Insulin is a major adipogen. It stimulates the uptake of glucose, which is then converted into triglycerides and stored in fat droplets leading to adipocyte maturation; insulin also promotes the differentiation of preadipocytes in the stroma of the adipose tissue into adipocytes. Although paracrine autacoids as well as transcription factors and signaling cascades have been identified to be involved in adipocyte differentiation and insulin signaling, these complex processes are still incompletely understood. 

We hypothesize that insulin elicits a prolonged increase in ROS formation, which is mediated by an increase in the expression of constitutively active Nox4. Moreover, we investigated whether or not this induction of Nox4 is required for long-term processes in response to insulin, such as proliferation and differentiation, and determined the underlying mechanisms.

**Methods**

For a detailed Material and Methods section please see the supplemental materials (available online at http://atvb.ahajournals.org).

**Cell Culture**

Human preadipocytes were isolated from the stroma vascular fraction of liposuctions by collagenase digestion. 3T3-L1 fibroblasts were obtained from ATCC. Differentiation was induced by standard protocol. Unless otherwise indicated, experiments were conducted at day 10 (3T3-L1) and 12 (human preadipocytes) after confluence. For transfection, Gene-Eraser was used for siRNAs and Gene-Jammer was used for plasmids (Stratagene). Overexpression of Nox4 was achieved by lentiviral transduction. Differentiation was determined by AdipoRed staining and quantified by fluorescence-activated-cell sorter (FACS) or microplate fluorimeter measurements. Proliferation was determined by cell counting or BrdU incorporation.

**Immunoblotting and Immunoprecipitation**

Extractions for Western blots and immunoprecipitations were performed with Nonidet (1%) lysis buffer containing protease and phosphatase inhibitors. The anti-Nox4 antibody was kindly provided by A.M. Shah (King’s College, London, UK).
**Results**

**Insulin-Induced Differentiation Depends on ROS**

Insulin induced the differentiation of 3T3-L1 cells into adipocytes as observed by fat droplet formation (Figure 1A), downregulation of the preadipocyte marker pref-1 (Figure 1B) as well as upregulation of the fatty-acid binding protein FABP4/aP2 (11% mean ± SEM, n = 3, *P < 0.05). Accumulation of fat droplets was considered to be the final step of differentiation succeeding a previous occurrence of changes in the gene expression. Therefore, in this study only cells with fat droplets were interpreted as fully differentiated. Insulin also induced a marked increase in ROS formation, which was apparent 4 hours after the beginning of insulin stimulation and still detectable 2 days after the onset of stimulation (Figure 1C). To test whether ROS are involved in differentiation, we stimulated 3T3-L1 cells and human preadipocytes with H₂O₂ (30 μmol/L, every other day) which resulted in differentiation even in the absence of insulin (Figure 1D). Accordingly, supplementation of catalase or overexpression of cytosolic catalase blocked the insulin-induced differentiation of preadipocytes (catalase overexpression: Figure 1E, catalase supplementation: solvent versus catalase with insulin 100 versus 49 ± 11%, mean ± SEM, n = 3, *P < 0.05). This effect was also reflected by the fact that catalase prevented the insulin-induced downregulation of pref-1 mRNA (% downregulation of pref-1 in response to insulin: control condition: 75.1 ± 10.2; catalase 17 ± 36.77, *P < 0.05 versus solvent; n = 3). Similarly, the nonspecific NADPH oxidase inhibitor diphenylene iodonium (10 μmol/L) blocked insulin-induced differentiation and pref-1 mRNA downregulation (data not shown).

**Insulin-Induced ROS Formation Is Mediated by an Induction of Nox4**

Quantitative RT-PCR was performed to identify the NADPH oxidase homologue responsible for the sustained increase of ROS formation in response to insulin. 3T3-L1 fibroblasts expressed Nox1, Nox2, as well as Nox4, which is the predominant homologue on the mRNA level (Figure 2A). Nox3 and Nox5 were undetectable in 3T3-L1 and human preadipocytes. Duox1 and 2 expression was detected at very low level only (ct values of 34 and 35, respectively). In the

**H₂O₂ Production**

H₂O₂ was measured by an Amplex red/Horseradish peroxide assay.

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**References**

1. Some references are missing or not provided in the text.

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**Figure 1.** Reactive oxygen species (ROS) are involved in insulin-induced differentiation. (A) Microscopic images of 3T3-L1 cell at day 12 of differentiation. (B) qRT-PCR for pref-1 and 18S (C) ROS-formation determined by Amplex red assay (D&E) Adipored staining (F) insulin-induced proliferation. For detailed figure legends see the supplemental materials.

**Figure 2.** Nox4 is involved in insulin-induced ROS formation. (A) q-RT-PCR (B&C) Nox4 protein expression (D) ROS-formation determined by Amplex red assay; lower part: representative gel of Nox4 RT-PCR. For detailed figure legends see the supplemental materials.
course of insulin-induced differentiation, the expression of Nox1 and Nox4 increased, whereas Nox2 mRNA expression remained unchanged (Figure 2A). Accordingly, Nox4 protein expression was observed to be increased in differentiated as compared to undifferentiated 3T3-L1 cells (Figure 2B).

We next determined the contribution of Nox1 and Nox4 to insulin-stimulated ROS production. A Nox4 siRNA was developed which decreased Nox4 protein expression by ≈70% (Figure 2C). Using this tool, it was observed that the constitutive ROS production in differentiating preadipocytes was predominantly mediated by Nox4: whereas siRNA against Nox1 had only a small, yet significant effect, Nox4 siRNA largely attenuated ROS formation at day 12 of differentiation (Nox1: 33 ± 20%, Nox4: 73 ± 27%, mean ± SEM, n = 5, P < 0.05). Subsequently, the effect of Nox4 siRNA on the induced ROS production after 48 hours of insulin stimulation was determined. Nox4 siRNA prevented the insulin-induced mRNA expression of Nox4 and blocked the insulin-induced increase in ROS production (Figure 2D).

Nox4 Mediates Differentiation but Impairs Proliferation in Response to Insulin

SiRNA was used to test the contribution of Nox1 and Nox4 to insulin-induced differentiation. SiRNA directed against Nox4 but not Nox1 siRNA blocked the insulin-induced accumulation of fat droplets in 3T3-L1 cells (Figure 3A). SiNox4 also prevented the induction of aP2 in the course of differentiation (scrambled siRNA 2 ± 1.4; Nox1 siRNA; 6.9 ± 5; Nox4 siRNA 0.9 ± 0.7; fold induction determined by the 2−ΔΔct method; mean ± SEM, n = 3, P < 0.05 scrambled siRNA versus Nox4 siRNA). Intriguingly, overexpression of Nox4 in human preadipocytes promoted the accumulation of fat droplets even in the absence of insulin, suggesting that Nox4 is a direct mediator of insulin-induced differentiation (Figure 3B). Pref-1 is not only a marker for preadipocytes but also maintains these cells in an undifferentiated state. We therefore determined the effect of Nox4 on pref-1 expression. Insulin-induced differentiation was associated with a marked reduction in pref-1 mRNA expression and this effect was almost completely prevented by siRNA directed against Nox4 (Figure 3C). Likewise, lentiviral-mediated overexpression of Nox4 induced a drastic downregulation of pref-1 even in the absence of insulin (Figure 3D). These observations suggest that pref-1 is an effector of Nox4.

Differentiation and proliferation represent dichotomous aspects of cellular function and proliferation is frequently associated with dedifferentiation. As insulin is known to stimulate proliferation of fibroblasts, the role of Nox4 in this process was determined. Insulin stimulated the proliferation of 3T3-L1 cells, and, importantly, this effect was potentiated by siRNA directed against Nox4 whereas Nox1 siRNA was without effect (BrdU incorporation: Figure 3E, determination of the increase in total cell number in response to insulin: scrambled siRNA 15.5 ± 3.4%, Nox4 siRNA 33.2 ± 9.4%, *P < 0.05 scrambled versus Nox4 siRNA). Intriguingly, overexpression of catalase enhanced the insulin-induced proliferation of 3T3-L1, suggesting that the effects of Nox4 are mediated by alterations in ROS level (Figure 1F).

Nox4 Attenuates MAP Kinase Signaling by Inducing MKP-1

Proliferation in response to insulin is mediated by the Ras-Raf-MEK-ERK1/2 pathway. Indeed, the basal and insulin-stimulated phosphorylation or ERK1/2 was enhanced when Nox4 was downregulated by siRNA, whereas it was attenuated when Nox4 was overexpressed in human preadipocytes (Figure 4A). As activation of ERK1/2 is limited by the MAP kinase phosphatase-1 (MKP-1), we tested the effect of Nox4 on this protein. MKP-1 expression paralleled that of Nox4: whereas downregulation of Nox4 attenuated MKP-1 expression, overexpression of the NADPH oxidase in human preadipocytes increased it. Importantly, Nox4 had no effect on the expression of other important phosphatases such as PP2A and PTP1B (Figure 4B). Moreover, we observed that Nox4 overexpression did not inhibit MKP-1 activity (Schröder K, unpublished observation, 2008) and siRNA directed against Nox1 did not alter MKP-1 expression or insulin-induced ERK1/2 activation in 3T3-L1 cells (data not shown). These observations identify Nox4, by controlling MKP-1 expression, as a negative regulator of ERK1/2 signaling.
Nox4 Facilitates Insulin Receptor Substrate-1–Mediated Insulin Signaling

On insulin-binding, the insulin receptor tyrosine phosphorylates insulin receptor substrate-1 (IRS-1), which then leads to differentiation via PI-3 kinase, Akt, and protein kinase C isoforms. Downregulation of Nox4 attenuated the insulin-induced tyrosine phosphorylation of IRS-1, whereas Nox4 overexpression in human preadipocytes enhanced it. Another phosphorylation site of IRS-1 is Ser612, which is known to hamper IRS-1 tyrosine phosphorylation. IRS-1 phosphorylation on Ser612 occurs by ERK1/2. Importantly, Nox4 attenuated the insulin-dependent phosphorylation at this site (Figure 4A). These data indicate that Nox4 promotes insulin-signaling through IRS-1 by attenuating the ERK1/2-mediated phosphorylation at the inhibitory serine site of this protein.

MKP-1 Is a Mediator of the Effects of Nox4

According to the above mentioned results, MKP-1 and ERK1/2 should essentially determine the balance between insulin-induced differentiation and proliferation. Inhibition of extracellular signal regulated kinase (ERK) 1/2 activation by PD98059 (10 μmol/L) or overexpression of MKP-1 enhanced insulin-induced differentiation (Figure 5A and 5B). Overexpression of MKP-1 prevented insulin-induced ERK1/2 phosphorylation in 3T3-L1 cells (insulin-induced increase in ERK1/2 phosphorylation: GFP control 5 ± 0.1; MKP-1 plasmid: 1.2 ± 0.1; n = 5, P < 0.05). Accordingly, PD98059 or overexpression of MKP-1 both inhibited insulin-induced proliferation (Figure 5C and 5D). To test the contribution of endogenous MKP-1 in this process siRNA was used. Indeed, MKP-1 siRNA more than tripled insulin-induced proliferation (Figure 5E). This demonstrates that endogenous MKP-1, which is controlled by Nox4, is central in attenuating insulin-induced proliferation. Unfortunately, because of the pronounced effect of MKP-1 siRNA on proliferation, the effect of this tool on the slow process of differentiation, which requires 12 days, could not be determined: the substantial changes in cell number interfered with the speed of the differentiation process.

Discussion

In this study we provide evidence that Nox4 acts as a switch from proliferation to differentiation. Nox4, through an indirect effect, facilitates the tyrosine phosphorylation of IRS-1, which is required for the insulin-induced differentiation. This process appears to involve MKP-1: Nox4 controls the expression of MKP-1 and thereby limits the contribution of the proliferative Ras–Raf-ERK1/2 pathway to insulin signaling. ERK1/2 phosphorylates IRS-1 on serine-residues and thereby prevents IRS-1 tyrosine phosphorylation. The Nox4-dependent induction of MKP-1 prevents this effect and therefore promotes insulin-induced differentiation but attenuates insulin-induced proliferation.

Nox4 expression has been demonstrated to be regulated, and differentiating factors such as transforming growth factor (TGF) β1 are known to induce this NADPH oxidase, as also observed in the present study. Interestingly, high glucose also appears to induce Nox4 (Schröder K. et al, unpublished observation, 2008). Thus, it is appealing to speculate that Nox4 promotes the development of obesity in scenarios of combined hyperglycemia and hyperinsulinemia, like type II diabetes.
To induce differentiation into adipocytes, 2 different protocols were used for human and mouse cells, which contain several compounds which interfere with signal transduction and potentially even NADPH oxidase expression. Ciglizone, which was used for human preadipocyte differentiation, has been shown to downregulate expression of several NADPH oxidases and to reduce ROS production in endothelial cells.\textsuperscript{11} Rosiglitazone is known to downregulate p47phox in monocytes and macrophages, and thereby it attenuates the respiratory burst in these cells.\textsuperscript{12} In 3T3-L1 cells, dexamethasone was used instead of ciglizone; dexamethasone also induces PPAR gamma.\textsuperscript{13} Moreover, we have previously observed that glucocorticoids downregulate p22phox in vascular smooth muscle cell (VSMC).\textsuperscript{14} On the other hand, high concentrations of glucocorticoids may even increase oxidative stress in endothelial cells, although the underlying mechanism is unclear.\textsuperscript{15} Finally, IBMX, which is given for 2 days in the course of differentiation, has been shown to downregulate p47phox phosphorylation in leukocytes.\textsuperscript{16} These observations could suggest that the process of differentiation requires a reduction in cellular ROS formation. This assumption, however, is in contrast to the fact that hydrogen peroxide or overexpression of Nox4 per se promotes differentiation. Moreover, it has consistently been demonstrated that the process of differentiation is associated with an increased ROS production: Serum-deprivation induced differentiation of smooth muscle cells\textsuperscript{17} as well as TGF\alpha-induced differentiation of human fibroblasts into myofibroblasts\textsuperscript{18} were both associated with an increased ROS production. These observations might indicate that effects of the glucocorticoids and PPAR\gamma agonists are unrelated to their actions on ROS production.

The main factor for differentiation into adipocytes and accumulation of fat droplets, however, is insulin. Priming for differentiation using the above mentioned protocols in the absence of insulin did not result in fat accumulation. Indeed, a robust link between insulin and increased cellular ROS production exists: First reports demonstrated that insulin acutely increases the hydrogen peroxide formation in membrane preparations of adipocytes in an NADPH-dependent manner.\textsuperscript{19} Using the DCHF assay, also an acute insulin-induced formation of peroxides was detected in intact 3T3-L1 fibroblasts. Interestingly, the insulin-induced ROS formation was prevented when cells were infected by a dominant negative Nox4 construct lacking parts of the cytosolic tail of the enzyme.\textsuperscript{20} On the basis of these observations, it might however be premature to conclude that the insulin-induced ROS formation is mediated by Nox4 as such a fragment can induce different types of unspecific actions.\textsuperscript{21,22}

In the present study, insulin stimulated the cellular ROS production within 4 hours from the onset of exposure, and this effect was maintained for several days. We assume that ROS production is a consequence of an insulin-mediated induction of the protein. Indeed, such a short time from induction until ROS production was recently demonstrated using tetracycline-inducible Nox4 expression plasmids.\textsuperscript{23} We observed that insulin stimulated the mRNA expression of Nox1 as well as of Nox4, suggesting that either Nox1 or Nox4 contributes to the increased ROS production in response to insulin. Indeed, siRNA directed against Nox1 and even more prominent against Nox4 attenuated the basal ROS-production in 3T3-L1 cells and also prevented the insulin-induced prolonged increase in radical formation. These observations are compatible with previous reports in other cellular systems, suggesting that Nox4 mediates the basal constitutive ROS production in endothelial cells,\textsuperscript{24} smooth muscle cells,\textsuperscript{25} and renal mesangial cells,\textsuperscript{26} and that the basal ROS production correlates with the expression of Nox4.\textsuperscript{27} The mechanism of acute insulin-induced ROS production, which was not a focus of this article, however, still needs to be identified. Although siRNA against Nox1 slightly attenuated the basal ROS production in 3T3-L1 cells, differentiation and insulin signaling were completely unaffected by changing Nox1 expression, and therefore the exact contribution of this NADPH oxidase to the function of preadipocytes remains to be determined.

The observations of the present work suggest that insulin-stimulated differentiation involves the induction of Nox4. Recently an article appeared which suggested that Nox4 is downregulated in the course of differentiation.\textsuperscript{28} Despite this, the authors describe a dramatically higher ROS production in mature adipocytes as compared to undifferentiated 3T3-L1 cells. However, this contradicts the decrease in Nox4 expression described by the authors. Interestingly, in the same article it was reported that adipose tissue of obese but not lean mice contains high level of Nox4. As a consequence those observations rather fit into the model proposed in the present study than into a concept of downregulation of Nox4 during differentiation.

**How Does Nox4 Control Differentiation?**

The undifferentiated state of preadipocytes is maintained by the EGF-like protein pref-1, which is released from preadipocytes themselves. Pref-1 expression is completely lost in the course of adipocyte differentiation, and overexpression of pref-1 in preadipocytes completely prevents differentiation.\textsuperscript{29} Pref-1 activates the MEK/ERK-pathway to block the induction of PPAR\gamma-2, which is required for further differentiation.\textsuperscript{30} Interestingly, pref-1 mRNA itself is governed by transcription factors dependent on the MEK/ERK1/2 pathway such as EGR-1,\textsuperscript{31} suggesting a positive feedback mechanism to maintain preadipocytes in undifferentiated state. In the present study we observed that insulin-induced downregulation of pref-1 was prevented by Nox4 siRNA. Nox4 siRNA also blocked the insulin-induced differentiation embodied in the prevention of apo2 expression and fat accumulation. This might suggest that Nox4 contributes to the downregulation of pref-1, potentially by interfering with pathways controlling pref-1 expression such as the MEK/ERK-pathway. Indeed, we could observe that Nox4 overexpression not only promoted differentiation per se but also decreased the phosphorylation of ERK1/2. Conversely, downregulation of Nox4 increased the phosphorylation of ERK1/2. This effect was probably mediated by MKP-1, the expression of which was dependent on Nox4 in the present study. As MKP-1 dephosphorylates MAPKs, ERK1/2 phosphorylation inversely correlates with Nox4 expression.
It is generally assumed that ROS promote proliferation. Apparently ROS derived from Nox4 lack this property, and it is unknown whether this is a consequence of the specific localization of Nox4 in the cell or the constitutive way in which Nox4 generates ROS. With the identification of MKP-1 induction by Nox4, we however provide a possible explanation why Nox4 does not promote proliferation.

**MKP-1 Is an Effector of Nox4**

Despite its role in pref-1 expression, Nox4-dependent regulation of MKP-1 expression directly controls insulin receptor signaling toward differentiation. After ligand binding the insulin receptor phosphorylates IRS-1 through its intrinsic tyrosine kinase activity, which subsequently activates PKC isoforms and Akt to induce differentiation. Simultaneously, the insulin receptor activates Ras, with consequent activation of MEK and ERK1/2, which is usually depressed by Nox4-mediated induction of MKP-1. ERK1/2 is known to directly phosphorylate IRS-1 on serine 612, and this phosphorylation attenuates the direct insulin-receptor tyrosine kinase-dependent tyrosine phosphorylation of IRS-1. Indeed, downregulation of Nox4 attenuated the insulin-induced IRS-1 tyrosine phosphorylation but increased the IRS-1 serine phosphorylation. As a consequence, Nox4 guides the insulin signal toward differentiation by promoting the IRS-1 pathway and by attenuating the ERK1/2-pathway. Loss of Nox4, however, would favor ERK1/2-signaling, which not only inhibits differentiation but also should promote proliferation as observed in the present study. Indeed, we observed that MKP-1 downregulation massively promotes proliferation, whereas overexpression of the protein promoted differentiation. Accordingly, it was observed that mice lacking MKP-1 are resistant to diet-induced obesity.

Although we provide evidence for a Nox4-dependent induction of MKP-1 in the present study, the underlying molecular mechanisms still remain to be determined. MKP-1 is an immediate early gene product which is rapidly and strongly induced by H2O2. Therefore it is attractive to speculate that ROS generated by Nox4 directly control MKP-1 expression. A possible explanation for such a mechanism might be that ROS are known to inactivate phosphatases, and indeed it has been demonstrated that insulin-stimulated ROS production inhibits the phosphatase PTP1B.

In summary, we have demonstrated that by controlling MKP-1 expression, Nox4 attenuates ERK1/2-signaling in preadipocytes. Through this mechanism, Nox4 tips the balance from insulin-induced proliferation toward differentiation.

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**Disclosures**

None.

**References**


Nox4 Acts as a Switch Between Differentiation and Proliferation in Preadipocytes
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Nox4 acts as a switch between differentiation and proliferation in preadipocytes
Katrin Schröder, Katalin Wandzioch, Ina Helmcke, Ralf P. Brandes

**Material and Methods**

**Cell culture**

Human preadipocytes were isolated from the stroma vascular fraction of liposuctions by collagenase digestion as described before. Patients were adult female with a BMI of 25±3. 3T3-L1 fibroblasts were obtained from ATCC and cultured in Dulbecco’s Modified Eagle’s Medium with nutrient mix F-12 (DMEM/F12) supplemented with 8% fetal calf serum (FCS) and gentamycin. Differentiation was induced by the following protocol: 3T3-L1 fibroblasts were grown until day 2 postconfluency and stimulated with DMEM high glucose (4.5 mg/ml) supplemented with Insulin (1 µg/ml = 174 nmol/l), dexamethason (DEXA 1 µmol/l) and isomethylbutylxanthine (IBMX 0.5 mmol/l). After 2 days medium was exchanged to DMEM high glucose (4.5 mg/ml) supplemented only with Insulin (1 µg/ml). Unless otherwise indicated, experiments were conducted at day 10 after confluence. Differentiation of human preadipocytes was performed according to the United States Patent 7001746. Briefly, 24 hours after seeding the stroma vascular fraction (2x10^5 cells/cm^2) the cell culture medium (DMEM/F12) was changed and supplemented with gentamycin (50µg/ml), dexamethason (DEXA 1 µmol/l), and Insulin (174 nmol/l), panthoten (17 µmol/l), insulin (174 nmol/l), triiodothyronine (1 nmol/l), cortisol (100 nmol/l), transferrin (10 µg/ml) and ciglitazon (1 µg/ml). Control conditions received the same differentiation procedure with the omission of insulin. Differentiation was carried out for 12 days. Cells were serum deprived overnight prior to the experiments.

**siRNA transfection**

SiRNAs were designed according to the database sequences and synthesized by Eurogentec (Seraing, Belgium). The following sequences were used: sense Nox1 5'-GGU CGU GAU UAC CAA GGU UTT-3' and antisense Nox1 5'-AAC CUU GGU AAU CAC GAC CTT-3'; sense mNox4 5'-GAC CUG ACU UUG UGA ACA UTT-3' and antisense mNox4 5'-AUG UUC ACA AAG UCG GGU CTT-3'. siRNA experiments were controlled using a universal negative siRNA control from Eurogentec. Transfection was carried out with the aid of the GeneEraser reagent (Stratagene, La Jolla, CA) according to the manufactures instructions with modifications: Cells were serum-deprived 4 hours prior to transfection with 50 nmol/l siRNA. After 4 additional hours medium containing 16% FCS was added to yield a final FCS concentration of 8%. Specificity of the siRNAs for the individual Nox subunit targeted was validated by quantitative RT-PCR and ROS measurement. Out of the 9 siRNAs directed against murine Nox4 test, we identified 6 siRNAs that down-regulated the mRNA Nox4 but only one of those did not also down-regulate Nox1. This siRNA was used for the study. For the downregulation of MKP-1 a combination of the following two sequences was used, which yield a more than 80% reduction in the protein expression: 5'-GUU UCA ACG AGG CUA UUG ATT-3', reverse: 5'-UCA AUA GCC UCG UUG AAC CTT-3' and 5'-CAC GCU UCU CGG AAG GAU ATT-3' and 5'-UAU CCA UCC GAG AAG CTT-3'.

**Lentiviral-based expression of Nox4**

The human Nox4CDNA, a generous gift from T. Leto, was cloned into the Lentil-V5-D-Topo vector and viruses were generated using the ViraPower packaging mix (Invitrogen). All virus-mediated overexpression experiments were performed using human preadipocytes. Infection efficiency was around 40% as judged by FACS analysis using the GFP-viruses. No clonal selection was performed and infection efficiency was kept relatively low not to overwhelm the cells with Nox4-derived radicals.

**Overexpression studies**

The mouse MKP-1 expression plasmid was obtained from Imagenes, Berlin. The rat catalase plasmid was a kind gift from W. Gwinner, Nephrology, Hannover Medical School. In order to direct the overexpressed catalase to the cytoplasm, the signal peptide was removed by site directed mutagenesis. Transfection was carried out using the Gene Jammer reagent (Stratagene, La Jolla, CA) according to the manufactures instructions with modifications: Cells were serum-deprived 4 hours prior to transfection. After 4 additional hours medium containing 16% FCS was added to yield a final FCS concentration of 8%.

**Immunoblotting and Immunoprecipitation**

Extractions for western blots and immunoprecipitations using protein A/G sepharose were performed using Nonidet (1%) lysis buffer with the following composition: (concentrations in mmol/l), pH 7.4, Tris-HCl (50), NaCl (150), sodium pyrophosphate (10), sodium fluoride (20), nonidet NP40 (1%), desoxycholate (0.5%), proteinase inhibitor mix, phenylmethylsulfonyl fluoride (1), orthovanadate (2), calcixyn A (0.1), okadaic acid (0.01). Proteins were separated by SDS-PAGE and
following Western blot proteins were detected using appropriate primary antibodies and infrared-fluorescent labeled secondary antibody for infrared-based fluorimetric detection by the Odyssey system (Licor, Bad Homburg, Germany). The anti-Nox4 antibody was kindly provided by A.M. Shah (King’s college, London). The commercially available antibodies were used: mouse anti-phosphotyrosine (Upstate, via Chemicon Munich, Germany), IRS-1 and phospho IRS-1 Ser612, ERK1/2 and phospho-ERK1/2, MKP-1 (Cell signaling) and infrared-dye-conjugated secondary antibodies were obtained from Licor (Bad Homburg).

Amplex red assay for H$_2$O$_2$ production

Cells were grown on 12 well dishes to 90% confluence and incubated with or without insulin for 1 hour in phenol-red free medium. Then, the cells were incubated in HEPES-modified Tyrode’s solution (300 µl) containing amplex red (50 µmol/l, Invitrogen), horse-radish peroxidase (2 U/ml) and no BSA. After 60 min the supernatant was transferred to 96 well plates and H$_2$O$_2$-dependent oxidation of amplex red was measured by a microplate fluorimeter (excitation 540 nm, emission 580 nm).

AdipoRed assay for differentiation

Adipocytes were incubated with the AdipoRed assay reagent (Cambrex Bioscience Walkersville, 2% in PBS; 10 min at 37°C) after differentiation with or without insulin. Subsequently, fluorescence was measured using a microplate fluorimeter (Victor, Perkin-Elmer, Rodgau-Jügenheim, Germany) (excitation 485 nm, emission 572 nm) or a FACS (BD, Heidelberg, Germany).

qRT-PCR

Primers were designed using the sequence information of the NCBI database. The following primers were used: pref-1 fw 5'-CAACCTGCCTACA ACCACATG-3', pref-1 rev 5'-GAACGCTGCTTAGAT CTCCCTC-3', aP2 fw 5'-tgg aagacagctcctcctcg-3', aP2 rev 5'-cataacacaccttcaccaccg-3', mNox1 fw 5'-CAGCAGAAGGTCGTGATTACCAAG-3', mNox1 rev 5'-TGACCCCAATCCCTGCCCCAACCAACCA-3', mNox4 fw 5'-TGACCCCAATCCCTGCCCCAACCAACCA-3', mNox4 rev 5'-TGGACACCTTGATTACCAAG-3', mNox4 rev 5'-AGGGACCTTCTGTGATCCTCG-3', hNox4 fw 5'-TGGACACCTTGATTACCAAG-3', hNox4 rev 5'-CCATTCCGATTTCCGACAT-3'. Total RNA was isolated using "Absolutely RNA RT PCR Miniprep kit" (Stratagene Europe) and the extracted total RNA (between 250 and 350 ng) was used for reverse transcription (Superscript III RT, Invitrogen) with random hexamer primers (Amersham/Pharmacia) in a total volume of 25 µl. Aliquots of the RT reaction were analyzed using a SYBR green mix (Abgene) and the MX4000 real time PCR machine (Stratagene). The PCR conditions were as follows: initial denaturation 95°C, 15 minutes; 40-45 cycles of denaturation (95°C, 30 seconds), annealing (55-62°C, 30-60 seconds), and elongation (72°C, 30-60 seconds). Data were normalized against 18S mRNA expression using the delta-delta-CT method.

Proliferation-Assay

Proliferation of 3T3-L1 cells was analyzed using a BrdU incorporation kit (Roche) according to the manufacture’s instruction with minor variations. Cells were seeded into 12 well plates and transfected with siRNA or infected with lentivirus the day after seeding. When cells reached 100% confluence (usually 2 days after transfection) serum was deprived over night. The next day cells were stimulated with insulin (1µg/ml = 174 nmol/l) for 8 hours. Then BrdU was added and cells were allowed to incorporate the substrate for 4 hours. Subsequently, cells were fixed and the assay was performed according to the manufacture’s instruction. In order to validate the BrdU incorporation, absolute cell number was determined in sub-studies. After serum-deprivation at 100% confluence, cells were incubated with insulin (1µg/ml) for 2 days. Then cells were trypsinized and cell number was determined using an automatic cell counter system (casy, Schärfe System).

Statistics Analysis

All values are mean ± SEM. Statistical analysis was carried out using ANOVA followed by LSD post hoc testing. Densitometry was performed using the odyssey-software. A p-value of less than 0.05 was considered statistically significant.

Reference List


Extended Figure Legends

Fig. 1: Reactive oxygen species (ROS) are involved in insulin-induced differentiation. (A) Exemplary microscopic images (left: phase contrast; right: Adipored fluorescence) of 3T3-L1 at day 12 of differentiation. Cells were treated with or without insulin (174 nmol/l) from day 2 to day 6. (B) Statistical analysis and exemplary DNA gel stain of quantitative real-time RT-PCR data (q-RT-PCR) for pref-1 and 18S of 3T3-L1 cells differentiated in the presence or absence of insulin. n=5, *p<0.05. (C) ROS-formation determined by Amplex red / horse radish peroxidase assay. 3T3-L1 cells were treated without (CTL) or with insulin for 4 (4hrs) or 48 (48hrs) hours. n=5; *p<0.05 vs. CTL. (D) Adipored staining of 3T3-L1 cells at day 12 differentiated without (CTL) or with H2O2 (30 µmol/l every other day) in the absence of insulin. n=6 *p<0.05. (E) Adipored staining of 3T3-L1 cells transfected with plasmids coding for catalase or GFP at day 12 differentiated with or without insulin n=6; *p<0.05 inhibition GFP vs. catalase. (F) Insulin-induced proliferation as measured by the relative increase in cell number in 3T3-L1 cells transfected with GFP or catalase. n=6, **p<0.05.

Fig. 2: Nox4 is involved in insulin-induced ROS formation. (A) Statistical analysis of q-RT-PCR for Nox1, 2 and 4 and 18S in 3T3-L1 cells at basal level and after 12 days differentiation with insulin. (n=4) and exemplary DNA gel stain of Nox1, 2 and 4 and 18S in human preadipocytes differentiated in the presence or absence of insulin. Numbers below the lanes indicate the results of the qRT-PCR. n=3, *p<0.05 with vs. without insulin. (B&C) Expression of Nox4 protein as determined by Western blot analysis in 3T3-L1 cells: (B) Comparison of undifferentiated (w/o) and differentiated cells (with) 3T3-L1 cells (C) Effect of scrambled and Nox4-siRNA in cell with and without stimulation with transforming growth factor β-1 (TGF-β1, 10 µg/ml, 12 hours). TGF-β1 was used as known stimulus for Nox4 to ascertain the identity of the Nox4 band. Pos. ctl = Hek293 cells overexpressing the murine Nox4. n=3, *p<0.05 (D) ROS-formation determined by Amplex red / HRP assay. 3T3-L1 cells transfected with siRNA against Nox4 or the scrambled control (scr) were treated with or without (CTL) insulin for 48 hours. n=4 *p<0.05 with vs. without insulin; #p<0.05 scr vs. Nox4 siRNA. In the lower part a representative gel staining of a Nox4 RT-PCR is shown.

Fig. 3: Nox4 mediates insulin-stimulated differentiation but attenuates insulin-induced proliferation. (A) Statistical analysis of adipored fluorescence in 3T3-L1 at day 12 of differentiation. 3T3-L1 cells were transfected with scrambled control (scr) siRNA or Nox1 and Nox4 siRNA or (B) human preadipocytes were transfected with lentivirus for green fluorescence protein (Lenti-GFP) or Nox4 (Lenti-Nox4) and subsequently treated without (CTL) or with insulin from day 2 to day 6. n=7; *p<0.05 with vs. without insulin and Lenti-GFP vs. Lenti-Nox4. (C&D) Statistical analysis and exemplary DNA gel stain of q-RT-PCR for pref-1 and 18S of 3T3-L1 cells (C) transfected with scrambled (scr)- Nox1- or Nox4 siRNA stimulated without or with insulin. Human preadipocytes (D) transfused with lentivirus for green fluorescence protein (Lenti-GFP) or Nox4 (Lenti-Nox4) in the absence of insulin. n=5, *p<0.05. (E&F): Proliferation in the presence or absence of insulin in 3T3-L1 cells transfected with scr siRNA, Nox1 siRNA or Nox4 siRNA using BrdU incorporation (E) and in human preadipocytes transduced with Lenti-GFP or Lenti-Nox4 (F). n=7; *p<0.05 with vs. without insulin using determination of cell number. #p<0.05 scr vs. Nox4 siRNA

Fig. 4: Role of Nox4 in insulin-stimulated signal transduction. (A) Immunoprecipitation (IP) for phosphor tyrosine residues and Western blot (WB) for IRS-1; IP for IRS-1 and WB for phospho-IRS-1-serine612 as well as WB for phosho-ERK1/2 and total ERK1/2. 3T3-L1 cells (left panel) were transfected with scrambled (scr)- Nox1- or Nox4 siRNA and human preadipocytes (right panel) were transfused with lentivirus for green fluorescence protein (Lenti-GFP) or Nox4 (Lenti-Nox4) and subsequently stimulated without or with insulin (174 nmol/l) for 5 min. Numbers below the lanes indicate the results of the relative densitometry standardized to the control condition treated with scr-siRNA. Densitometrical analysis was performed for non-stimulated cell and cell stimulated with insulin for 5 minutes. Mean ± SEM. n=3 *p<0.05 without vs. with insulin; #p<0.05 scr and Nox1 siRNA with insulin vs. Nox4 siRNA with insulin; $p<0.05 without vs. with insulin; %p<0.05 Lenti-GFP with insulin vs. Lenti-Nox4 with insulin (B) WB of MKP-1, PP2A and PTP1B. 3T3-L1 cells (left panel) transfected with scr- Nox1- or Nox4 siRNA and human preadipocytes (right panel) transfused with lentivirus for green fluorescence protein (Lenti-GFP) or Nox4 (Lenti-Nox4). All blots shown are representative for at least 3 independent similar experiments. n=3 *p<0.05 scr vs. Nox4 siRNA; %p<0.05 Lenti-GFP vs. Lenti-Nox4

Fig. 5: Role of MAP kinase phosphatase-1 for insulin-stimulated differentiation and proliferation. (A&B): Insulin-induced differentiation in 3T3-L1 as determined by adipored fluorescence in cells treated with PD98059 (10 µmol/L) or the solvent DMSO (A) or cells transfected with an expression plasmid for MKP-1 or the control LacZ (B) at the day of the initiation of differentiation. n=9, *p<0.05 with vs. without insulin, #p<0.05 DMSO vs. PD98059 and LacZ vs. MKP-1 plasmid. (C-E): Effect of PD980589 (C, 10 µmol/L, PD), the MKP-1 expression plasmid (D) and siRNA directed against MKP1 (si-MKP1, E) on the insulin-induced proliferation as determined by cell counting of 3T3-L1. n=6, *p<0.05 vs. the appropriate control.