Effects of hyperoxia exposure on metabolic markers and gene expression in 3T3-L1 adipocytes

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ABSTRACT

Adipose tissue often becomes poorly oxygenated in obese subjects. This feature may provide cellular mechanisms involving chronic inflammation processes such as the release of proinflammatory cytokines and macrophage infiltration. In this context, the purpose of the present study was to determine whether a hyperoxia exposure on mature adipocytes may influence the expression of some adipokines and involve favorable changes in specific metabolic variables. 3T3-L1 adipocytes (14 days differentiated) were treated with 95% oxygen for 24 h. Cell viability, intra and extracellular reactive oxygen especies (ROS) content, glucose uptake and lactate and glycerol concentrations were measured in the culture media. Also, mRNA levels of HIF-1α, leptin, IL-6, MCP-1, PPAR-γ, adiponectin, and ANGPTL-4 were analyzed. Hyperoxia treatment increased intra and extracellular ROS content, reduced glucose uptake and lactate release and increased glucose release. It also led to an upregulation of the expression of IL-6, MCP-1 and PPAR-γ, while ANGPTL4 was downregulated in the hyperoxia group with respect to control. The present data shows that hyperoxia treatment seems to provoke an inflammatory response due to the release of ROS and the upregulation of pro-inflammatory adipokines, such as IL-6 and MCP-1. On the other hand, hyperoxia may have an indirect effect on the improvement of insulin sensitivity, due to the upregulation of PPAR-γ gene expression as well as a possible modulation of both glucose and lipid metabolic markers. To our knownledge, this is the first study analyzing the effect of hyperoxia in 3T3-L1 adipocytes.

Key words: hyperoxia, cell culture, inflammation, hypoxia, obesity

INTRODUCTION

Obesity is a major metabolic disorder associated to an excessive fat accumulation [4]. The most common causes for the increase in the prevalence of this disease are over-nutrition and a reduction in physical activity, leading to a chronic positive balance between energy intake and energy expenditure. However, other factors such as endocrine disruptions, perinatal malnutrition, environmental effects or epigenetic dysregulations, can also contribute to obesity [6, 36]. This disease often courses with a low-grade chronic inflammation [33], characterized by changes in the release of inflammation-related adipokines and macrophage infiltration within the white adipose tissue, which may play an important role in the onset and development of obesity-related diseases [23, 55, 63]. This could be a target for understanding the etiology and complications of some causes of obesity [20]. Indeed, several hypotheses have been proposed as the cause for the initiation of inflammatory processes during obesity, including oxidative stress [14, 23], endoplasmic reticulum stress [21, 24, 38], and adipose tissue hypoxia [48, 59]. Therapies trying to manage and counterbalance some of these adipose tissue pro-inflammatory conditions are under investigation [16, 39, 50]. Nevertheless, to our knowledge there are no studies trying to ameliorate the hypoxic state within adipose tissue. Indeed, recent investigations have suggested that adipose tissue hypoxia provides a cellular explanation for chronic inflammation and macrophage infiltration in white adipose tissue in obesity [22, 60], which have been associated to some complications accompanying obesity-related diseases.

In this context, oxygen is used in current medicine as a treatment for several conditions such as apnea, migraine and wounds [2, 29, 31]. Moreover, some animal studies have demonstrated that treatment with hyperoxia might produce beneficial effects in different metabolic disorders, such as protecting the rat brain tissue against ischemia reperfusion injury [5], reducing severity of colitis [11] or ameliorating hemorrhagic shock-induced renal failure by decreasing intrarenal hypoxia and improving renal functions [10]. Additionally, several studies have shown that hyperoxia can decrease the expression of some pro-inflammatory genes in different organs and cell types. Hence, Desmarquest et al. observed a decrease in several inflammatory markers in alveolar macrophages exposed to hyperoxia [9]. This effect seems to be consistent with hypobaric oxygen therapy (HBOT) studies carried out in *ex vivo* cell cultures [3, 30, 54]. These findings are also in agreement with studies

demonstrating that HBOT attenuates pro-inflammatory cytokine production of systemic inflammation in animal models [32, 57, 58].

Taking all this information into account, and in relation to the hypothesis exposed by our group in 2010 [43] where we proposed hyperoxia as a novel therapeutic intervention for the improvement of obesity state, we performed the current study to determine whether an exposure of mature adipocytes to hyperoxia may regulate the expression of some adipokines and involve favorable changes in specific metabolic variables.

MATERIAL AND METHODS

Cell culture and treatment

3T3-L1 mouse preadipocytes were cultured with Dulbecco's minimal essential media (DMEM) containing 4.5 g/L glucose and supplemented with 10% calf bovine serum (CBS) as described elsewhere [13]. Two days after full confluence, cells were cultured in twelve-well plates. Their differentiation into adipocytes was induced by treating cells for 2 days with 0.5 mM isobutylmethylxanthine (IBMX), 1 μM dexamethasone (Dex) and 10 μg/ml insulin, in DMEM supplemented with 10% fetal bovine serum (FBS), and then for 2 days with 10 μg/ml insulin in the same media. Thereafter, cells were maintained and re-fed every 2 or 3 days with FBS without any hormones until 14 days after differentiation induction, when between 80 and 90% of the cells exhibited the adipocyte phenotype. Media had 100 units/ml penicillin, and 100 μg/ml streptomycin, and cells were always maintained at 37ºC in a humidified atmosphere containing 5% CO_2.

Twenty-four hours before treatment, cells were serum-deprived. After 14 days post-differentiation, cells were exposed to 95% O_2 for up to 24 hours. The cells exposed to hyperoxia were placed in an MIC-101 incubator chamber (Billups-Rosenberg, Del Mar, CA, USA) with an inside concentration of 95% O_2/5% CO_2 at 37ºC. Control cells were cultured in a standard incubator (21% O_2/5% CO_2) at 37ºC. Culture media were collected and stored at -80ºC for further measurements.

Cell viability assay and ROS determination

Cell viability was measured with the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit at 24 h according to manufacturer`s instructions (Cayman Chemical Company, Ann Arbor, USA).

For intracellular and extracellular reactive oxygen species (ROS) concentration determinations, 2',7'-dichlorfluorescein (DCFH) was used following a protocol described elsewhere [15]. Briefly, cells were incubated with 10 mM DCFH for 40 min at 37ºC in 5% CO_2, frozen for at least 1 h at -80ºC, and then lysed with 1000 µl lysis buffer (150 mM NaCl, 0.1% Triton, and 10 mM Tris). Finally, 200 µl of each lysate were loaded on a 96-well black plate. For extracellular ROS determinations, after treatment 300 µl of culture media of each sample were also incubated with 10 mM DCFH at 37ºC in 5% CO_2 for 40 min, frozen for at least 1 h at -80ºC, and then 200 µl of this incubation mix was loaded on to a 96-well black plate. Finally, fluorescence intensity was measured using a POLARstar spectrofluorometer plate reader (BMG Labtechnologies, Offenburg, Germany) at an excitation wavelength of 485 nm and at an emission of 530 nm.

Measurement of metabolic markers

The glucose (HK-CP kit; Horiba, Montpellier, France), lactate (ABX Diagnostic, Montpellier, France) and glycerol (GLY 105; Randox Laboratories, Antrim, UK) concentrations were measured from culture medium samples with a PENTRA C200 auto-analyzer (Horiba, Montpellier, France) after the 24 h treatment. The adipocyte glucose uptake was estimated by the difference between the content of glucose in the culture media at the beginning and at the end of the experiment.

RT-PCR analysis

Total RNA was isolated from all samples using Trizol (Invitrogen, Paisley, UK), according to the manufacture's instructions. Thus, purified total RNA from adipocytes was then treated with DNAse (DNAfree kit; Ambion Inc., Austin, USA) and used to generate cDNA with M-MLV reverse transcriptase (Invitrogen, Paisley, UK). Real-time PCR was performed in an ABI PRISM 7000 HT Sequence Detection System (Applied Biosystems, California, USA). Taqman probes for mouse HIF-1α, leptin, IL-6, MCP-1, PPAR-γ, adiponectin, and ANGPTL-4 were also supplied by

Applied Biosystems (California, USA). All the expression levels of the target genes studied were normalized by the expression of 18s as the selected internal control, as it demonstrated that no significant changes in its expression were detected after 95% O_2 exposition against control (data not shown). All procedures were performed according to a previous described protocol [16].

Statistical analysis

The results are expressed as mean ± SD. Statistical significance between groups was assessed by U Mann-Whitney test. A probability of $p<0.05$ was set up for determining statistically significant differences. The statistical analyses were performed using SPSS 20.0 (Chicago, USA) and Graphpad Prism 5.0a (San Diego, USA) software for Mac.

RESULTS

Cell viability and ROS

The activity of LDH was determined in the conditioned cell media in order to investigate the potential cytotoxicity of the applied treatment. The 24 h hyperoxic treatment did not decrease significantly cell integrity with respect to control (Fig. 1). Furthermore, intracellular and extracellular ROS secretions were both significantly increased by the hyperoxia exposure (Fig. 2a, b). Both ROS measurements were corrected by cell viability.

Culture media determinations

Glucose uptake, lactate production, and glycerol release of isolated adipocytes from both groups were determined after the 24 h treatment. Glucose uptake as well as lactate production were significantly reduced ($p<0.05$ and $p<0.01$, respectively), while glycerol release was significantly increased ($p<0.01$) in the hyperoxia group when compared to the control one (Fig. 3). These results were corrected by cell viability.

Gene expression

Regarding gene expression, a significant increase in the expression of IL-6, MCP-1 and PPAR-γ ($p<0.05$, $p<0.01$ and $p<0.01$, respectively) and a significant decrease in the expression of ANGPTL4 ($p<0.01$) were observed. No statistically

significant differences were found in HIF-1α, leptin and adiponectin mRNA expression between the experimental groups (Fig. 4).

DISCUSSION

It is widely recognized that obese subjects often present a low-degree chronic inflammation within the adipose tissue [27, 33]. Furthermore, it has been reported that this tissue is under chronic hypoxic conditions during the development of obesity [22, 48], although a situation of decreased adipose tissue oxygen tension in obese compared to lean men may not always occur [18], which would be explained by a lower adipose tissue oxygen consumption. This hypoxic state is linked to an increase in pro-inflammatory cytokine release in adipocytes, while anti-inflammatory adipokines such as adiponectin are often decreased [60]. In the current study, the effects of hyperoxia in adipocytes were investigated as a potential approach trying to counteract the hypoxic state within an excessively expanded adipose tissue.

Several studies have reported a positive relationship between low oxygen concentrations and cell death in adipocytes and other cell types [61, 64]. In our experiment, 3T3-L1 adipocytes incubated under hyperoxic conditions (95% O_2) did not show a significant decrease or increase in cell death. However, an enhanced generation of both intracellular and extracellular ROS was observed after a 24-hour exposure. This finding seems to be consistent with another study that found an increase of ROS production in 3T3/J2 fibroblasts exposed to HBOT [8], where it was suggested that intracellular ROS generation could be directly related to cell apoptosis.

In adipocytes, it has been reported that low oxygen concentrations induce an increase on lipolysis [61]. In our study, hyperoxia also increased glycerol release, an episode that perhaps may lead to pro-inflammatory responses inducing ROS production, as it has been shown in other studies [53]. Furthermore, Yin et al. [61] demonstrated that hypoxia increases glucose metabolism in adipocytes through both insulin–dependent and -independent pathways. In our experiment, glucose uptake was significantly inhibited by hyperoxic conditions compared to control. Thus, decreased glucose utilization in hyperoxia would be expected to result in a lower production and release of lactate, as it was observed in the present results. This seems to go in the same direction as some studies have observed in blood and other tissues [19, 34, 47].

A possible explanation for this finding could be that an excessive amount of oxygen might induce the adenosine-5'-triphosphate production fully through mitochondrial respiration, leading to suppression of the anaerobic pathway and therefore resulting in a minor lactate release. In obesity, it has been found that adipocyte-derived lactate by the hypoxic state may constitute another link between this disease and its associated pathologies [40]. In this sense, hyperoxia might decrease lactate release in hypoxic adipocytes and, therefore, ameliorate some obesity-associated complications.

Several groups have investigated the effects of normobaric (NBOT) and hypobaric (HBOT) oxygen therapy in both, animal and cell culture models, regarding the expression of some inflammatory genes. Thus, Desmarquest et al. [9] observed a decrease in TNF-α, IL-1β and IL-6 expression in alveolar macrophages exposed to 48 h hyperoxia. This effect seems to be consistent with HBOT studies carried out in *ex vivo* cell cultures. In this context, macrophages were isolated from patients with Crohn's disease treated with 90 min of HBOT, secreting less IL-1, IL-6 and TNF-α than cells obtained prior to the treatment [54]. Moreover, Lahat et al. [30] also observed a decrease in the secretion of TNF-α in macrophages of rats exposed to HBOT for 90 min. Furthermore, in a study by Benson et al. [3], IL-1β and TNF-α synthesis in macrophages was inhibited by a 90-min HBOT exposure. All these findings are also in agreement with studies demonstrating that HBOT attenuates pro-inflammatory cytokine production in animal models of systemic inflammation. Thus, Yamashita and Yamashita [57] observed that HBOT reduced inflammatory cytokine induction by improving liver ischemia. In another study, TNF-α levels were reduced in rats after the treatment of an inflammatory state with HBOT [32]. Furthermore, Yang et al. [58] also demonstrated the inhibition of TNF-α production in a rat model of intestinal injury treated with HBOT. However, significant increases in the expression of IL-6, IL-1 and TNF-α have been also reported [1, 25, 26, 41, 49]. Interestingly, these effects were not evident until the animals were treated for at least 48 h of hyperoxia, suggesting that inflammation is dependent on the duration of the oxygen exposure. Overall, these studies suggest that oxygen availability may improve oxygen utilization by body organs.

In adipose tissue, there is no data apparently available regarding the effect of hyperoxia on gene expression of isolated adipocytes. However, in adipose cells exposed to hypoxia, it has been shown that pro-inflammatory adipokines are

increased, while anti-inflammatory adipokines are decreased [28, 60, 62]. In our study, mRNA levels of pro-inflammatory markers IL-6 and MCP-1 were upregulated by hyperoxia. In this context, it has been demonstrated that lipolysis can produce inflammatory responses in endothelial cells [52]; it is therefore possible that FFA may induce the expression of pro-inflammatory markers in 3T3-L1 adipocytes. Interestingly, a strong correlation between MCP-1 mRNA expression and ROS release was found, which is in accordance with other observations that showed ROS production could increase MCP-1 expression [42]. It is known that IL-6 suppresses adiponectin gene expression [7, 12, 51]. However, mRNA levels of this anti-inflammatory adipokine did not show a significant change. Adiponectin is also an important selective controlled modulator of insulin sensitivity and it has been demonstrated that its expression is enhanced by PPAR-γ [46]. In our experiment, we could observed an increase of PPAR-γ mRNA expression and perhaps this may prevent the decrease of adiponectin expression by IL-6. Indeed, PPAR-γ is a transcription factor preferentially expressed in adipose tissue [37] and it is known that its activation, as we have observed in adipocytes exposed to hyperoxia, could improve insulin resistance [46]. HIF-1α leptin are important regulators of hundreds of target genes involved in several biological functions, such as cellular metabolism, cell growth and apoptosis and restoration of the oxygen supply [44, 45]; nevertheless, their expression was not modified by hyperoxia. Finally, ANGPTL4 is a gene involved in glucose and lipid metabolism, mainly involved in the regulation of plasma triacylglycerides metabolism by inhibition of LPL [35]. It has been observed that hypoxia stimulates its expression and secretion in human adipocytes [17]. In contrast, a significant decrease in ANGPTL4 mRNA expression was observed in mouse adipocytes exposed to hyperoxia. This outcome proves that oxygen negatively regulates ANGPTL4 mRNA expression in adipocytes. Yamada et al. also demonstrated a down-regulation of ANGPTL4 induced by insulin [56], suggesting the possibility that elevated ANGPTL4 expression might be involved in hypertriglyceridemia in insulin resistant states within 3T3-L1 cells. Thus, a down-regulation in ANGPTL4, as it occurs with hyperoxia, might contribute to ameliorate these metabolic disorders.

In summary, the current study shows for the first time the effect of hyperoxia on 3T3-L1 adipocytes. The exposure to 95% O_2 seemed to provoke an inflammatory

response due to the release of ROS and the upregulation of pro-inflammatory adipokines such as IL-6 and MCP-1. Perhaps, these toxic effects were produced by the high amount of oxygen used. On the other hand, hyperoxia may play an indirect role in the improvement of insulin sensitivity, due to the upregulation of PPAR-γ gene expression, as well as a possible modulation of both glucose and lipid metabolism. A possible explanation for these contradictory effects might be that hyperoxia could have a beneficial effect on glucose metabolism, which may contribute to understand and treat the inflammatory processes associated to obesity.

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Author disclosure statement

All the authors declare that there are no competing financial interests regarding the contents of this article.

Figure 1.

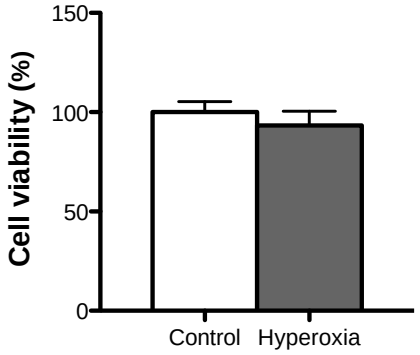


Figure 2.

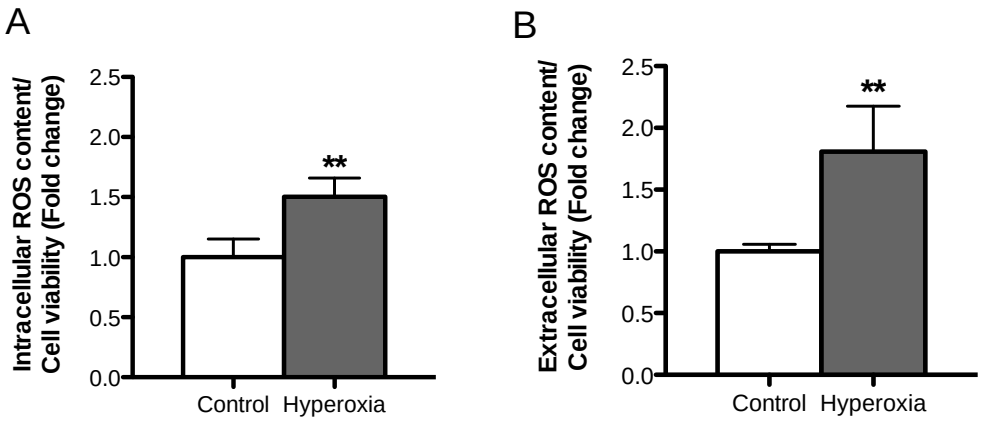


Figure 3.

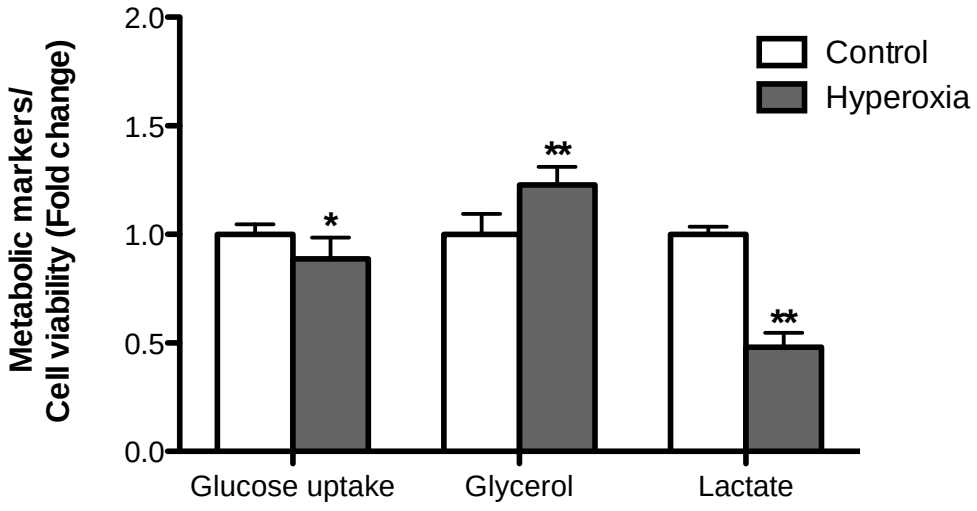
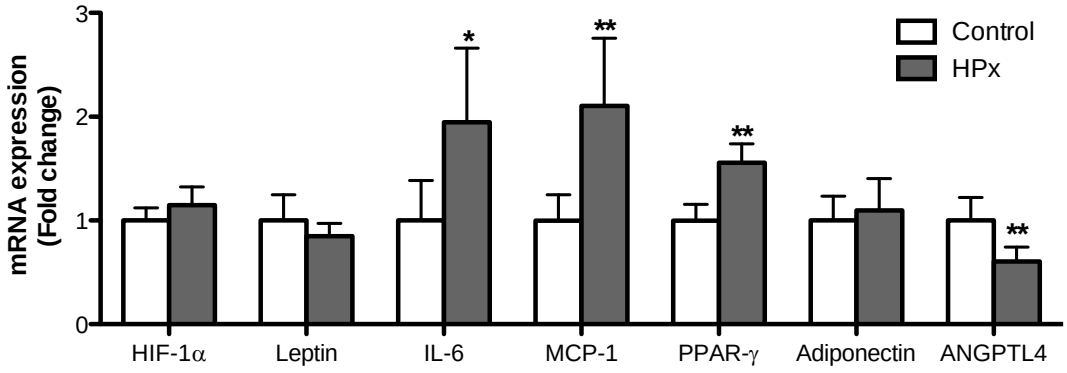


Figure 4.


mRNA expression
(Fold change)
3
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Control
HPx
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HIF-1α
Leptin
IL-6
MCP-1
PPAR-γ
Adiponectin
ANGPTL4

Figure 1. Cellular integrity in control and treated (95% oxygen) 3T3-L1 adipocytes (14 days post-differentiation) was measured at 24 h. White bars show control group and grey bars hyperoxia group. Data (n=6) are expressed as mean ± SD. U Mann Whitney test was performed to identify statistical effects.

Figure 2. Intracellular and extracellular ROS content in control and hyperoxia treatments at 24 h in 3T3-L1 adipocytes (14 days post-differentiation). White bar shows control group and grey bar hyperoxia group. Data (n=6) are expressed as mean ± SD. U Mann Whitney test was performed to identify statistical effects. **P<0.01 vs untreated cells.

Figure 3. Effect of 95% oxygen exposure on glucose uptake, glycerol and lactate release in 3T3-L1 adipocytes (14 days post-differentiation). White bars show control group and grey bars hyperoxia group. Data (n=6) are expressed as mean ± SD. U Mann Whitney test was performed to identify statistical effects. *P<0.05 and **P<0.001 vs untreated cells.

Figure 4. Gene expression analysis of HIF-1α, leptin, IL-6, MCP-1, PPAR-γ, Adiponectin and ANGPTL4 at 24 h of 95% oxygen treatment in 3T3-L1 adipocytes (14 days post-differentiation). White bars show control group and grey bars hyperoxia group (HPx). A mean value of triplicates was used for relative mRNA level. Data (n=6) are expressed as mean ± SD. U Mann Whitney test was performed to identify statistical effects. *P<0.05 and **P<0.001 vs untreated cells.

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