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

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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 prostaglandinE_2 were elevated accordingly. NS398, a selective COX-2 inhibitor, reduced nitrite levels. The expression of PGC-1α, a transcriptional co-activator involved in mitochondrial biogenesis, and PPARγ, 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.

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, Hotamsigil et al., demonstrated that the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) expression 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 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 μm. Since the diffusion limit of oxygen is considered at 100 μm, 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)

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and, CC-chemokine ligand 2 (CCL2; also known as MCP1) [14,15]. Alternatively, inflammation can also drive hypoxia and indeed 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 (Biochrom 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 μM), insulin (10 μg/ml) and rosiglitazone (2 μM) in DMEM with 10% FBS. After 3 days, the media was replaced with DMEM plus 10% FBS and insulin (1 μg/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% O_2) 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 μM), 3-isobutyl-1 methylxanthine (0.5 mM), insulin (10 μg/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 μg/ml) and T3 (1 nM). The differentiation media was changed after every 2 days. 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% O_2) 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 perfomed 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 μl 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 mM), EDTA (0.5 mM), 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 mM), 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 μl of lysis supernatant was pre-cleaned for non-specific binding by incubating with 50 μl 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 μl 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 μM; Cayman chemical) or COX-2 specific inhibitor NS398 (10 μM; Enzo Life Sciences, Lausen, Switzerland). The culture supernatants were collected to measure the released nitric oxide (NO). Levels of NO, in form of nitrite (NO_2^-) 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 PGE_2 production

PGE_2 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 μM) or NS398 (10 μM) 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 prostaglandinE_2 (PGE_2) levels.

2.7. Statistical analysis

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

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