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Rapid report

Reduced expression of the NADPH oxidase NOX4 is a hallmark of adipocyte differentiation

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Abstract

Adipocyte differentiation is a complex process regulated among other factors by insulin and the production of reactive oxygen species (ROS). NOX4 is a ROS generating NADPH oxidase enzyme mediating insulin′s action in 3T3L1 adipocytes. In the present paper we show that NOX4 is expressed at high levels both in white and brown preadipocytes and that differentiation into adipocytes results in a decrease in their NOX4 mRNA content. These in vitro results were confirmed in vivo by demonstrating that in intact adipose tissue the majority of NOX4 expressing cells are localized within the preadipocyte containing stromal/vascular fraction, rather than in the portion consisting of mature adipocytes. In line with these observations, quantification of NOX4 mRNA in fat derived from different rodent models of insulin resistance indicated that alteration in NOX4 expression reflects changes in the ratio of adipocyte/interstitial fractions. In conclusion, we reveal that decreased NOX4 mRNA content is a hallmark of adipocyte differentiation and that NOX4 expression measured in whole adipose tissue is not an unequivocal indicator of intact or impaired insulin action.

Keywords: Reactive oxygen species; NADPH oxidase; NOX4; Insulin signaling; Adipocyte differentiation

Reactive oxygen species (ROS) are highly reactive molecules that are generated during physiological cellular metabolic processes as well as under various stress conditions [1]. Excess, uncontrolled production of oxygen radicals is involved in the development of insulin resistance [2–7]. By contrast, tightly regulated and targeted ROS production is an essential component of insulin signaling [8–13]. The major sources of ROS production within the cell are the mitochondria, different metabolic and detoxifying enzymes and various NAD(P)H oxidases [1,14]. The classical NADPH oxidase isoform (gp91-^{phox}/NOX2) is found in phagocytes and is involved in bacterial killing. Recently several homologues of NOX2 have been described, but their function and regulation is still poorly understood [14]. NOX1 is present in colon epithelial cells, NOX3 in human embryonic kidney and mouse inner ear, while expression of NOX5 has been shown to be restricted to the testis and lymphatic tissues. NOX4, originally termed “renox”, is most highly expressed in the kidney [15]. However, more recently NOX4 expression has been demonstrated in other tissues and cell types, including diverse embryonic tissues [16], vascular smooth muscle (VSMC) and endothelial cells [17,18], melanocytes [19], osteoclasts [20], hepatocytes [21], cardiac fibroblasts [22], adipocytes [23] and corneal cells [24]. It has been suggested that in these cells NOX4 is involved in cell cycle

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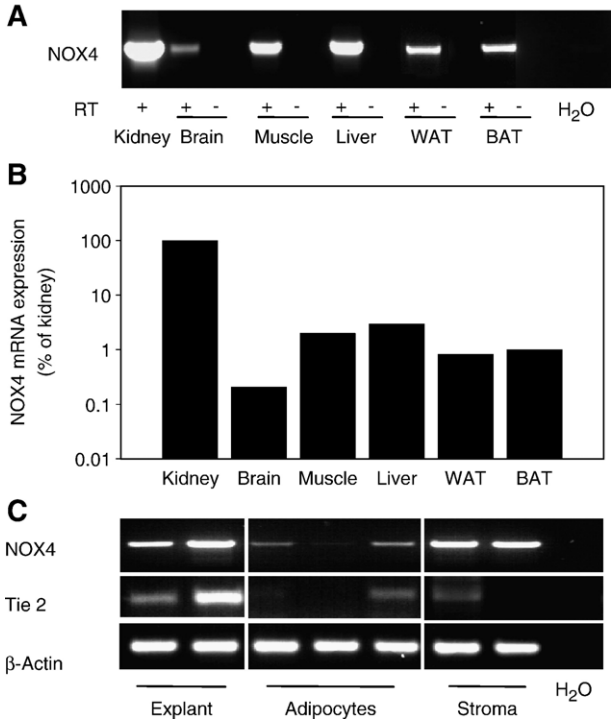

Fig. 1. Detection of NOX4 mRNA in mouse tissues and human adipose tissue fractions. (A) RT-PCR shows detectable levels of NOX4 expression in all insulin responsive tissues. Total RNA was prepared using TRIZOL Reagent (Invitrogen, Basel, Switzerland) or Ultraspec RNA Isolation System (Biotecx, Houston, TX) and 2 μg of DNA-free RNA was reverse-transcribed by Superscript II Reverse Transcriptase (Invitrogen, Basel, Switzerland). Mouse NOX4 primers and PCR conditions were as described in [30]. WAT: white adipose tissue, BAT: brown adipose tissue RT+: sample with reverse transcription reaction, RT−: sample without reverse transcription. H_2O denotes PCR reaction devoid of template cDNA. (B) Real-time PCR quantification of NOX4 expression in mouse tissues relative to the amount of 18S rRNA. Primers, conditions and the method of quantification were identical to [30] and are listed in Table 1. Results are shown as percentage of the expression in kidney. (C) NOX4 expression is localized in the stromal fraction of human adipose tissue. Human subcutaneous adipose tissue was obtained from surgical specimens of patients undergoing plastic surgery with the authorization of the Ethical Commission of the University Hospitals of Geneva and the Centre Hospitalier Universitaire Vaudois, Switzerland and the informed consents from the patients. Separation of the stromal/vascular fraction from adipocytes was carried out by digesting 0.6 g adipose tissue with type 1 collagenase (Worthington Biochemical Corporation, Lakewood, USA) (600 U/g adipose tissue) in low glucose DMEM containing penicillin and streptomycin, as well as the bovine albumin fraction V (20 mg/ml) (Sigma, Buchs, Switzerland) at 37 °C in a rocking bath. After 30 min of incubation, the digestion was stopped by the addition of FBS (0.3 ml/ml digestion medium). Adipocytes and the stromal fraction were then separated from undigested tissue by filtration through a 350 μm nylon mesh and centrifugation (10 min at 200×*g*). Adipocytes were resuspended in 2 ml of low glucose DMEM (10% FBS, glutamine 200 mM and 1% penicillin and streptomycin). The pellet containing the stromal fraction was resuspended in low glucose DMEM containing 10% FBS, 1% glutamine and 1% penicillin and streptomycin to promote cell adhesion. 24 h after plating, the medium was changed and cells were grown for 4 days before lysis and RNA preparation. NOX4 expression was mainly localized in the stromal/vascular fraction as assessed by RT-PCR. Tie 2 PCR indicates the presence of endothelial cells. β-actin expression was amplified to verify mRNA integrity, and equal rate of cDNA synthesis and input in all fractions. H_2O signifies PCR reaction lacking cDNA. The primers for human NOX4, Tie 2 and β-actin are listed in Table 1.

Table 1
RT-PCR and real-time PCR primers used in this study

Primer RT-PCR	Sequences of forward (F) and reverse (R) primers
NOX1 mouse	TGAACAACAGCACTCACCAATGCC(F)
	TCATTGTCCCACATTGGTCTCCCA(R)
NOX2 mouse	TGTCATTCTGGTGTGGTTGG(F)
	CCCCTTCAGGGTTCTTGATT(R)
NOX3 mouse	TTGTGGCACACTTGTTCAACCTGG(F)
	TCACACGCATACAAGACCACAGGA(R)
NOX4 mouse	CCTCATGGTTACAGCTTCTACCTACGC(F)
	TGACTGAGGTACAGCTGGATGTTCAC(R)
NOX4 human	AGGAGAACCAGGAGATTGTTGGATAAA(F)
	ATCTGAGGGATGACTTATGACCGAAAT
Tie 2 human	CCTTAATGAACCAGCACCAGG(F)
	ACTTCTGGGCTTCACATCTCCG(R)
β-Actin human	ATGGGTCAGAAGGATTCCTATGT(F)
	GAAGGTCTCAAACATGATCTGGG(R)
DUOX1 mouse	TGTCAGGCTACGAGATGGTG(F)
	GTTGCTGGACAGGATGAGGT(R)
DUOX2 mouse	GCCAGCTGCCATGGATGATG(F)
	CATAGCTGCCATGGATGATG(R)
Cyclophillin mouse	GAGCTGTTTGCAGACAAAGTTCCA(F)
	ATCTTCTTGCTGGTCTTGCCATTC(R)
Primer Real-time PCR	Sequences of forward (F) and reverse (R) primers
NOX4 mouse	TTGCCTGGAAGAACCCAAGT(F)
	TCCGCACAATAAAGGCACAA(R)
Cyclophillin mouse	CAAATGCTGGACCAAACACAA(F)
	GCCATCCAGCCATTCAGTCT(R)
$p22^{phox}$ mouse	TGCCCTCCACTTCCTGTT(F)
	GCAGATAGATCACACTGGCAAT(R)

regulation, vasodilatation and TGF-β and insulin signaling. Importantly, NOX4 has been shown to produce H_2O_2 continuously in NOX4 overexpressing HEK293 cells and endothelial cells [15,25,26]. Moreover, increased activity of NOX4 has been linked to elevated NOX4 mRNA expression levels in vivo [15,22,25], while a decrease in activity was associated with a downregulation of its expression [27]. Thus, the regulation of NOX4 transcription is an important step in modulating NOX4 activity. In murine 3T3L1 adipocytes NOX4 has been shown to be essential for proper insulin receptor phosphorylation and insulin-induced glucose transport, while in human skin fibroblasts the lack of NOX4 resulted in decreased PDGF and EGF receptor phosphorylation [23,27]. By contrast, increased whole adipose tissue NOX4 expression has been linked to oxidative stress and insulin resistance [28]. Therefore, the goal of our study was to gain a better insight in the relationship between NOX4 expression and insulin signaling in vitro and in vivo. Since insulin is an important regulator of adipogenesis, we have characterized the changes in NOX4 expression during brown and white adipocyte differentiation in vitro and in the adipose tissues of rodent models of altered insulin signaling in vivo.

The NADPH oxidase isoform NOX4 has been identified in the kidney by Northern blot analysis [15]. To evaluate the expression of NOX4 in insulin responsive tissues by a more sensitive method we performed RT-PCR and real-time quantitative PCR analysis using mouse brain, muscle, liver, and white and brown adipose tissues (Fig. 1A and B, respectively). All tissues examined showed expression of NOX4 mRNA.

Quantitative real-time PCR showed that NOX4 expression in the muscle, liver and the white and brown adipose tissues was between 2 and 8% of that of the kidney, explaining the lack of detection by Northern-blot analysis. White and brown adipose tissue showed similar quantities of NOX4 mRNA expression. To identify the NOX4 containing cells within the adipose tissue we separated the stromal–vascular and adipocyte fraction derived from explants of human subcutaneous fat depot. Semiquantitative RT-PCR detected high levels of NOX4 expression in the total explant. However, upon separation of adipocytes from the preadipocyte/stromal/vascular fraction, NOX4 was predominantly found in the latter part with only marginally detectable levels in adipocytes. As the stromal fraction contains endothelial cells known to express NOX4 we assessed the mRNA levels of the endothelial cell specific receptor Tie 2 in the same three fractions. High expression of Tie 2 was detected in the intact explant, but was diminished in the isolated stromal fraction under tissue culture conditions. By contrast, NOX4 mRNA was readily detected in this fraction indicating the presence of other NOX4 expressing cells most likely fibroblasts and pre-adipocytes dominating the culture over the endothelial cells (Fig. 1C).

To further localize the expression of NOX4 in intact white adipose tissue we performed in situ hybridization and immunohistochemical staining for NOX4. The NOX4 antisense probe strongly marked the stromal areas (Fig. 2A, left panel), while there was no signal detected when hybridized with the sense probe (Fig. 2A, right panel). Localization was confirmed and refined by immunohistochemistry, where arteriole walls, capillaries, stromal fibroblasts/preadipocytes showed clear staining with only a weak signal present in the adipocytes (Fig. 2B, left panel). The rabbit polyclonal anti-NOX4 antibody used in these experiments has been shown to be specific for NOX4 and in a previous study we have successfully applied it on mouse histochemical sections [29,30]. No staining was obtained in the absence of primary antibody (Fig. 2B, right panel). Brown adipose tissue shares several molecular characteristics with the white adipose tissue but their functions are distinct. To examine the adipose tissue specificity of NOX4 expression we performed the same experiments on mouse brown adipose tissue slides. As shown in Fig. 2C and D, images similar to those seen in white adipose tissue sections were observed: adipocytes and stromal elements were strongly labelled with the NOX4 antisense probe (Fig. 2C, left panel) but no signal was obtained when hybridized with the sense probe (Fig. 2C, right panel). Immunohistochemical staining showed a corresponding picture with clear labelling both in brown adipocytes and the stroma, while omission of the first antibody resulted in the lack of detectable signal (Fig. 2D left and right panels, respectively).

The isolated stromal fraction of human adipose tissue, where RT-PCR revealed high level of NOX4 expression, contains preadipocytes capable to differentiate into adipocytes. In addition, we detected similar stromal localization of NOX4 expression in intact white and brown adipose tissues. To determine if a relationship between NOX4 and adipogenesis exits, we first decided to investigate the expression of the members of the NOX enzyme family during differentiation in two well-characterized fat cell lines: the 3T3L1 mouse white adipose cell line and in an immortalized mouse brown adipose cell line [31]. As shown by semiquantitative RT-PCR only NOX4 showed identical differentiation-related changes in both white and brown adipose cells with a high expression level in preadipocytes and a decreased expression in differentiated

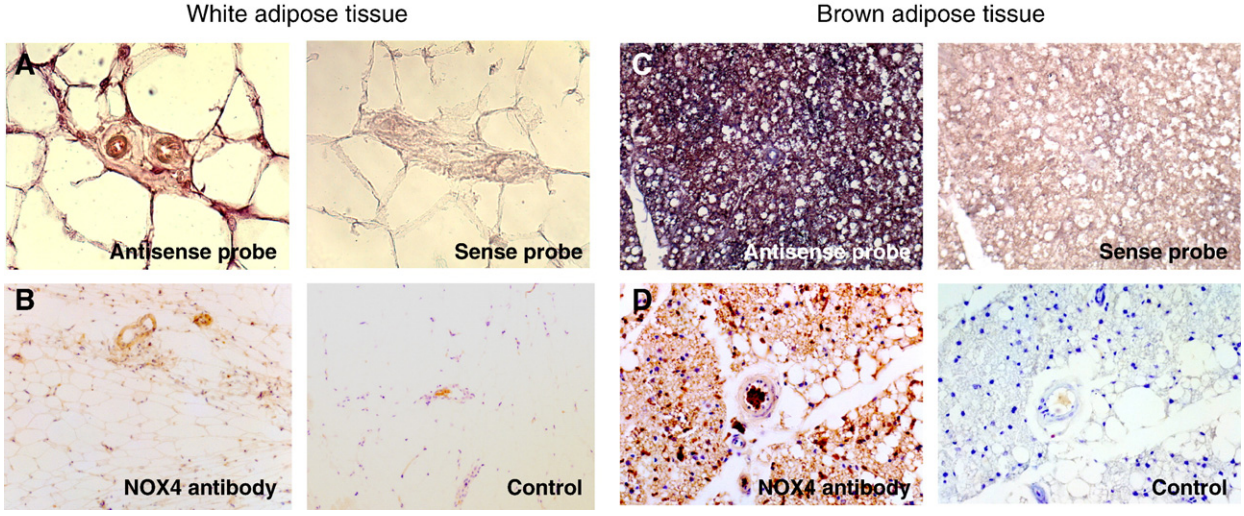

Fig. 2. Localization of NOX4 expressing cells in mouse white and brown adipose tissues. (A) In situ hybridization with NOX4 antisense probe shows NOX4 expression mainly in cells within the stromal fraction of white adipose tissue (left panel, antisense probe). No signal is obtained when staining is performed using the sense probe (right panel, sense probe). (B) Immunohistochemical detection using antiNOX4 antibody shows strong staining in vascular cells and stromal fibroblasts. Weaker signal is present in adipocytes (left panel, NOX4 antibody). The specificity of the antibody has been demonstrated in NOX4 siRNA knock-down cells [29] and in our previous publication [30]. Absence of reaction upon omission of the primary antibody (right panel, control). (C) In situ hybridization with NOX4 antisense probe detects NOX4 expression in brown adipocytes (left panel, antisense probe) and lack of signal when labelling with NOX4 sense probe (right panel, sense probe). (D) Immunohistochemical staining with NOX4 antibody showing labelling in brown adipocytes and the surrounding white adipocytes (left panel, NOX4 antibody). Omission of the first antibody results in the ablation of staining (right panel, control).

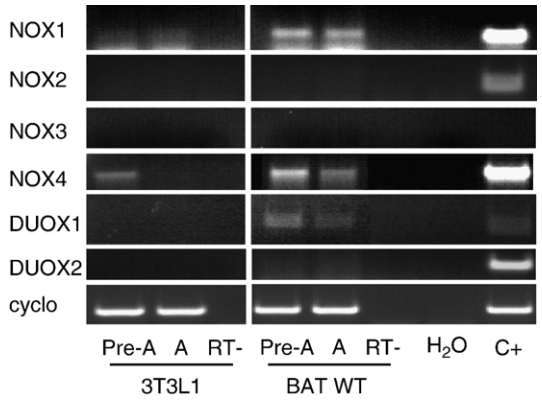

Fig. 3. NOX enzyme expression in 3T3L1 and wild type brown adipocytes (BAT WT) upon differentiation. Only NOX4 displays similar differentiation-related decrease in 3T3L1 and brown adipose cells. The PCR primers used are listed in Table 1. Pre-A=preadipocytes, A=adipocyte, RT− signifies PCR reaction devoid of reverse transcription, and H_2O marks PCR reaction devoid of added cDNA. +C signifies positive control reaction: mouse cDNA prepared from colon for NOX1, spleen for NOX2, kidney for NOX4, cerebellum for DUOX1 and colon for DUOX2. NOX3 PCR was performed using the primers as in [54] but no positive control mouse inner ear cDNA could be obtained. Amplification of cyclophillin serves as control for cDNA integrity.

adipocytes (Fig. 3). To better characterize the changes of NOX4 expression in adipogenesis we first examined the mRNA and protein expression of this enzyme in 3T3L1 cells by real-time PCR, conventional RT-PCR and Western blot analysis. Differentiation of 3T3L1 preadipocytes was induced using a standard protocol by a cocktail containing 1 μg/ml insulin, 0.25 μM dexamethasone and 0.5 mM isobutylmethylxanthane (IBMX) for 96 h. Thereafter cells were cultured in high-glucose DMEM supplemented with 1 μg/ml insulin, 10% FBS and penicillin–streptomycin and were used between days 10 and 12. Real-time PCR detected high level of NOX4 expression in confluent preadipocytes, which then decreased significantly by the end of the induction stage (day 4), and further declined in the fully differentiated adipocytes (days 10–12) (Fig. 4A, upper panel). In the a human lung cancer cell line, four isoforms of NOX4 have been described with different expression levels and ROS producing capacity, but their physiological function and regulation have not been further examined [32]. Conventional RT-PCR reactions performed with primers designed to detect a potential differentiation-induced change in isoform expression revealed only one transcript for NOX4, thus argues against a hypothetical switch between the full length and the truncated dominant negative NOX4 transcripts (Fig. 4A, lower panel). To assess the changes in NOX4 protein amount we performed Western blot analysis using a rabbit polyclonal anti-NOX4 antibody that has been successfully employed to monitor siRNA mediated NOX4 gene knock-down in mouse embryonic bodies [33]. Quantification of the blots confirmed that NOX4 protein levels were diminished during adipogenesis mirroring the alterations in mRNA transcription (Fig. 4B).

Brown preadipocytes may be induced to undergo a differentiation process similar in several aspects to that of white preadipocytes. To assess if NOX4 mRNA expression is regulated during brown adipogenesis, we compared NOX4 mRNA levels in undifferentiated and differentiated state using immortalized brown preadipocytes derived from different insulin receptor substrate (IRS) knock-out mice. Differentiation of confluent dishes of brown preadipocytes was induced by supplementing the differentiation medium (high glucose DMEM supplemented with 10% FBS and penicillin–streptomycin, 20 nM insulin and 1 nM 3,3,5-triiodo-L-thyronine (T3)) with 0.5 mM isobutylmethylxanthin (IBMX), 0.5 μM dexamethasone and 0.125 mM indomethacin for 48 h. Then cells were placed back to differentiation medium for 96 h and were used for experimentation [31]. These various IRS knock-out brown adipose cell lines are known to exhibit divers capacity to differentiate into mature adipocytes with the decreasing order of wild type, IRS-4 KO, IRS-2 KO, IRS-3 KO and IRS-1KO cells [31]. As assessed by real-time PCR and RT-PCR analysis in the undifferentiated stage all cell lines expressed high amount of NOX4. In those cells which differentiated, (WT, IRS-2 and IRS-4 KO) NOX4 mRNA levels declined dramatically (60–80%) upon differentiation. By contrast, NOX4 expression levels remained high in those cell lines which displayed reduced differentiating capacity (IRS-1 and IRS-3 KO) (Fig. 4C, upper panel). None of the cell lines exhibited altered NOX4 isoform expression as judged by conventional RT-PCR (Fig. 4C, lower panel). Thus, similar to the process observed during white adipocyte differentiation, brown adipose cell lines display again a tight inverse correlation between their NOX4 mRNA levels and their degree of differentiation. By contrast, differentiation-related changes in NOX4 mRNA and protein levels were less coordinated in brown adipocytes than in 3T3L1 cells. All brown adipose cell lines displayed high NOX4 protein expression in the undifferentiated state. This high expression was significantly downregulated both in the wild type and in all the various IRS knock-out cell lines when cells were subjected to the standard differentiation protocol (Fig. 4D). This apparent discrepancy between the regulation of NOX4 mRNA and protein levels suggests a more complex regulatory mechanism compared to 3T3L1 adipocytes. Of specific interest is the explicit opposite regulation of NOX4 mRNA and proteins levels in the IRS-3 knock-out cells arguing for a multifaceted regulatory pathway possibly involving a feed-back regulatory mechanism in these cells. Indeed, a similar contradictory regulation can be observed in the expression of IRS-2 in differentiating wild type brown adipocytes where a decline in IRS-2 mRNA expression is accompanied by an elevation in protein expression, likely through an alternative regulation of IRS-2 protein by the proteasome pathway [31,34]. Stabilization and maturation of the NOX4 homologue molecules NOX2 and NOX3 is highly dependent on the presence of $p22^{phox}$, a membrane bound component of several NOX enzyme complexes [35–37]. In addition, $p22^{phox}$ mRNA levels exhibited parallel changes to NOX4 mRNA expression in the white adipose tissue of the insulin resistant KKAy mice and in the kidney of Streptozotocin-induced diabetic rats [28,38]. In our study we did not observe a statistically significant differentiation-dependent change in $p22^{phox}$ mRNA expression in any of the cells studied (data not shown). Whether some other brown adipocyte specific post-transcriptional regulatory pathway is responsible for the

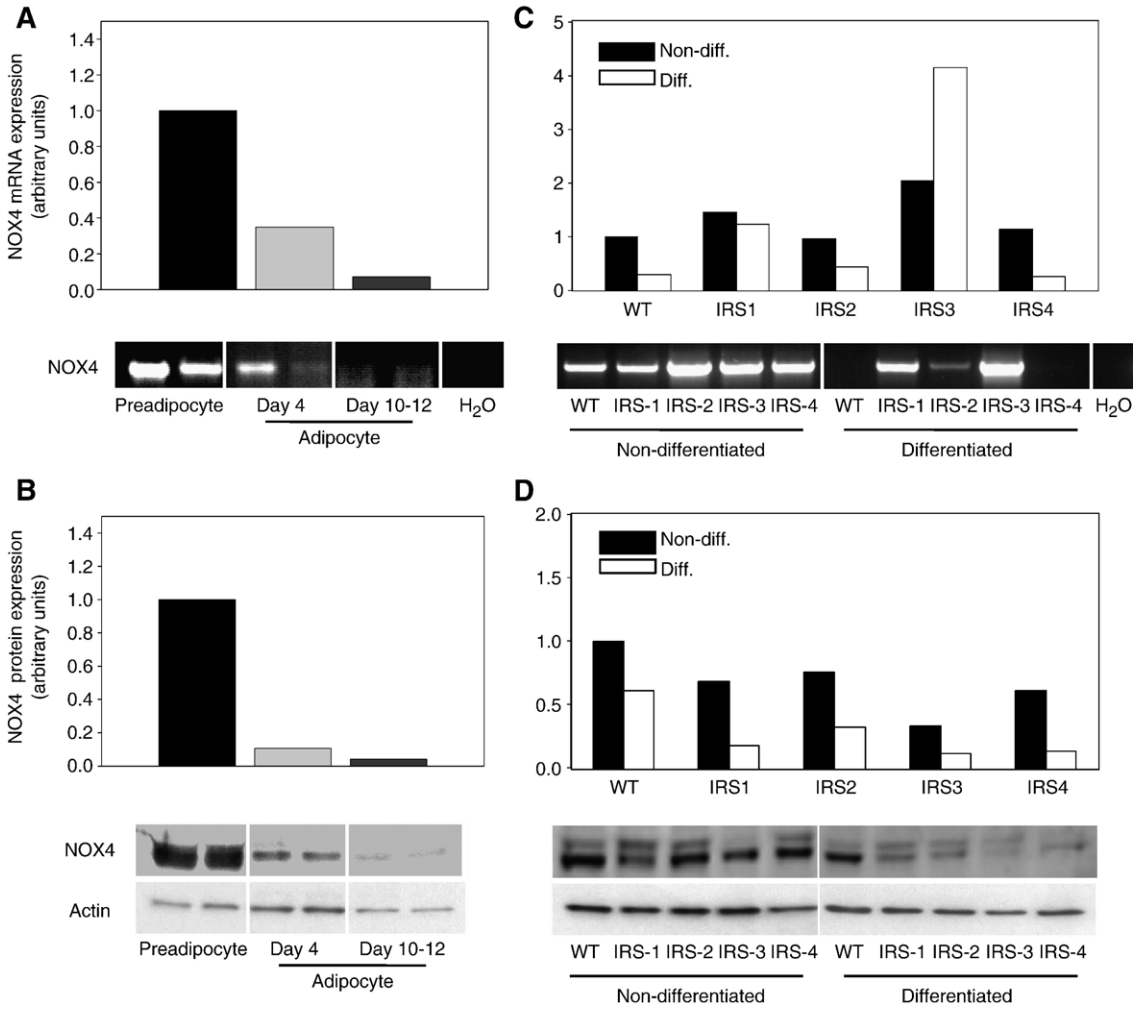

Fig. 4. NOX4 expression is down-regulated in differentiated 3T3L1 and brown adipocytes. (A) Upper panel: downregulation of NOX4 expression during white adipocyte differentiation. Real-time PCR quantification of NOX4 mRNA expression in 3T3L1 preadipocytes (day 0), at the end of the induction phase (day 4) and after complete differentiation (days 10–12). NOX4 mRNA levels are shown as arbitrary units relative to the expression in preadipocytes. Lower panel: semiquantitative RT-PCR detection of NOX4 in 3T3L1 preadipocytes and differentiated adipocytes. H_2O represents PCR reaction in the absence of cDNA. (B) NOX4 protein amount is down-regulated during differentiation. Upper panel: Quantification of NOX4 protein expression in 3T3L1 adipocytes. Signal intensity was quantified by ChemiDoc software (Biorad). Protein expression is shown as the NOX4 protein amount relative to actin and is expressed as arbitrary unit compared to preadipocytes. Lower panel: Representative Western blot of 3T3L1 cells before and after the induction of differentiation. Equal protein charge was verified by blotting with an anti-actin antibody. The specificity of the rabbit polyclonal anti-NOX4 antibody was previously demonstrated in NOX4 knock-down cells [33]. (C) Upper panel: downregulation of NOX4 expression during brown adipocyte differentiation. Real-time PCR analysis of NOX4 expression in wild type (WT) and different IRS protein (IRS1–4) knock-out brown adipocyte cells before (solid bars) and after (empty bars) differentiation. Results are expressed as arbitrary units compared to NOX4 expression in wild type preadipocytes. Lower panel: semi-quantitative RT-PCR detection of NOX4 expression in the same cell lines in non-differentiated and differentiated state. H_2O signifies PCR reaction lacking cDNA. Real-time PCR and RT-PCR reactions were carried out as described previously [30], the primers are listed in Table 1. (D) Western blot analysis of NOX4 protein expression in non-differentiated and differentiated wild type and diverse IRS protein knock-out brown adipose cell lines. Upper panel: quantitative analysis of NOX4 protein expression relative to actin and expressed as arbitrary units compared to the expression in wild type cells. Lower panel: representative Western blot for NOX4 and actin. Anti-NOX4 antibody was identical as in Fig. 4B.

differences observed between NOX4 mRNA and protein levels is an intriguing question and should be addressed in a detailed fashion in further studies with a special emphasis on the role of IRS-3, most abundantly expressed in the adipose tissue.

Taken all the above data together, they suggest that NOX4 is expressed at high levels in white and brown preadipocytes and that its mRNA and protein expression is subjected to a tight and complex regulation during adipogenesis. Moreover, expression of NOX4 or the lack of it might modify insulin receptor signaling in related cell types such as preadipocytes and adipocytes, and be an important regulatory element in mediating insulin′s effect on cell differentiation in cells with low levels of insulin receptor expression. In support of a role for NOX4 in adipocyte differentiation, a recent study reported the downregulation of NOX4 mRNA in endothelial cells in response to PPARγ ligands, agents known to be major regulators of adipocyte differentiation [39]. In addition, as a more general aspect of cell differentiation another study reported the requirement for NOX4 in regulating vascular smooth muscle specific gene expression [40].

Studies conducted in 3T3L1 cells have demonstrated a link between adipogenesis and ROS production [12,41]. NOX4 has been shown to be constitutively active when overexpressed in

HEK293 cells, and several studies have suggested that the regulation of NOX4 expression is a major controller of NOX4 activity [15,25,26]. These data raise the possibility that changes in NOX4 expression might be a link between adipogenesis and production of reactive oxygen species. NOX4-related intracellular ROS production has been detected by CM-H_2DCFDA fluorescence and nitroblue tetrazolium (NBT) reduction assays [9,27,28,32]. More recently, NOX4-derived extracellular ROS

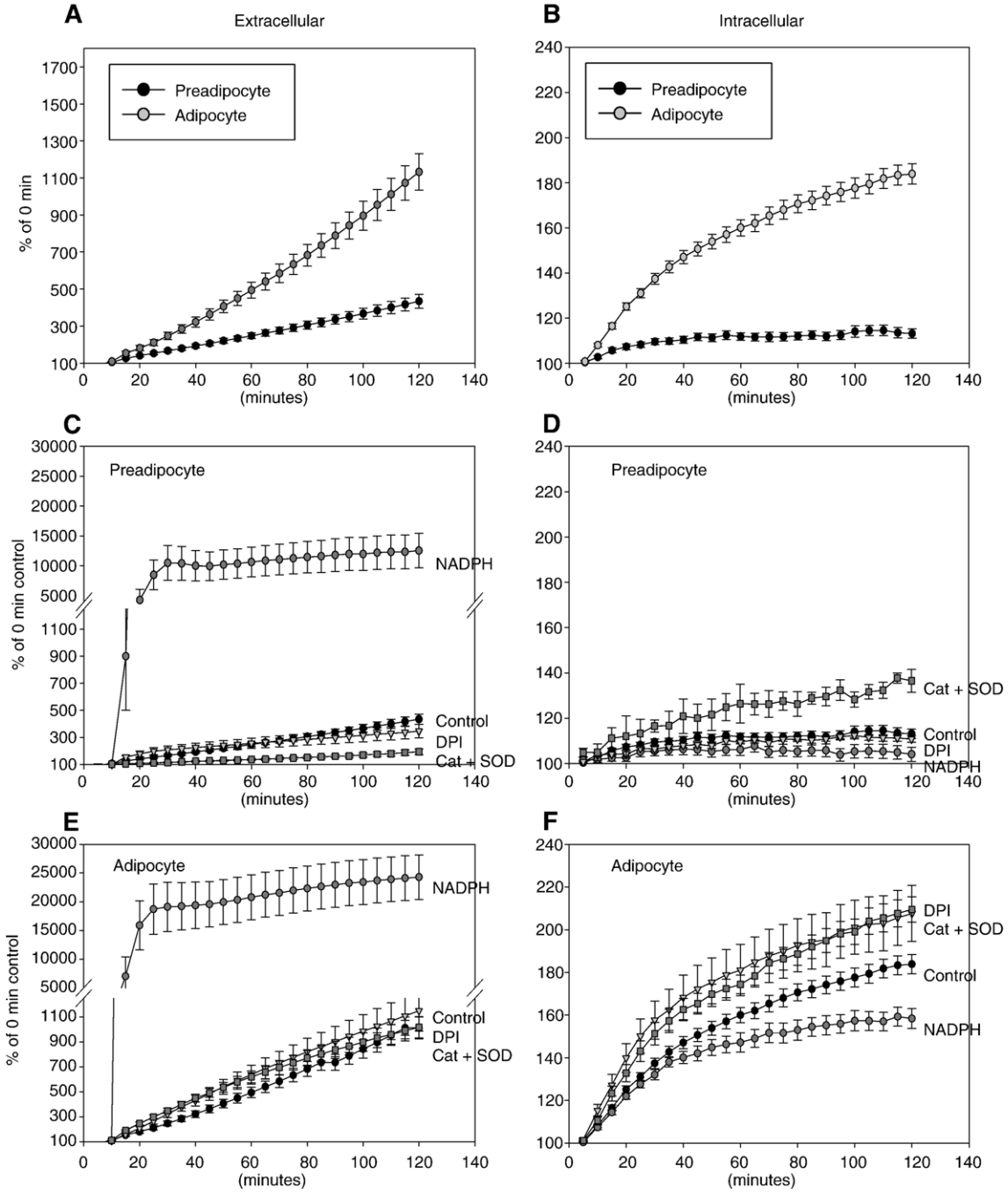

Fig. 5. NADPH stimulates extracellular but not intracellular ROS production in 3T3L1 preadipocytes and adipocytes. Spontaneous extracellular (A) and intracellular (B) ROS production was recorded in preadipocytes (black circle) and adipocytes (gray circle). Reactive oxygen species were detected in the extra- and intracellular space using fluorescence assays. H_2O_2 released from the cells was measured by HRP-dependent oxidation of N-acetyl-dihydrophenoxanine (Amplex Red Hydrogen Peroxide/Peroxidase Assay kit). Intracellular release of ROS was measured by the membrane permeable dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA). Cells were starved for 14–16 h before measurements. Plates were read in a thermostat controlled (37 °C) plate reader. Basal fluorescence of individual wells was measured for 5–10 min followed by the addition of test compounds and fluorescence was recorded sequentially over a 2-h period. Results are expressed as the percentage of 0 min production of the respective cells and shown as mean±S.E.M., derived from triplicate measurements from 6 to 8 independent experiments. (C) Extracellular and (D) intracellular ROS production in preadipocytes in the absence (control) or presence of NADPH (0.25 mM), DPI (10 μM) or SOD (20 U/ml) and catalase (Cat) (100 U/ml). Results are expressed as the percentage of production of non-treated (control) cells at 0 min. (E) Extracellular and (F) intracellular ROS production in differentiated adipocytes in the presence of NADPH, DPI or SOD and catalase.

production has been detected using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Method [33]. Therefore we decided to measure ROS production detectable in the intra- and extracellular space in the absence or presence of different stimulatory agents in 3T3L1 and wild type brown adipose cells before and after differentiation looking for a relationship between NOX4 expression and ROS production (Figs. 5A–F, 6A–F, 7A–F, and 8A–F, respectively).

First we characterized spontaneous ROS production in non-differentiated and differentiated 3T3L1 cells and found striking differences between preadipocytes and adipocytes, regarding spontaneous free radical production. Preadipocytes showed a moderate production of extracellular ROS but little accumulation intracellularly. By contrast, adipocytes had a relatively higher rate of spontaneous intra- and extracellular ROS production compared to the preadipocytes (Fig. 5 A and B, note also the different scale). This increased basal free radical production in adipocytes is unexpected in view of the decreased NOX4 expression in these cells. Noteworthy, however, that a similar increase in ROS production upon the depletion of a NOX enzyme have been previously reported in NOX-3 siRNA knock-down HepG2 cells [42]. The extra- but not the intracellular H_2O_2 generation was inducible by NADPH ($p < 0.001$ vs. control) pointing towards the differential in-

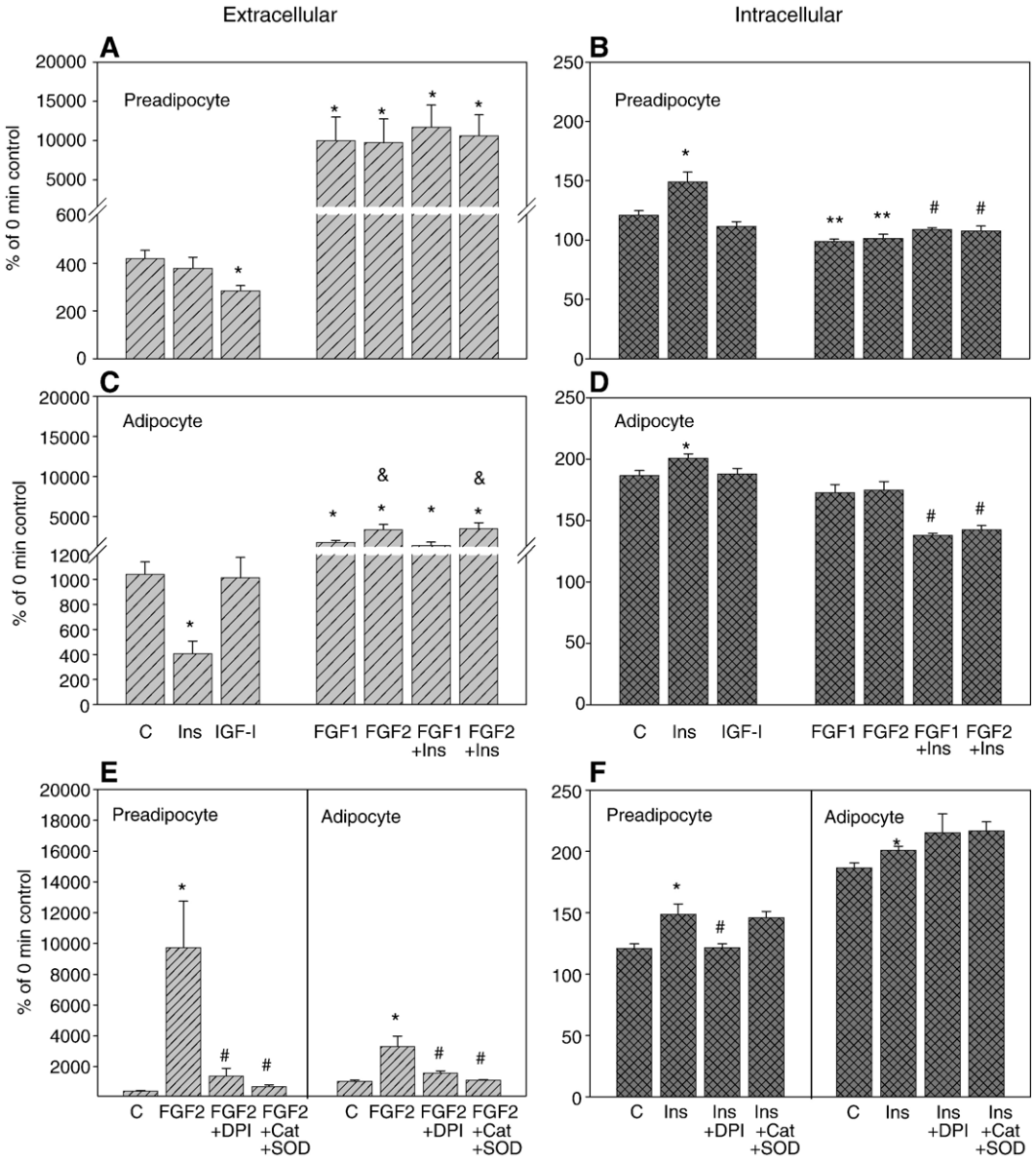

Fig. 6. Fibroblast growth factor (FGF) induces extracellular, while insulin induces intracellular ROS production in both 3T3L1 preadipocytes and adipocytes. Extracellular (A) and intracellular (B) ROS production was measured in preadipocytes in the presence or absence of the insulin (100 nM), IGF-1 (100 ng/ml), FGF-1 and 2 (1 nM). Results are shown after 120 min of stimulation and expressed as the percentage of control cells at 0 min. Statistical analysis was performed using Student's *t* test, $*p < 0.05$ vs. control, $**p < 0.01$ vs. control, $^{\#}p < 0.05$ vs. insulin stimulation. Extracellular (C) and intracellular (D) ROS production was measured in differentiated adipocytes using the same agents as in (A) and (B). Student's *t* test, $*p < 0.05$ vs. control, $**p < 0.01$ vs. control, $^{\#}p < 0.01$ vs. insulin stimulation, $^{*\&}p < 0.05$ vs. FGF-1 stimulation. Inhibition of extracellular (E) and intracellular (F) ROS production by DPI or SOD and catalase in preadipocytes and adipocytes. $*p < 0.05$ vs. control, $**p < 0.01$ vs. control, $^{\#}p < 0.05$ vs. stimulated cells.

volvement of an NADPH oxidase enzyme in the two processes. Spontaneous extracellular ROS production was unaffected by the addition of the flavoprotein inhibitor DPI or the H_2O_2 scavangers catalase and superoxide dismutase (SOD) both in preadipocytes (Fig. 5C and D) and in adipocytes as well (Fig. 5E and F). By contrast, intracellular free radical concentration was enhanced by catalase and superoxide dismutase (SOD) in both differentiation stages (Fig. 5D and F).

ROS production in 3T3L1 cells is inducible by a diverse array of cytokines and growth factors which are also important modulators of adipogenesis [41,43]. To assess the relationship between ROS production and adipogenesis we compared free radical production induced by these different stimuli, namely fibroblast growth factors 1 and 2 (FGF-1 and FGF-2), insulin and insulin-like growth factor 1 (IGF-1). When studying the effects of FGF-1 and FGF-2 we found that both cytokines increased extracellular H_2O_2 production, but with a significantly higher magnitude in preadipocytes than in adipocytes (Fig. 6A and C, respectively; also compare scales). This difference can be readily explained by differences in FGF receptor expression which is much greater in preadipocytes as compared to adipocytes. By contrast, both FGFs affected intracellular ROS generation in a contradictory fashion as they inhibited insulin′s action in both cell types and blocked the low amount of spontaneous production in preadipocytes (Fig. 6B and D, respectively).

The importance of intracellular free radical generation has been underlined by the data published recently by Mahadev et al. who described a complex interaction between ROS production and the classical insulin signaling pathways [44]. Using adenovirus mediated expression of dominant negative NOX4 and siRNA transfections they argued that in differentiated 3T3L1 adipocytes NOX4 mediates insulin induced intracellular ROS production [23]. Our results also support the formation of intracellular ROS upon insulin stimulation in adipocytes. We found however, that preadipocytes actually responded to insulin to a higher degree than adipocytes (Fig. 6B and D, respectively). Insulin receptor expression is low in preadipocytes and high in adipocytes [45], thus variances in the number of receptors cannot account for this difference. In this respect the data reported by Krieger-Bauer et al. is of special interest as they described the presence of an H_2O_2 generating system in human fat cells that was activated by insulin through a mechanism bypassing the receptor kinase [11]. If a similar mechanism links insulin stimulation and ROS generation in preadipocytes is an intriguing possibility. It is also noteworthy that while DPI blocked insulin stimulated ROS production in preadipocytes it had no such effect in adipocytes (Fig. 6F).

Taken together all the above data they suggest an appealing scenario where in preadipocytes an initial insulin-induced "burst" in ROS production may trigger signaling events leading to the initialization of the differentiation process and eventually to the downregulation of NOX4 and the upregulation of insulin receptor expression. Indeed, the tightly regulated timing of insulin receptor expression was shown to be essential for successful adipocyte generation as both insulin receptor deficient and overexpressing preadipocytes failed to differentiate [43]. There are different observations concerning the exact cellular localization of NOX4. It has been described to be present in conjunction with focal adhesions and in the nucleus in dedifferentiated rat vascular smooth muscle cells [46]. By contrast, in differentiated muscle cells NOX4 showed a prominent distribution along α-actin fibers [47]. Others observed NOX4 in association with the membrane fraction in rabbit corneal epithelial and stromal cells [24]. Thus NOX4-mediated ROS could be released intracellularly triggering adipogenesis in preadipocytes or extracellularly providing a mean of communication between preadipocytes and other cells such as endothelial cells or adipocytes. The signaling network mediating the insulin-induced ROS production in preadipocytes and its link to NOX4 is a worthy topic for future studies.

In our next set of experiments we characterized ROS production in brown preadipocytes and adipocytes with a similar approach. In contrast to 3T3L1 cells there was no difference in the rate of spontaneous ROS production between brown preadipocytes and adipocytes (Figs 7A and B, and compare to Fig. 5A and B). Addition of NADPH selectively increased extracellular ROS production, a feature similar to 3T3L1 cells, though the magnitude of this effect was significantly lower (Fig. 7C–F and compare to Fig. 5C–F). Addition of superoxide dismutase and catalase resulted in a robust augmentation of intracellular ROS generation in adipocytes likely reflecting enhanced mitochondrial superoxide production. When investigating the effects of different growth factors we noted that brown preadipocytes responded to different stimuli to a much lesser degree compared to 3T3L1 preadipocytes (Fig. 8A, B and E left panel) suggesting perhaps a less important role of ROS generation in their initial differentiation program. This finding is in line with other studies showing that in brown adipocytes FGFs are potent mitogens but do not hamper differentiation, by contrast they interfere with differentiation in 3T3L1 preadipocytes [41,48]. Brown adipocytes responded to insulin with an increase and to norepinephrine with a decrease in their ROS production. None of these two stimuli were sensitive to DPI making the involvement of an NADPH oxidase in these effects unlikely (Fig. 8C–E right panel). Taken all the above data together, they indicate the presence of a complex network of ROS producing molecules in both 3T3L1 and brown adipocytes preventing to determine a clear link between cellular NOX4 content and the observed spontaneous or induced ROS generation. In a previous study NOX4 activity in NOX4 overexpressing cells was measured by the Amplex Red method and displayed good correlation with NOX4 mRNA and protein levels [33]. In our non-overexpressing cells we cannot confirm these data. In line with our observation other studies used the oxidation of the fluorescence dye dihydroethidiumbromide quantified by high performance liquid chromatography to measure NOX4 related intracellular superoxide release in primary human cardiac fibroblasts and rat vascular smooth muscle cells [22,47]. Establishing a direct connection between NOX4 expression and cellular ROS production will be addressed in NOX4 knock-down preadipocytes and adipocytes in our future experiments.

Obesity is closely linked to adipocyte differentiation and in several but not all cases to insulin resistance [49–52]. Previous studies favored a conclusion of associating elevated NOX4

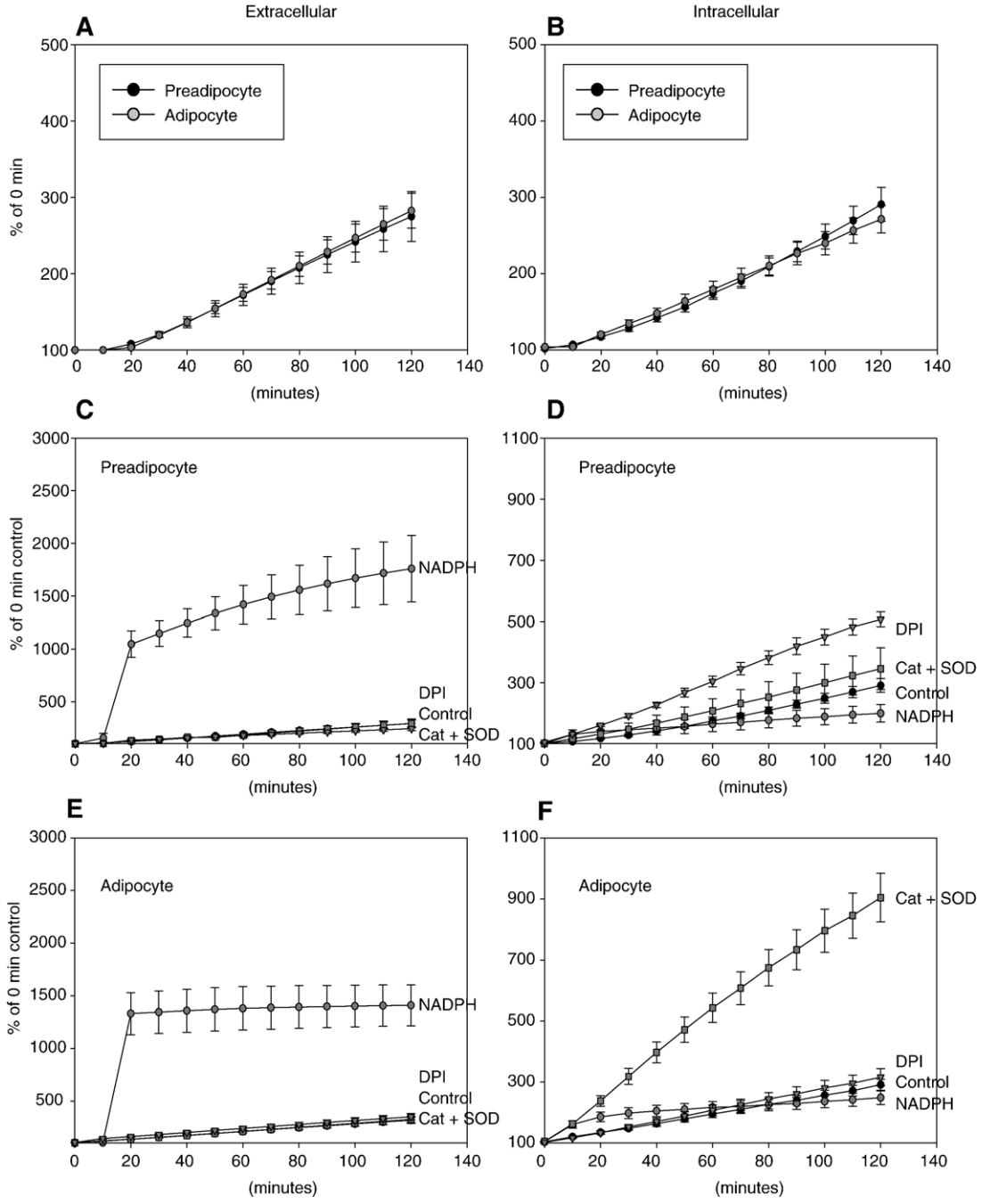

Fig. 7. NADPH stimulates extracellular but not intracellular ROS production in brown preadipocytes and adipocytes. Spontaneous extracellular (A) and intracellular (B) ROS production was recorded in preadipocytes (black circle) and adipocytes (gray circle) as described in Fig. 5. Results are expressed as the percentage of 0 min production of the respective cells and shown as mean±S.E.M., derived from triplicate wells from 4 to 6 independent experiments. (C) Extracellular and (D) intracellular ROS production in preadipocytes in the absence (control) or presence of NADPH, DPI or SOD and catalase (Cat). Results are expressed as the percentage of production of non-treated (control) cells at 0 min. (E) Extracellular and (F) intracellular ROS production in differentiated adipocytes in the presence of NADPH, DPI or SOD and catalase.

expression with insulin resistance [27,28]. To explore the relationship between NOX4 expression and adipocyte differentiation and/or insulin resistance in vivo, we examined NOX4 mRNA levels in different animal models of altered insulin receptor signaling using RT-PCR and quantitative RT-PCR. Long-term (8 months) high fat diet feeding in mice resulted in massive obesity with adipocyte hypertrophy and hyperplasia and a significant decrease in NOX4 expression (Fig. 9A). This low level of NOX4 mRNA expression reflects a significantly elevated adipocyte/stroma ratio compared to control mice. Surprisingly, however, NOX4 protein expression was not correlated with these results showing a sustained protein amount despite lower mRNA levels (Fig. 9B). While the exact mechanism of this stable NOX4 protein expression cannot be explored in the frame of this study, these results suggest a cell-specific, complex and multilevel regulation of NOX4

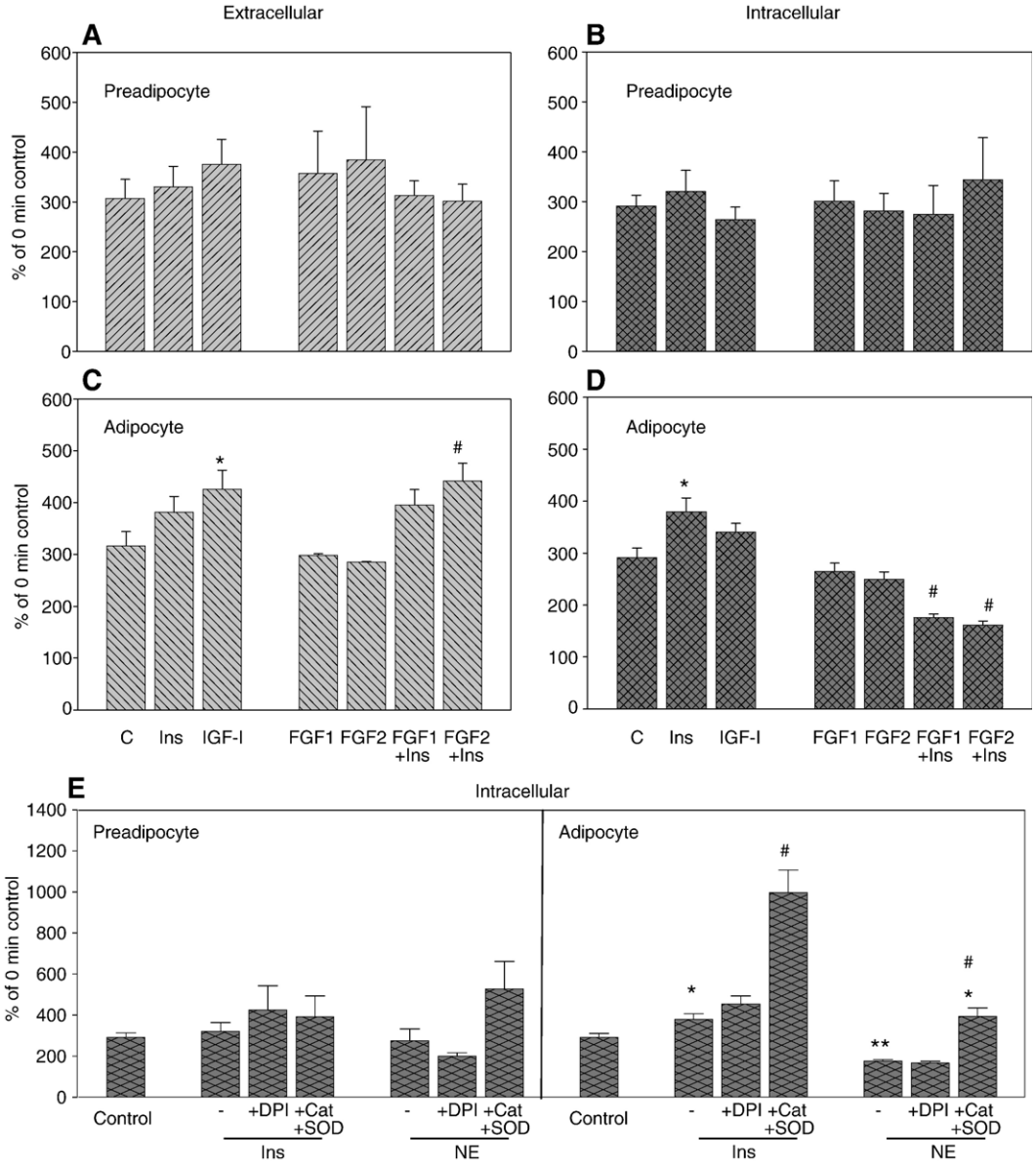

Fig. 8. Extra- and intracellular ROS production in non-differentiated and differentiated brown adipocytes in response to insulin, insulin-like growth factor 1 (IGF-1) and fibroblast growth factor 1 and 2 (FGF-1 and FGF-2, respectively). Extracellular (A) and intracellular (B) ROS production was measured in preadipocytes in the presence or absence of the different agents as marked. Results are shown after 120 min of stimulation and expressed as the percentage of control cells at 0 min. Extracellular (C) and intracellular (D) ROS production was measured in differentiated adipocytes using the same agents as in (A) and (B). Statistical analysis was performed using Student's *t* test, $*p < 0.05$ vs. control. (E) Reversal of the effect of insulin and norepinephrine (10 μM) on intracellular ROS production by SOD and catalase in non-differentiated and differentiated wild type brown adipocytes, $*p < 0.05$ vs. control, $**p < 0.01$ vs. control, $^{\#}p < 0.05$ vs. insulin alone, $*\,^{\#}p < 0.001$ vs. norepinephrine alone.

expression involving both transcriptional and post-transcriptional mechanisms in vivo.

To further explore a possible interaction between insulin sensitivity and adipose tissue NOX4 expression we extended our investigation to the well-characterized murine model of insulin resistance, the leptin deficient ob/ob mice. In this model again we found dissociation between NOX4 mRNA and protein levels with a three-fold increase in protein amount but no significant change in mRNA expression (Fig. 9C and D). These observations are in agreement with previous data derived from another model of insulin resistance, the mice carrying the obesity-related agouti yellow mutation (KKAy mice). In these mice an increase of NOX4 expression unrelated to the onset of diabetes was described in 7-week-old obese, but not yet diabetic and in 13-week-old obese and overtly diabetic mice as well [28].

The study mentioned above reported also an upregulation of NOX4 mRNA content in the white adipose tissue of high fat diet fed mice as another model of insulin resistance [28]. As our results (Fig. 9A) were obtained from mice with diet induced obesity that were kept on high fat diet for an unusually long period we decided to extend our investigations to shorter time frames in order to better characterize the changes in NOX4 expression associated with short term metabolic challenges. To reach this goal we analyzed NOX4 mRNA expression in the

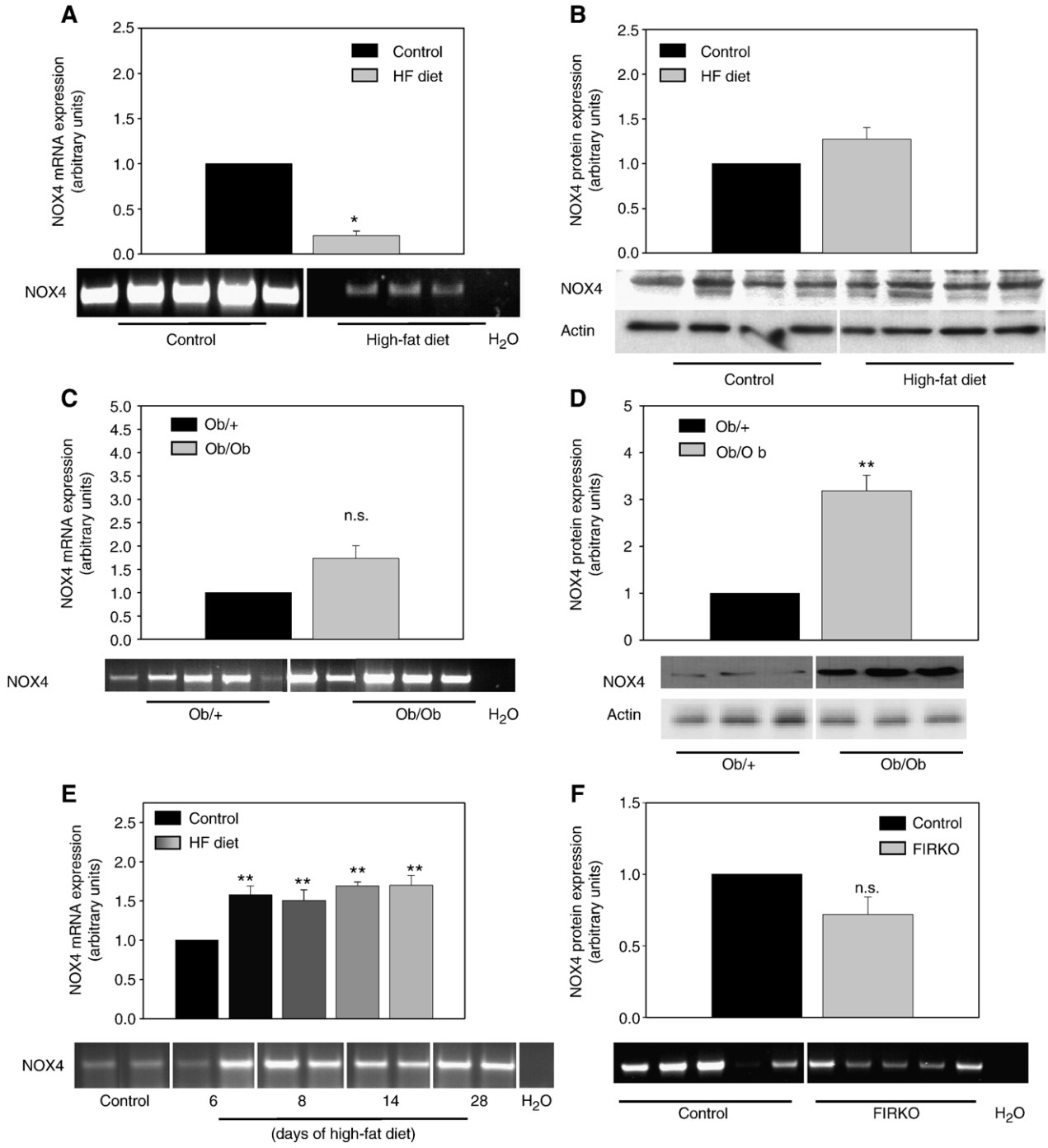

Fig. 9. Differential regulation of NOX4 mRNA and protein expression in adipose tissue of mice with altered insulin signaling. (A) Obesity was induced in C57Bl6 male mice fed a high fat containing diet (4.31 kcal/g fat) for a period of 8 months. Control mice were fed a standard chow diet (3 kcal/g fat). The protocol was approved by the Veterinary Office of the Canton of Geneva (Geneva, Switzerland). All mice had ad libitum access to water. Mice were sacrificed by decapitation and adipose tissues were harvested and snap frozen in liquid nitrogen. NOX4 mRNA expression was assessed by quantitative real-time PCR. NOX4 values are normalized to cyclophillin and expressed as arbitrary units compared to control diet fed littermates. $n=4$–6 mice/group, $^{*}p<0.05$ vs. control. The image shows amplification of NOX4 from the same mice by semiquantitative RT-PCR. H_2O denotes PCR reaction devoid of added cDNA. (B) NOX4 protein expression in the white adipose tissue of the same group of mice as in panel (A). The graph shows the quantification of NOX4 protein expression relative to that of actin. n.s.=non significant ($p>0.05$). The image shows a representative Western blot of NOX4 and actin. (C) Nine-week-old ob/ob and control ob/+ male mice were purchased from Elevage J anvier (Le Genest-St-Isle, France). NOX4 mRNA expression in the white adipose tissue was determined by real-time PCR and semiquantitative RT-PCR as in (A). $n=6$ mice/group, n.s.=non-significant ($p>0.05$) as determined by Student's t test. (D) Quantification of NOX4 protein in the white adipose tissue of the same group of ob/ob and ob/+ mice as in (C). $^{**}p<0.01$ as determined by Student's t test. (E) Quantification of NOX4 mRNA by real-time PCR in the white adipose tissue of C56Bl6 male mice fed a high fat containing (HF diet) or regular chow diet (control) for 6,8,14 or 28 days. $n=3$–4 mice/group, $^{*}p<0.05$, $^{**}p<0.01$ vs. control as determined by Student's t test. The gel below the graph represents RT-PCR detection of the NOX4 transcript from the same mice. (F) Comparison of NOX4 mRNA expression between control mice and mice with fat specific ablation of the insulin receptor (FIRKO) as in (E). $n=5$ mice/group, n.s.=non significant. Fat-specific insulin receptor knock-out mice [49] were kept at the animal facility of Joslin Diabetes Center (Boston, MA) according to the guidelines of the local Ethical Committee.

white adipose tissue from mice subjected to high fat diet for the periods of 6, 8, 14 and 28 days. As shown in Fig. 9E, short term diet was linked to an increase in adipose tissue NOX4 mRNA content. As the duration of the high fat diet and the real-time PCR primers applied in the study of Furukawa et al. mentioned above were not indicated we cannot directly compare our results, however the magnitude of changes (~2 fold) correlates with our findings. Noteworthy, however, that in our experiment no change in NOX4 mRNA expression was observed when comparing the shortest and longest period (6 and 28 days, respectively), indicating again a distance between the development of insulin resistance and whole adipose tissue NOX4 mRNA content.

The most straightforward way of relating adipose tissue insulin resistance to NOX4 expression is to compare NOX4 mRNA levels between mice with fat tissue specific ablation of the insulin receptor (FIRKO mice) and their littermates carrying only the flox allele. FIRKO mice lack insulin signaling in adipose tissue, exhibit reduced fat mass with heterogenicity of cell size and are protected from obesity and obesity related glucose intolerance [49]. WAT of FIRKO mice contained slightly decreased amount of NOX4 when compared to control, however this change was not statistically significant (Fig. 9F).

Taken together all the above in vivo data they indicate that while NOX4-deficient adipocyte cell lines show impaired insulin signaling [23], the NOX4 content of whole adipose tissue does not directly correlate with insulin sensitivity but rather changes in the ratio between the stromal/vascular fraction and adipocytes or in the "differentiation stage" of the adipose tissue as shown in the mice challenged by short term high fat diet. Adipose tissue is a remarkably active tissue with a high remodelling capacity. In this adaptative remodelling the interaction between endothelial cells and preadipocytes plays an important role as demonstrated by a recent study where blocking of vascular endothelial growth factor (VEGF) receptor resulted in decreased angiogenesis with a concomitant inhibition of adipocyte differentiation [53]. As endothelial cell and preadipocytes both express high levels of NOX4 mRNA their contribution to the whole tissue NOX4 mRNA content should be taken into consideration.

In summary, our study investigated the regulation of NOX4, an NADPH oxidase enzyme during adipogenesis in in vitro models of white and brown adipocytes. Our results suggest that NOX4 mRNA is regulated during adipogenesis in the same fashion as antiadipogenic molecules. Protein expression did not always correlate with the alterations in gene transcription, evoking a tight and complex regulatory mechanism and reinforcing the notion about the importance of NOX4 in adipogenesis. Using animal models we demonstrate that in vivo whole adipose tissue NOX4 content is not directly linked to insulin resistance but should rather be viewed as a hallmark for relative adipocyte mass and differentiation.

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