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Running title: SmadL integrates all GPCR signalling

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Keywords:
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Abstract

Protease activated receptors (PARs) transactivate both epidermal growth factor receptors (EGFR) and transforming growth factor (TGF)-β receptors (TGFBR1) in vascular smooth muscle leading to the increased expression of genes (CHST11 and CHSY1) which are rate limiting for the enzymes that mediate hyperelongation of glycosaminoglycan (GAG) chains on the lipid-binding proteoglycan, biglycan. This is an excellent model to investigate mechanisms of transactivation as the processes are biochemically distinct. EGFR transactivation is dependent on the classical matrix metalloprotease (MMP) based triple membrane bypass mechanism and TGFBR1 transactivation is dependent on Rho/ROCK signalling and integrins. We have shown that all kinase receptor signalling is targeted towards phosphorylation of the linker region of the transcription factor, Smad2. We investigated the mechanisms of thrombin mediated kinase receptor transactivation signalling using anti-phospho antibodies and Western blotting and gene expression by RT-PCR. Thrombin stimulation of phospho-Smad2 (Ser 245/250/255) and of phospho-Smad2(Thr220) via EGFR transactivation commences quickly and extends out to at least 4 hr whereas transactivation via TGFBR1 is delayed for 120 min but also persists for at least 4 hr. Signalling of thrombin stimulated Smad linker region phosphorylation is approximately equally inhibited by the MMP inhibitor, GM6001 and the ROCK inhibitor, Y27632, and similarly expression of CHST11 and CHSY1 is approximately equally inhibited by GM6001 and Y27632. The data establishes Smad linker region phosphorylation as a central target of all transactivation signalling of GAG gene expression and thus an upstream kinase may be a target to prevent all transactivation signalling and its pathophysiological consequences.

Introduction:

G protein coupled receptors (GPCRs) have long been a major interest of pharmacology studies as they are involved in diverse physiological pathways and functions. GPCRs currently constitute the largest family of proteins targeted by approved drugs [1] accounting for more than 27% of the therapeutic global market [2]. The identification and exploitation of new targets is warranted for diseases with unmet clinical needs. There is a need to elucidate GPCR signalling to its full extent. Classic GPCR signalling involves transactivation independent pathways [3, 4] passing from the agonist to the receptor, leading to activation of G proteins and signal propagation via a plethora of downstream mechanisms.

For over two decades it has been known that GPCRs can transactivate cell surface protein tyrosine kinase receptors (PTKR) [5] and over this time it was also established that GPCRs can also transactivate serine/threonine kinase receptors (S/TKR) [6, 7]. Since then there has been considerable work on the mechanism(s) of transactivation [3]. In the context of human vascular smooth muscle cells (VSMCs), protease activated receptor (PAR)-1 agonist thrombin transactivates the PTKR epidermal growth factor receptor (EGFR) via the "triple membrane bypass" pathway which involves activating a membrane bound matrix metalloproteinase (MMP) resulting in the cleavage and release of EGFR ligand which subsequently activates its cognate receptor [8, 9]. The PAR-1 receptor can also transactivate the S/TKR, transforming growth factor (TGF)-β type 1 receptor (TGFBR1) via cytoskeletal rearrangement which activates ROCK signalling, leading to the activation of cell surface integrins and ultimately activation of large latent complex which holds TGF-β near the surface that subsequently is rearranged and activates the TGFBR1 [3, 4, 9, 10].

Transactivation signalling has emerged to be equally important to classic signalling when measured by the ability to stimulate gene expression in array studies [11]. Transactivation dependent signalling accounts for approximately 50 percent of the total genes regulated by the GPCR, PAR-1[11]. Thus GPCR transactivation dependent signalling significantly broadens the GPCR signalling paradigm [11, 12]. Thrombin transactivation of the EGFR and TGFBR1 lead to enhanced synthesis of extracellular matrix (ECM), proteoglycans [6] and the genes involved in the elongation of the glycosaminoglycan (GAG) chains covalently attached to the proteoglycan core protein [8, 13, 14]. The modification of proteoglycan structure predominantly with the elongation of the GAG chains results in an increased binding to lipids in vitro [15-17] leading to the progression of atherosclerosis in the vessel wall. Hence exploitation of transactivation dependent signalling is of great interest as the identification of a common intermediate could lead to a potential therapeutic target for atherosclerosis [11].

The canonical TGF-β signalling pathway involves direct carboxy-terminal phosphorylation of Smad2 by TGFBR1 [18]. In addition to carboxy-terminal phosphorylation, Smads can also be phosphorylated in the linker region [19-21]. Downstream of the TGFBR1, serine/threonine kinases are activated which phosphorylate the Smad2 linker region that drives the synthesis and expression of genes for the enzymes involved in the elongation of the GAG chains [22-25]. The paradigm for Smad signalling is evolving [26]. Recent observations by our lab show that in keratinocytes, the GPCR agonist thrombin transactivates the EGFR to phosphorylate the Smad2 linker region [27]. Smad2 linker region phosphorylation drives proteoglycan synthesis [22] and GAG gene expression [23-25] hence we investigated the mechanisms of Smad2 linker phosphorylation as a central intermediate in thrombin mediated GAG gene expression. A central integrating point for all GPCR mediated transactivation of the two kinase receptors leading to GAG hyperelongation has the potential to be developed as a vessel wall directed therapeutic target to prevent atherosclerosis and cardiovascular disease.

This project investigates the mechanisms involved in Smad2 linker region phosphorylation via thrombin transactivation of both the EGFR and TGFBR1. We report that thrombin stimulates Smad2 linker region phosphorylation which occurs via transactivation of the EGFR and TGFBR1. The combined mechanisms involving these signalling pathways account for all thrombin stimulated gene expression of GAG synthesizing enzymes chondroitin 4-sulfotransferase-1 (CHST11) and chondroitin synthase-1 (CHSY-1). This data points towards the Smad2 linker region as a central integrating point for all GPCR transactivation dependent signalling leading to GAG chain hyperelongation and thus upstream kinases represent a potential vessel wall directed therapeutic target to prevent development of atherosclerosis and cardiovascular disease.

Methods and Materials:

Materials

Foetal bovine serum (FBS) was purchased from Invitro Technologies Pty. Ltd. (VIC, Australia). Dulbecco's Modified Eagle Medium (DMEM) (0 and 25mM glucose), Trypsin-Versene, penicillin and streptomycin were from Thermo Scientific (VIC, Aus). Human TGF-β1, anti-pSmad2(Ser245/250/255), anti-rabbit IgG horseradish peroxidase (HRP) anti-GAPDH antibodies were purchased from Cell Signalling Technology (MA, USA). Flavopiridol, SP600125, SB202190 and LY294002 were purchased from Sigma-Aldrich (St Louis, USA). UO126 was from Promega (WI, USA). Amersham ECL Prime chemiluminescence detection reagent was purchased from GE Healthcare (NSW, Aus). The primers (forward and reverse) for CHST11, CHSY1 and 18S, RNeasy Mini Kit, the QuantiTect reverse transcription kit, QuantiFast SYBR green PCR kit and the Rotor Gene Q series software were from Qiagen (VIC, Aus). Anti-phospho-Smad2L(Thr220) rabbit IgG polyclonal was a gift from Professor Koichi Matsuzaki (Kansai Medical University, Osaka, Japan)

Cell culture

Primary cultures of human vascular smooth muscle cells (VSMCs) were obtained from discarded sections of saphenous veins from patients undergoing surgery at the Alfred Hospital (Melbourne, Australia) and the acquisition of the vessels was approved by the Alfred Hospital Ethics Committee. VSMCs were grown in DMEM (5mM glucose, 10% FBS and 1% antibiotics at 37°C in 5% CO_2). VSMCs were seeded in 60 mm dishes and 6 well-plates. Cells were grown to confluence then rendered quiescent by serum deprivation for 48 h. Cells were pre-incubated with inhibitors for 30 min prior to treatment with agonists. Incubation times and concentrations are given in detail in the figure legends.

Western blotting

Whole cell lysates (30μg of protein) were resolved on 10% SDS-PAGE and semi-dry transferred onto PVDF. Membranes were blocked with 5% bovine serum albumin and incubated with primary antibody targeting protein of interest followed by HRP-anti-rabbit IgG and ECL detection. The protein of interest was normalised with GAPDH to determine equal loading. Blots were imaged using the Bio-Rad gel documentation system and densitometry analysis was performed with Quantity One imaging software.

Assessing mRNA gene expression

The mRNA level of the GAG enzymes was determined by quantitative real-time polymerase chain reaction (RT-PCR). Total RNA was isolated from VSMCs treated as described. RNA was extracted from 5×10^5 cells using RNeasy Mini kit (Qiagen) according to the instructions of the manufacturer. RNA purity was checked by spectrophotometry (260/280 nm) using Nanodrop2000 spectrophotometer (Thermo Fisher Scientific). First strand cDNA was synthesized from 1µg RNA using Quantitect reverse transcriptase kit (Qiagen) according to the instructions of the manufacturer. Quantitative RT-PCR was performed using Qiagen Rotor Gene Q and QuantiFast SYBR green PCR master mix kit (Qiagen). Data was normalised to the ribosomal 18S housekeeping gene to adjust for control variations between individual experiments. Relative expression of mRNA levels was quantified using comparative delta delta Ct method.

Statistical analysis

Normalised data is expressed as the mean ± standard error of the mean of three or more independent experiments, unless stated otherwise. A one-way ANOVA was used to calculate statistical significance of normalised data as stated followed by least significant difference *post-hoc* analysis. Results were considered significant when the probability was less than 0.05 (*$p<0.05$) and 0.01 (**$p<0.01$).

Results:

Thrombin mediated Smad2 linker region phosphorylation occurs through the transactivation of the EGFR

Our earlier work showed that thrombin via its receptor PAR-1 transactivates both the EGFR and TGFBR1 to modify proteoglycan synthesis [6, 9] and mediate GAG chain elongation [8, 14] via completely distinct biochemical mechanisms. In the context of TGF-β signalling, Smad2 linker region phosphorylation is associated with GAG gene expression [23, 24] and GAG hyperelongation [28]. We investigated the role of Smad2 linker region phosphorylation as a central point for all thrombin mediated GAG chain hyperelongation. To address this question we carried out a concentration-dependent study in VSMCs using the EGFR antagonist, AG1478 (0.5-10μM) and TGFBR1 antagonist, SB431542 (0.3-30μM), in the presence of thrombin for 30 min on the phosphorylation of the cluster serine residues (Ser245/250/255) and the threonine site (Thr220) of the Smad2 linker region.

In VSMCs thrombin treatment stimulated phosphorylation of the Thr220 residue which was dose dependently inhibited by AG1478 at concentrations of 0.5-10μM ($p<0.01$) (Fig 1A). Treatment with EGF (100nM) showed a 2.5-fold increase in the phosphorylation of Thr220 and as expected this response was abolished in the presence of AG1478 (Fig 1A). Thrombin treated VSMCs showed a 7.7-fold increase in the phosphorylation of the Ser245/250/255 of the Smad2 linker region (Fig 1B). In the presence of AG1478 a partial inhibition to approximately 4-fold stimulation (0.1-5μM)($p<0.01$) was seen (Fig 1B). EGF treated VSMCs showed a 5-fold increase in the phosphorylation of Ser245/250/255 and as expected this was completely abolished in the presence of AG1478 ($p<0.01$) (Fig 1B). The data demonstrates that at the relatively early time point of 30 min treatment with thrombin transactivates the EGFR to phosphorylate the Ser245/250/255 and Thr220 residues of the Smad2 linker region.

We then evaluated the role of TGFBR1 transactivation in thrombin stimulated phosphorylation of Smad linker region sites. Thrombin treated VSMCs showed a 5-fold ($p<0.01$) increase in the phosphorylation of the Thr220 residue (Fig 1C). This response was unaffected by treatment with SB431542 (0.3-30μM). VSMCs stimulated with TGF-β showed a 10-fold increase in the phosphorylation of the Thr220 residue and this was abolished in the presence of SB431542 (Fig 1C). Thrombin treated VSMCs showed an increase in the phosphorylation of the Ser245/250/255 by 12-fold ($p<0.01$) this was unaffected by treatment with SB431542 (0.3-30μM) (Fig 1D). TGF-β treatment increased the phosphorylation of Ser245/250/255 and this was completely inhibited ($p<0.01$) by SB431542 (Fig 1D). These results indicate that at 30 min thrombin stimulated Smad2 linker region phosphorylation is occurring via the transactivation of the EGFR and not the TGFBR1.

Thrombin mediated transactivation of the TGFBR1 leading to phosphorylation of the Smad2 linker region is time dependent.

In bovine aortic endothelial cells (BAECs) TGF-β mediated phosphorylation of the Smad2 linker region residues peaked at 60 min [29]. Similarly in VSMCs, TGF-β mediated phosphorylation of the Smad2 linker region is maximal at 60 min [22-24]. The hypothesis tested here is if thrombin transactivation of the TGFBR1 leading to Smad2 linker region phosphorylation has a delayed component when compared to thrombin transactivation of the EGFR. VSMCs were treated with thrombin for 60, 120 and 240 min in the presence and absence of SB431542 (10μM). Thrombin treatment of VSMCs at 60 min caused a 2.2-fold increase in the phosphorylation of Thr220, this was unaffected in the presence of SB431542 (Fig 2A). However at 120 and 240 min thrombin mediated phosphorylation of Thr220 was increased to approximately 4-fold and this response was inhibited ($p<0.01$) by more than 50 per cent in the presence of SB431542 (Fig 2A). The TGFBR1 antagonist abolished ($p<0.01$) TGF-β mediated phosphorylation of the Thr220 in the Smad2 linker region (Fig 2A).

The thrombin mediated phosphorylation of Ser245/250/255 followed a similar pattern of stimulation and inhibition to that observed for the phosphorylation of the Thr220 residue. At 60 min treatment with thrombin there was a 2.2-fold increase in level of the phosphorylation of Ser245/250/255 and this response was unaffected by SB431542 (Fig 2B). However, VSMCs treated with thrombin for 120 and 240 min showed an increase in the phosphorylation of Ser245/250/255 to 3.8 and 4 fold, respectively ($p<0.01$) (Fig 2B) and treatment with SB431542 inhibited this response by 50-55% ($p<0.01$) (Fig 2B). As the control, TGF-β treated VSMCs caused a large increase in the phosphorylation of the serine residues which was abolished by SB431542 (Fig 2B). These results indicate that in VSMCs, thrombin stimulation of the phosphorylation of the Smad2 linker region via the transactivation of the TGFBR1 occurs but only after a time delay of 60 min. Thrombin transactivation of the EGFR occurs within 30 min however transactivation of the TGFBR1 leading to Smad2 linker phosphorylation is activated at 120 min and is sustained for 240 min. The implication of these results is consistent with our earlier reports that these two cellular responses occur by distinct biochemical mechanisms.

Thrombin mediated transactivation of the EGFR leading to the phosphorylation of the Smad2 linker region is sustained for 4 hr.

We have shown that in VSMCs thrombin mediated transactivation of the TGFBR1 leading to Smad2 linker region phosphorylation is time dependent. The onset of GPCR mediated transactivation of EGFR is rapid [30, 31] as compared to transactivation of the TGFBR1. Thrombin stimulates the phosphorylation of the Smad2 linker region via EGFR transactivation within 30 min (Fig 1C-D), hence we investigated whether this response is sustained for 4 hr. To address this question VSMCs were treated with thrombin over a time course (30-240 min) in the presence and absence of AG1478 (5μM). EGF stimulation at 15 min in the presence of AG1478 was used as a positive control. Thrombin treatment stimulated the phosphorylation of Thr220 at 60, 120 and 240 min (4-6 fold) which was inhibited 50-70%

in the presence of AG1478 (Fig 3A). EGF treatment stimulated the phosphorylation of Thr220 which was abolished in the presence of AG1478 (Fig 3A). These results show thrombin stimulation of the phosphorylation of the Thr220 residue occurs via transactivation of the EGFR and is sustained for at least 4 hr. VSMCs treated with thrombin stimulated the phosphorylation of Ser245/250/255 to 2.5-fold at 60, 120 and 240 min ($p<0.01$)(Fig 3B). At the three time points the phosphorylation was inhibited by 50-60% ($p<0.01$) in the presence of AG1478 (Fig 3B). These results indicate that Smad2 linker region phosphorylation occurring via PAR-1 transactivation of the EGFR occurs within 30 min and is sustained up to 4 hr. Thus, mechanistically, both EFGR and TGFBR1 transactivation pathways show sustained stimulation out to at least 4 hr but the TGFBR1 pathway shows a delayed onset of 60-120 min. These temporal differences in the response profiles most likely arise from the distinct biochemical mechanisms of transactivation so we proceeded to probe deeper into the mechanisms

Thrombin mediated Smad2 linker region requires the activation of matrix metalloproteinase and cytoskeletal rearrangement

The transactivation of PTKR, but not S/TKR, is largely dependent on the triple membrane by-pass system that involves the stimulation of cell surface matrix metalloproteinase (MMP) leading to the generation of the ligand that binds to the EGF receptor [30]. In contrast, the transactivation of S/TKRs is achieved by cytoskeletal rearrangement which activates ROCK signalling leading to the activation of cell surface integrins which allow for the latent ligand to bind its receptor [32]. To investigate whether PAR-1 mediated phosphorylation of Smad2 linker region is a common signalling point to both transactivation dependent pathways we used pharmacological inhibitors, GM6001 as a broad spectrum MMP inhibitor and Y27632 as a ROCK antagonist. VSMCs were treated with thrombin for 4 hr in the presence and absence of respective antagonists (Fig 4). Thrombin treatment stimulated the phosphorylation of

Thr220 to 1.5-fold ($p<0.01$) (Fig 4A). This response was inhibited by greater than 50 percent by either MMP ($p<0.05$) or ROCK ($p<0.01$) antagonists (Fig 4A). VSMCs treated with thrombin for 4 hr increased the phosphorylation of Ser245/250/255 to 2.4 fold ($p<0.01$) (Fig 4B). In presence of MMP and ROCK antagonist this stimulation was by more than 50 percent inhibited ($p<0.05$) (Fig 4B). Taken together these results indicate that thrombin mediated Smad2 linker region phosphorylation is mediated by transactivation of the EGFR and TGFBR1.

Thrombin mediated GAG chain elongation is dependent on matrix metalloproteinase activation and cytoskeletal rearrangement

We explored if the biochemical mechanisms which define EGFR and TGFBR1 transactivation that determine Smad linker region phosphorylation extent to the effects on thrombin stimulated expression of the mRNA of the genes which are rate limiting for GAG elongation on the proteoglycan, biglycan [8, 13]. Thrombin treatment stimulated the mRNA expression of CHST11 to 4-fold ($p<0.01$) (Fig 5A). In the presence of the MMP inhibitor, GM6001, and ROCK antagonist, Y27632, this response was inhibited by 50 percent ($p<0.01$) (Fig 5A). VSMCs treated with thrombin showed an increased expression of CHSY-1 mRNA to 3.2-fold ($p<0.01$) (Fig 5B) and treatment with MMP and ROCK antagonists inhibited 60 and 45 percent, respectively (Fig 5B). Taken together these results indicate that thrombin mediated Smad2 linker region phosphorylation is mediated by transactivation of EGFR and TGFBR1, these results are consistent with our earlier findings related to hyperelongation of GAG chains [9] and the expression of genes for GAG elongation [8].

Discussion

The exploitation of the signalling pathways which regulate proteoglycan synthesis and specifically the length of the GAG chains on the proteoglycan represents a potential therapeutic target for the prevention of lipid deposition diseases such as atherosclerosis and aortic valve disease [33-35]. In this study we investigated the role of Smad linker region phosphorylation in GPCR transactivation dependent signalling pathways involving both EGFR and TGFBR1. We show that thrombin mediated Smad2 linker region phosphorylation occurs via transactivation of both EGFR and TGFBR1.Transactivation signalling via the TGFBR1 is delayed (by30-60min) when compared to transactivation of the EGFR but both pathways are activated for at least 4 hr. Thrombin mediated Smad2 linker region phosphorylation is dependent on MMP and ROCK signalling pathways. When examining the genes associated with the elongation of the GAG chains, we observed a partial role for MMPs and ROCK dependent pathways in stimulation of the mRNA expression of CHST11 and CHSY1, MMPs reflect signalling via EGFR and ROCK is involved in TGFBR1 transactivation. Consistent with our earlier results both transactivation dependent pathways mediated GAG elongation [8], however we also demonstrate the role for the Smad linker region as a common intermediate for all signalling occurring via transactivation dependent pathways.

Thrombin transactivation of the EGFR has a rapid 15 min onset and is sustained for at least 4 hr, however transactivation of the TGFBR1, mediated Smad2 linker region phosphorylation, requires 120 mins. GPCR agonists such as lysophosphatidic acid (LPA) [36-38], thryrotropin releasing hormone [39], endothelin-1 [5, 40] and thrombin [9, 41, 42] rapidly induce the transactivation of the PTKR, EGFR. GPCR-mediated transactivation of the EGFR occurs via the activation of a membrane bound MMP, resulting in the cleavage and release of the EGF ligand which subsequently activates its cognate receptor in an autocrine manner [43-45]. Although a convoluted pathway, surprisingly this occurs very quickly (within minutes). The results presented in this study reveal that thrombin transactivation of the TGFBR1 leading to the phosphorylation of the Smad2 linker region is time dependent in that it occurs after a lag period of about 60 min. GPCR-mediated transactivation of the TGFBR1 is time dependent under various other conditions. For example, in mouse embryonic fibroblasts transfected with β6 integrin, LPA-mediated transactivation of the TGFBR1, assessed by the phosphorylation of the downstream intermediate Smad2, was evident at 2-4 hr post treatment [46]. In human airway smooth muscle cells, LPA and methacholine treated cells resulted in a delayed increase in the phosphorylation of the Smad2 protein signifying a delay in GPCR-mediated transactivation of the TGFBR1 [47]. In VSMCs, the protracted time course in thrombin-mediated transactivation of the TGFBR1 is not due to new protein synthesis. The inhibitor of translation and transcription, cycloheximide, has no effect on thrombin-mediated phosphorylation of the Smad2 [9]. GPCR transactivation of the TGFBR1 occurs via cytoskeletal rearrangement which activates ROCK signalling, resulting in the activation of cell surface integrins which bind to the latent TGF-β complex. This results in the exposure of the TGF-β ligand to the receptor [48]. The cell surface integrin activation which is involved in the transactivation of TGFBR1 is hypothesized to be the rate limiting event which explains why this transactivation cascade is delayed. Integrins signal via inside out, allowing for the talin and kindlin to bind to the cytoplasmic end of the β subunit [9, 48]. Compared to GPCR transactivation of EGFR, signalling via transactivation of TGFBR1 leading to Smad2 linker region phosphorylation is delayed due to the cell surface mechanisms that are involved in transactivation dependent signalling. These cellular processes need to be evaluated in detail to identify the molecular events of PAR-1 mediated transactivation of TGFBR1.

The Smad linker region is associated with a number of pathophysiological conditions, including but not limited to cancer development [20, 49], cardiovascular disease [22, 23, 28, 50] and Alzheimer's [51]. The work presented here demonstrates that GPCR transactivation of the EGFR and TGFBR1 share Smad2 linker as a common signalling target. A genome wide study published by our group shows that transactivation dependent signalling accounts for 50% of all GPCR mediated signalling [11]. The transactivation dependent signalling pathways are equally important, with approximately the same number of genes regulated by either pathway.

GPCR transactivation of a PTKR involves a mechanism that is distinct from transactivation of S/TKR. Together, these findings demonstrate that the Smad2 linker region is a common intermediate between the two pathways, serving as a mediator of biological processes occurring via GPCR transactivation dependent signalling

In summary, GPCR transactivation of EGFR and TGFBR1 signal via Smad2 linker region to increase the expression of genes associated with GAG chain elongation on proteoglycans. This represents an example and a model of a concurrent signalling cascade and a kinase upstream of the Smad linker region might provide a therapeutic target for various human diseases.

Figure 1: Early thrombin mediated linker region phosphorylation is through the transactivation EGFR. VSMCs were treated with thrombin for 30 min, EGF for 15 min or TGF-β for 30 min, in the presence and absence of **A-B** EGFR antagonist AG1478 (0.5-10μM) or **C-D** TGFBR1 antagonist SB431542 (0.3-30μM). Blots were probed with antibodies specific to **A,C.** phospho-Smad2/3(Thr220/179) or **B,C.** phospho-Smad2(Ser245/250/255). Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis ##,$p<0.01$ versus basal and **, $p<0.01$ agonist versus antagonist.

Disclosure and funding

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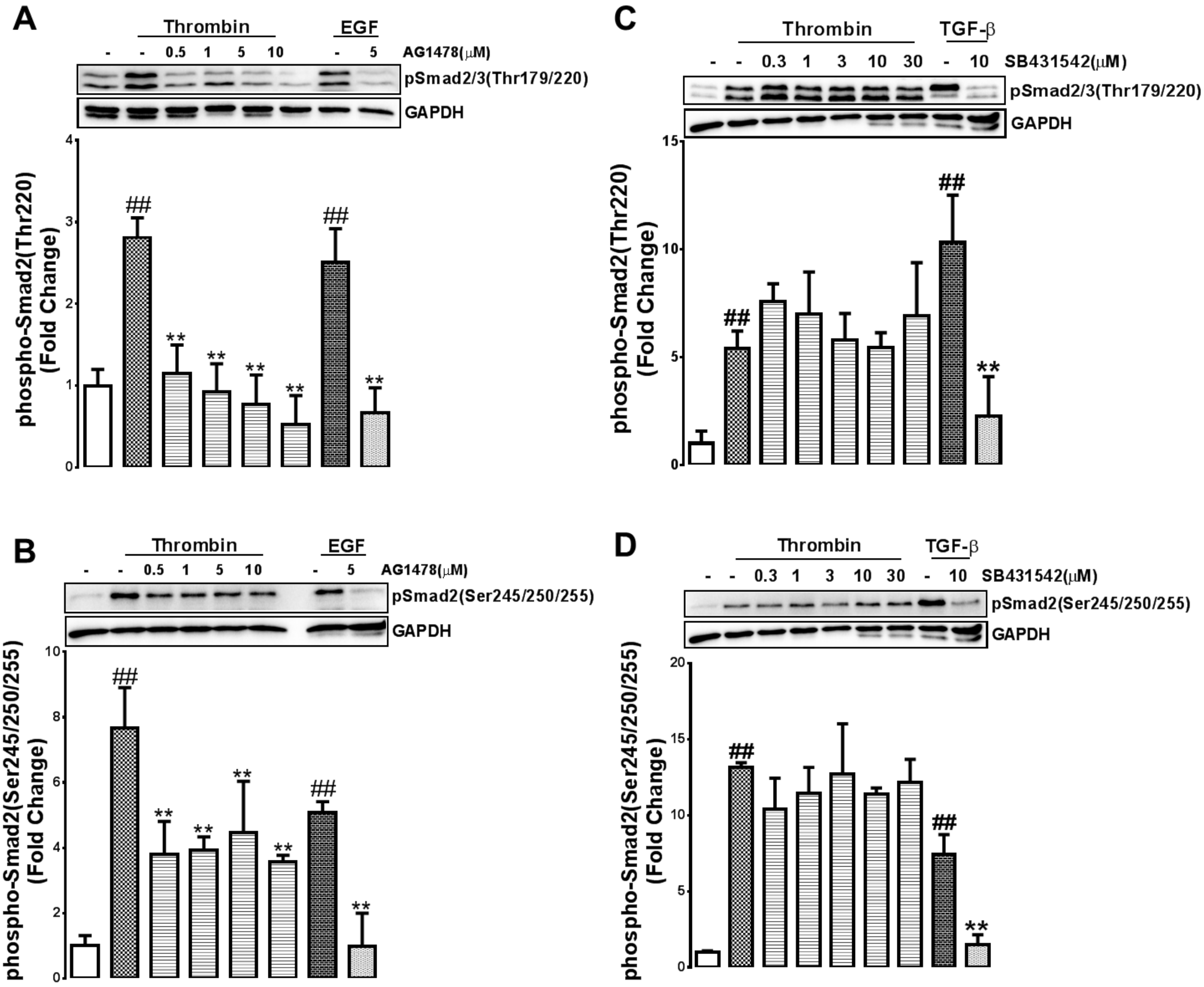
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Thrombin
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pSmad2/3(Thr179/220)
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pSmad2(Ser245/250/255)
GAPDH
phospho-Smad2(Ser245/250/255) (Fold Change)
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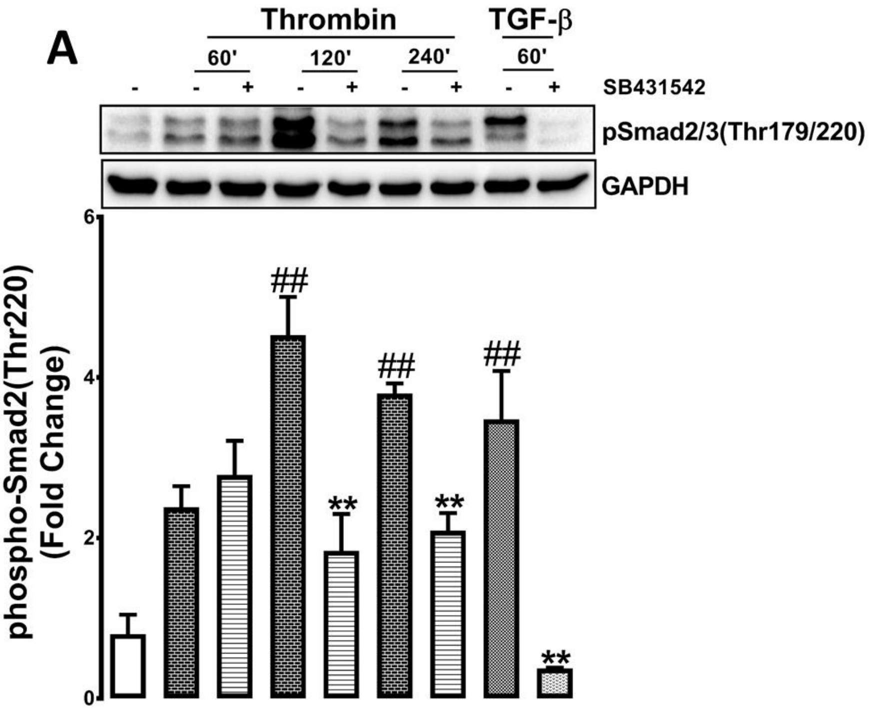
SB431542(μM)
- - 0.3 1 3 10 30 - 10
pSmad2(Ser245/250/255)
GAPDH
phospho-Smad2(Ser245/250/255) (Fold Change)

Figure 2: Thrombin mediated Smad2 linker region phosphorylation via the TGFBR1 is time dependent. VSMCs were treated with thrombin over 240 min time course or TGF-β for 60 min in the presence and absence of TGFBR1 antagonist SB431542 (10μM). Blots were probed with antibodies specific to **A.** phospho-Smad2/3(Thr220/179) or **B.** phospho-Smad2(Ser245/250/255). Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis ##,$p<0.01$ versus basal and **, $p<0.01$ agonist versus antagonist.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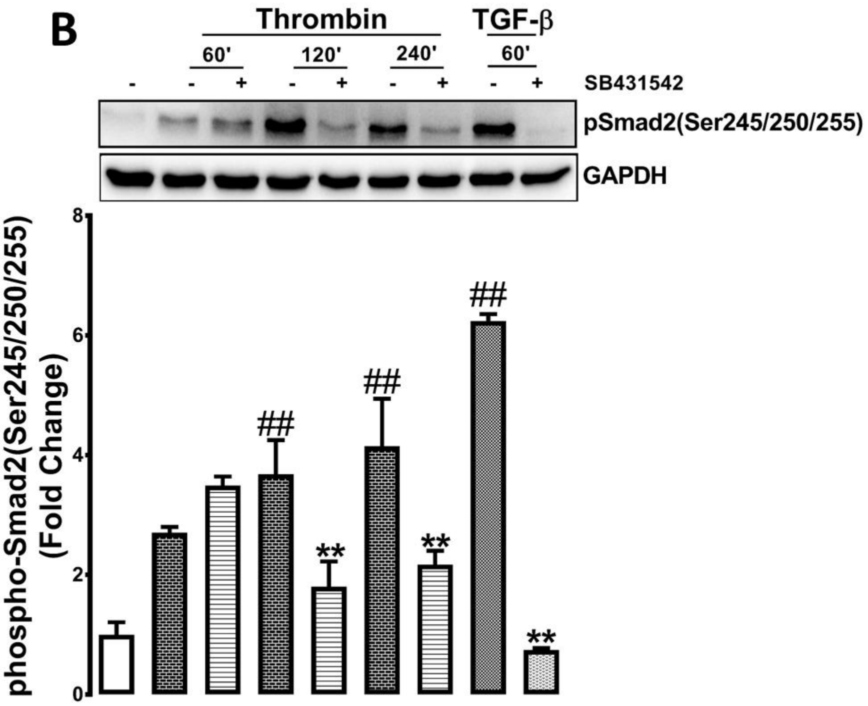
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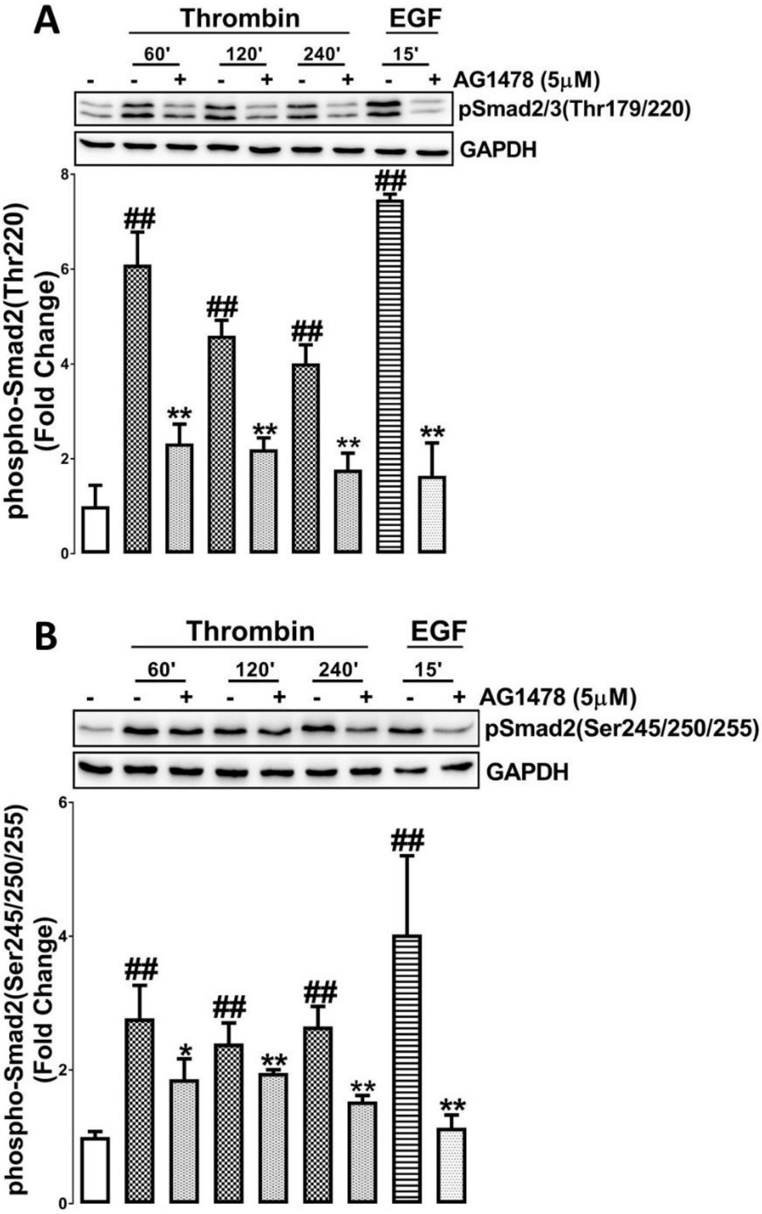
Figure 3: Thrombin mediated Smad2 linker region phosphorylation via the EGFR is sustained for four hr. VSMCs were treated with thrombin over 240 min time course or EGF for 15 min in the presence and absence of EGFR antagonist AG1478. Blots were probed with antibodies specific to **A.** phospho-Smad2/3(Thr220/179) or **B.** phospho-Smad2(Ser245/250/255). Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis ##,$p<0.01$ versus basal and **, $p<0.01$ agonist versus antagonist.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Thrombin
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(Fold Change)
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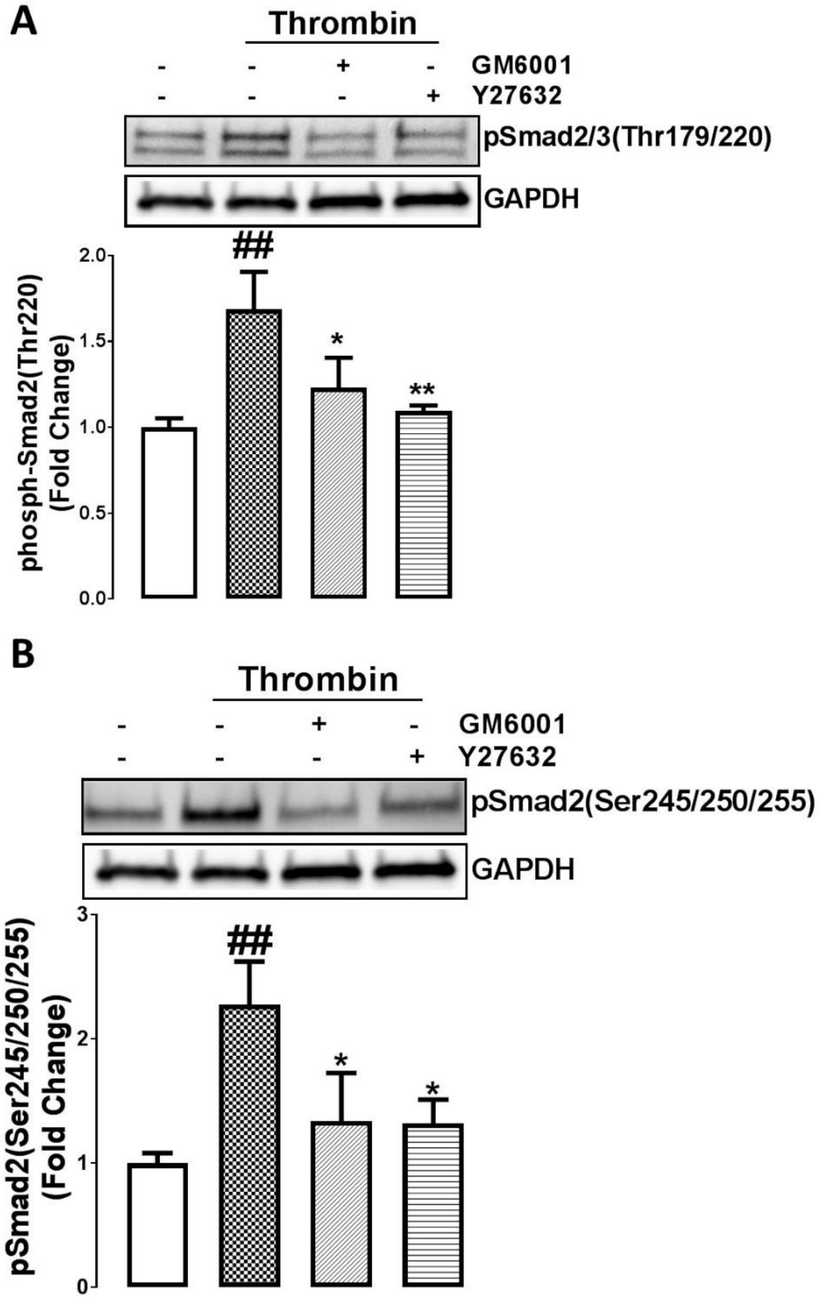
EGF
60'
120'
240'
15'
AG1478 (5μM)
pSmad2(Ser245/250/255)
GAPDH
phospho-Smad2(Ser245/250/255)
(Fold Change)

Figure 4: An intact cytoskeleton and MMPs are required for thrombin mediated Smad2 phosphorylation. VSMCs were pre-incubated with treated MMP inhibitor, GM6001 (10µM) and ROCK inhibitor Y27632 (10µM) and then exposed to thrombin for 120 min. Blots were probed with antibodies specific to **A.** phospho-Smad2/3(Thr220/179) or **B.** phospho-Smad2(Ser245/250/255). Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis ##,$p<0.01$ versus basal and **, $p<0.01$ agonist versus antagonist.



A
Thrombin
GM6001
Y27632
pSmad2/3(Thr179/220)
GAPDH
phosph-Smad2(Thr220)
(Fold Change)
2.0
1.5
1.0
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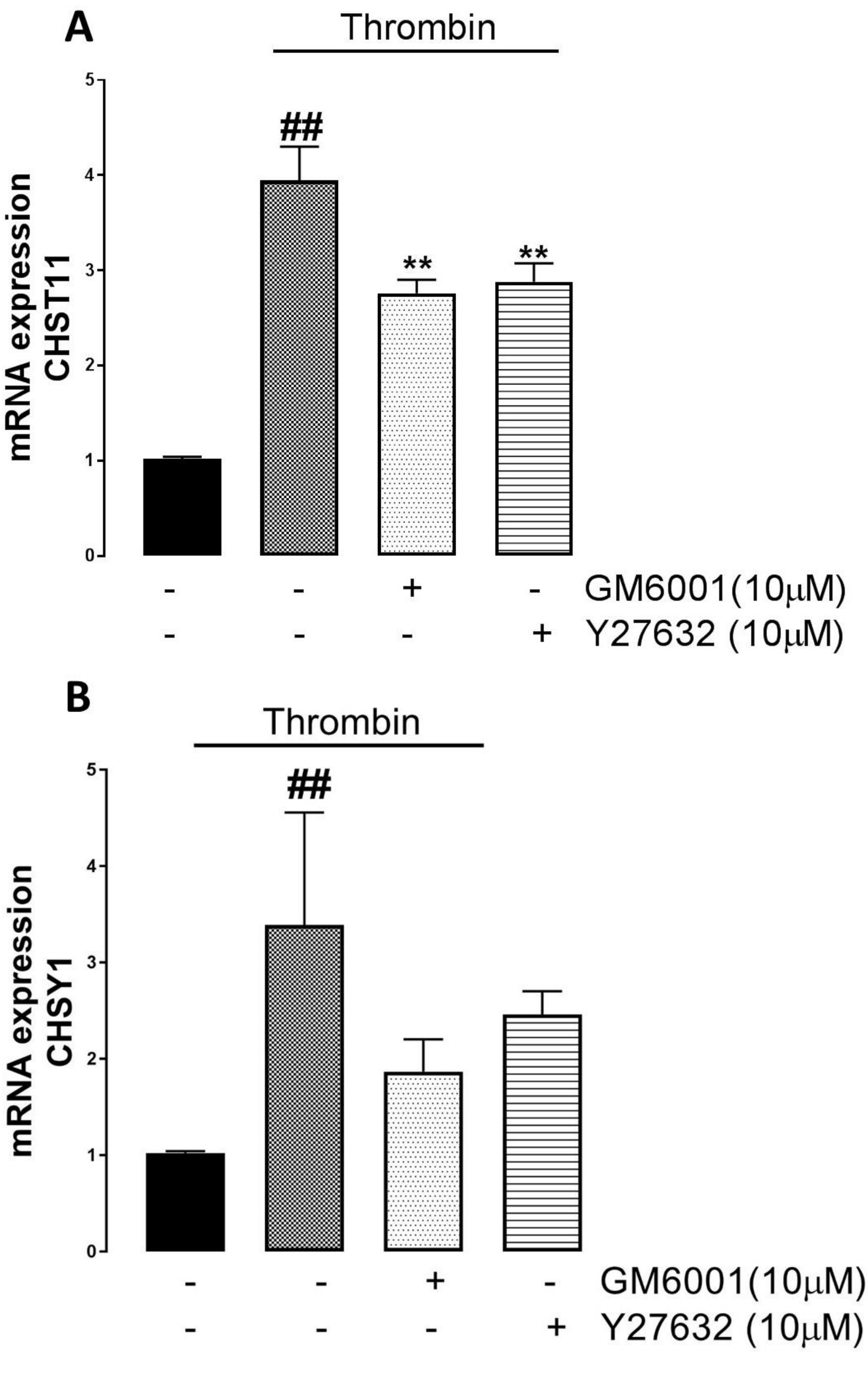
**
B
Thrombin
GM6001
Y27632
pSmad2(Ser245/250/255)
GAPDH
pSmad2(Ser245/250/255)
(Fold Change)
3
2
1
0
##
*
*

Figure 5: Thrombin mediated mRNA expression of GAG synthesizing genes CHST11 and CHSY1 are dependent on MMP activation and an intact cytoskeleton. VSMCs were pre-incubated with treated MMP inhibitor, GM6001 (10µM) and ROCK inhibitor Y27632 (10µM) and then exposed to thrombin for 6 hr to study the effects on the mRNA expression of **A.** CHST11 and **B.** CHSY1. Data are expressed as the means ± standard error from three experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. ##$p<0.01$ basal versus thrombin and *$p<0.05$ thrombin versus inhibitor was used for statistical analysis.


A
Thrombin
mRNA expression
CHST11
##
**
**
5
4
3
2
1
0
- - + - GM6001(10μM)
- - - + Y27632 (10μM)
B
Thrombin
mRNA expression
CHSY1
##
5
4
3
2
1
0
- - + - GM6001(10μM)
- - - + Y27632 (10μM)

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