TITLE: Dietary-induced obesity alters signaling pathways and induces atrophy and apoptosis in skeletal muscle in a prediabetic rat model.

AUTHORS: Balindiwe Sishi*, Ben Loos*, Beverly Ellis*, Wayne Smith#, Eugene F du Toit#, Anna-Mart Engelbrecht*

ADDRESSES: Department of Physiological Sciences, Stellenbosch*,
Department of Biomedical Sciences, Tygerberg#,
University of Stellenbosch, South Africa.

CORRESPONDING AUTHOR: Dr A-M Engelbrecht
Department of Physiological Sciences
P O Box X1
Matieland, Stellenbosch, 7600
South Africa

e-mail: ame@sun.ac.za
fax: 27-21-8083145
tel: 27-21-8084573

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ABSTRACT

Pro-inflammatory and stress activated signaling pathways are important role players in the pathogenesis of obesity and insulin resistance. This is supported by the fact that obesity and type II diabetes are associated with chronic, low-grade inflammation and elevated TNF-α levels. There is increasing evidence to suggest that TNF-α may play a critical role in skeletal muscle atrophy. However, the effects of obesity-induced insulin resistance on these signaling pathways in skeletal muscle are poorly understood. Therefore, the present study investigated the effects of a cafeteria (high caloric) diet which induces obesity and insulin resistance on the activity of the ubiquitin ligases, NF-κB, p38 MAPK- and PI3-kinase signaling pathways in the gastrocnemius muscle and compared these with muscle of lean control rats fed a standard rat chow diet. Male Wistar rats were randomly allocated to a control diet (standard commercial chow – 60% carbohydrates, 30% protein and 10% fat) or a cafeteria diet (65% carbohydrates, 19% protein and 16% fat) and maintained on their respective diets for 16 weeks. Blood analysis were conducted to determine the impact of the model of obesity on circulating insulin, glucose, free fatty acids, TNF-α and angiotensin II concentrations. The experimental animals were 18% heavier and had a 68% greater visceral fat mass than their control littersmates. The animals receiving the experimental diet were also dyslipidemic with increased levels of triglycerides and free fatty acid concentrations as well as elevated TNF-α and angiotensin II. Significant increases in the ubiquitin ligases, MURF-1 and MAFbx, as well as in caspase-3- and PARP cleavage were observed in the muscle of obese animals compared to the controls. We propose that dyslipidemia may be a mechanism for the activation of pro-inflammatory/stress activated signaling pathways.
in obesity and type II diabetes which will lead to apoptosis and atrophy in skeletal muscle.

**INTRODUCTION**

Obesity has developed into a considerable health problem in both westernized societies and in urban developing communities over the past 20 years. The increased prevalence of obesity and its associated life-style diseases can primarily be attributed to the increased availability and consumption of energy dense foods and physical inactivity (Kahn & Flier, 2000; Spiegelman & Flier, 2001; Haffner & Taegmeyer, 2003). This observation has lead to the development and characterization of numerous animal models of diet induced obesity in which these disease conditions can be studied (Turpin *et al.*, 2009; Kim *et al.*, 2010).

Pro-inflammatory and stress activated signaling pathways are important contributors in the pathogenesis of obesity and insulin resistance. Both obesity and insulin resistance are associated with chronic, low-grade inflammation (a phenomenon recently termed metabolically triggered inflammation or metaflammation) and elevated blood TNF-α levels (Hotamisligil, 2006). TNF-α is a pleiotrophic, inflammatory cytokine with increased expression in lipid overloaded tissue. Although it is well known that elevated TNF-α levels correlate positively with insulin resistance, the mechanisms mediating these effects are poorly understood (Steinberg *et al.*, 2006).
There is increasing evidence suggesting that TNF-α may also play a critical role in skeletal muscle atrophy in a number of clinical settings (Dirks & Leeuwenburgh, 2006; Vescovo & Libera, 2006). Unique mechanisms are induced during skeletal muscle atrophy which may contribute to the reduction of muscle fiber size. One such mechanism is the ubiquitin/proteosome pathway in which TNF-α is also actively involved (Tracey, 2002; Glass, 2005). Ubiquitin targets specific proteins for degradation and usually involves the action of E1-ubiquitin-activating enzyme, E2-ubiquitin-conjugating enzyme and E3-ubiquitin protein ligase. Two ubiquitin-protein ligases which are specifically involved in myofibrillar protein degradation are, MAFbx, (also known as mouse atrophy gene-1 or atrogin-1), and MuRF-1 (Bodine et al., 2001; Gomes et al., 2001).

Apoptosis is another mechanism which may play a role in atrophy of skeletal muscle. However, this is a complicated scenario since single muscle cells contain hundreds of myonuclei, while apoptosis only assists in the elimination of fiber segments and not the entire fiber (Allen et al., 1997; Borisov & Carlson, 2000). A decrease in the mean number of myonuclei per fiber has been reported in atrophying muscle under several experimental conditions (Allen et al., 1997; Fitts et al., 2001).

TNF-α can also induce catabolic actions and is a potent stimulator of the NF-κB pathway through various mechanisms (Kandarian & Jackman, 2006). Possible mechanisms include the direct action of TNF-α on skeletal muscle to induce muscle catabolism or indirectly by modifying the hormones such as IGF-1 that regulate protein turn-over (Granado et al., 2006). TNF-α signaling is mediated through its two receptors TNF-R1 (or p55 in rodents, p60 in humans) and TNF-R2 (p75 in rodents, p80 in humans) (Qi &
Pekala, 2000), which then causes activation of NF-κB. Activation of NF-κB is tightly regulated by IKK. When IκB is phosphorylated by IKK, it (IκB) is ubiquitinated and subjected to the proteasome for degradation, resulting in the activation of NF-κB. Activated NF-κB serves as the trigger which induces transcriptional up-regulation of the E3 ubiquitin ligase MuRF-1. Another E3 ligase, MAFbx, is also up-regulated albeit through a different trigger, namely the p38-MAPK (Fitts et al., 2001; Schieven, 2005). Both of these pathways lead to muscle breakdown known as “ubiquitin dependent protein wastage” (Granado et al., 2006).

Skeletal muscle mass is determined by the balance between anabolic and catabolic stimuli. The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is now recognized as one of the most critical pathways involved in maintaining skeletal muscle mass (Brunet et al., 1999; Song et al., 2005). Akt is a serine/threonine kinase which is mostly activated in response to survival signals from growth factors via mechanisms involving PI3K. Once Akt is fully activated it dissociates from the plasma membrane and phosphorylates both cytoplasmic and nuclear target proteins which include FOXO proteins, a subgroup of the Forkhead transcription factors. Phosphorylation of FOXO proteins by Akt promotes FOXO sequestration by 14-3-3 proteins in the cytoplasm leading to inhibition of their transcriptional functions. On the other hand, dephosphorylation of FOXO leads to nuclear entry and growth suppression or apoptosis (Brunet et al., 1999; Tran et al., 2003). Another target protein which Akt can phosphorylate and activate is mTOR. Activated mTOR blocks the up-regulation of both MAFbx and MuRF-1, ultimately inhibiting protein breakdown and increasing protein
synthesis via phosphorylation of p70S6 kinase (Glass, 2005; Kandarian & Jackman, 2006).

Atrophy in skeletal muscle is a highly regulated process that may serve distinct functions under different pathological conditions; therefore a study to better understand the signaling pathways implicated and the role of apoptosis during atrophy in muscle is warranted. Furthermore, there is to our knowledge no data about the effects of diet induced obesity and insulin resistance on these signaling pathways in skeletal muscle.

The aims of this study were therefore twofold: 1) to determine whether diet-induced obesity induces atrophy and apoptosis in the gastrocnemius muscle, and 2) to elucidate some of the intracellular signaling events in this muscle in our model of diet-induced obesity.

MATERIALS AND METHODS

Ethical approval for this project was obtained from the research ethics committee of the University of Stellenbosch (Faculty of Natural Science) and conforms to the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH publication No. 85-23, revised 1985).

Experimental animals

Forty adult male Wistar rats (average body mass of 376 ± 40 g at the start of the intervention protocol) were used in this study. All rats were housed at 22.0 ± 0.5°C in
standard cages with a 12 h:12 h light:dark cycle (lights on 7 am). The animals were fed standard rat chow and tap water ad libitum. Male rats weighing 200 ± 5 grams were separated into two groups (10 animals/group). One group, represented the control animals, received standard rat chow, while the other received a diet which contains elevated levels of CHOs and fats and resembles a typical Western-type diet (du Toit et al., 2008). The diet contains 65% CHOs, 19% protein and 16% fat in comparison to those of the control diet who received 60% CHO’s, 30% protein and 10% fat for a period of 16 weeks. The diet was designed to induce hyperphagia (du Toit et al., 2008), resulting in a greater energy intake (570 ± 23 kJ/day) as compared with the control group which only consumed 371±18 kJ/day. Differences in micronutrient (vitamins and minerals) intake produced by the dilution of the diet by the addition of CHOs and fats do not modify the outcomes of the present study as a separate group of rats pair-fed to the micronutrient intake of the diet fed group were also assessed previously (du Toit et al., 2008). Blood pressure measurements were performed using standard tail-cuff techniques employing a photo-electric diode to detect the tail pulse (Harvard Apparatus) at different time points during the study.

**Sample collection**

At the end of the supplementation protocol, all rats were sacrificed between 11:00 and 13:00 by injection with intraperitoneal pentobarbitone sodium (12 mgkg⁻¹). The animals were taken from the housing cage one at a time, placed into a weighing basket and carried to another room where they were weighed and sacrificed. The right Gastrocnemius muscles were removed, and divided into two pieces by a medial section through the mid-belly. One piece was snap-frozen in liquid nitrogen, and stored at -80°C for subsequent
analyses. The other piece was frozen, embedded in mounting media (Tissue-Tek OCT, Miles, Naperville, Illinois, USA) and stored at -80°C for subsequent histological and morphometric analysis.

**Serum Chemistry**

Blood analysis was conducted to determine the impact of the model of obesity on circulating insulin, blood glucose, NEFAs, TNF-α and angiotensin II concentrations as well as percentage glycosylated hemoglobin (HbA1c). Fasting blood samples were obtained from the thoracic cavity immediately after extirpation of the muscle. Samples were collected in EDTA or serum separation tubes and were centrifuged at 3000 rpm at 4°C within 30 minutes of collection. For the assessment of TNF-α and angiotensin II concentrations, serum samples were stored at -20°C until assays were performed. Angiotensin II was extracted using solid phase extraction (SepPak C18 cartridges) and the plasma concentrations determined using a commercially available radioimmunoassay (Euro Diagnostica, Malmo, Sweden). TNF-α concentrations were determined by ELISA (OptEIA\textsuperscript{TM}, PharMingen, USA). Insulin concentrations were determined using a commercially available radioimmunoassay (Diagnostic Products Corporation, USA). Blood glucose was measured using the glucose meter (Gluco Plus\textsuperscript{TM}, distributed by CIPLA DIBCARE, Bellville, South Africa). Non-esterified free fatty acid concentrations were determined using a colorimetric assay (Roche Diagnostics, Penzberg, Germany). Serum lipid levels were determined using CardioCheck\textsuperscript{P} PA analyser. After collection, 40 µl of flesh blood was placed on a lipid test strip (PTS Panels P\textsuperscript{TM}; Polymer technology, IN, USA) and levels (in mmol/L) of total cholesterol, HDL-cholesterol, triglycerides were obtained.
Insulin resistance (IR) was assessed with the homeostasis model: HOMA-IR = fasting glucose level (mg/dl) x fasting insulin level (ng/dl) ÷ 22.5 as described by Matthews and co-workers (1985).

**Histology and morphometry**

Cryosections of 10 µm were made at -18°C, mounted onto slides with poly-L-lysine and then stained with haematoxylin and eosin. Digital images of the sections were taken and fibers were analyzed semi-automatically for cross-sectional area using image analysis software (Simple PCI). A minimum of 100 and maximum of 200 fibers per sample were counted. All individual fiber cross-sectional area measurements for each rat were included in the statistical comparison, according to previously published methods (Legerlotz et al., 2008).

**Immunofluorescence Microscopy**

Serial muscle cross sections (7 µm) were incubated with the primary antibodies, and diluted in blocking solution [10% newborn calf serum, 1% BSA, and 0.05% Tween-20 in TBS (TBST)] for 2 h at room temperature or overnight at 4°C. The sections were incubated for 45–60 min with an appropriate secondary antibody (fluorochrome-labeled) and mounted with coverslips (Fluoromount-G, Chemi-Teknik AS, Oslo, Norway, or ProLong Gold Antifade Reagent with DAPI, Invitrogen-Molecular Probes, Eugene, OR). The muscle sections were washed in TBST between each step. Tissue sections were observed through a Nikon Eclipse E 400 fluorescence microscope equipped with a Nikon DMX1200 CCD Camera 200. Image acquisition for analysis was carried using the ACT-
1 software with the same camera settings (sensitivity and exposure time). Tissue was imaged using a Nikon Plan Fluor 100x/1.3 Oil objective and several myofibers per field in 6 random fields in 4 independent experiments were analysed using Simple PCI (C-Imaging Systems) software. Data were expressed in arbitrary pixel values. Pictures were taken with 20x, 40x, and 100x magnification objectives. Co-localisation was calculated using the CellR software (Olympus Soft Imaging Systems) and represented as a false colour images.

**Western-blot analysis**

Skeletal muscle proteins were extracted with a lysis buffer, pH 7.4, containing (in mM): Tris 20, p-nitrophenylphosphate 20, EGTA 1, sodium fluoride (NaF) 50, sodium orthovanadate 0.1, phenylmethyl sulphonyl fluoride (PMSF) 1, dithiothreitol (DTT) 1, aprotinin 10 µg/ml, leupeptin 10 µg/ml. Mitogen-activated protein kinase phosphatase-1 (MKP-1) protein was extracted with a lysis buffer, pH 7.4 containing (in mM): Hepes 50, EDTA 10, EGTA 10, PMSF 1, Aprotinin (1 ug/ml), Leupeptin (1 ug/ml) and Triton (0.5%). The tissue lysates were diluted in Laemmli sample buffer, boiled for 5 minutes and 20 µg protein for kinases, or 50 µg protein (caspase-3, PARP) were separated by 10% PAGE-SDS-gel electrophoresis. The lysate protein content was determined using the Bradford technique (1976). The separated proteins were transferred to a PVDF membrane (Immobilon™ P, Millipore), which were routinely stained with Ponceau Red for visualization of proteins and stripped and reprobed with anti-actin antibody to ensure equal loading. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline-0.1% Tween 20 (TBST) and then incubated with the primary antibodies that recognize phosphospecific and total p38-MAPK (Thr180/Tyr182),
PTEN, PKB Ser^{473}, FKHR, mTOR, MURF-1, MAFbx, PI3-K p85 and p110, caspase-3 (p17 fragment pAb) and PARP (p85 fragment pAb). Membranes were subsequently washed with large volumes of TBST (5X5 min) and the immobilized antibody conjugated with a diluted horseradish peroxidase-labelled secondary antibody (Amersham LIFE SCIENCE). After thorough washing with TBST, membranes were covered with ECL™ detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through a non-radioactive method (ECL™ Western blotting). Films were densitometrically analysed (UN-SCAN-IT, Silkscience) and phosphorylated protein values were corrected for minor differences in protein loading, if required. Experiments were performed to ensure that all signals were within the linear range of detection on the autoradiographs under our assay and gel loading conditions (data not shown).

**Statistical analysis**

All results are expressed as means ± SEM. The unpaired Student’s *t*-test was used to determined statistical significance. P values < 0.05 were regarded as significant. The number of experiments is indicated in the figure legends.

**RESULTS**

**The effect of diet–induced obesity on biometric and blood parameters (Table 1).**

The body weight was significantly increased in the obese group compared with the control group. The obese group also displayed a significant reduction in the muscle fiber cross-sectional area of the *gastrocnemius*. Fasting blood glucose concentrations were
similar, but insulin concentrations were elevated in the obese rats. The obese animals had an elevated HOMA index compared to the controls. Although there was a non-significant trend for HbA1c levels to increase in the diet fed rats as compared to the control rats, all values obtained in the experimental group were well below the threshold levels of 6% for the detection of abnormal blood glucose control and a similar number of rats in each group had values above the upper 95% confidence intervals for the control group (50% of obese as compared to 39% of control rats). The animals receiving the experimental diet were dyslipideamic with increased triglyceride and NEFA levels. The experimental group also had elevated serum TNF-α and angiotensin II concentrations.

The effect of diet-induced obesity on MURF-1 and MAFBx, two markers of the ubiquitin proteasome pathway, in gastrocnemius muscle (Figures 2a & b and 3a & b).

A significant increase in MURF-1 (197.4 ± 17.91%, p=0.0056) was observed in the obese group. Although there was an increase in MAFBx, it was not significant (p=0.06). The immunofluorescence imaging data confirmed the western blot results.

The effect of diet-induced obesity on parameters of apoptosis in gastrocnemius muscle (Figures 4a & b).

Diet-induced obesity caused a significant increase in cleavage of both caspase-3 (172.4 ± 13.31%, p=0.012) as well as of poly-ADP-ribose-polymerase (PARP) (112.5 ± 1.55%, p=0.004).

The effect of diet-induced obesity on TNF-alpha receptors levels (Fig 5)
There were significant increases in both TNF-α receptor 1 (380.6 ± 53.9%, p=0.002) and TNF-α receptor 2 (332.6 ± 24.91%, p<0.0001) levels in the obese group.

**The effect of diet-induced obesity on NF-κB and p38 phosphorylation (Fig 6a & b)**

Although obesity caused a significant increase in NF-κB phosphorylation (120.6 ± 6.73%, p=0.022), no significant differences were observed in p38 phosphorylation.

**The effect of diet-induced obesity on the PI3-Kinase signaling pathway (Fig 7a, b, c & d)**

The obese group showed significant reductions in PI3-kinase activity (83.0 ± 2.62%, p=0.0013) as well as in PKB/Akt(Ser^{473})- (79.33 ± 3.48%, p=0.0022) Fox01- (86.72 ± 2.59%, p=0.007) and mTOR phosphorylation (50.0 ± 4.72%, p=0.0005).

**DISCUSSION**

Although obesity and diabetes have become two of the most common metabolic disorders worldwide, their effects on skeletal muscle and the underlying molecular mechanisms for their effects have not been elucidated.

The high caloric (high carbohydrate and fat) diet used in the current study successfully induced obesity and generated a model which closely resembles metabolic syndrome that is characterized by insulin resistance, dyslipidaemia (high triglycerides, low HDL cholesterol levels), hypertension and visceral obesity (Miranda et al., 2005). The obese rats used in this study were viscerally obese, insulin resistant and dyslipidaemic.
Skeletal muscle atrophy occurs as a consequence of many different stimuli, which include aging, denervation, bed rest and inactivity, glucocorticoid treatment, sepsis and cancer. We have demonstrated that diet-induced obesity also resulted in significant atrophy of the gastrocnemius muscle as indicated by a reduction in fiber cross sectional area. (Table 1). Although a variety of diverse stimuli induce muscle atrophy, there are a number of similarities in intracellular responses (Jagoe et al., 2002; Glass, 2003; Lecker et al., 2004). Bodine and co-workers found that although several genes were upregulated in a rat model of atrophy, only a small subset was universal in all atrophy models. Two of these genes encode ubiquitin ligases, namely MuRF1 and MAFbx. The overexpression of MAFbx in myotubes produced atrophy, whereas mice lacking either MAFbx or MuRF1 were found to be resistant to atrophy (Bodine et al., 2001). We have demonstrated for the first time that diet-induced obesity in rats caused a upregulation of both of these ubiquitin ligases in the obese rat group (Fig 1).

Our data also illustrates a significant increase in apoptosis in the skeletal muscle of the obese group. Although apoptosis is necessary for maintaining the integrity of highly proliferative tissue such as epithelial cells, the role of apoptosis in post-mitotic tissue such as skeletal muscle is less well defined. Apoptosis of myonuclei likely contributes to the loss of muscle mass, but the mechanisms underlying this process are largely unknown. The important role apoptosis plays in skeletal muscle atrophy is evident from the observations showing that it is increased in a number of pathological and physiological conditions such as muscular dystrophy, motor neuron disorders, spinal cord injury and skeletal muscle atrophy due to hind limb restraint. We have demonstrated a
significant increase in cleaved caspase-3, a hallmark of the apoptotic pathway, in the skeletal muscle of the obese group. In addition, PARP was also cleaved to its proteolyzed products, a phenomenon that is well known to result from caspase-3 activation. Normal protein turnover in skeletal muscle under healthy conditions is not linked to caspase-3 mediated protein breakdown, however, in pathological catabolic conditions excessive myofibrilar protein breakdown can be blocked by the caspase inhibitor, Ac-DEVD-CHO (Du et al., 2004).

The elevated plasma TNF-α levels observed in our obese rodent model have also been reported by several other researchers (Lo et al, 2007; Dzienis-Straczkowska et al 2003; Uysal et al 1998; Liu et al 2006). This altered blood parameter has been associated with decreased skeletal muscle IκB levels (Bhatt et al, 2006). Furthermore, not only is TNF-α thought to be central in obesity-induced insulin resistance, TNF-α has also been implicated in the propagation of skeletal muscle wasting. Possible mechanisms for this wasting include that TNF-α acts directly through its receptors on skeletal muscle to induce muscle catabolism. Alternatively, it may act indirectly by modifying hormones that regulate protein turn-over, such as IGF-1 (Granado et al, 2006). We have demonstrated significant increases in TNF-R1 and TNF-R2 levels in the obese animals which might be associated with the activation of the NF-κB pathway and the subsequent induction of the E3 ligase, MuRF-1, which might ultimately lead to muscle atrophy. Romanatto and co-workers (2009) also observed that TNFR1 KO mice, when fed a high-fat diet, do not become obese, even in the face of increased caloric intake.
Interestingly, the role of p38, a serine-threonine MAPK in inflammatory responses, has only recently been highlighted (Scheiven, 2005; Shoelson et al, 2007 and Wu et al, 2006). The p38-α MAPK isoform has been closely linked to the pathogenesis of obesity and diabetes (Wu et al, 2006). Once activated by a series of activators, including TNF-α, p38 MAPK phosphorylates downstream substrates which initiate signaling cascades that regulate the synthesis of various mediators. Although several authors have reported an upregulation of p38 MAPK, we were unable to see significant differences in either phospho- or total p38 MAPK. p38 MAPK has also been identified as a likely mediator of catabolic signaling in skeletal muscle (Tracey, 2002). Di Giovanni and co-workers (2004) reported elevated p38 phosphorylation in atrophic muscles of patients with acute quadriplegic myopathy. The involvement of p38 in muscle atrophy was further supported by the results of Li and co-workers (2005) who demonstrated that the atrogin1/MAFbx gene is a downstream target of p38 MAPK signaling. The contradictory results we observed in p38 MAPK phosphorylation in the obese group might be due to the fact that the diet caused a transient induction of p38 which returned to control levels at the time the skeletal muscle sample was collected.

An intriguing aspect of PI3-kinase signaling is the wide range of cellular functions which are mediated by this pathway. It was also demonstrated by Amirouche and co-workers (2009) that myostatin overexpression induced atrophy in tibialis anterior muscle through negative regulation of the Akt/mTOR signaling pathway. Although the PI3-Kinase/Akt signaling pathway is linked with both synthesis and degradation of muscle proteins, recent data indicates that the Akt/mTORC1 pathway specifically controls protein synthesis, whereas the Akt/mTORC2 pathway controls protein degradation (Zhao et al.,
mTOR is part of two multiprotein complexes: mTORC1, contains raptor, is rapamycin sensitive and is required for signaling to S6K and 4E-BP1. On the other hand, mTORC2 contains rictor and is required for signaling to FoxO (Guertin et al, 2006). Our results demonstrated a significant decrease in PI3K activity in the obese group. This decrease in PI3K activity was associated with subsequent decreases in phosphorylation of downstream targets of PI3K, namely PKB/Akt, mTOR and FoxO in the obese groups. All the members of the FoxO family contain PKB phosphorylation sequences, which can be effectively phosphorylated by PKB in vitro (Biggs, 1999; Rena, 1999). Phosphorylation of FoxO by PKB alters its subcellular location. FoxO phosphorylation was significantly inhibited in the obese group, therefore these proteins remains predominantly in the nucleus where they are able to promote transcription of atrophy- and apoptosis related genes through specific DNA elements in their promotors (Brunet et al., 1999; Tran et al., 2003). On the other hand, increased phosphorylation of FoxO by PKB leads to its export form the nucleus and its accumulation and sequestration by 14-3-3 proteins in the cytoplasm; thus inhibiting apoptosis and atrophy.

CONCLUSION

We have demonstrated that obesity and its systemic metabolic consequences induces molecular/signaling changes in skeletal muscle which promote apoptosis and atrophy of the muscle. The close relationship between obesity and insulin resistance and the progression to type II diabetes is an enormous health problem in both developed and developing countries. The identification and characterisation of humoral factors and molecular mechanisms that might contribute to obesity induced loss of muscle mass and the development of insulin resistance in peripheral tissue such as skeletal muscle is
therefore essential. This knowledge would be invaluable to expedite the development of new generation therapies that could minimize obesity induced insulin resistance and muscle atrophy and wasting.
DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


LEGENDS:

Figure 1: Scheme demonstrating possible mechanisms for obesity-induced muscle apoptosis and atrophy.

Figures 2a & b: Skeletal muscle atrophy in obese rats. Mixed gastrocnemius muscle was excised from rats fed a high-fat diet and a control diet for 16 weeks. (a) Muscle was homogenized and protein expression of MURF-1 was determined, p=0.0056, n=6. (b) Images were acquired with a 100x oil immersion objective (scale bar 5 µm). The figure shows: (A) control tissue (B) tissue from obese rats. Sections were labeled with (1) Hoechst 33342, displayed in blue, (2) MURF/FITC displayed in green, (3) both signals overlayed, and (4) colocalized. The images shown are representative for each experiment.

Figures 3a & b: Skeletal muscle atrophy in obese rats. Mixed gastrocnemius muscle was excised from rats fed a high-fat diet and a control diet for 16 weeks. (a) Muscle was homogenized and protein expression of MAFbx was determined, p=0.06, n=6. (b) Images were acquired with a 100x oil immersion objective (scale bar 5 µm). The figure shows: (A) control tissue (B) tissue from obese rats. Sections were labeled with (1) Hoechst 33342, displayed in blue, (2) MAFbx/FITC displayed in green, (3) both signals overlayed and (4) colocalized. The images shown are representative for each experiment.

Figures 4a & b: Skeletal muscle apoptosis in obese rats. Mixed gastrocnemius muscle was excised from rats fed a high-fat diet and a control diet for 16 weeks. Muscle was homogenized and (a) cleavage of caspase-3 (p=0.012, n=6) and (b) PARP (p=0.004, n=6) were determined.
Figure 5: The effect of diet-induce obesity on TNF-alpha receptors levels. Mixed gastrocnemius muscle was excised from rats fed a high-fat diet and a control diet for 16 weeks. Muscle was homogenized and TNF-R1 (p=0.002, n=6) and TNF-R2 (p<0.0001, n=6) were determined.

Figure 6a & b: The effect of diet-induce obesity on NF-κB and p38 phosphorylation. Mixed gastrocnemius muscle was excised from rats fed a high-fat diet and a control diet for 16 weeks. Muscle was homogenized and NF-κB (p=0.022, n=6) and p38-MAPK (n=6) phosphorylation were determined.

Figures 7a-d. The effect of diet-induce obesity on the PI3-Kinase signaling pathway. Mixed gastrocnemius muscle was excised from rats fed a high-fat diet and a control diet for 16 weeks. Muscle was homogenized and PI3-kinase activity (p=0.013, n=6) as well as PKB/Akt(Ser473)- (p=0.0022, n=6) Fox01- (p=0.007, n=6) and mTOR- (p=0.0005, n=6) phosphorylation were determined.
Table 1: Biometric- and blood parameters for rats fed a control or high fat diet for 16 weeks.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>OBESE</th>
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<tr>
<td>Body weight (grams)</td>
<td>513 ± 11 (n=11)</td>
<td>603 ± 11 *(n=10)</td>
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<tr>
<td>Visceral fat weight (grams)</td>
<td>29.8 ± 1.58 (n=11)</td>
<td>49.5 ± 2.2 *(n=10)</td>
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<tr>
<td>Blood pressure (mm Hg)</td>
<td>141 ± 9 (n=8)</td>
<td>141 ± 6 (n=10)</td>
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<tr>
<td>Muscle fiber cross-sectional area (µm²)</td>
<td>3986.1 ±20.5</td>
<td>3072 ±10.9*</td>
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<td>Glucose (mmol/L)</td>
<td>4.82 ± 0.18 (n=6)</td>
<td>5.82 ± 0.15 (n=6)</td>
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<tr>
<td>Insulin (µIU/ml)</td>
<td>31.4 ± 2.8 (n=11)</td>
<td>49.5 ± 6.2 *(n=11)</td>
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<td>HOMA</td>
<td>6.2 ± 0.7 (n=6)</td>
<td>12.5 ± 2.0 *(n=6)</td>
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<td>HbA1c (%)</td>
<td>3.97 ± 0.18 (n=13)</td>
<td>4.36 ± 0.13 (n=13)</td>
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<td>Triglycerides (mmol/L)</td>
<td>0.72 ± 0.07 (n=13)</td>
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<td>Total cholesterol (mmol/L)</td>
<td>1.43 ± 0.05 (n=13)</td>
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<td>HDL cholesterol (mmol/L)</td>
<td>0.87 ± 0.03 (n=13)</td>
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<td>NEFA (mmol/L)</td>
<td>0.79 ± 0.10 (n=8)</td>
<td>1.695 ± 0.31 #(n=8)</td>
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<td>Angiotensin II (mmol/L)</td>
<td>27.07 ±4.66 (n=10)</td>
<td>43.96 ±5.18 *(n=10)</td>
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<td>TNF-α (pg/ml)</td>
<td>13.27 ±2.09 (n=6)</td>
<td>42.80 ±5.93 *(n=6)</td>
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Obesity-induced metabolic related factors, eg TNF-α

Sarcolemma

RTK

PI(4,5)P₂

PI(3,4,5)P₃

PDK1

GF

p85

p110

PTEN

P

p38

NFκB

mitochondrion

Cytochrome c

Caspase-3 & PARP cleavage

↑ MURF-1

↑ MAFbx

FoxO

nucleus

↑ MURF-1

↑ MAFbx

mTOR

p70S6k

S6

Protein synthesis

ATROPHY

APOPTOSIS

Akt

Protein synthesis

mTOR

p70S6k

S6

Protein synthesis

Obesity-induced metabolic related factors, eg TNF-α

Fig 1
Figure 2a
Figure 2b
Figure 3a

- β-actin
- MAFbx

Bar chart showing MAFbx expression levels between Control and Obese groups.
Figure 4b
Figure 5
Figure 6a
Figure 6b
Figure 7a
Figure 7b
Figure 7d