

Visfatin induces oxidative stress in differentiated C2C12 myotubes in an Akt- and MAPK-independent, NF κ B-dependent manner

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Abstract Adipose tissue is an important endocrine and metabolic tissue that is actively involved in cross-talk with peripheral organs such as skeletal muscle. It is likely that adipose-derived factors may underlie the development of insulin resistance in muscle. Thus, the cross-talk between adipose and muscle may be important for the propagation of obesity-related diseases. Visfatin (Pre-B-cell colony-enhancing factor 1 homolog/Nampt) is a recently discovered adipokine with pleiotropic functions. The aim of this study was to examine the effect of visfatin on cellular stress responses and signalling pathways in skeletal muscle. Visfatin treatment of differentiated C2C12 myotubes generated reactive oxygen species (ROS) comprising both superoxide and hydrogen peroxide that was dependent on de novo transcription and translation. In differentiated C2C12 myoblasts, visfatin had no effects on insulin-stimulated Akt phosphorylation nor on activation of the Akt signalling pathway. Additionally, visfatin-induced oxidative stress occurred independent of activation of the stress-activated protein kinases (MAPKs) ERK and p38. In contrast, phosphorylation of NF κ B was associated with visfatin-mediated generation of ROS and blockade of this pathway via selective IKK inhibition led to a partial reduction in oxidative stress. Furthermore, the generation of ROS following visfatin treatment was highly dependent on both de novo transcription and translation. Taken

together, these findings provide novel insights for the unique pathophysiological role of visfatin in skeletal muscle.

Keywords Adipokine · Inflammation · ROS · Insulin resistance · Obesity · Skeletal muscle

Introduction

Once considered to be just a lipid storage depot, adipose tissue is now widely accepted to function as both a secretory and signalling tissue [17]. The finding that both obese and lipodystrophic patients develop insulin resistance and diabetes demonstrates the critical importance of normal adipose function on whole-body physiology [44, 47]. Adipose tissue releases a number of protein factors named adipokines (including adiponectin, resistin, leptin, IL-6 and visfatin) many of which have been demonstrated to act in both an autocrine or paracrine manner [40] as well as in an endocrine fashion [42]. In fact changes in adipose tissue physiology that modify adipokine expression and/or secretion have been demonstrated to associate with insulin resistance [37] metabolic syndrome [50], obesity [9] and diabetes [4].

Due to its major role in insulin-stimulated glucose disposal, skeletal muscle is likely to be a major peripheral target of adipokines. In fact, it is believed that crosstalk between adipose tissue and skeletal muscle is involved in the pathogenesis of insulin resistance and type 2 diabetes. For example, it is now thought that low-level inflammation in skeletal muscle—a key contributor to the pathogenesis of obesity-related disease—is partly generated by changes in plasma adipokine levels [reviewed in [17]]. Furthermore, experiments involving co-culture of adipocytes with myocytes or those in which myocytes were treated with

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adipocyte-conditioned media demonstrated induction of insulin resistance in muscle cells concomitant with decreased activity of key insulin signalling pathways [10, 11, 45, 46, 48]. Reciprocally, a similar study reported protective effects of adiponectin, a beneficial adipokine, on insulin-responses in myocytes [12]. However, to date, with the exception of adiponectin, TNF α and IL-6 [19, 35, 46], few individual cytokines that are known to be released by adipose tissue have been demonstrated to mediate physiological processes in skeletal muscle.

Visfatin/PBEF/Nampt is a recently described adipokine with diverse and complex functions. Initially named PBEF (pre-B-cell colony-enhancing factor) [43], it was later found to have similarities with the nicotinamide phosphoribosyl-transferase *nadv* gene from the prokaryote *Haemophilus ducreyi* [31]. In addition to an almost ubiquitously expressed intracellular form, the discovery of an extracellular form in the plasma that is released from visceral adipose tissue—visfatin—led to its recent inclusion in the adipokine family [16]. In contrast to the well-described functions of Nampt in modulating intracellular NAD⁺ levels [39, 41], the role of extracellular visfatin is not entirely clear. The previously reported function of visfatin as an insulin-mimetic adipokine is at present contentious and highly debated [14]. However, a role for visfatin in the propagation of inflammation and oxidative stress responses is beginning to emerge. In human pulmonary vascular endothelial cells, visfatin was demonstrated to interact with several proteins mediating oxidative stress and inflammation such as ND1, ferritin light chain and IFITM3, leading to increased levels of reactive oxygen species (ROS) [59]. In another study, the treatment of endothelial cells with visfatin led to activation of NF κ B and subsequent transcriptional activation of genes associated with oxidative stress and inflammation including VEGFA and MMP2 [3]. Furthermore, visfatin was also found to increase expression of the inflammatory adhesion molecules VCAM-1 and ICAM-1 in vascular endothelial cells in a ROS-dependent manner through NF κ B signalling [21]. In another study, blocking Nampt/visfatin enzyme function by using its specific inhibitor FK-866 reduced both the production of pro-inflammatory TNF α plasma levels and its associated pathology in an animal model of inflammatory arthritis [8]. Taken together, these studies demonstrate a role for visfatin in mediating oxidative stress and inflammation via activation of NF κ B and other stress-related signalling pathways in a cell-type dependent manner. Whether visfatin exerts similar effects in myocytes has not previously been explored.

In the current study, in order to understand the effects of visfatin on skeletal muscle, we treated differentiated C2C12 myotubes with exogenous visfatin and measured its effects on oxidative stress responses and cellular signalling pathways,

focussing on its effects on the Akt-, mitogen-activated protein kinase (MAPK)- and NF κ B-dependent signalling cascades. The role of visfatin in regulating skeletal muscle physiology is of particular interest because in addition to its secretion by adipocytes, visfatin is also highly expressed in the skeletal muscle [22]. Thus elucidating the role of visfatin in muscle physiology is of critical importance. This study contributes to a better understanding of the role of visfatin locally and in the cross-talk between adipose tissue and muscle and provides insight to both its normal function in muscle and in pathological conditions such as obesity where its secretion and/or activity may be altered.

Materials and methods

Cell culture

C2C12 cells were purchased from European Collection of Cell Culture (ECCC, catalogue number 91031101) and proliferated as myoblasts in 4.5 g/L glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), glutamine (2 mM) and FCS (15%; Sigma, St. Louis, MO, USA). The medium was changed every 2 days and the semi-confluent cultures were seeded at a density of 2,000/cm². To differentiate the myoblasts into myotubes, 95% confluent myoblasts were grown for 4–5 days in DMEM supplemented with glutamine (2 mM) and horse serum (5%; Sigma, St. Louis, MO, USA). After fusion, differentiated cells were cultured in antibiotic-free and serum-free medium for an additional 24 h before being subjected to experimental procedures.

Visfatin and inhibitor treatment

All visfatin and blocking treatments were performed in antibiotic-free and serum-free media. Mouse recombinant visfatin (Biovision, catalogue number 4908-50) was resuspended in phosphate-buffered saline (PBS) at a concentration of 0.5 mg/ml. Time course (0–48 h) and dose response studies (0–200 ng/ml) indicated that a working concentration of 100 ng/ml for 18 h generated highest levels of oxidative stress with low levels of toxicity (data not shown). For the inhibition experiments, inhibitors were added to the C2C12-differentiated myotubes 1 h prior to visfatin treatment: 50 μ M LY294002 (Cell Signal, catalogue number 9901) was used to block PI3K; 10 μ M U0126 was used to block MEK1/2 (Cell Signal, catalogue number 9903); 10 μ g/ml of the InSolutionTM p38 MAP Kinase Inhibitor III (Calbiochem, catalogue number 506148) was used to block p38; 0.1 μ M of Cycloheximide Ready-Made Solution (Sigma, catalogue C4859) was used

to block translation; 1 $\mu\text{g}/\text{ml}$ of Actinomycin D (Sigma, catalogue number A9415) was used to block transcription; 50 $\mu\text{g}/\text{ml}$ I κ B Kinase Inhibitor Peptide (Calbiochem, catalogue number 401477) was used to inhibit IKK α/β and 1 mM *N*-acetyl-L-cysteine (NAC; Sigma, A8199), a glutathione precursor, was used to inhibit the production of reactive oxygen species.

Measurement of ROS production

After treatment, cells were washed twice in PBS and incubated at 37°C with the following fluorescent probes for the indicated times: 5 μM Mitosox Red (Invitrogen, M36008) for 20 min, used for detection of superoxide at 510/580 nm; 25 μM carboxy-H₂DCFDA (Invitrogen, I36007) for 30 min, used for the detection of reactive oxygen species at 495/529 nm; 1 $\mu\text{g}/\text{ml}$ of pentafluorobenzenesulfonyl fluorescein (Cayman Chemicals, 10005983) for 30 min, which specifically recognises hydrogen peroxide species at 480/530 nm. Fluorescence was measured using a Leica DFC420 camera (Leica Microsystems, Wetzlar, Germany), attached to a Leica DMIRBE microscope with the following filter cubes: A, I3 and N2.1. In order to visualise the nuclei 1 μM Hoechst 33342 (Sigma, St Louis, MO, USA) was added in the last 5 min of incubation. Image analysis was performed with the Image J 1.37c (Wayne Rasband, NIH, <http://rsb.info.nih.gov/ij/plugins/imagej-updater.html>). For imaging experiments the quantification of the fluorescent signal was measured, compared to the background/noise, and the mean and standard deviation were computed. Student's *t* tests were applied to determine the significance of variance between control and treated cells.

Western blotting

Differentiated C2C12 myotubes grown in six-well plates were washed twice in ice-cold PBS and then lysed by incubating for 5 min on ice with 400 μl of ice-cold cell lysis buffer (Cell Signal, catalogue number 9803) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄ and 1 $\mu\text{g}/\text{ml}$ leupeptin supplemented with 1 mM PMSF and 5 mM NaF (Sigma, St. Louis, MO, USA). Cells were scraped from the wells and incubated for an additional 40 min on ice with inversion of the tubes every 10 min. Cell lysates were centrifuged at 14,000 rpm at 4°C for 15 min and protein content of the supernatant was calculated using a BCA kit (Perbio Science, catalogue number 23225).

For the detection of the phospho-proteins the following kits were used: phospho-Akt sampler kit (Cell Signal,

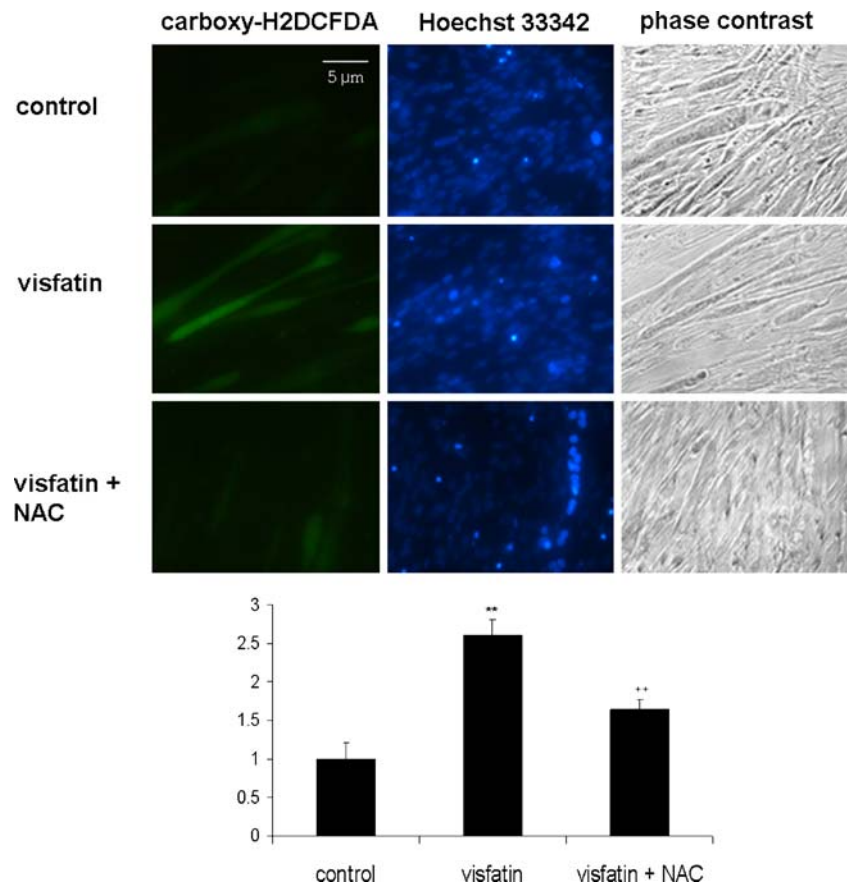
catalogue number 9916), NF κ B pathway sampler kit (Cell Signal, catalogue number 9936) and phospho-MAPK family sampler kit (Cell Signal, catalogue number 9910). Briefly, 60 μg of protein was boiled in NuPage LDS sample buffer (Invitrogen, catalogue number NP0007) for 10 min at 70°C and then separated on Novex Bis-Tris 4–12% gels (Invitrogen, Paisley, UK). The proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) which was further washed for 5 min in TBS before being blocked for 1 h in 1 \times TBS with 5% BSA and 0.1% Tween-20. The primary antibodies used for detection of phospho-proteins were diluted 1:1,000 in 1 \times TBS with 5% BSA and 0.1% Tween-20 and incubated with the blot overnight at 4°C with gentle rocking (30 rpm). The membrane was washed with TBST three times for 15 min each, and then incubated for 1 h with the appropriate secondary antibody provided by each kit, diluted 1:2,000 in 1 \times TBS with 5% BSA and 0.1% Tween-20. The membrane was later washed three times in TBST for 15 min each, followed by another wash for 5 min in TBS. The chemiluminescent signal was detected using SuperSignal West Pico substrate (Perbio Science, 34077) and a ChemiDoc XRS system (Bio-Rad). Images were captured using Quantity One software version 4.7 (Bio-Rad). As a loading control, the HRP-bound GAPDH antibody was used (Abcam, ab9482), diluted 1:5,000 in 1 \times TBS with 5% BSA and 0.1% Tween-20.

Results

Exogenous visfatin generates oxidative stress in differentiated C2C12 myotubes

The aim of this study was to investigate the effects of exogenous visfatin on oxidative stress responses and signalling pathways. In order to assess the effects of increased visfatin on oxidative stress responses in muscle, differentiated C2C12 myocytes were incubated with recombinant mouse visfatin. Dose response (0–200 ng/ml) and time course (0–48 h) studies indicated that a working concentration of 100 ng/ml for 18 h generated highest levels of oxidative stress with low levels of toxicity (data not shown). Additionally, 100 ng/ml was the minimum dose of visfatin shown to activate cellular stress signalling in studies of endothelial cells and cardiomyocytes [26, 30, 57]. Figure 1 shows that the addition of 100 ng/ml mouse recombinant visfatin for 18 h generates ROS in myotubes, which were partially inhibited by the antioxidant NAC (reduced by approximately 40%). In order to ensure that increased ROS observed following visfatin treatment were not specific to the particular recombinant protein used in this study, the effects of other forms of recombinant visfatin

Fig. 1 Incubation of differentiated myocytes with visfatin induces oxidative stress. Differentiated C2C12 myotubes were incubated for 18 h with 100 ng/ml recombinant visfatin following pre-treatment in the presence or absence of 1 mM NAC for 1 h. Cells were incubated with 25 μ M carboxy-H2DCFDA for 25 min to detect ROS followed by 5 min incubation with 1 μ M Hoechst 33342 to stain the nuclei. Relative expression compared to control data is shown. Data shown are the average of quadruplicate samples per experiment, from three independent experiments \pm SEM. **Denotes $p < 0.01$ compared to control. ++Denotes $p < 0.01$ compared to visfatin treatment



on ROS generation were assessed. HEK-produced human recombinant visfatin (Axxora UK Ltd., Nottingham, UK) and another form of bacteria-produced mouse recombinant visfatin (MBL International, Caltag-MedSystems Ltd., Buckingham, UK) generated similar levels of ROS in C2C12 myocytes (data not shown).

In order to determine what specific types of ROS were generated by visfatin exposure, dyes were used which specifically recognise superoxide and hydrogen peroxide molecules (MitoSOXTM Red and pentafluorobenzenesulfonyl fluorescein, respectively). Figure 2 shows a significant increase of 1.9-fold in production of superoxide ions (Fig. 2a) and 1.7-fold increase in the generation of hydrogen peroxide (Fig. 2b) following treatment with visfatin compared to control.

The generation of oxidative stress is independent from Akt signalling

It has been reported in many cell types [3, 26, 30, 49, 55–57] that extracellular visfatin binds to the insulin receptor and activates insulin-dependent Akt signalling. However, whether visfatin exerts similar effects in skeletal myocytes is presently unclear. Since skeletal muscle is the major

organ responsible for glucose uptake, we hypothesised that visfatin may alter insulin-stimulated Akt phosphorylation in differentiated C2C12 myotubes. Figure 3a shows that while 5 min of insulin treatment resulted in rapid and substantive phosphorylation of Akt, 5 min visfatin treatment alone failed to induce Akt Ser472 phosphorylation, compared to control. Additionally, we failed to observe any effect of visfatin on insulin-stimulated Akt phosphorylation. Whilst we could see higher basal phosphorylation of Akt in control samples when blots were not saturated with the high levels of insulin-stimulated Akt phosphorylation, longer exposure to visfatin (5–15 min) had no effect on Akt Ser472 and Thr308 phosphorylation (Fig. 3b). Moreover, we did not see any changes after visfatin treatment in the phosphorylation of upstream regulators of Akt, PDK1 and PTEN, nor in the phosphorylation of Akt downstream targets like c-Raf and GSK-3 β (Fig. 3b). These data further indicate that in contrast to effects observed in other cell types, visfatin exerts no effects on the Akt signalling pathway in differentiated mouse myocytes.

Although we were unable to demonstrate activation of the Akt pathway following visfatin treatment, it remains possible Akt may be activated at later time points through pathways independent of insulin-receptor signalling, and in

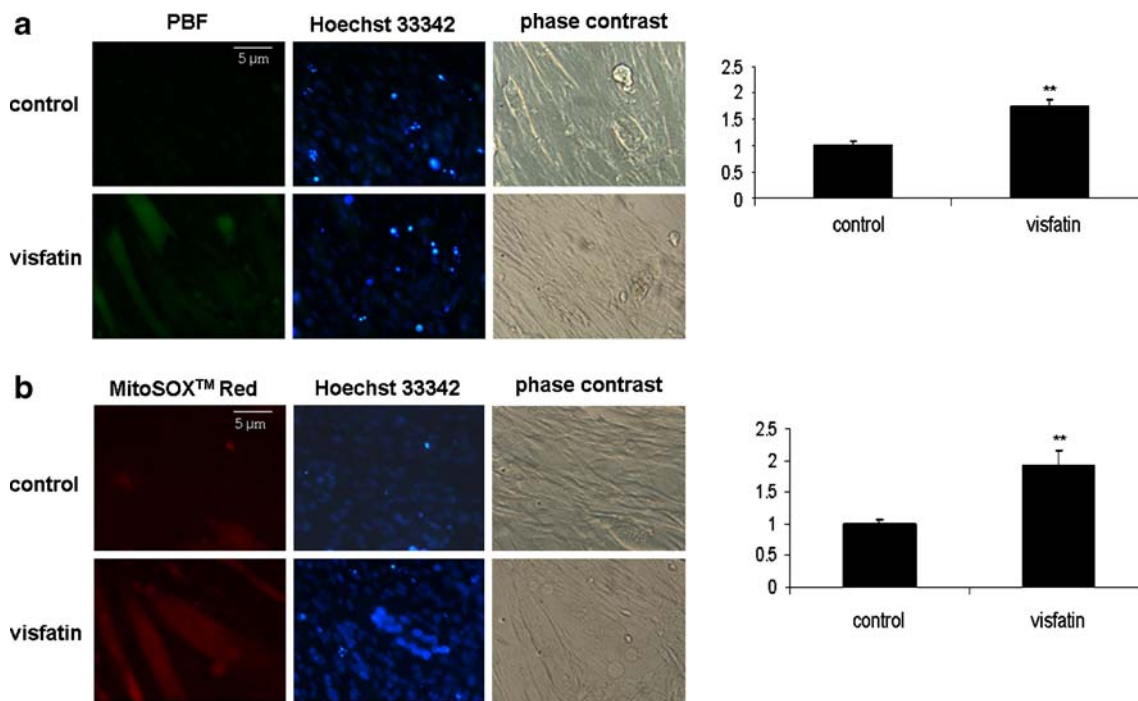


Fig. 2 Visfatin generates both superoxide and hydrogen peroxide species. C2C12 differentiated myotubes were incubated for 18 h with 100 ng/ml of visfatin. **a** Production of hydrogen peroxide was detected by incubating myoblasts with 1 μ g/ml of pentafluorobenzene sulfonamide fluorescein for 25 min followed by 5 min incubation with 1 μ M Hoechst 33342 (to stain the nuclei). **b** Superoxide generation was

detected by incubating differentiated cells with 5 μ M MitoSOX™ Red for 15 min followed by 5 min incubation with 1 μ M Hoechst 33342. Relative expression compared to control is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments \pm SEM. **Denotes $p < 0.01$ compared to control

this way modulate ROS levels. Thus, in order to test whether the Akt signalling is involved in the generation of oxidative stress responses in differentiated C2C12 cells we blocked the upstream regulator of Akt, phosphatidylinositol-3-kinase (PI3K), thereby blocking all pathways that may lead to activation of Akt. Figure 3c shows that pre-treatment of cells with the PI3K inhibitor LY294002 1 h prior to the addition of visfatin did not significantly reduce the visfatin-mediated ROS production. These data suggest that visfatin-induced oxidative stress occurs independently of Akt and PI3K signalling.

Visfatin-induced generation of oxidative stress occurs independent of mitogen-activated protein kinase signalling

Many studies have reported the role of stress-activated protein kinases such as p38/MAPK [18, 23, 25, 52] and ERK [1, 34, 54] in the generation and modulation of oxidative stress responses. In order to investigate whether visfatin-induced oxidative stress in myocytes was associated with activation of MAPK signalling, we investigated phosphorylation of p38 and ERK proteins by Western blot analysis. We failed to detect increased phosphorylation of p38 and ERK (Fig. 4a) kinases as previously reported [15, 57] and, furthermore, the pre-treatment of cells with

selective p38 and ERK inhibitors did not reduce the oxidative stress levels induced by visfatin (Fig. 4b). Taken together, these data suggest that visfatin-induced oxidative stress occurs independently of MAPK signalling.

Visfatin-induced generation of reactive oxygen species is partially dependent on NF κ B signalling and de novo transcription and translation

Since it was previously reported that visfatin induces a pro-inflammatory response through the activation of NF κ B [3, 21, 24, 32] and that NF κ B can further mediate ROS production through the synthesis of pro-inflammatory cytokines [5, 27, 33], we tested the hypothesis that visfatin-mediated activation of NF κ B in myocytes may lead to the generation of oxidative stress. In order to test this hypothesis, we pre-treated differentiated myotubes with a selective IKK-inhibitor peptide that blocks the phosphorylation and degradation of I κ B, thus leading to reduced NF κ B pathway activation. Figure 5a shows that inhibition of the NF κ B pathway reduced visfatin-induced ROS levels by 25%. Next we assessed whether visfatin-induced generation of oxidative stress correlated with activation of the NF κ B protein. Figure 5b shows a twofold increase in the phosphorylated p65 subunit of NF κ B following visfatin treatment. These

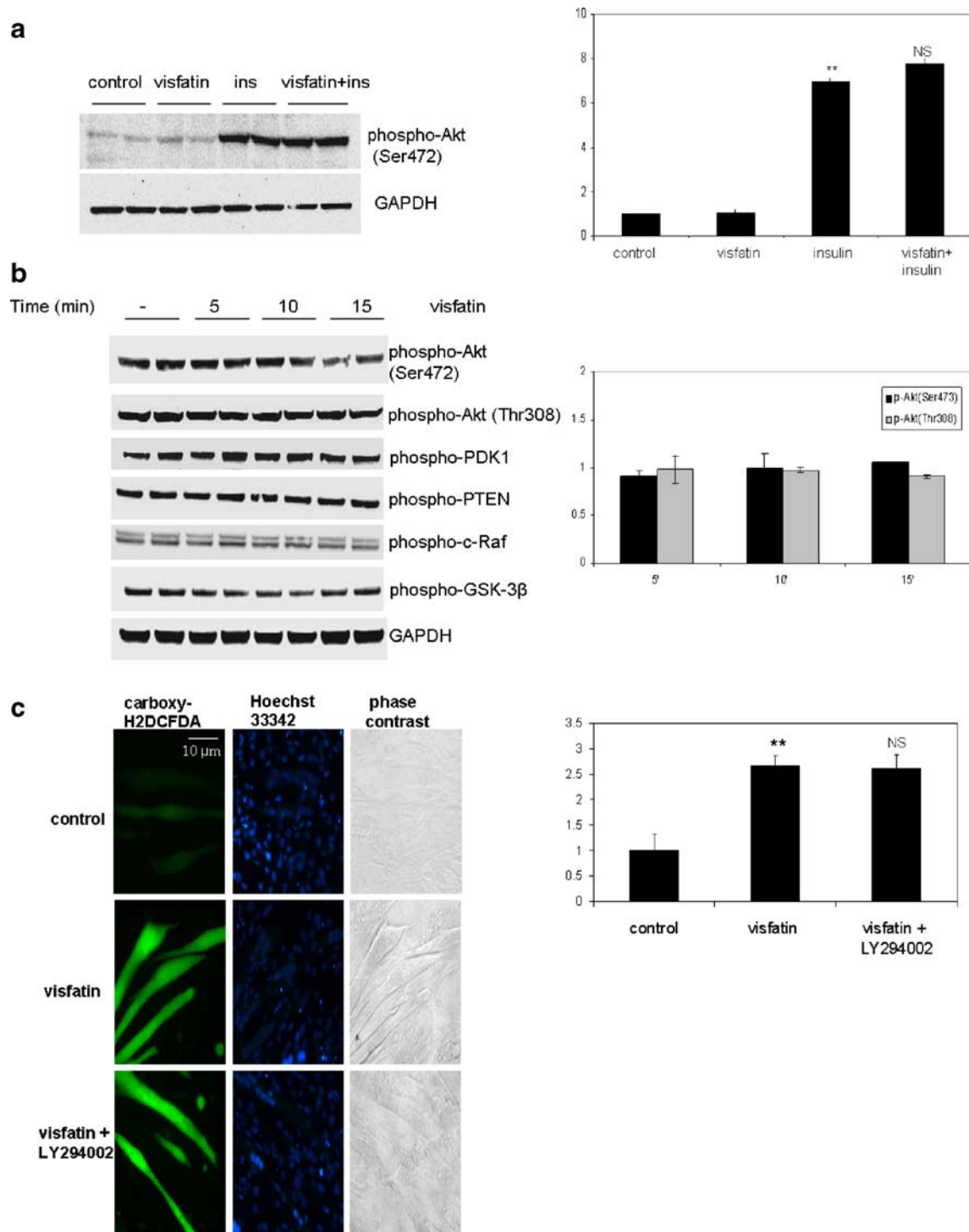


Fig. 3 Visfatin-induced oxidative stress occurs independent of Akt signalling. Activation of the Akt signalling pathway in C2C12 differentiated myotubes was assessed by Western blotting. **a** Phosphorylation of Akt at Ser472 residue was detected at baseline, following visfatin exposure (100 ng/ml) for 18 h, following insulin stimulation for 5 min and following treatment with visfatin and insulin for 5 min. **b** Western blot analysis was performed detecting activation of the Akt signalling pathway by phosphorylation of Akt at Ser472 or Thr308 residues, the phosphorylation of Akt upstream regulators (PDK1 and PTEN) or of its downstream targets (c-Raf and GSK-3 β) following visfatin treatment (no treatment, 5, 10 and 15 min).

Quantitation of Akt phosphorylation at Ser472 or Thr308 was performed, compared to control. **c**. Differentiated C2C12 cells were pre-treated for 1 h with 50 μ M LY294002, an inhibitor of PI3K. Cells were incubated with 25 μ M carboxy-H2DCFDA for 25 min (to detect ROS) followed by 5 min incubation with 1 μ M Hoechst 33342 to stain the nuclei. Relative expression compared to control is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments \pm SEM. *Denotes $p < 0.05$ compared to control. **Denotes $p < 0.01$ compared to control. NS denotes no significant difference between visfatin and insulin treatment compared to insulin alone

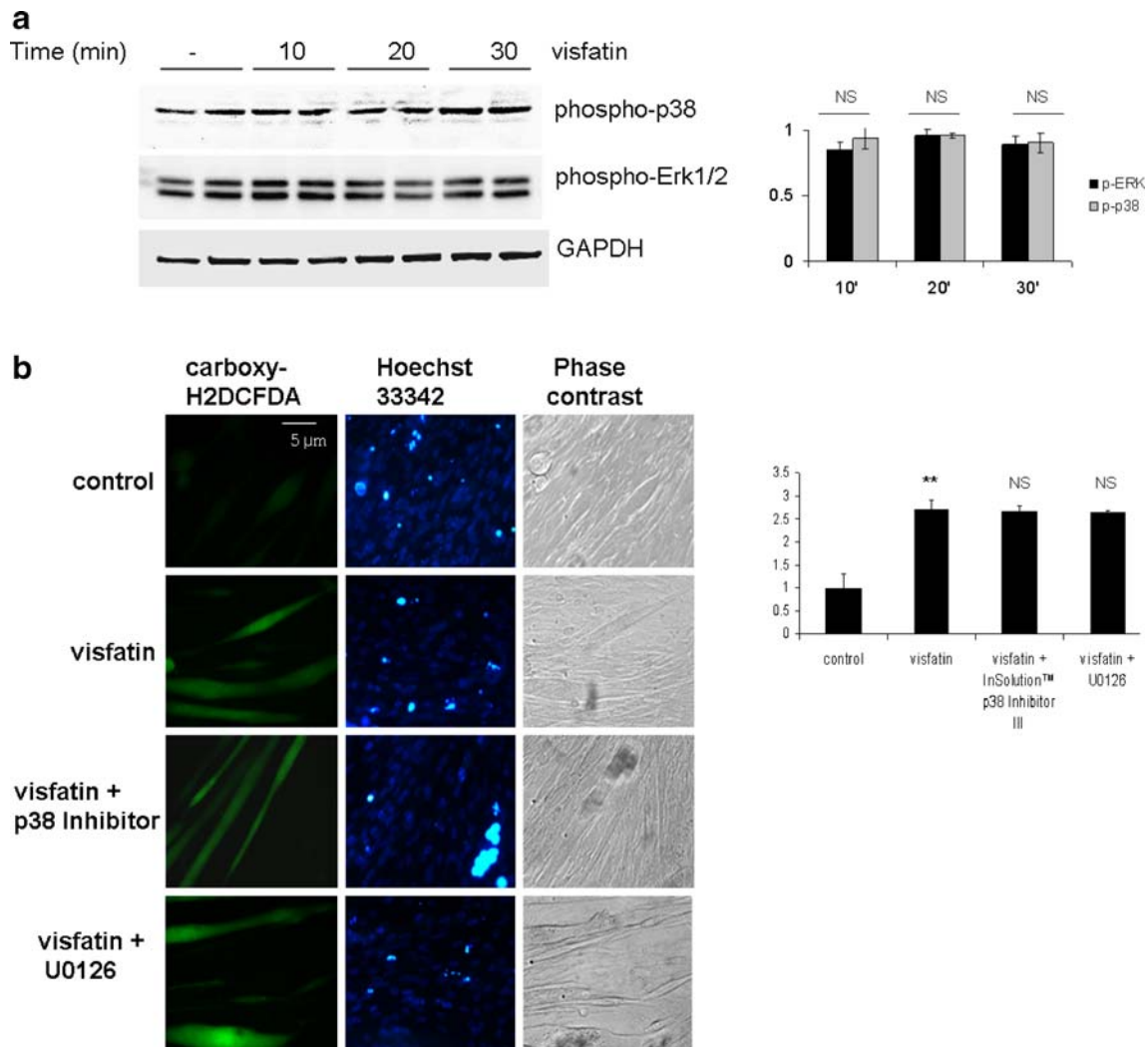


Fig. 4 The generation of oxidative stress is independent of MAPK signalling. Differentiated C2C12 myotubes were incubated in the presence or absence of 100 ng/ml visfatin. **a** Western blot analysis was performed to detect activation of ERK and p38 signalling pathways by phosphorylation (p-ERK and p-p38, respectively) following 10, 20 and 30 min of visfatin exposure. **b** Prior to visfatin exposure cells were pre-treated for 1 h with 10 μ g/ml of the InSolution™ p38 MAP Kinase Inhibitor II, an inhibitor of p38 or with 10 μ M U0126, an

inhibitor of MEK. Generation of ROS was determined by incubation with 25 μ M carboxy-H2DCFDA for 25 min followed by 5 min incubation with 1 μ M Hoechst 33342 to stain the nuclei. Relative expression compared to control data is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments \pm SEM. *Denotes $p < 0.01$ compared to control. NS denotes no significant change from visfatin treatment alone

data indicate that visfatin-induced ROS generation is partially dependent on activation of the NF κ B pathway.

Finally, to further characterise the mechanisms that underlie visfatin-mediated stress responses we pre-treated C2C12 myotubes with cycloheximide or actinomycin D prior to exposure to visfatin in order to understand whether visfatin-mediated stress responses are dependent on de novo transcription and/or translation. Figure 6 shows that pre-treatment with either transcription or translation blocker completely reversed the visfatin-mediated generation of ROS suggesting that in addition to effecting the NF κ B signalling pathway via post-translational modifications, visfatin-induced generation of reactive oxygen species is

highly dependent on both de novo transcription and translation.

Discussion

In the present study, we show that exposure of differentiated murine C2C12 skeletal myocytes to visfatin generates both superoxide anion and hydrogen peroxide ROS. Visfatin-induced generation of oxidative stress was found to be mediated by de novo transcription and translation. We additionally found that whilst the induction of oxidative stress was not dependent on either the Akt-, Erk1/2- nor

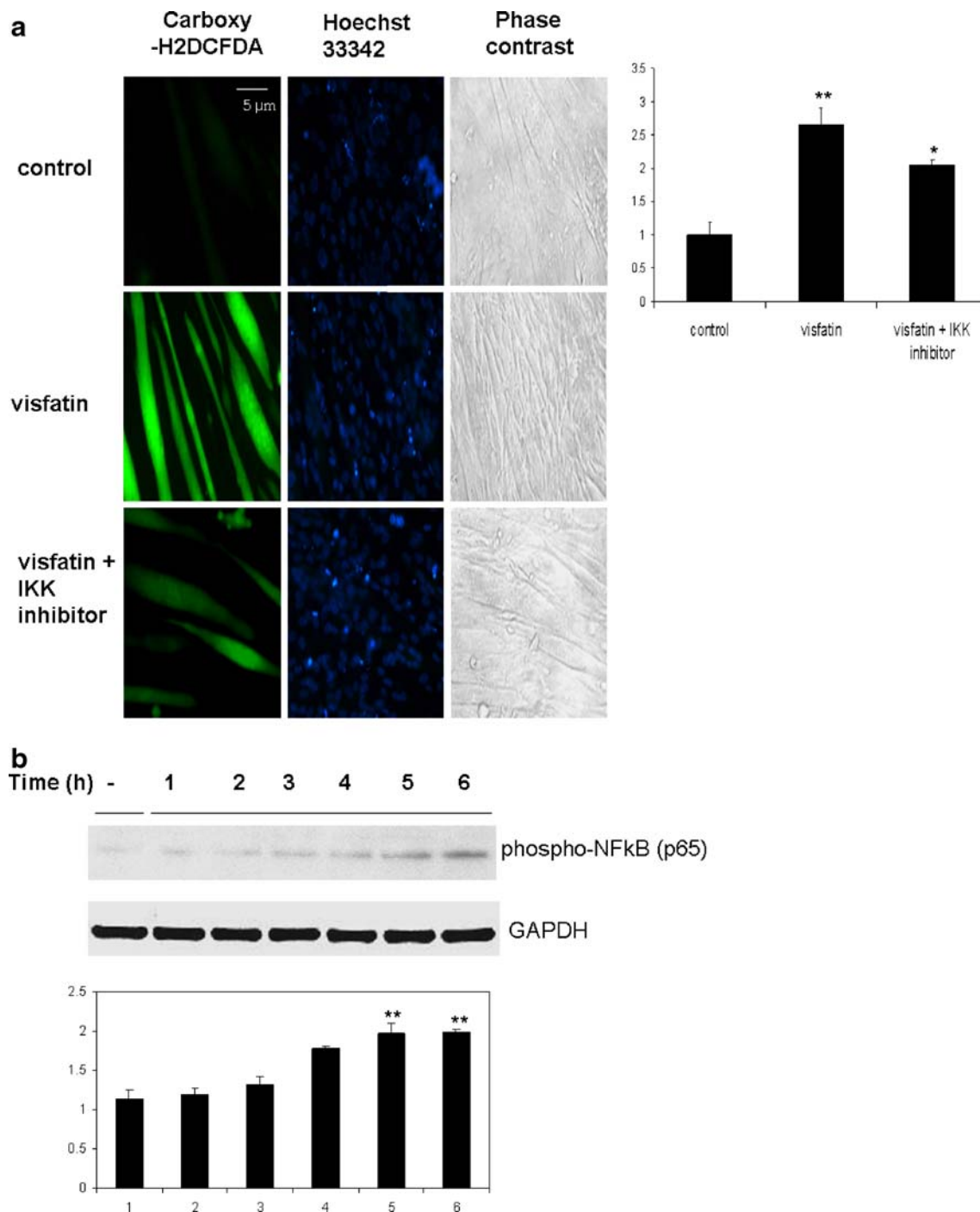


Fig. 5 Visfatin-induced ROS production is dependent on NF κ B signalling. **a** Prior to 18 h visfatin exposure, C2C12 cells were pretreated for 1 h with 50 $\mu\text{g}/\text{ml}$ I κ B Kinase Inhibitor Peptide, which blocks the activation of NF κ B. To visualise and quantify the ROS production cells were incubated with 25 μM carboxy-H2DCFDA for 25 min followed by 5 min incubation with 1 μM Hoechst 33342. **b** Differentiated C2C12 myotubes were incubated in the absence or

presence of 100 ng/ml recombinant visfatin for 1, 2, 3, 4, 5, or 6 h and phosphorylation of the p65 subunit of NF κ B was detected by Western blot. Relative expression compared to control data is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments \pm SEM. *Denotes $p < 0.05$ compared to visfatin treatment; **denotes $p < 0.01$ compared to control

p38-dependent signalling pathways, it was mediated in part via activation of the NF κ B signalling cascade. Taken together these data suggest a novel role for the adipokine visfatin as a generator of oxidative stress in skeletal

myocytes. This hypothesis is supported by recent findings that adipose tissue visfatin mRNA increases following acute exercise, which correlates with an inflammatory response in skeletal muscle [13].

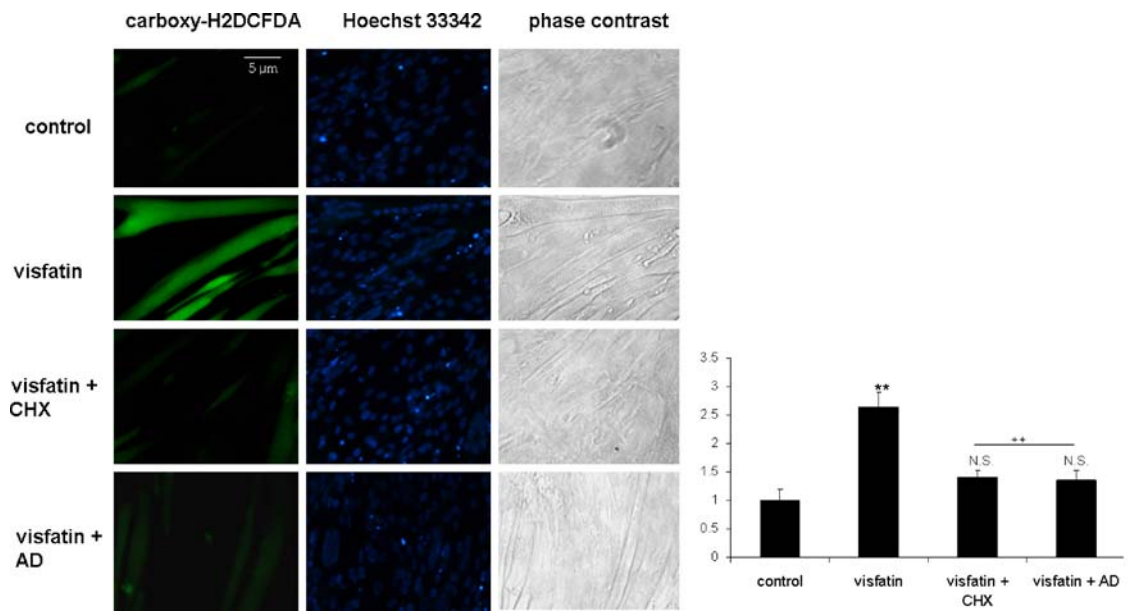


Fig. 6 Visfatin-induced ROS production is dependent on de novo transcription and translation. C2C12 cells were incubated with 1 μ g/ml actinomycin D to block transcription, or with 0.1 μ M cycloheximide ready-made solution to block de novo translation before treatment with 100 ng/ml visfatin for 18 h. ROS production was measured by incubating the cells with 25 μ M carboxy-H2DCFDA for 25 min followed by 5 min incubation with 1 μ M Hoechst 33342 to stain the nuclei. Relative

expression compared to control data is shown. Each data point is the average of quadruplicate wells per experiment from three independent experiments. *CHX* cycloheximide, *AD* actinomycin D. **Denotes $p < 0.05$ compared to control. ++Denotes $p < 0.05$ compared to visfatin treatment. *NS* denotes no significant difference between *CHX* and control nor between *AD* and control

The fact that adipokines such as adiponectin, $\text{TNF}\alpha$, resistin or leptin released by subcutaneous or visceral fat depots modulate physiology of peripheral tissues is well established [38, 58] and these effects have been implicated in the development of pathological conditions such as insulin resistance [29] and metabolic syndrome [53]. Similarly, the association of visfatin with pathological conditions involving oxidative stress and inflammation is also well-supported by both human and animal studies. For example, in obese individuals, plasma visfatin correlated with inflammatory markers such as CRP, IL-6 and $\text{TNF}\alpha$ [7, 28]. Additionally, polymorphisms within the visfatin promoter are associated with inflammation and acute lung injury susceptibility [6, 59]. However, the mechanisms by which visfatin may induce stress responses in pathological conditions are unclear and appear to differ in a cell-type- and tissue-dependent manner.

In the present study, in accordance with results obtained in vascular endothelial cells [51] we demonstrate that in differentiated mouse myocytes, visfatin treatment generates ROS. It is likely that visfatin-induced ROS are produced by both the mitochondria and peroxisomes because we observed generation of both superoxide and hydrogen peroxide species. Despite the lack of detailed mechanistic studies, there are a few possible explanations for the role of visfatin in the generation of stress responses. First, it may be possible that visfatin-induced generation of free radicals

and inflammation could occur as a consequence of NAD^+ metabolism. This concept is supported by a recent study in which adipose-derived plasma visfatin-generated oxidative stress through the synthesis of NAD^+ and the further activation of the extracellular, membrane-bound NADPH oxidases [2].

Alternatively, visfatin-induced oxidative stress could depend on activation of protein kinases such as PI3K [26], the MAPKs p38 [3] and ERK [20], or signalling pathways such as the $\text{NF}\kappa\text{B}$ pathway [24]. In the current study, we were unable to demonstrate any effects of visfatin on phosphorylation of Akt, its upstream regulators and downstream targets, nor on insulin-stimulated Akt activation. However, our observed lack of an effect on Akt is relatively unsurprising as the role of visfatin on skeletal muscle insulin signalling is disputed. Although visfatin was initially proposed to have insulin-like functions due to its ability to bind the insulin receptor and activate the PI3K/Akt pathway in endothelial cells, this observation was later retracted and subsequent investigations failed to confirm the insulin-mimetic properties of visfatin [14, 39]. Our study is the first to demonstrate that in cultured myocytes which are responsive to insulin, treatment with 100 ng/ml visfatin fails to elicit an activation of PI3K/Akt signalling. We further failed to see increased phosphorylation of the Ser472 position of Akt following treatment with higher concentration of recombinant visfatin (100–500 ng/ml, data

not shown). These data are relatively unsurprising as it is likely that visfatin has divergent effects in different tissues; analysis of the PBEF gene has revealed the presence of two distinct promoters, suggesting that the gene and associated protein product(s) may be differentially expressed in tissues [36].

Previous reports in endothelial cells, monocytes and vascular smooth muscle cells have shown that visfatin activates members of the MAPK signalling pathway involved in stress responses including ERK [3, 20, 26, 27] and p38 [3, 15]. Interestingly, in contrast to results reported in monocytes and smooth muscle cells we did not observe activation of p38 and ERK following short-term incubation of skeletal myocytes with visfatin. Moreover, the pre-incubation of skeletal myotubes with specific inhibitors against p38 and ERK had no effect on ROS levels, further providing evidence that MAPKs are not involved in visfatin-induced ROS generation in mouse skeletal myocytes.

Another transcription factor, NF κ B, regulates the inflammatory response to a variety of stimuli by regulating pro-inflammatory cytokines such as IL-6 and TNF α . Interestingly, the distal promoter region of the PBEF gene that encodes visfatin contains a NF κ B binding site, as does the third intron of the gene [36] suggesting that NF κ B may itself regulate visfatin expression and/or activity in either a positive or negative fashion. Furthermore, visfatin is known to activate NF κ B signalling in endothelial cells, upregulating the levels of its pro-inflammatory transcriptional targets ICAM-1 and VCAM-1 and causing endothelial dysfunction [21]. These data link NF κ B and visfatin to inflammatory and stress responses through NF κ B signalling. In concordance with these studies, here, we demonstrate that visfatin-induced generation of oxidative stress is partially dependent on both NF κ B signalling and de novo transcription and translation. Blockade of the NF κ B pathway led to a significant reduction in ROS generation, further suggesting that visfatin-induced stress responses may be mediated in part via NF κ B signalling in skeletal myocytes. These data raise the possibility that once activated by visfatin (in an ROS-dependent or independent manner) NF κ B may transcriptionally regulate some as yet unknown target genes which will further activate a positive feed-back loop resulting in ROS generation. Whilst this is a possible mechanism by which NF κ B signalling may be involved in visfatin-induced generation of ROS, inhibition of this pathway did not entirely abrogate ROS generation suggesting that other signalling pathways or molecules may be involved. In this study we observed a modest increase (1.45-fold compared to control) of iNOS—an important NF κ B transcriptional target that is known to generate oxidative stress through the production of peroxynitrate—following visfatin treatment (data not shown) suggesting that NF κ B-dependent transcriptional regulation may also play a role in visfatin-induced

generation of ROS. This hypothesis would be consistent by our findings that inhibition of de novo transcription blocked visfatin-induced ROS generation. Taken together, these data support a potential role for NF κ B-dependent transcriptional regulation in ROS generation and/or propagation of oxidative stress responses, but this hypothesis needs to be further tested. Furthermore, the precise molecules and pathways regulated by NF κ B that may facilitate visfatin-induced ROS generation and the physiological implications of this oxidative stress are only a matter of speculation at the present. However, due to the association of visfatin with a number of pathological conditions, it remains important to fully elucidate the mechanism by which visfatin effects physiological processes in different tissues.

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References

1. Aam BB, Myhre O, Fonnum F (2003) Transcellular signalling pathways and TNF-alpha release involved in formation of reactive oxygen species in rat alveolar macrophages exposed to tert-butylcyclohexane. *Arch Toxicol* 77(12):678–684
2. Adams JD Jr (2008) Alzheimer's disease, ceramide, visfatin and NAD. *CNS Neurol Disord Drug Targets* 7(6):492–498
3. Adya R, Tan BK, Punn A et al (2008) Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis. *Cardiovasc Res* 78(2):356–365
4. Aldhahi W, Hamdy O (2003) Adipokines, inflammation, and the endothelium in diabetes. *Curr Diab Rep* 3(4):293–298
5. Anrather J, Racchumi G, Iadecola C (2006) NF-kappaB regulates phagocytic NADPH oxidase by inducing the expression of gp91phox. *J Biol Chem* 281(9):5657–5667
6. Bajwa EK, Yu CL, Gong MN et al (2007) Pre-B-cell colony-enhancing factor gene polymorphisms and risk of acute respiratory distress syndrome. *Crit Care Med* 35(5):1290–1295
7. Bo S, Ciccone G, Baldi I et al (2009) Plasma visfatin concentrations after a lifestyle intervention were directly associated with inflammatory markers. *Nutr Metab Cardiovasc Dis* 19(6):423–430
8. Busso N, Karababa M, Nobile M et al (2008) Pharmacological inhibition of nicotinamide phosphoribosyltransferase/visfatin enzymatic activity identifies a new inflammatory pathway linked to NAD. *PLoS ONE* 3(5):e2267
9. Chaldakov GN, Stankulov IS, Hristova M et al (2003) Adipobiology of disease: adipokines and adipokine-targeted pharmacology. *Curr Pharm Des* 9(12):1023–1031
10. Dietze D, Koenen M, Röhrig K et al (2002) Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. *Diabetes* 51(8):2369–2376
11. Dietze D, Ramrath S, Ritzeler O et al (2004) Inhibitor kappaB kinase is involved in the paracrine crosstalk between human fat and muscle cells. *Int J Obes Relat Metab Disord* 28(8):985–992
12. Dietze-Schroeder D, Sell H, Uhlig M et al (2005) Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 54(7):2003–2011
13. Frydelund-Larsen L, Akerstrom T, Nielsen S et al (2007) Visfatin mRNA expression in human subcutaneous adipose tissue is

- regulated by exercise. *Am J Physiol Endocrinol Metab* 292(1): E24–E31
14. Garten A, Petzold S, Körner A et al (2009) Namp1: linking NAD biology, metabolism and cancer. *Trends Endocrinol Metab* 20(3):130–138
 15. Hong SB, Huang Y, Moreno-Vinasco L et al (2008) Essential role of pre-B-cell colony enhancing factor in ventilator-induced lung injury. *Am J Respir Crit Care Med* 178(6):605–617
 16. Hug C, Lodish HF (2005) Medicine. Visfatin: a new adipokine. *Science* 307(5708):366–367
 17. Jensen MD (2008) Role of body fat distribution and the metabolic complications of obesity. *J Clin Endocrinol Metab* 93(11 Suppl 1): S57–S63
 18. Kamiya T, Hara H, Yamada H et al (2008) Cobalt chloride decreases EC-SOD expression through intracellular ROS generation and p38-MAPK pathways in COS7 cells. *Free Radic Res* 42(11–12):949–956
 19. Kim JH, Bachmann RA, Chen J (2009) Interleukin-6 and insulin resistance. *Vitam Horm* 80:613–633
 20. Kim SR, Bae SK, Choi KS et al (2007) Visfatin promotes angiogenesis by activation of extracellular signal-regulated kinase 1/2. *Biochem Biophys Res Commun* 357(1):150–156
 21. Kim SR, Bae YH, Bae SK et al (2008) Visfatin enhances ICAM-1 and VCAM-1 expression through ROS-dependent NF- κ B activation in endothelial cells. *Biochim Biophys Acta* 1783(5):886–895
 22. Krzysik-Walker SM, Ocón-Grove OM, Maddineni SR et al (2008) Is visfatin an adipokine or myokine? Evidence for greater visfatin expression in skeletal muscle than visceral fat in chickens. *Endocrinology* 149(4):1543–1550
 23. Ku BM, Lee YK, Jeong JY et al (2007) Ethanol-induced oxidative stress is mediated by p38 MAPK pathway in mouse hippocampal cells. *Neurosci Lett* 419(1):64–67
 24. Lee WJ, Wu CS, Lin H et al (2009) Visfatin-induced expression of inflammatory mediators in human endothelial cells through the NF- κ B pathway. *Int J Obes (Lond)* 33(4):465–472
 25. Li X, Rong Y, Zhang M et al (2009) Up-regulation of thioredoxin interacting protein (Txnip) by p38 MAPK and FOXO1 contributes to the impaired thioredoxin activity and increased ROS in glucose-treated endothelial cells. *Biochem Biophys Res Commun* 381(4):660–665
 26. Lim SY, Davidson SM, Paramanathan AJ et al (2008) The novel adipocytokine visfatin exerts direct cardioprotective effects. *J Cell Mol Med* 12(4):1395–1403
 27. Lin BR, Yu CJ, Chen WC et al (2009) Green tea extract supplement reduces D-galactosamine-induced acute liver injury by inhibition of apoptotic and proinflammatory signalling. *J Biomed Sci* 16(1):35
 28. Liu SW, Qiao SB, Yuan JS et al (2009) Visfatin stimulates production of monocyte chemoattractant protein-1 and interleukin-6 in human vein umbilical endothelial cells. *Horm Metab Res* 41(4):281–286
 29. Lorenzo M, Fernández-Veledo S, Vila-Bedmar R et al (2008) Insulin resistance induced by tumor necrosis factor- α in myocytes and brown adipocytes. *J Anim Sci* 86(14 Suppl):E94–E104
 30. Lovren F, Pan Y, Shukla PC et al (2009) Visfatin activates eNOS via Akt and MAP kinases and improves endothelial function. *Am J Physiol Endocrinol Metab* 296(6):E1440–E1449
 31. Martin PR, Shea RJ, Mulks MH (2001) Identification of a plasmid-encoded gene from *Haemophilus ducreyi* which confers NAD independence. *J Bacteriol* 183(4):1168–1174
 32. Moschen AR, Kaser A, Enrich B et al (2007) Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. *J Immunol* 178(3):1748–1758
 33. Munhoz CD, García-Bueno B, Madrigal JL et al (2008) Stress-induced neuroinflammation: mechanisms and new pharmacological targets. *Braz J Med Biol Res* 41(12):1037–1046
 34. Myhre O, Sterri SH, Bogen IL et al (2004) Erk1/2 phosphorylation and reactive oxygen species formation via nitric oxide and Akt-1/Raf-1 crosstalk in cultured rat cerebellar granule cells exposed to the organic solvent 1, 2, 4-trimethylcyclohexane. *Toxicol Sci* 80(2):296–303
 35. Nieto-Vazquez I, Fernández-Veledo S, Krämer DK (2008) Insulin resistance associated to obesity: the link TNF- α . *Arch Physiol Biochem* 114(3):183–194 Review
 36. Ognjanovic S, Bao S, Yamamoto SY (2001) Genomic organization of the gene coding for human pre-B-cell colony enhancing factor and expression in human fetal membranes. *J Mol Endocrinol* 26(2):107–117
 37. Pajvani UB, Scherer PE (2003) Adiponectin: systemic contributor to insulin sensitivity. *Curr Diab Rep* 3(3):207–213
 38. Rabe K, Lehrke M, Parhofer KG et al (2008) Adipokines and insulin resistance. *Mol Med* 14(11–12):741–751
 39. Revollo JR, Körner A, Mills KF et al (2007) Namp1/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* 6(5):363–375
 40. Roncari DA, Hamilton BS (1993) Cellular and molecular factors in adipose tissue growth and obesity. *Adv Exp Med Biol* 334:269–277
 41. Rongvaux A, Andris F, Van Gool F et al (2003) Reconstructing eukaryotic NAD metabolism. *Bioessays* 25(7):683–690 Review
 42. Ronti T, Lupattelli G, Mannarino E (2006) The endocrine function of adipose tissue: an update. *Clin Endocrinol (Oxf)* 64(4):355–365
 43. Samal B, Sun Y, Stearns G et al (1994) Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol Cell Biol* 14(2):1431–1437
 44. Seip M, Trygstad O (1996) Generalized lipodystrophy, congenital and acquired (lipatrophy). *Acta Paediatr Suppl* 413:2–28
 45. Sell H, Eckardt K, Taube A (2008) Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility. *Am J Physiol Endocrinol Metab* 294(6):E1070–E1077
 46. Sell H, Eckel J, Dietze-Schroeder D, 2 (2006) Pathways leading to muscle insulin resistance—the muscle—fat connection. *Arch Physiol Biochem* 112(2):105–113 Review
 47. Sharma AM (2006) The obese patient with diabetes mellitus: from research targets to treatment options. *Am J Med* 119(5 Suppl 1): S17–S23
 48. Skurk T, Alberti-Huber C, Herder C et al (2007) Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 92(3):1023–1033
 49. Song HK, Lee MH, Kim BK et al (2008) Visfatin: a new player in mesangial cell physiology and diabetic nephropathy. *Am J Physiol Renal Physiol* 295(5):F1485–F1494
 50. Sonnenberg GE, Krakower GR, Kissebah AH (2004) A novel pathway to the manifestations of metabolic syndrome. *Obes Res* 12(2):180–186
 51. Takebayashi K, Suetsugu M, Wakabayashi S et al (2007) Association between plasma visfatin and vascular endothelial function in patients with type 2 diabetes mellitus. *Metabolism* 56(4):451–458
 52. Taki K, Shimozono R, Kusano H et al (2008) Apoptosis signal-regulating kinase 1 is crucial for oxidative stress-induced but not for osmotic stress-induced hepatocyte cell death. *Life Sci* 83(25–26):859–864
 53. Viollet B, Mounier R, Leclerc J et al (2007) Targeting AMP-activated protein kinase as a novel therapeutic approach for the treatment of metabolic disorders. *Diabetes Metab* 33(6):395–402

54. Windelborn JA, Lipton P (2008) Lysosomal release of cathepsins causes ischemic damage in the rat hippocampal slice and depends on NMDA-mediated calcium influx, arachidonic acid metabolism, and free radical production. *J Neurochem* 106(1):56–69
55. Xiao J, Xiao ZJ, Liu ZG et al (2009) Involvement of dimethylarginine dimethylaminohydrolase-2 in visfatin-enhanced angiogenic function of endothelial cells. *Diabetes Metab Res Rev* 25(3):242–249
56. Xie H, Tang SY, Luo XH et al (2007) Insulin-like effects of visfatin on human osteoblasts. *Calcif Tissue Int* 80(3):201–210
57. Yamawaki H, Hara N, Okada M et al (2009) Visfatin causes endothelium-dependent relaxation in isolated blood vessels. *Biochem Biophys Res Commun* 383(4):503–508
58. Yu YH, Ginsberg HN (2005) Adipocyte signaling and lipid homeostasis: sequelae of insulin-resistant adipose tissue. *Circ Res* 96(10):1042–1052
59. Zhang LQ, Adyshev DM, Singleton P et al (2008) Interactions between PBEF and oxidative stress proteins—a potential new mechanism underlying PBEF in the pathogenesis of acute lung injury. *FEBS Lett* 582(13):1802–1808