



Hypoxia potentiates tumor necrosis factor- α induced expression of inducible nitric oxide synthase and cyclooxygenase-2 in white and brown adipocytes

Indranil Bhattacharya^{a,b,*}, Ana Pérez Domínguez^a, Katja Dräger^{a,b}, Rok Humar^{a,b}, Elvira Haas^{a,b}, Edouard J. Battagay^{a,b,c}

^a Department of Internal Medicine, University Hospital of Zurich, Switzerland

^b Center of Competence Multimorbidity and University Research Priority Program "Dynamics of Healthy Aging", University of Zurich, Switzerland

^c Zurich Center for Integrative Human Physiology, CH-8057, Zurich, Switzerland

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ABSTRACT

Obesity involves hypoxic adipose tissue and low-grade chronic inflammation. We investigated the impact of hypoxia on inflammatory response to TNF- α in white and brown adipocytes. In response to TNF- α , the expression of the inducible enzymes iNOS and COX-2 was prominently and selectively potentiated during hypoxia while only moderately under normoxia. Levels of their products, nitrite and prostaglandinE₂ were elevated accordingly. NS398, a selective COX-2 inhibitor, reduced nitrite levels. The expression of PGC-1 α , a transcriptional co-activator involved in mitochondrial biogenesis, and PPAR γ , a transcription factor involved in adipocyte homeostasis, was reduced by TNF- α during hypoxia. These results suggest that hypoxia potentiates the inflammatory response by TNF- α in both white and brown adipocytes and downregulates the transcription factors involved in adipocyte function.

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1. Introduction

Obesity, which is defined as excessive fat accumulation, is characterized by low-grade chronic inflammation of adipose tissue [1,2]. The obesity-driven inflammation in adipose tissue can progress to other metabolic tissues such as liver, pancreatic islets and muscles and mediates insulin-resistance [3].

In 1993, Hotamisigil et al., demonstrated that the expression of pro-inflammatory cytokine, tumor necrosis factor- α (TNF- α) is elevated in adipose tissue of obese rodents [4]. Later, macrophages located in the adipose tissue were identified as the source of the TNF- α and also of inducible nitric oxide synthase (iNOS) [2]. TNF- α down-regulates the insulin-sensitive glucose transporter Glut4 in adipocytes [4], and indeed both pro-inflammatory proteins TNF- α and inducible nitric oxide synthase (iNOS) contribute to the development of obesity-induced insulin resistance in mice [5,6]. During normoxia stimulation with TNF- α in several cell lines led to

a co-induction of iNOS and cyclooxygenase-2 (COX-2) [7,8]. Both iNOS and COX-2 are inducible enzymes that generate nitric oxide (NO) and prostaglandin, respectively and both act cooperatively and synergistically in pathological conditions [9]. In 1993 Salvemini and colleagues showed that a cross-talk exists between COX and NOS pathways and that NO activates COX enzymes to produce increased amounts of prostaglandins [10].

The presence of hypoxia in obese adipose tissue and its contribution to the initiation and progression of inflammation appears significant [11]. Hypoxia occurs when oxygen availability does not match the demand of the surrounding tissue, resulting in decreased oxygen tension [12]. In obese adipose tissue, the adipocytes become hypertrophied and the diameter of adipocytes increases up to 150–200 μ m. Since the diffusion limit of oxygen is considered at 100 μ m, hence pockets of hypoxia develop within the adipose tissue [11]. Moreover, in obesity vascularization in adipose tissue is compromised which further restricts the oxygen availability to the adipocytes [13]. Hypoxia plays an underpinning role in triggering the infiltration of macrophages and secretion of pro-inflammatory cytokines such as tumor-necrosis factor (TNF), interleukin 6 (IL 6) and, CC-chemokine ligand 2 (CCL2; also known as MCP1) [14,15]. Alternatively, inflammation can also drive hypoxia and indeed

* Corresponding author. Department of Internal Medicine, University Hospital of Zurich, Switzerland.

E-mail address: Indranil.bhattacharya@usz.ch (I. Bhattacharya).

inflammatory disease states are often characterized by tissue hypoxia or stabilization of hypoxic markers such as hypoxia-inducible factor (HIF) [16]. Thus, hypoxia and inflammation are intertwined at the molecular, cellular, and clinical levels [16].

In the present study, using an in-vitro cell culture model of white and brown adipocytes, we characterized the effect of hypoxia on the inflammatory response. In particular, we investigated the molecules that are perturbed after treatment with TNF- α during hypoxia.

2. Materials and methods

2.1. Cell culture and differentiation

Murine 3T3-L1 preadipocytes (from ATCC, Manassas, VA, USA) at low passage were cultured in DMEM growth medium (Biobrom AG, Berlin, Germany) and differentiated to adipocytes as done previously [17]. Briefly, 3T3-L1 cells were grown to confluence in DMEM with 10% NCS. The cells were then given the differentiation cocktail comprising of 3-isobutyl-1 methylxanthine (0.5 mM), dexamethasone (0.25 μ M), insulin (10 μ g/ml) and rosiglitazone (2 μ M) in DMEM with 10% FBS. After 3 days, the media was replaced with DMEM plus 10% FBS and insulin (1 μ g/ml). After every 2nd day, the media was changed and on 11th day 80–90% of preadipocytes were differentiated. The differentiated 3T3-L1 adipocytes were then starved overnight in DMEM with FBS (0.5%) and adipocytes were then stimulated with TNF- α (10 ng/ml) in the presence of normoxia or hypoxia (1% O₂) for 24 h.

Immortalized preadipocytes (were provided by Prof. Dr. Johannes Klein) when differentiated resemble brown adipocytes [18]. The cells were grown in DMEM with 20% FBS. At 80% confluence, the cells were treated with induction media which comprised of T3 (1 nM), indomethacin (0.125 mM), dexamethasone (0.25 μ M), 3-isobutyl-1 methylxanthine (0.5 mM), insulin (10 μ g/ml) in DMEM with 20% FBS for 24 h. Cells were then changed to differentiation media which comprised of DMEM with 20% FBS, insulin (10 μ g/ml) and T3 (1 nM). The differentiation media was changed after every 2 day. Differentiation was stopped on the 10th day. Around 80–90% cells were differentiated to adipocytes and were starved overnight in DMEM with FBS (0.5%). The cells were then stimulated with TNF- α (10 ng/ml) in the presence of normoxia or hypoxia (1% O₂) for 24 h.

2.2. RNA isolation and quantitative PCR

RNA from cells was extracted using RNeasy® lipid tissue kit according to the protocol of the manufacturer (Qiagen, Hombrechtikon, Switzerland). RNA quantity was determined using Nanodrop 2000 Spectrophotometer. Reverse transcription of 50 ng RNA was performed using iScript™ Reverse Transcription Supermix according manufacture's recommendations (BioRad, Hercules, CA, USA). For quantitative RT-PCR, final cDNA concentration was adjusted to 10 ng in a 20 μ l reaction volume. Each reaction was performed in duplicates using Bio-Rad CFX96 Real-Time System and iQ™ SYBR® Green Supermix (BioRad, Hercules, CA, USA) using specific primers. Gene was normalized to the reference gene *acidic ribosomal phosphoprotein (Arbp)* using the comparative C(T) method [19].

2.3. Western blotting

Cells were homogenized using the lysis buffer containing Tris.Cl (50 mmol/L, pH 8.0), NaCl (150 mmol/L), EDTA (0.5 mmol/L), Triton X-100 (1%), protease inhibitor, and phosphatase inhibitor

(Sigma, Buchs, Switzerland). Equal amounts of protein were loaded for SDS-PAGE, transferred to nitrocellulose and analyzed with the indicated antibodies. Immunoblotting was performed using the antibodies against iNOS, ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), COX-2 and COX-1 (Cayman Chemical Company, Ann Arbor, Michigan USA). The membranes were then incubated with the corresponding HRP-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK). Labeled proteins were visualized on X-ray films using a chemiluminescence reaction.

2.4. Immunoprecipitation

3T3-L1 adipocytes were lysed with Tris.Cl (100 mmol/L, pH 7.4), NaCl (150 mmol/L), glycerol (10%), Triton X-100 (1%), protease inhibitor, and phosphatase inhibitor (Sigma, Buchs, Switzerland). The co-immunoprecipitation was performed as mentioned before [20]. Briefly, 800 μ l of lysis supernatant was pre-cleaned for non-specific binding by incubating with 50 μ l protein A-Sepharose. After a quick spin, the supernatant was pipetted into a new eppendorf tube and incubated for overnight with COX-2 antibody at 4 °C on a rotator. Subsequently, 80 μ l of Protein A-Sepharose beads were added for 2 h under rotation at 4 °C. The pellet was washed 3 times with lysis buffer and was boiled in SDS sample buffer and loaded to an 8% SDS PAGE. Immunoblotting was performed using iNOS antibody. The membranes were then incubated with the corresponding HRP-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK). Labeled proteins were visualized on X-ray films using a chemiluminescence reaction.

2.5. Measurement of nitrite

Adipocytes kept in normoxia or hypoxia incubators were treated with or without TNF- α (10 ng/ml) for 24 h. The cells were pre-treated either with iNOS specific inhibitor 1400W (10 μ M; Cayman chemical) or COX-2 specific inhibitor NS398 (10 μ M; Enzo Life Sciences, Lausen, Switzerland). The culture supernatants were collected to measure the released nitric oxide (NO). Levels of NO, in form of nitrite (NO₂⁻) were determined using Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid) as done previously [21]. The absorbance was measured at 570 nm with a multi-mode microplate reader (Molecular Devices, Spectra Max M2, Bucher Biotec, Basel, Switzerland).

2.6. Determination of PGE₂ production

PGE₂ was measured in the culture media by competitive enzyme immunoassay according to the manufacturer's protocol (Cayman Chemical Co., Ann Arbor, MI, USA). Briefly, cells in normoxia or hypoxia were pre-treated either with 1400W (10 μ M) or NS398 (10 μ M) along with or without TNF- α (10 ng/ml) for 24 h. The cell culture medium was collected and centrifuged at 500 g for 5 min. The supernatant was used to measure the prostaglandinE₂ (PGE₂) levels.

2.7. Statistical analysis

Student's *t* test was used to compare data between two groups. Values are expressed as means \pm SEM of three independent experiments. *P* < 0.05 was considered statistically significant.

3. Results

3.1. *TNF- α* increases the expression of iNOS and COX-2 in hypoxic adipocytes

Around 80% of 3T3-L1 preadipocytes were differentiated to adipocytes (Fig. 1A). In 3T3-L1 preadipocytes, stimulation with *TNF- α* increased the gene expression of iNOS in normoxia and to a greater extent in hypoxia; however they were non-significant (Fig. 1B). The gene expression of COX-2 was minutely increased with *TNF- α* and the increase was similar between normoxic and hypoxic conditions (Fig. 1B). When differentiated 3T3-L1 adipocytes were used, stimulation with *TNF- α* increased the gene expression of iNOS in normoxia and its expression was increased by 7-fold in hypoxia (Fig. 1C). The COX-2 gene expression was also increased by 7-fold in adipocytes treated with *TNF- α* in the presence of hypoxia compared with normoxia (Fig. 1C). When brown adipocytes were used, treatment with *TNF- α* increased the expression of iNOS gene and its expression was around 40-fold higher in

hypoxia as compared with normoxia (Fig. 1D). The expression of COX-2 gene in the presence of *TNF- α* and hypoxia was increased in brown adipocytes but was not significant compared with normoxia (Fig. 1D).

3T3-L1 adipocytes treated with *TNF- α* increased the protein expression of iNOS in normoxia. Adipocytes in hypoxia treated with *TNF- α* caused a robust increase in the protein expression of iNOS and COX-2 (Fig. 1E) but not of COX-1 (data not shown). A significant increase in expression of iNOS and COX-2 was also observed in brown adipocytes under hypoxia and *TNF- α* (Fig. 1F). Stimulation of 3T3-L1 preadipocytes with *TNF- α* in the presence of hypoxia did not increase the expression of iNOS and COX-2 (Fig. 1G). Similarly, in endothelial cells iNOS and COX-2 protein expression was not increased by hypoxia and *TNF- α* (Fig. 1H).

Since iNOS and COX-2 are co-induced in adipocytes in the presence of hypoxia and *TNF- α* , an immunoprecipitation experiment was performed to determine whether iNOS physically interacts with COX-2 (Supplementary Figure 1). The data suggest that in endogenous condition, iNOS does not bind to COX-2 in adipocytes.

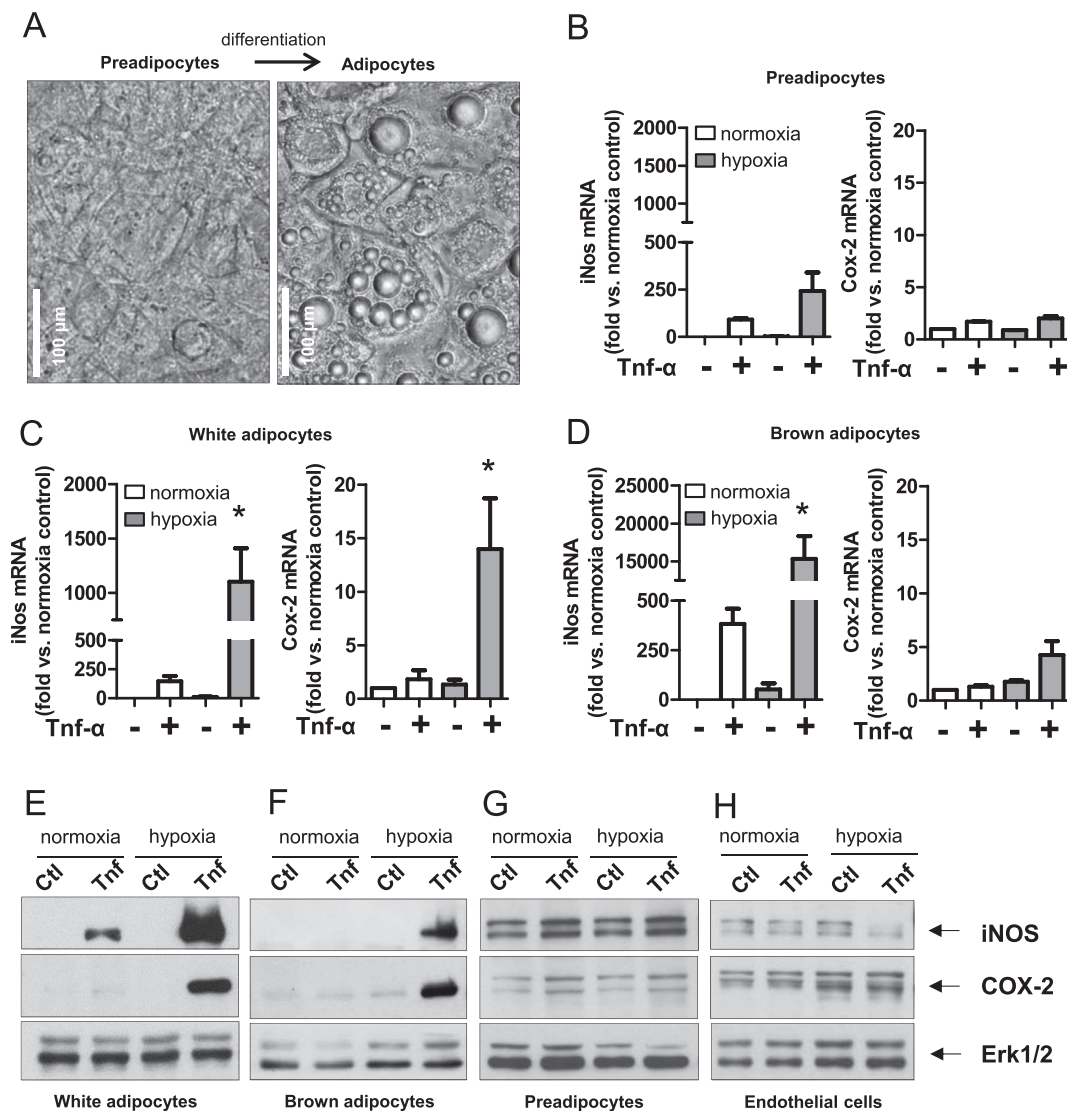


Fig. 1. Gene and protein Expression of iNOS and COX-2. (A) Differentiation of 3T3-L1 preadipocytes to adipocytes. (B) 3T3-L1 preadipocytes. (C) 3T3-L1 adipocytes and (D) brown adipocytes were stimulated without or with *TNF- α* for 24 h in the presence of normoxia (21% O_2) or hypoxia (1% O_2). Gene expression of iNOS and COX-2 were examined using real time qPCR. * $p < 0.05$ vs. normoxia and *TNF- α* . N = 3–5. (E) Cell lysates from 3T3-L1 adipocytes, (F) brown adipocytes, (G) 3T3-L1 preadipocytes and (H) mouse aortic endothelial cells were immunoblotted with specific iNOS or COX-2 antibodies. The protein loading was checked using Erk2 antibody. N = 3.

3.2. TNF-α alters the gene expression of cytokines in normoxia and hypoxia

In 3T3-L1 adipocytes, the gene expression of pro-inflammatory genes such as interleukin 6 (IL 6), serum amyloid A1 (SAA1), regulated on activation, normal T cell expressed and secreted (Rantes) and monocyte chemoattractant protein1 (MCP1) was similarly increased after stimulation with TNF-α in normoxic and hypoxic conditions (Fig. 2). In contrast, the gene expression of anti-inflammatory adiponectin and leptin was decreased in the presence of TNF-α in normoxia and to a same extent by hypoxia. Hypoxia alone increased the gene expression of plasminogen activator inhibitor type 1(PAI-1) by 3-fold and the expression levels were similar when cells were treated with TNF-α in hypoxia (Supplementary Figure 2A). The gene expression of vascular endothelial growth factor (VEGF), a pro-angiogenic molecule, was increased only by hypoxia and TNF-α had no effect on its expression (Supplementary Figure 2B).

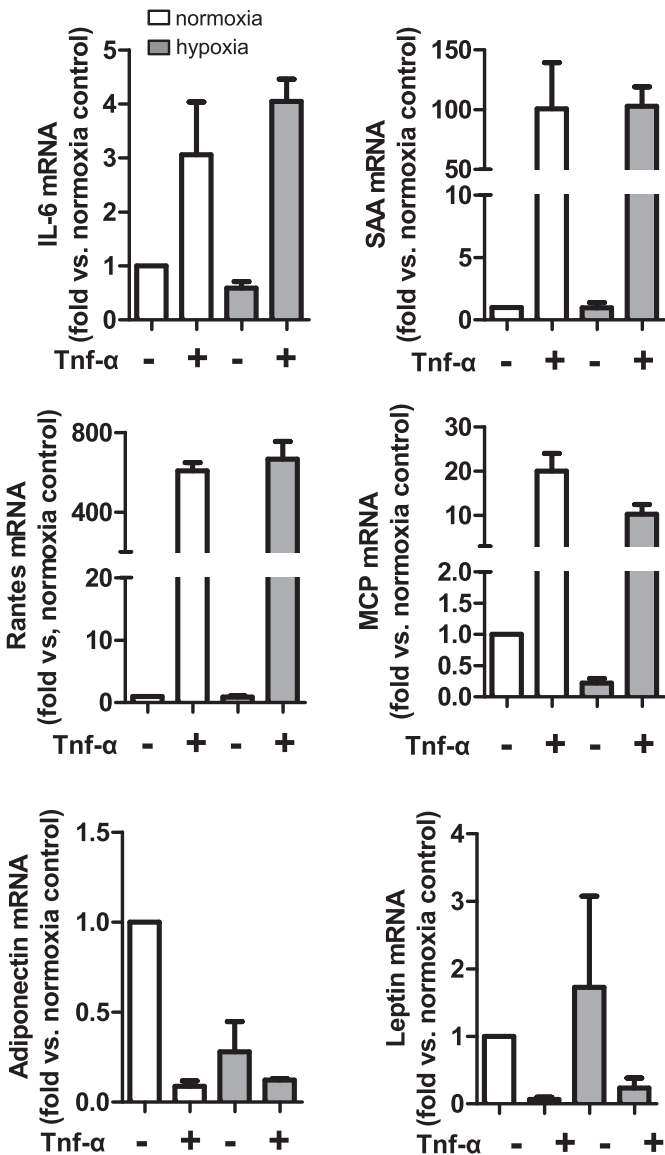


Fig. 2. Gene expression of cytokines. 3T3-L1 adipocytes were stimulated without or with TNF-α for 24 h in the presence of normoxia (21% O₂) or hypoxia (1% O₂). Gene expression was examined using real time qPCR. N = 3–4.

3.3. iNOS and COX-2 inhibitors reduce nitrite levels

Since iNOS was significantly increased in adipocytes by TNF-α during hypoxia, the nitrite levels were measured in 3T3-L1 adipocytes under these conditions. As compared with only hypoxia, the nitrite levels were not significantly increased in presence of hypoxia with TNF-α (Fig. 3A). The increased nitrite levels in hypoxia were significantly reduced by iNOS specific inhibitor 1400W (Fig. 3A). Interestingly, the COX-2 specific inhibitor NS398 also reduced the nitrite level (Fig. 3B).

3.4. TNF-α and hypoxia increases PGE₂ levels

The expression of COX-2 was significantly upregulated by TNF-α during hypoxia, hence the functional activity of COX-2 was evaluated and its enzymatic product PGE₂ was measured in similar conditions. Treatment of adipocytes with TNF-α in the presence of hypoxia significantly increased the levels of PGE₂. In the presence of iNOS specific inhibitor, 1400W, the PGE₂ levels were not significantly reduced (Fig. 3C). In contrast, the selective COX-2 inhibitor, NS398, significantly reduced the increased levels of PGE₂ in hypoxia (Fig. 3D).

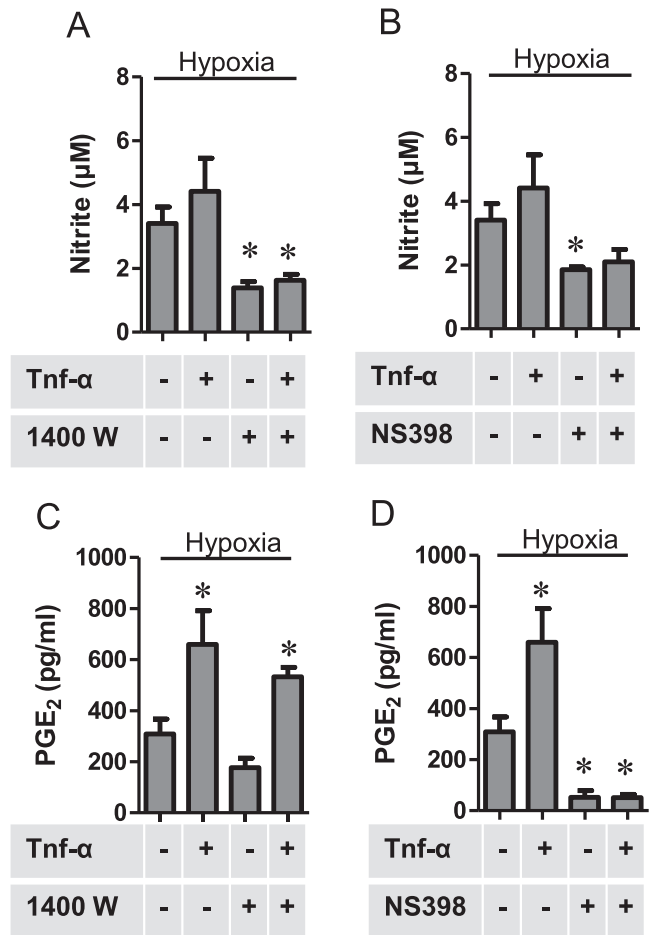


Fig. 3. Measurement of nitrite and prostaglandin E₂. The levels of nitrite were measured in the conditioned media using Griess reagent. (A) The 3T3-L1 adipocytes in hypoxia were pre-treated either with 1400W or (B) with NS398 in the presence or absence of TNF-α for 24 h *p < 0.05 vs. hypoxia. N = 3. The levels of prostaglandin E₂ (PGE₂) were measured from the conditioned media. (C) The 3T3-L1 adipocytes in hypoxia were pre-treated either with 1400W or with (D) NS398 in the presence or absence of TNF-α for 24 h *p < 0.05 vs. hypoxia. N = 3.

3.5. $\text{TNF-}\alpha$ and hypoxia decreases $\text{PGC-1}\alpha$ mRNA

We then investigated the gene expression of some pro-inflammatory transcription factors and its co-activators. ATF3 , a pro-inflammatory transcription factor was increased by $\text{TNF-}\alpha$ in the presence of normoxia (Fig. 4A). Hypoxia alone or together with $\text{TNF-}\alpha$ increased the gene expression of ATF3 and the expression levels were similar. The gene expression of $\text{PPAR}\gamma$, a transcription factor required for adipocyte homeostasis, was reduced by 2-fold upon $\text{TNF-}\alpha$ treatment in the presence of normoxia and was further reduced, albeit non-significantly, with hypoxia (Fig. 4B). Nrf-1 , a transcription factor involved in mitochondrial biogenesis, was reduced by 1.6-fold with $\text{TNF-}\alpha$ in the presence of normoxia and to the same extent by hypoxia (Fig. 4C). The gene expression of $\text{PGC-1}\alpha$, a co-activator of transcription factor which is also involved in mitochondrial biogenesis, was reduced by 4-fold after treatment with $\text{TNF-}\alpha$ in the presence of normoxia (Fig. 4D). Hypoxic condition reduced the gene expression of $\text{PGC-1}\alpha$ to the same extent. Interestingly, treatment of adipocytes with $\text{TNF-}\alpha$ during hypoxia further decreased the expression of $\text{PGC-1}\alpha$ (by 3-fold) in comparison with normoxia and $\text{TNF-}\alpha$.

4. Discussion

Development of adverse pathophysiological conditions in obesity involves inflammation [3,22]. Heightened levels of $\text{TNF-}\alpha$ in obese individuals triggers adipocyte dysfunction [23] and mediates insulin resistance [4,22,24]. Macrophages within the adipose tissue

are supposedly the source of $\text{TNF-}\alpha$ [2]. In this study, we mimicked the pro-inflammatory environment in adipose tissue by stimulating adipocytes under normoxia and hypoxia with exogenous $\text{TNF-}\alpha$. In both normoxic and hypoxic conditions, $\text{TNF-}\alpha$ increased the expression of several pro-inflammatory and acute-phase molecules such as IL-6, SAA, Rantes, MCP-1, PAI-1 and reduced the expression of cytokines such as adiponectin and leptin. Furthermore, $\text{TNF-}\alpha$ stimulated the expression of iNOS and COX-2 in normoxia and interestingly, hypoxia potentiated the effect of $\text{TNF-}\alpha$ in inducing iNOS and COX-2 expression in both white and brown adipocytes.

It is well established that hypoxia in obese adipose tissue triggers inflammation and pockets of hypoxia colocalize with the presence of macrophages [11,15,25]. The robust increase in the expression of iNOS and COX-2 after $\text{TNF-}\alpha$ stimulation during hypoxia was observed specifically in adipocytes (white and brown) and not in preadipocytes and endothelial cells. Cardiomyocytes treated with IL-1 β in the presence of hypoxia reduced the expression of iNOS and displayed reduced levels of nitrite [26]. This suggests that inflammatory stimuli and hypoxia driven induction of iNOS and COX-2 is evident in adipocytes and is clearly cell type dependent. A crosstalk between iNOS and COX-2 has been reported and a physical interaction between the molecules was shown using an overexpression system in HEK293T cells [20]. However, in our work, we could not detect a physical interaction under endogenous setting even with high expression of iNOS and COX-2. Murine macrophages stimulated with IFN- γ increased the expression of iNOS mRNA however nitrite did not accumulate in culture supernatant [27]. In our experiments, hypoxia caused a significant rise in nitrite level even with low iNOS expression. However, hypoxia plus $\text{TNF-}\alpha$ did not significantly augment the nitrite levels further even with elevated iNOS expression levels. Since oxygenation is critical for nitric oxide formation and it has been suggested that oxygenation mediates the accumulation of nitrite in culture supernatant [27]. It is thus possible that the conserved release of nitrite in presence of hypoxia and $\text{TNF-}\alpha$ was due to the absence of re-oxygenation in our experimental setting. The inhibitor studies using iNOS selective inhibitor, 1400W, indicate that the iNOS was involved in nitric oxide release in hypoxia. Surprisingly, the levels of nitrite were lowered when COX-2 specific inhibitor NS398 was used. The COX-2 product prostaglandin E_2 was only lowered by NS398 and not by 1400W. These findings suggest that in differentiated adipocytes COX-2 may be upstream of iNOS in the signaling cascade.

Decreased mitochondrial biogenesis increases iNOS expression in adipocytes [28] and $\text{PGC-1}\alpha$, a transcriptional coactivator, is important for mitochondrial biogenesis [29]. Chronic exposure of adipocytes to $\text{TNF-}\alpha$ under normoxia causes insulin resistance and decreases $\text{PGC-1}\alpha$ expression [24]. In our study, the expression of $\text{PGC-1}\alpha$ was significantly lowered after adipocytes were exposed to hypoxia in the presence of $\text{TNF-}\alpha$. In white adipose tissue, $\text{PGC-1}\alpha$ stimulates its own transcription by coactivating the transcription factor $\text{PPAR}\gamma$ [30]. In our study, the expression of $\text{PPAR}\gamma$ was also decreased by hypoxia in the presence of $\text{TNF-}\alpha$. Thus, the lower expression of $\text{PPAR}\gamma$ could be the linked to the low $\text{PGC-1}\alpha$ expression. It has been shown that mice with iNOS deletion in brown adipose tissue have an enhanced expression of $\text{PGC-1}\alpha$ [31]. This can be extrapolated to our finding and suggest a probable link between the expression of iNOS and $\text{PGC-1}\alpha$. Moreover, leptin increases $\text{PGC-1}\alpha$ expression [32]. In our study, expression of leptin was decreased after adipocytes were exposed to hypoxia with $\text{TNF-}\alpha$. In adipocytes, the mitochondrial function is essential for adiponectin synthesis [33]. The low expression of adiponectin in our study indicates the presence of mitochondrial dysfunction by hypoxia and $\text{TNF-}\alpha$. Thus, the increased expression of iNOS and COX-2 along with decreased expression of adiponectin, leptin,

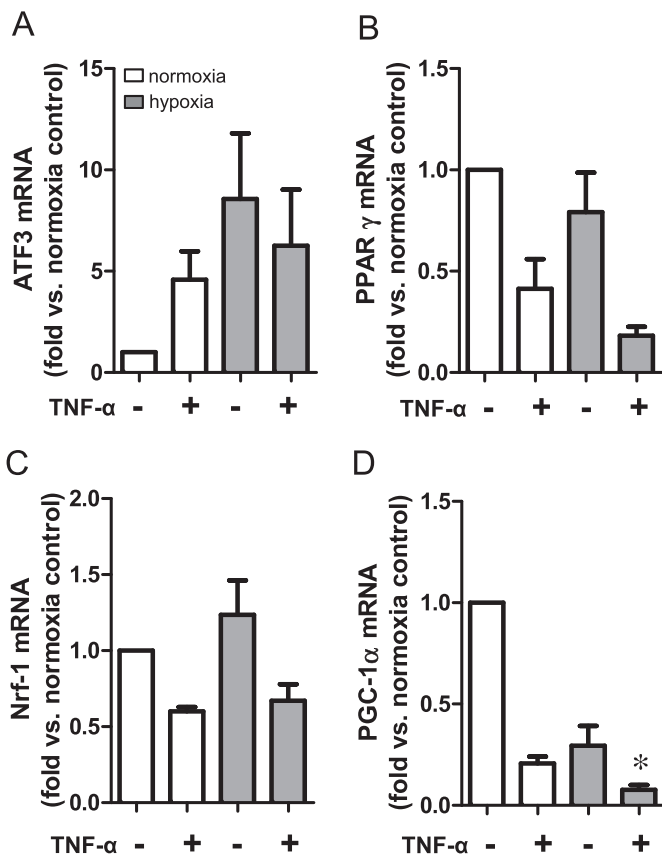


Fig. 4. Gene expression of transcription factors or its co-activators. 3T3-L1 adipocytes were stimulated without or with $\text{TNF-}\alpha$ for 24 h in the presence of normoxia (21% O_2) or hypoxia (1% O_2). (A) Gene expression of ATF3 , (B) $\text{PPAR}\gamma$, (C) Nrf-1 and (D) $\text{PGC-1}\alpha$ were examined using real time qPCR. * $p < 0.05$ vs. normoxia with $\text{TNF-}\alpha$. $N = 4-6$.

PPAR γ and PGC-1 α contribute to adipocyte dysfunction probably via altering the mitochondrial function.

At a pathophysiological level the in vitro findings in this work suggest that hypoxia within adipose depots in obese patients could further worsen an existent low-level inflammation by amplifying the inflammatory response. Therefore, methods to reduce hypoxia within obese adipose tissue may be of importance. In this regard, improving vascularization and thus oxygenation using anti-hypertensive drugs such as angiotensin-converting enzyme inhibitors which normalize rarefied microvessel density [34] could be an useful approach. Moreover, practicing healthy lifestyle can promote oxygenation and reduce the tissue inflammatory load. Thus, the reduction in hypoxia-mediated inflammation and lowering of iNOS and COX-2 expression in adipose tissue could prevent or even reverse the worsening of obesity-associated pathophysiological conditions.

Conflict of interest

The authors have declared there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2015.04.020>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2015.04.020>.

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