Influence of different oxygen supply on metabolic markers and gene response in murine adipocytes

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ABSTRACT

Obese subjects often present a low-grade chronic inflammation in the white adipose tissue, which seems to play an important role in the initiation and development of obesity-related diseases. It has been reported that this inflammatory process may be due to a hypoxic state occuring whithin this tissue. Oxygen is used in current medicine as a treatment for several conditions. The aim of this study was to analyze the effects of 95% O_2 on specific metabolic variables and on the expression of some adipokines on murine adipocytes. 3T3-L1 adipocytes were exposed during 48 h to different treatments: 95% O_2 hyperoxia (HPx group), $CoCl_2$ ($CoCl_2$ group), hyperoxia with $CoCl_2$ (HPx+$CoCl_2$ group) and 1%O_2 hypoxia (Hx group). Cell viability, intracellular ROS content, glucose utilization, lactate and glycerol concentrations were measured. Also, mRNA expression of HIF-1α, GLUT-1, ANGPTL4, PPAR-γ, adiponectin, IL-6 and MCP-1 genes was analyzed. Importantly, 95% O_2 decreased cell viability and increased intracellular ROS production. Also, glycerol and lactate release were significantly increased and decreased, respectively, in HPx treated cells. This treatment also provoked a down-regulation of GLUT-1 and ANGPTL-4, while IL-6 and MCP-1 were up-regulated. Exposure to a hyperoxia of 95% O_2 seemed to provoke an inflammatory response in adipocytes. The two hypoxia-inducing conditions ($CoCl_2$ and 1% O_2) produced different outcomes in metabolic measurements as well as in the expression of some genes (GLUT-1, ANPGTL4, PPAR-γ and adiponectin), while it remained similar in others (HIF-1α, IL-6 and MCP-1). Indeed, hyperoxia increased significantly the ROS levels and the lipolytic activity, while it reduced lactate production. In addition to the effects on inflammation, the changes in GLUT-1, ANGPTL4 and PPAR-γ genes let suppose that hyperoxia may be beneficial for the hypertrophied adipose tissues of obese subjects and for improving insulin sensitivity.

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

INTRODUCTION

Obesity is a major metabolic disorder commonly accompanied by the onset and development of other important diseases such as type 2 diabetes, artheriosclerosis and several common cancers (1). These events are often associated with a chronic low-grade inflammation in white adipose tissue. Indeed, is now recognized that obesity can induce an aberrant production of several inflammatory markers such as IL-6, MCP-1 or TNF-α, which seem to play a causative role in the development of obesity-associated insulin resistance and cardiovascular disorders (2, 3).

In this context, in 2004 it was reported that hypoxia occurs in adipose tissue in obesity (4). Further investigations have corroborated this hypothesis, demonstrating a lower partial pressure of O_2 in adipose tissue in the state of obesity in rodents and humans (5, 6). Indeed, white adipose tissue expansion in obesity appears to impair the growth of adipocytes in size and number, resulting in an increase distance to the vasculature and a reduction of O_2 tension whithin this tissue. In the following years, this adipose tissue hypoxia has been proposed as a possible factor that could play an important role in promoting inflammatory mechanisms in this tissue (7).

In this context, O_2 is used in current medicine as a treatment for several conditions (8, 9), as well as ozone (O_3) used under controlled conditions (10). Thus, normobaric and hyperbaric oxygen therapies (NBOT and HBOT, respectively) are important techniques under continuous investigation. Studies in animals have demonstrated that the treatment with hyperoxia might produce beneficial effects in different metabolic disorders, such as protecting the rat brain tissue against ischemia reperfusion injury (11), reducing severity of colitis (12) or ameliorating hemorrhagic shock-induced renal failure by decreasing intrarenal hypoxia and improving renal functions (13). Regarding inflammation, several studies have shown that hyperoxia can decrease the expression of some pro-inflammatory genes in different organs and cell types (14, 15). Some investigations have also demonstrated that HBOT attenuates pro-inflammatory cytokine production of systemic inflammation in animal models (16, 17). These findings are in agreement with HBOT studies carried out in *ex vivo* cell cultures (15, 18).

Taking all these studies into account, where high O_2 levels seem to produce beneficial effects on inflammatory markers, it was hypothesized that hyperoxia could be used as a

specific therapeutic intervention for the improvement of obesity state (19). To our knowledge, there are no available data regarding the effects of a hyperoxic treatment on murine adipocytes. Therefore, in this study we investigated the effects of 95% of O_2, alone or in combination with cobalt chloride (as a hypoxia-generating agent), in the expression of some genes and in the regulation of specific metabolic variables in hypoxic adipocytes. This research was designed in order to understand the possible role and mechanisms of hyperoxia in combating low O_2 tension in the adipose tissue of obese patients.

MATERIAL AND METHODS

Cell culture

This research was carried out in 3T3-L1 mouse preadipocytes, that were cultured with Dulbecco's minimal essential media (DMEM) containing 4.5 g/L glucose and supplemented with 10% calf bovine serum. 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, 1 μM dexamethasone 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 2-3 days with FBS without any hormones until 14 days after differentiation induction, when between 80 and 90% of the cells exhibited the adipocyte phenotype. Also, 100 units/ml penicillin and 100 μg/ml streptomycin were added to all media. Cells were always maintained in a CytoGROW GLP incubator (Sanyo, San Diego, CA, USA) at 37ºC in a humidified atmosphere containing 5% CO_2.

Treatments

After 14 days post-differentiation, adipocytes were exposed to four different experimental treatments: environmental hyperoxia with 95% O_2/5% CO_2 (HPx group), $CoCl_2$ (a hypoxia mimetic which stabilizes HIF-1α (20)). Besides, another group with environment hypoxia (1% O_2) was added to compare whether $CoCl_2$ could mimic the effects produced by real hypoxia.

at a concentration of 100 μM ($CoCl_2$ group), hyperoxia with $CoCl_2$ (HPx+$CoCl_2$ group) and ambient hypoxia with 1% O_2/94% N_2/5% CO_2 (Hx group). Each of these treatments

was generated in sealed chambers (Billups-Rosenberg, Del Mar, CA, USA) at 37ºC during 48 h. Non-treated cells placed in a standard incubator with 21% O_2/5% CO_2 were used as reference (Control group). After the experimental treatments, 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 48 h according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, USA).

To determine intracellular ROS concentration, 2',7'-dichlorofluorescein (DCFH) was used following a protocol described elsewhere (21). This protocol, which measures H_2O_2 production, is commonly used as a surrogate of ROS generation (22). 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 and fluorescence intensity was measured using a Fluoroskan Ascent FL (Thermo Fisher Scientific, Waltham, MA) 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 48 h treatment. The adipocyte glucose utilization was estimated by the difference between the content of glucose in the culture media at the beginning and at the end of the experiment, as previously described (21).

RT-PCR analyses

Total RNA was isolated from all samples using Trizol according to manufacturer's instructions (Invitrogen, Paisley, UK). 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, GLUT-1, ANGPTL4, PPAR-γ, adiponectin, IL-6 and MCP-1 were also supplied by Applied Biosystems (California, USA). Moreover, it has been previously established that hypoxia can alter mRNA expression levels of some standard genes (23). Therefore, we considered necessary to characterize the suitability of various housekeeping genes to serve as internal RNA controls. Thus, the expression of 18s, cyclophilin, RNA polymerase II and 28s was determined, the latter being selected as the internal control because it demonstrated no significant expression changes after different treatments (data not shown). All procedures were performed according to a previously described protocol (24).

Statistical analysis

All data was evaluated using the Kruskal-Wallis test. When interaction was detected, the U Mann-Whitney test was used for group comparisons. All results are expressed as mean ± standard deviation (SD). Overall, 6 cell culture experiments were performed and the reported data come from a representative experiment with n = 6 as stated in all figures. Association analyses for selected biochemical markers and gene expression values were performed using the Spearman test. A probability of $p<0.05$ was set up for determining statistically significant differences. The statistical analyses were performed using SPSS 15 (Chicago, USA) and GraphPad Prism 5.0 (San Diego, USA) software.

RESULTS

Cell viability and ROS assessments

The activity of LDH was determined in the conditioned cell media in order to investigate the potential cytotoxicity of the applied treatments. As shown in Fig. 1, cell viability of 3T3-L1 adipocytes significantly decreased with both hyperoxia and hypoxia treatments. However, $CoCl_2$ did not show differences when compared to the control group and seemed to be protective against cell death when cells were treated with hyperoxia.

FIGURE 1

Furthermore, intracellular ROS production, corrected by cell viability, was significantly increased only by the hyperoxia exposure, while the other treatments did not show differences when compared to control (Fig. 2).

FIGURE 2

Culture media determinations

Glucose utilization, lactate production, and glycerol release of isolated adipocytes were determined after the 48 h treatment and were corrected by cell viability. Glycerol production was enhanced by both hyperoxia and hypoxia treatments, while it was significantly decreased by $CoCl_2$ (Fig. 3A). Glucose utilization was decreased by the $CoCl_2$ treatment and increased by hypoxia (Fig. 3B). Moreover, there was a significant elevation in lactate release in HPx+$CoCl_2$ and Hx groups, while HPx and $CoCl_2$ treatments significantly decreased it (Fig. 3C). In all three biochemical markers, hyperoxia counteracted the effect of $CoCl_2$. A high association between glucose utilization and lactate release was found ($r=0.607$; $p<0.01$). Moreover, these two biochemical parameters were also strongly correlated with GLUT-1 gene expression ($r=0.486$; $p<0.01$ and $r=0.639$; $p<0.001$, respectively).

FIGURE 3

Gene expression

Regarding gene expression, no changes were observed in HIF-1α mRNA levels in each of the four treatments when compared to control (Fig. 4A). A significant decrease in the expression of GLUT-1 was observed in HPx, while it was up-regulated in HPx+$CoCl_2$ and Hx groups. There was also a down-regulation of ANGPTL4 mRNA when treated with HPx, HPx+$CoCl_2$ and Hx, while no changes were observed with $CoCl_2$. PPAR-γ mRNA expression was down-regulated only by the hypoxia treatment (Fig. 4A). An association between GLUT-1 and glucose utilization ($r=0.9$; $p<0.03$) was found in the HPx group.

To determine whether these treatments induced changes on inflammatory adipokines, mRNA expression of adiponectin, MCP-1 and IL-6 was measured (Fig. 4B). Thus, adiponectin expression was down-regulated by Hx and HPx+$CoCl_2$, but no changes were observed with HPx and $CoCl_2$ treatments separately, showing that these two factors could provoke different situations if they act together. Finally, MCP-1 and IL-6 mRNA expression were both up-regulated by HPx. Unexpectedly, these two proinflammatory adipokines were down-regulated by the Hx group. $CoCl_2$ treatment decreased the expression of MCP-1, but did not change IL-6 expression. In this two proinflammatory adipokines, HPx seemed to revert the effect of $CoCl_2$, as it can be observed in the HPx+$CoCl_2$ group. Interestingly, a highly positive correlation between IL-6 and MCP-1 mRNA levels and glycerol release was found ($r=0.864$; $p<0.001$ and $r=0.845$; $p<0.001$, respectively) considering C and HPx groups. Furthermore, associations between the expression of PPAR-γ with HIF-1 ($r=0.597$; $p<0.001$) and IL-6 ($r=0.577$; $p<0.001$) were found.

FIGURES 4A and 4B

DISCUSSION

The adipose tissue becomes poorly oxygenated in obese subjects leading to inflammatory processes and metabolic syndrome features in these subjects (7), although a decrease in adipose tissue O_2 tension may not always occur (25). In the current trial, the effects of hyperoxia in chemical-hypoxic adipocytes were investigated an approach to counteract the hypoxic alterations that often occur in an expanded adipose tissue.

The 48-h hyperoxia exposure produced a significant cell death respect to control. In other experiment of our group, a 24-h exposure did not cause significant differences in cell death (26). Thus, cell damage caused by hyperoxia exposure could be related to the duration of the treatment. On the other hand, $CoCl_2$ did not affect cell viability. Interestingly, $CoCl_2$ has been observed to be protective to cell death in some conditions (27). In this experiment, $CoCl_2$ also reverted the cell death of adipocytes induced by hyperoxia. Furthermore, 1% O_2 hypoxia significantly decreased this variable as previously reported (28).

The enhanced generation of intracellular ROS by hyperoxia may lead to the cell death observed (29). Nevertheless, the HPx+$CoCl_2$ group did not show significant differences on intracellular ROS release, being consistent with the cell viability result. Although it has been established that both environmental and chemical hypoxia increase ROS generation in adipocytes (30, 31), no differences in intracellular ROS release were found in Hx and $CoCl_2$ treatments. Regarding $CoCl_2$, this observation could be explained by the time and dose used (31).

The Hx group induced an increase on lipolysis, glucose utilization and lactate release, as it has been previously described (28, 32). It is known that in anaerobic conditions the cell is not able to oxidize glucose fully through mitochondrial oxidation and in consequence, there is a lower adenosine-5'-triphosphate (ATP) production with respect to respiration. To compensate this lack of ATP, cells may increase their glucose uptake. This mechanism could explain the response of glucose utilization observed with hyperoxia. Probably, a higher O_2 availability might induce the ATP production mainly through the aerobic metabolism and therefore, no compensatory pathways need to be activated. This outcome seems to be in accordance with the significant reduction observed in lactate production in hyperoxic conditions, and goes in the same direction as other investigations observed in other tissues (33). 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 (32). In this sense, since hyperoxia decreased lactate release in adipocytes, it might ameliorate some obesity-associated complications. Furthermore, a high association between glucose utilization and lactate release was found, confirming the relationship of these two metabolic pathways with O_2 tension. Contrary to expectations, the hypoxic mimetic $CoCl_2$ did not follow the tendency observed with 1% O_2 on these biochemical parameters.

It is known that glucose uptake and GLUT-1 mRNA expression are increased in hypoxia (28, 34), as we observed in our experiment. Under hyperoxic conditions, GLUT-1 expression was significantly decreased by adipocytes. Interestingly, the expression of this glucose transporter did not change with the occurrence of $CoCl_2$, although it was significantly increased when both conditions were placed together (HPx+$CoCl_2$ group). An association of the expression of this gene with glucose utilization was also found, suggesting that a higher O_2 tension might also be able to

participate on glucose metabolism in adipose tissue, as previously observed in muscle cells (35). Furthermore, ANGPTL4 is a gene involved in the regulation of plasma triacylglycerides metabolism (36). Thus, a down-regulation in ANGPTL4, as it occurred with hyperoxia, may contribute to ameliorate some metabolic disorders. The analysis of Hx and $CoCl_2$ groups revealed a decrease in the expression of this gene. Nevertheless, these results should be taken with caution, since they differ from experiments in human adipocytes (37).

Several trials have confirmed that the exposure to high O_2 concentrations seems to produce beneficial effects on inflammatory markers in both, cell culture (15) and animal models (17). Although a significant increase in the expression of these type of cytokines has been also reported (38, 39). Nevertheless, 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 O_2 exposure. Contrary to our initial expectations, mRNA levels of pro-inflammatory markers IL-6 and MCP-1 were up-regulated by hyperoxia. The induction of adipocyte death may be a mechanism by which hyperoxia could contribute to up-regulate the expression of these genes in 3T3-L1 adipocytes. Another possible explanation could be that free fatty acids released by lipolysis might contribute to this inflammatory response, as demonstrated in other cell types (40). This outcome is in accordance with a strong relationship observed in the current experiment between the expression of IL-6 and MCP-1 genes and glycerol release concerning C and HPx groups. Furthermore, the HPx+$CoCl_2$ group increased MCP-1 and IL-6 gene expressions with respect to $CoCl_2$ group, suggesting that hyperoxia could exert a dominant effect in the expression of these genes in hypoxic conditions. Even though hyperoxia provoked the up-regulation of pro-inflammatory adipokines, this treatment did not modify the expression of adiponectin, known to be down-regulated by IL-6 (41). However, since the expression of this adipokine did not change with HPx and $CoCl_2$ independently, it was significantly decreased in the HPx+$CoCl_2$ group, probably due to cell stress produced by the combined effect of both conditions.

Adiponectin is also an important selective controlled modulator of insulin sensitivity whose expression is enhanced by PPAR-γ (42). In our experiment, PPAR-γ mRNA expression tended to increase in hyperoxia and this event may prevent the decrease of adiponectin expression by IL-6. Furthermore, it has been proposed that HBOT could

increase insulin sensitivity in type 2 diabetes patients (43). On the other hand, Hx treatment produced a down-regulation of mRNA levels of PPAR-γ. This outcome is in agreement with the results from Kim et al., who suggested that hypoxic condition attenuates adipocyte differentiation inhibiting PPAR-γ expression by the activation of AMPK, which impairs clonal expansion phase (44).

Contradictory data has been found regarding the effects of $CoCl_2$ and ambient hypoxia. Perhaps the concentration of this hypoxia mimetic (100 μM) was not high enough to produce the same effects in 3T3-L1 mature adipocytes as 1% O_2 did. Since not always mRNA levels and protein secretion are related, data at the protein level could complement the value of this research, which was specifically focused on the transcriptomic effects of O_2 supply in adipocytes.

In summary, to the authors knowledge this is the first study to combine the measurements of metabolic markers and the expression of genes involved in glucose and lipid metabolism as well as the inflammatory response in 3T3-L1 adipocytes exposed to hyperoxia. Indeed, hyperoxia increased significantly the ROS levels and the lipolytic activity, while it reduced lactate production. The changes in genes not involved in inflammation such as GLUT-1 and ANGPTL4 and PPAR-γ let suppose that hyperoxia may be beneficial for the hypertrophied adipose tissues of obese subjects and for improving insulin sensitivity.

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CONFLICT OF INTERESTS

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

FIGURE LEGENDS

Figure 1. Cellular integrity in control and treated groups was measured at 48 h. One symbol means $p<0.05$, two symbols $p<0.01$. * Treatment groups vs Control; Δ HPx vs $CoCl_2$; ϕ HPx vs HPx+$CoCl_2$; Ω $CoCl_2$ vs Hx. Data (n=6) are expressed as mean ± SD.

Figure 2. Intracellular ROS content at 48 h in 3T3-L1 adipocytes (14 days post-differentiation). Two symbols mean $p<0.01$. * Treatment groups vs Control; Δ HPx vs $CoCl_2$; ϕ HPx vs HPx+$CoCl_2$; ψ HPx vs Hx. Data (n=6) are expressed as mean ± SD.

Figure 3. Effect of different treatments on glucose utilization, glycerol and lactate release in 3T3-L1 adipocytes (14 days post-differentiation). One symbol means $p<0.05$, two symbols $p<0.01$. * Treatment groups vs Control; Δ HPx vs $CoCl_2$; ϕ HPx vs HPx+$CoCl_2$; # $CoCl_2$ vs HPx+$CoCl_2$; ψ HPx vs Hx; Ω $CoCl_2$ vs Hx. Data (n=6) are expressed as mean ± SD.

Figure 4. (A) Gene expression analysis of HIF-1α, GLUT-1, ANGPTL4 and PPAR-γ and (B) of inflammatory genes IL-6, MCP-1 and adiponectin at 48 h in 3T3-L1 adipocytes (14 days post-differentiation). Two symbols mean $p<0.01$. * Treatment groups vs Control; Δ HPx vs $CoCl_2$; ϕ HPx vs HPx+$CoCl_2$; # $CoCl_2$ vs HPx+$CoCl_2$; ψ HPx vs Hx; Ω $CoCl_2$ vs Hx. Data (n=6) are expressed as mean ± SD.

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