade of ET and oxidant injury in this model. Since hearts are blood free, anti-neutrophil actions of A_3ARs and NHE inhibition are also unimportant [592,752]. As reviewed by Baxter recently [8], the weight of evidence now suggests neutrophils are not primary mediators of most forms of ischaemia-reperfusion injury, and indicates vascular injury occurs prior to neutrophil accumulation. Perhaps of greater relevance, endothelial Ca^{2+} overload may interfere with NO generation [753]. As reported by Maczewski and Beresewicz [754], prevention of Ca^{2+} overload by NHE inhibition limits excessive Ca^{2+}-dependent NO generation. Importantly, A_3AR activation also modulates cytosolic Ca^{2+} by reducing ryanodine binding in myocytes, delaying intracellular Ca^{2+} overload in a similar manner to NHE inhibition [542].

Relation Between Myocardial Injury And Vascular Dysfunction

Relevance of coronary dysfunction to myocardial recovery is important to consider. Prior studies verify vascular dysfunction contributes to myocardial contractile depression and reveal impaired reflow is a key determinant of infarct size [5]. Tight coupling between reflow and ischaemic damage, while not surprising, highlights the importance of vascular dysfunction in dictating post-ischaemic phenotype. In the current model, a weaker link between vascular dysfunction and myocardial injury is observed. While coronary dysfunction is reduced by A_3AR agonism and NHE inhibition, reflow does not differ from untreated hearts (Fig. 6.5A). Thus, reflow in this model is not crucial to the cardiac protection observed, consistent with prior findings from our lab [686,755], and others [756], which collectively indicate coronary reflow in isolated hearts is not a critical determinant of cardiac injury. It is thus probable "no-reflow", shown to contribute to infarct size, is mediated via blood cell components and

involves vascular plugging not evident in blood free models.

Correlations between reflow and myocardial outcome also raise a less obvious possibility that changes in myocardial damage (or myocardial pathways) may impact on coronary injury [5]. This possibility was assessed by examining effects of a cardiac specific intervention, transgenic A_1AR overexpression in cardiomyocytes [555]. This strategy markedly limits cardiac injury (Fig. 6.5), in agreement with prior data [555], yet failed to modify coronary dysfunction (Fig. 6.6) despite the fact endogenous A_1AR activation limited vascular injury (Fig. 6.1A). Additionally, BIIB-513 concentration dependently limited myocardial injury (Fig. 6.5), but not coronary dysfunction (Fig. 6.4A). Finally, the cardiac protective effects of BIIB-513 and Cl-IB-MECA were identical whereas vasoprotective responses differed (Fig. 6.4A). These varied data show extent of cardiac injury does not impact on vascular dysfunction, and also show that A_1AR mediated vasoprotection must occur via processes unrelated to cardiac A_1ARs (ie. activation of non-cardiac A_1ARs).

Conclusions

In summary, the current data verify that activation of A_1ARs by endogenous adenosine, and reveals activation of A_3ARs by exogenous agonists, significantly attenuates post-ischaemic coronary endothelial dysfunction in intact mouse hearts. Activation of A_3ARs also reduces effects of ischaemia-reperfusion on coronary reactive hyperaemia. Inhibition of Na^+/H^+ exchange is vasoprotective, with effects not being additive with those of A_3AR agonism. This provides preliminary support for common signalling via A_3ARs and NHE inhibition. Given the importance of vascular integrity and function in outcome from ischaemic insult, the genesis of this coronary dysfunction, and the precise mechanism of pronounced protection via A_3 (and A_1) adenosine receptors deserves further investigation.

CHAPTER 7

GENERAL CONCLUSION

Coronary heart disease leading to acute myocardial infarction is the number one killer of people in the industrialised world [757]. In 2000 alone, coronary heart disease accounted for 39% of all deaths, totalling 49,741 deaths Australia wide [757,758]. Thus understanding mechanisms involved in the pathogenesis of coronary heart disease remains critical in reducing the number of potentially preventable deaths, improving our quality of life and reducing the growing cost of healthcare. Importantly, endothelial dysfunction is a hallmark of the leading form of coronary heart disease, atherosclerosis, which if untreated ultimately results in myocardial infarction [532,533]. In addition, endothelial dysfunction is also observed following routine clinical procedures, such as, thrombolysis, recannulisation and revascularisation, due to an unavoidable episode of ischaemia-reperfusion. Therefore, understanding the mechanisms involved in the manifestation of endothelial dysfunction, may be the cornerstone for effective treatment and management in the incidence of vascular pathologies and the increased risk of death from resultant myocardial infarction.

The studies presented within this thesis attempted to not only increase our understanding of adenosine's role in normal vascular function, but focused on the impact of ischaemia-reperfusion on coronary vascular function in an asanguinous model. Additional studies also assessed the potential of adenosinergic therapy and other vasoprotective strategies as a step towards understanding the mechanisms involved in the manifestation of post-ischaemic vascular injury. Furthermore, the relation between flow and contractility in post-ischaemic blood-free hearts was identified and the potential of myocardial injury impacting upon vascular dysfunction in the absence of inflammatory responses was assessed.

The studies described demonstrate that adenosine plays a significant role in the control of normal coronary vascular function in the murine model. Activation of

A_{2A}ARs mediates coronary dilation in mouse, whereas A_{2B}ARs mediate dilation in rat. Responses in mouse occur largely via NO-independent mechanisms, however, a sensitive NO-dependent mechanism also contributes to coronary dilation (when adenosine concentrations are low). These finding indicate that endothelial responses may play an important role under more physiological conditions. This is consistent with findings of Hein and Kuo [136] that the endothelium is highly sensitive to varying concentrations of adenosine. Though not investigated here, the increased sensitivity of endothelium to adenosine is thought to involve G-proteins (coupled to adenosine receptors on endothelium) amplifying cellular signalling linked to K_{ATP} channels [136].

Subsequent studies described identified that K_{ATP} channels and NO are primarily involved in mediating sustained elevations in flow during reactive hyperaemic responses in isolated mouse heart, irrespective of occlusion duration (5-40 s). However, K_{ATP} channels appear to be predominantly involved in modulating initial flow adjustments following brief (5-10 s) occlusions, whereas K_{ATP} (and NO) independent processes are increasingly important with longer (20-40 s) occlusion. Data acquired support a modest role for A_{2A}ARs in mediating reactive hyperaemia, with a negligible role for prostanoids. Interestingly, compensatory changes in K_{ATP} and/or NO mediated dilation occur when one path is blocked. This may have important implications post-ischaemia when endothelial mechanisms may be impaired. Importantly, co-treatment studies with K_{ATP} channel, NO, prostanoid and A_{2A}AR antagonism were unable to completely abolish changes in coronary flow reserve. Therefore, additional mechanisms are involved in reactive hyperaemic responses. Future studies will examine a role for EDHF, since there is a growing body of support for a role for EDHF-mediated dilation in the regulation of coronary blood flow *in vitro* and *in vivo* [699,700].

Limited studies have assessed the impact of ischaemia-reperfusion in asanguinous hearts. The research studies undertaken in this thesis verified that endothelial dysfunction is manifest post-ischaemia in buffer perfused hearts. Furthermore, endogenous activation of A_1ARs protects against this vascular injury. In support of these findings, Maczewski and Beresewicz [715] observe vascular protection via A_1 receptors independent of blood-born elements. Additionally, data presented here support a key role for A_{2A}ARs in controlling coronary flow post-ischaemia. However, we find no direct evidence of impaired reflow relative to work performed post-ischaemia in this model. Thus, coronary reflow does not appear to be critical determinant of myocardial injury in isolated buffer perfused hearts.

To the best of our knowledge the potential of the adenosine receptor system in modifying post-ischaemic vascular injury has not been previously examined. Importantly, data presented here demonstrate that post-ischaemic endothelial dysfunction is significantly reduced by intrinsic A_1AR activation and that a superior degree of vasoprotection is afforded by exogenous A_3AR activation. Interestingly, protection appears unrelated to inhibition of ET or oxidant stress. However, evidence supports a role for Ca^{2+} (based on effects of the Ca^{2+} desensitiser, BDM) as well as NHE (based on effects of NHE inhibitor) in vasoprotection. Furthermore, preliminary data suggest A_3AR vasoprotection may share signalling with NHE inhibition. Finally, this study demonstrates that post-ischaemic myocardial injury does not impact upon the severity of vascular dysfunction. Since strategies that limit myocardial injury, such as A_1AR overexpression [555], failed to modify vascular dysfunction.

In summary, these studies show that adenosine has a significant role in mediating coronary dilation and reactive hyperaemic responses via a sensitive NO-dependent and K_{ATP}-dependent mechanism. Ischaemia-reperfusion modifies vascular

control and induces significant endothelial dysfunction in the absence of blood, and endogenous adenosine affords intrinsic protection against this injury via A_1AR activation. Adenosinergic therapy via exogenous A_3AR activation represents a new strategy for directly protecting against post-ischaemic vascular injury.

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Note: * denotes publications which include Thesis material

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