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# Resveratrol has inhibitory effects on the hypoxia-induced inflammation and angiogenesis in human adipose tissue *in vitro*

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ABSTRACT

Hypoxia modulates the production of proteins involved in e.g. inflammation, angiogenesis and glucose utilization and hypoxia may therefore be an important factor underlying adipose tissue dysfunction in obesity. Resveratrol (RSV) is a natural polyphenolic compound and has been shown to have powerful anti-inflammatory effects and beneficial effects on several obesity-related complications. Thus, in the present study we investigated whether RSV has effects on hypoxic markers (GLUT-1, VEGF), hypoxia-induced key markers of inflammation (IL8, IL6), and leptin in human adipose tissue *in vitro*. Hypoxia was induced by incubating human adipose tissue fragments with 1% $O_2$ for 24 h as compared to 21% $O_2$ The gene expressions were investigated by RT-PCR and protein release by Elisa. Hypoxia increases the expression of glucose transporter-1 (GLUT-1) (19-fold, $p < 0.001$), vascular endothelial growth factor (VEGF) (10-fold, $p < 0.05$), interleukin-8 (IL8) (8-fold, $p < 0.05$), interleukin-6 (IL6) (5-fold, $p < 0.05$) and leptin (9-fold). The protein levels of VEGF released to the medium was increased (8-fold, $p < 0.01$) by hypoxia. RSV dose-dependently inhibited several of these hypoxia-induced expressions and at a concentration of 50 μM RSV almost completely inhibited the hypoxic responses at the above mentioned gene expression levels ($p < 0.05$–$p < 0.001$) and significantly attenuated the hypoxia-induced protein releases by 50–60%. These results demonstrate that hypoxia induces extensive changes in human adipose tissue in the expression and release of inflammation and angiogenesis-related adipokines. In addition the inhibition of hypoxia-mediated inflammation and angiogenesis might represent a novel mechanism of RSV in preventing obesity-related pathologies.



## 1. Introduction

Inflammation in adipose tissue is increasingly considered to be of importance for the development of the diseases associated with obesity, such as type 2 diabetes and the metabolic syndrome. However, the mechanistic basis for the inflammatory response in the expanding adipose tissue is still unknown but suggestions include involvement of free fatty acid, endoplasmatic reticulum stress, reactive oxidative species, adipocyte death and hypoxia (Ye, 2009; Wood et al., 2007). Thus, in several studies hypoxia has been proposed as an important underlying cause of the inflammatory response in adipose tissue in the obese state (Trayhurn and Wood, 2004; Trayhurn et al., 2008a,b; Wang et al., 2007). It has been shown, that hypoxia inhibits differentiation of preadipocytes and stimulates the secretion of vascular endothelial growth factor (VEGF) from mature adipocytes *in vitro* (Lolmede et al., 2003; Ye et al., 2007; Hosogai et al., 2007; Yun et al., 2002; Chen et al., 2006; Wang et al., 2007). Therefore, hypoxia could be one of the factors for the link between an enlarged adipose tissue with adipocyte hypertrophy and impaired adipocyte function, because hypertrophic adipocytes (cell diameter of >150 μm) are larger than the normal diffusion distance of $O_2$ within tissue resulting in local hypoxic conditions (Lolmede et al., 2003). The cellular response to low $O_2$ levels is accomplished through the activation of specific transcription factors. The best known of these factors is hypoxic inducible factor 1 (HIF-1) (Stuart et al., 2009). The key element of this heterodimer is the subunit HIF-1α, which is considered to be the molecular $O_2$ sensor. When cellular $O_2$ levels are sufficient, this protein, which is continuously synthesised, is immediately targeted for degradation, but under

*Abbreviations:* ANGPTL-4, angiopoietin like protein-4; AMPK, AMP-activated protein kinase; FAS, fatty acid synthase; GLUT, glucose transporter; HIF-1, hypoxic inducible factor-1; HSD, hydroxysteroid dehydrogenase; IL, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage inhibitory factor; PI3K, phosphatidylinositol 3-kinases; PPARγ, proliferator-activated receptor gamma; RSV, resveratrol; SIRT1, silent mating type information regulation 2 homolog 1; UCP2, mitochondrial uncoupling protein 2; VEGF, vascular endothelial growth factor.

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low $O_2$ levels HIF-1α is accumulating (Stuart et al., 2009). The second subunit, HIF-1β, a constitutively-expressed protein that is $O_2$ insensitive, is now able to bind to HIF-1α and form the active transcription factor HIF-1. HIF-1 is involved in the activation of over 70 genes directly via binding to hypoxic response elements. These HIF-dependent genes involve a multiplicity of functions including inflammation, angiogenesis, glucose metabolism, apoptosis, cellular stress and extracellular matrix re-modelling (Rocha, 2007; Stuart et al., 2009). Examples of specific hypoxia-sensitive genes that are transcriptionally regulated by HIF-1, in both murine and human adipose tissue, include leptin, angiopoietin-like protein-4 (ANGPTL-4), interleukin-6 (IL6), IL8, macrophage migration inhibitory factor (MIF), and vascular endothelial growth factor (VEGF) as well as the glucose transporter 1 (GLUT-1) and GLUT-3 (Forsythe et al., 1996; Rocha, 2007; Stuart et al., 2009).

The hypoxia-induced activation of various genes may be an adaptive/protective mechanism for the tissue or organism to survive ischemic conditions (Bento and Pereira, 2011), but as suggested hypoxic conditions in the expanded adipose tissue may also be involved in some of the inflammatory and metabolic complications seen in the obese state (Trayhurn and Wood, 2004; Trayhurn et al., 2008a,b; Wang et al., 2007).

Resveratrol (RSV) (trans-3,5,4-trihydroxystilbene) is a natural polyphenolic compound synthesized in a large number of plant species such as mulberries, peanuts, grapes, and is present in red wine (Rivera et al., 2009). Numerous beneficial effects of RSV described in the literature involve cardio-protective, anti-cancer, anti-inflammatory and anti-oxidative actions (Szkudelska and Szkudelski, 2010; Saiko et al., 2008; Baur and Sinclair, 2006). Research has also shown potential possibility of the use of RSV in preventing and/or treating both obesity and diabetes by stimulating glucose uptake, reducing glucose conversion to lipids, and reducing body weight and adiposity (Lagouge et al., 2006; Baur et al., 2006; Rivera et al., 2009; Su et al., 2006; Szkudelska et al., 2009). Recent studies have reported beneficial effects of RSV on metabolic diseases by mimicking calorie restriction (Hou et al., 2008; Lagouge et al., 2006; Baur et al., 2006; Howitz et al., 2003). The mechanism whereby RSV exerts its effect is proposed to be related to the activation of silent mating type information regulation 2 homolog 1 (SIRT1), but it seems that activation of SIRT1 is not the sole effect whereby RSV reduces the pathological consequences of obesity (Hou et al., 2008; Lagouge et al., 2006; Baur et al., 2006; Howitz et al., 2003). It has also been proposed that some of the beneficial effects of RSV result from phosphorylation/activation of AMP-activated protein kinase (AMPK), repressing proliferator-activated receptor gamma (PPARγ) and Phosphatidylinositol 3-kinases (PI3K)/Akt (Baile et al., 2011), as has been described in several tissues and cell lines (Hou et al., 2008; Lagouge et al., 2006; Baur et al., 2006; Howitz et al., 2003).

We and others have shown that RSV treatment improves the inflammatory status in the adipose tissue by reducing the expression and release of adipokines in rat and human adipocytes in cultures (Rivera et al., 2009; Kennedy et al., 2009; Zhu et al., 2008; Ahn et al., 2007; Olholm et al., 2010). These results indicates that RSV may have implications in the treatment of obesity and its complications (Rayalam et al., 2008).

In order to further identify possible treatment potential of RSV for obesity related complications we investigated in the present study whether the hypoxia-induced inflammation and angiogenesis in human adipose tissue could be affected by RSV *in vitro*.

## 2. Materials and methods

### 2.1. Whole adipose tissue cultures

Subcutaneous adipose tissue from the abdominal region was obtained from 10 non-obese women (mean age: 31 years and mean BMI: 23.8 ± 0.9 kg/m$^2$) undergoing liposuction for cosmetic reasons. None of the subjects received any medication known to influence adipose tissue metabolism. The adipose tissue was transported to the laboratory in a sterile container. The adipose tissue was treated essentially as previously described (Kristensen et al., 1999). In short, 500 mg adipose tissue was washed and incubated in medium 199. Medium 199 was supplemented with 25 mM HEPES; 1% bovine albumin; 1 nM insulin, antipain and leupeptin 20 mL/100 mL medium, penicillin and streptomycin (10,000 IU) 1 mL/100 mL medium. The adipose tissue was preincubated for 24 h. Then the medium was replaced with medium 199 or medium 199 containing 50 μM RSV (Cayman Chemical, Ann Arbor, MI, USA), before starting the experiment. The adipose tissue fragments were then moved to the respective incubators and incubated in either normoxia (21% $O_2$ and 5% $CO_2$) or severe hypoxia (1% $O_2$ and 5% $CO_2$) in a designated hypoxia workstation (Xvivo System, BioSpherix, NY) that was pre-balanced for the desired $O_2$ concentration. The incubation lasted for the time indicated (maximum 48 h). The samples that were exposed to re-oxygenation, was removed from 1% $O_2$ after 24 h and placed in the incubator with 21% $O_2$ for up to 4 h. All incubations were performed in duplicate, and each data point represented the mean of duplicate incubations from 3 to 10 individuals. The culture medium obtained from the different incubations was kept at −20 °C, and the adipose tissue was immediately frozen in liquid nitrogen and kept at −80 °C for RNA extraction.

### 2.2. Isolation of RNA from adipose tissue fragments

Total RNA was isolated from the adipose tissue fragments using TriZol reagent (Gibco BRL, Life Technologies, Roskilde, Denmark); RNA was quantified by measuring absorbance at 260 and 280 nm, and the inclusion criteria was a ratio ⩾2. Finally, the integrity of the RNA was checked by visual inspection of the two ribosomal RNAs, 18S and 28S, on an agarose gel.

### 2.3. Real-time reverse transcriptase PCR of inflammatory and angiogenic factors

For real-time reverse transcriptase PCR, complementary DNA was constructed using random hexamer primers as described by the manufacturer (GeneAmpRNAPCR Kit from Perkin–Elmer Cetus, Norwalk, CT). PCR-mastermix, containing the specific primers, HotStar Taq DNA polymerase (Qiagen, Valencia, CA) and SYBR-Green PCR buffer were added. The following primer pairs were used: VEGF sense primer 5′-GTTCATGGATGTCTATCAGCGCAGCTACTG-3′ and antisense primer 5′-GGGGCACACAGGATGGCTTGAAGATGTACT-3′. GLUT-1 sense primer 5′-CTCATGGGCTTCTCGAAACTGGGCAAGTCC-3′ and antisense primer 5′-GGTCAGGCCGCAGTACACAC CGATGATG-3′. IL8 sense primer 5′-TTGGCAGCCTTCCTGATTTC-3′ and antisense primer 5′-AACTTCTCCACAACCCTCTG-3′. IL6 sense primer 5′-AAATGCCAGCCTGCTGACGAAG-3′ and antisense primer 5′-AACA ACAATCTGAGGTGCCCATGCTAC-3′. Leptin sense primer 5′-GATGACACCAAAACCCTCATC-3′ and antisense primer 5′-GCCACCACC TCTGTGGAGTAG-3′. Adiponectin sense primer 5′-TGAATGCTGAGCG GTAT-3′ and antisense primer 5′-CATGACCAGGAAACCACGACT-3′. HIF-1α sense primer 5′-GCGCAAGTCC TCAAAGCACAGTTACA-3′ and antisense primer 5′-GTGGTAGTGGTGGCATTAGCAGTAGGTTC T-3′. The housekeeping gene, beta-2 microglobulin, was amplified using sense primer 5′-TCTCTCTTTCTGGCCTGGAG-3′ and antisense primer 5′-AATGTCGGATGGATGAAACC-3′. Real-time quantification of genes was performed by SYBR-green real-time reverse transcription PCR assay (Qiagen, Valencia, CA) using an ICycler from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). cDNA with specific primers was amplified in separate tubes, and the increase in fluorescence was measured in real time. The threshold cycle was calculated,