Hypoxia decreases insulin signaling pathways in adipocytes

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ABSTRACT

Objective: Obesity is characterized by an overgrowth of adipose tissue that leads to the formation of hypoxic areas within this tissue. We investigated if this phenomenon could be responsible for insulin resistance by studying the effect of hypoxia on insulin signaling pathway in adipocytes.

Research design and Methods: Hypoxic signaling pathway was modulated in adipocytes from human and murine origins through incubation under hypoxic condition (1% O2) or modulation of HIF expression. Insulin signaling was monitored through the phosphorylation state of several key partners of the pathway and glucose transport.

Results: In both human and murine adipocytes, hypoxia inhibits insulin signaling as revealed by a decrease in the phosphorylation of insulin receptor. In 3T3-L1 adipocytes, this inhibition of insulin receptor phosphorylation is followed by the decrease in the phosphorylation state of PKB and AS160, as well as an inhibition of glucose transport in response to insulin. These processes were reversible under normoxic conditions. The mechanism of inhibition seems independent of protein tyrosine phosphatases activities. Overexpression of HIF-1α or HIF-2α, or activation of HIF transcription factor with CoCl2 mimicked the effect of hypoxia on insulin signaling while downregulation of HIF-1α and HIF-2α by siRNA inhibited it.

Conclusion: We have demonstrated that hypoxia creates a state of insulin resistance in adipocytes which is dependent upon HIF transcription factor expression. Hypoxia could be envisioned as a new mechanism which participates in insulin resistance in adipose tissue of obese patients.
Obesity results from an imbalance between energy intake and energy expenditure. Abdominal obesity and adipose tissue dysfunction are major risk factors for chronic diseases, such as insulin resistance, type 2 diabetes and cardiovascular diseases. Insulin resistance is associated with alterations in glucose and lipid homeostasis. At the molecular level, insulin resistance is triggered by a dysregulation of the insulin signaling cascade. Insulin stimulates the tyrosine kinase activity of its receptor, leading to tyrosine phosphorylation of its substrates, such as IRS-1 and IRS-2 (Insulin Receptor Substrate) or Shc. They are upstream of two major signaling pathways: PI3K/PKB pathway, responsible for most of the metabolic actions of insulin, and the Ras-ERK pathway that regulates gene expression (1).

During the genesis of obesity, adipose tissue is one of the first tissues affected by insulin resistance. This phenomenon is closely associated with the development of a pro-inflammatory state within the adipose tissue. In addition to this pro-inflammatory state, obesity is associated with the formation of hypoxic areas within the tissue. This has been demonstrated in obese mice (ob/ob and dietary induced obesity) using various methods such as immunohistochemistry with pimonidazole, use of oxygen sensor probe and lactate detection (2-4).

Hypoxia, a deficiency in oxygen, is a major stimulus affecting a number of biological functions such as, angiogenesis, cell proliferation, apoptosis, inflammation and switches cell metabolism from aerobic respiration to anaerobic glycolysis (5-7). Hypoxia mediates its effect through the activation of HIF (Hypoxia Inducible Factor) a basic helix-loop-helix transcription factor composed of two subunits, HIFα and HIFβ. While HIFβ is constitutively expressed, HIFα protein level is regulated. In presence of oxygen, HIFα is subjected to proline hydroxylation leading to degradation by the proteasome. Hypoxia inactivates prolyl-hydroxylases leading to HIFα accumulation and formation of a functional heterodimeric transcription factor. Two α subunits, HIF-1α and HIF-2α, show similarities in structure and regulation, but they regulate distinct sets of genes, and are not redundant (5; 7; 8). HIF-1α and HIF-2α expression are also regulated by oxygen independent mechanisms, since growth factors and cytokines stimulate HIF-1α and HIF-2α protein synthesis via PI3K or ERK pathways (9-12). Since hypoxia produces profound changes in cell metabolism, we investigated its effect on insulin signaling.

In the present study, we demonstrated that hypoxia creates an insulin resistant state in adipocytes by inhibiting the insulin receptor tyrosine phosphorylation leading to a decrease in glucose transport. This phenomenon could contribute to the development of insulin resistance within the adipose tissue.

**RESEARCH DESIGNS AND METHODS**

**Materials.** Insulin was obtained from Lilly (Paris, France). Antibody to HIF-1α (clone H1α67) and HIF-2α were purchased from Novus Biologicals, Inc. (Littleton, CO). Antibodies to GLUT-1 and HIF-2α were obtained from Abcam (Paris, France). Antibodies to phosphotyrosine,
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phospho-S6K-1, pThrPKB, PKB, and GLUT-4 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibody to S6K-1 and IRβ, and siRNA (control, HIF-1α and HIF-2α) were purchased from Santa Cruz Biotechnology, Inc. (Tebu, France). Polyclonal IRS-1 and IRS-2 antibodies used in immunoprecipitation experiments were raised against a peptide corresponding to the last 14 amino acids of IRS-1 and a peptide corresponding to the last 16 amino acids of IRS-2 (Eurogentec, Seraing, Belgium). Polyclonal antibody directed against phospho-Ser632 IRS-1 has been described previously (13). Monoclonal anti-IRS-1 antibody used in immunoblotting experiments was purchased from BD Biosciences (PharMingen, San Diego, CA). Antibody to tubulin was purchased from Sigma-Aldrich. Culture media were from Invitrogen. DNA Plasmids. pEGFP-C1-HIF-1α-GFP has been described previously (10). pcDNA3-HIF-2α cDNA was obtained from Steve McKnight and Richard Bruick (University of Texas Southwestern Medical Center) (14).

Cell Culture. Human embryonic kidney cells (HEK-293) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% (v/v) fetal calf serum. 3T3-L1 fibroblasts were grown at 7% CO2 and 37°C in DMEM supplemented with 10% (v/v) calf serum and induced to differentiate as previously described (15). Human preadipocytes were obtained from Biopredic (Rennes, France), were grown and induced to differentiate as previously described (16). Transfection. HEK-293 cells were transiently transfected using FuGENE 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN), according to manufacturer’s instructions. SiRNA were transfected using INTERFERin siRNA transfection reagent according to manufacturer conditions (Polyplus Transfection, Ozyme). Briefly, differentiated 3T3-L1 adipocytes were trypsinized and seeded in 12-wells plates. After 24 h, siRNA (50 nM) directed against HIF-1α or HIF-2α were transfected using INTERFERin and used 48 h after transfection.

Hypoxia treatment. For hypoxic incubations, medium was replaced with DMEM containing 0.5% BSA (w/v) and adipocytes were incubated at 37°C in 95% air and 5% CO2 (normoxic conditions) or placed in a hypoxic chamber (Billups-Rothenberg, Dell Mar, USA) flushed for 10 min with gas mixture consisting of 1% O2, 5% CO2, 94% N2 (hypoxic conditions). Cells were incubated for 16 h at 37°C. Following hypoxic incubation, the chambers were opened in an anaerobic glove box (flushed with N2) to avoid reoxygenation. Cells were stimulated with ligands and cell lysates were prepared. Absence of cytotoxicity was assessed by measuring lactate deshydrogenase activity according manufacturer conditions (Roche). For short term hypoxia experiments, media were flushed with N2 within the anaerobic glove box, in order to remove oxygen from the medium prior hypoxic incubation.

Western Blot Analysis. Adipocytes were resuspended in lysis buffer (50 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM Na4P2O7, 100 mM NaF, 2 mM vanadate, protease inhibitor cocktail (Complete, Roche) 1% (v/v) Triton X-100) and immediately frozen in liquid
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Lysates were centrifuged (14,000 rpm) for 10 min at 4°C and protein concentration was determined using BCA protein assay reagent (Pierce). Cell lysates were either directly analyzed by western blot or were subjected to immunoprecipitation. Immunoblottings were performed as previously described (10), and were revealed using Fujifilm LAS-3000 imaging system. Quantifications were realized using MultiGauge software.

Measurement of ROS (Reactive Oxygen Species). 3T3-L1 adipocytes were incubated for 30 min with 10 μM DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) (Molecular Probes) in phosphate buffered saline. DCFH-DA was removed and adipocytes were incubated in normoxia or in hypoxia. After washes, adipocytes were sonicated in water. The fluorescence was determined at 485/520 nm and normalized to protein concentration.

Glucose transport. 3T3-L1 adipocytes were incubated for 16 h in normoxia or in hypoxia and were stimulated with 100 nM insulin for 20 min in normoxia or in hypoxia. Glucose transport was determined by the addition of 2-[3H]deoxyglucose (0.1 mM, 0.5 μCi/ml) as previously described (15).

Glycerol release, IL-1β and IL-6 measurement. Glycerol was measured in cell culture media using free glycerol reagent (Sigma-Aldrich, France) and IL-1β and IL-6 were measured in cell culture media using RayBio ELISA kit (Tebu, France) according to the manufacturer’s instructions.

Protein tyrosine Phosphatase (PTPase) activity. Adipocytes were incubated in normoxia or hypoxia and were homogenized inside an anaerobic glove box (17). Insulin stimulation, cell homogenization and PTPase assay were performed in anaerobic conditions to avoid any air oxidation. Cell were lysed in PTPase lysis buffer (150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM Hepes, pH 7.5 containing protease inhibitor cocktail (Complete, Roche) and 1% (v/v) Triton X-100) followed by a centrifugation at 14,000 rpm for 15 min. PTPase activity was determined at 30°C for 30 min in a reaction buffer containing 20 mM para-nitrophenyl phosphate in 50 mM Tris, (pH 7.5) 50 mM NaCl, 3mM DTT, and the absorption was determined at 410 nm (18). Positive control was performed using purified CIP (calf intestine phophatase).

Statistical analysis. Statistical differences between groups were analyzed by Student’s t-test and were considered significant when $p \leq 0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

RESULTS

Hypoxia inhibits insulin signaling pathway in human adipocytes and 3T3-L1 adipocytes. Since obesity is associated with the formation of hypoxic areas within the adipose tissue, we investigated the effect of hypoxia on insulin signaling pathway in adipocytes. Murine 3T3-L1 adipocytes were incubated in normoxia or hypoxia (1%) for 16 h before being stimulated with insulin for 5 min. 1% oxygen is a condition similar to the one found in the adipose tissue of obese mice (15.2 mmHg, 1-2% O2) (2; 3). Hypoxia induced an increase in HIF-1α and HIF-2α protein level in 3T3-L1 adipocytes (Figure 1A). In parallel, hypoxia inhibited the ability of insulin to stimulate the autophosphorylation of its receptor, as well as the phosphorylation of PKB (Figure 1B). 3T3-L1 adipocytes
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were incubated for 16 h in normoxia or in hypoxia before being stimulated with decreasing amount of insulin (ranging from 100 nM to 0.01 nM). The inhibition of insulin-induced insulin receptor phosphorylation by hypoxia was observed at maximal and submaximal insulin concentration. Moreover, hypoxia inhibited S6K phosphorylation in response to insulin (Figure 1C).

Specific immunoprecipitation of IRS-1 or IRS-2 revealed that hypoxia inhibited IRS-1 and IRS-2 tyrosine phosphorylation (Figure 1D). Since serine phosphorylation of IRS-1 is implicated in the inhibition of its tyrosine phosphorylation and in insulin resistance (19), we investigated the level of IRS-1 serine phosphorylation. Hypoxia inhibited the phosphorylation of serine 632 residue in response to insulin (Figure 1D). No effect of hypoxia on the phosphorylation on serine 307 and 789 was detected (data not shown). It is to be noted that hypoxia did not induce cell toxicity, as measured by lactate dehydrogenase activity, nor caspase 3 activity (data not shown). Since general tissue oxygenation is around 50 mmHg (7% O2), the ability of 3T3-L1 adipocytes to respond to insulin treatment after incubation in 21%, 7% or 1% O2 was examined. Adipocytes incubated in 21% or 7% O2 responded similarly to insulin stimulation (Online Appendix 1).

Hypoxia also inhibited insulin signaling pathway in human adipocytes (Figure 1E). Indeed, human adipocytes were incubated in normoxia (21% O2) or in hypoxia (1% O2) for 24 h, and stimulated with insulin for 5 min. Hypoxia stimulated HIF-1α and HIF-2α protein expression, and inhibited insulin-induced insulin receptor and IRS tyrosine phosphorylation. In conclusion, in both human and murine adipocytes, hypoxia inhibited the ability of insulin to induce the phosphorylation of its receptor, as well as IRS-1, IRS-2, PKB and S6K.

Inhibition of insulin induced-insulin receptor tyrosine phosphorylation by hypoxia is rapid and reversible. The time course of hypoxia-induced inhibition of insulin receptor phosphorylation was evaluated by incubating 3T3-L1 adipocytes in normoxia or in hypoxia, for 1, 4, 8 and 16 h before being stimulated with insulin for 5 min. As shown in figure 2A, the inhibitory effect of hypoxia on insulin receptor tyrosine phosphorylation was detected as soon as 1 hour. These effects were more pronounced after 8 h of hypoxia. These observations demonstrate that hypoxia rapidly regulated the inhibition of insulin signaling pathway.

To determine whether the effect of hypoxia is reversible, 3T3-L1 adipocytes were incubated in hypoxia for 16 h. Hypoxic adipocytes were stimulated directly after hypoxia or were reoxygenated for 15 and 45 min before insulin stimulation (Figure 2B). Control adipocytes were maintained in normoxia for 16 h and stimulated with insulin. Hypoxia impaired the ability of insulin to stimulate insulin receptor tyrosine phosphorylation, as well as PKB and AS160 phosphorylation. During reoxygenation, the ability of insulin to stimulate phosphorylation of insulin receptor and signaling proteins was restored after 45 min.

Hypoxia inhibits glucose transport. PKB and its substrate AS160 play an important role in insulin-induced GLUT4 translocation and glucose transport. We investigated whether hypoxia inhibited downstream response of insulin, such as
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3T3-L1 adipocytes were incubated in normoxia or in hypoxia for 16 h or reoxygenated for 1 hour. Glucose transport was determined by the captation of \(^{3}\)H deoxyglucose (Figure 3A). In normoxic adipocytes, insulin induced a 10-fold increase in glucose transport. Hypoxia upregulated basal glucose transport, but inhibited insulin-induced glucose transport. Reoxygenation of adipocytes restored the ability of insulin to stimulate glucose entry to level comparable to normoxic adipocytes. In reoxygenated adipocytes, basal glucose transport remained elevated. This increase in basal glucose uptake probably resulted from an increased GLUT1 expression which is maintained even after 1 hour of reoxygenation, without modifying GLUT4 protein level (Figure 3B).

**Hypoxia induces reactive oxygen species (ROS) generation in 3T3-L1 adipocytes.** Insulin resistance has been associated with the generation of reactive oxygen species. We investigated whether hypoxia induced ROS production in 3T3-L1 adipocytes. To this end, we monitored the formation of ROS detected by the oxidation of the 2',7'-dichlorodihydrofluorescein (DCFH) dye (20). Hypoxia stimulated the generation of ROS as soon as 1 hour, which is maintained up to 16 h (Fig. 4A). Formation of ROS was in part inhibited by the anti-oxidant, N-acetyl cysteine (NAC).

**Hypoxia does not inhibit insulin signaling through the activation of protein tyrosine phosphatases.** Attenuation of insulin signaling can occur through the dephosphorylation of the receptor by proteins tyrosine phosphatases (PTPase). PTP1B and LAR have been implicated in insulin receptor dephosphorylation (21). We determined whether hypoxia modulated total phosphatase activity by measuring the dephosphorylation of para-nitrophenyl phosphate (pNPP). Cell homogenization and measurement of phosphatase activities were performed in anaerobic conditions to avoid oxidation and their subsequent inhibition. No significant difference in total phosphatase activity was observed in hypoxia compared to normoxia (Fig. 4B).

To confirm the lack of implication of PTPases in hypoxia-mediated inhibition of insulin pathway, we studied whether vanadate, a potent tyrosine phosphatase inhibitor, could reverse the effect of hypoxia. Adipocytes were incubated in normoxia or in hypoxia in absence or in presence of vanadate for 16 h before insulin stimulation (Fig. 4C). While vanadate increased the level of protein tyrosine phosphorylation in normoxia, due to the inhibition of cellular phosphatases, it did not restore the ability of insulin to stimulate the tyrosine phosphorylation of its receptor in hypoxia. Similar results were obtained with H\(_2\)O\(_2\), which induces oxidation and subsequent inhibition of tyrosine phosphatases (data not shown). These results suggest that hypoxia did not mediate its action through the activation of tyrosine phosphatases activities.

**Hypoxia stimulates lipolysis and IL-6 secretion.** Free fatty acids and adipokines have been shown to be implicated in the development of insulin resistance. Thus, we investigated whether these factors could play a role in the inhibition of insulin signaling pathway by hypoxia. First, we measured the release of glycerol, and of two adipokines IL-1β
Hypoxia inhibits insulin signaling and IL-6 in the conditioned media from normoxic or hypoxic adipocytes. 3T3-L1 adipocytes were subjected to normoxia or hypoxia for 16 h, conditioned media were collected and glycerol (Figure 5A) and IL-1β and IL-6 (Figure 5B) were measured. Hypoxia induced a release of glycerol in the media (48 µg/ml ± 2 in hypoxia compared to 4 µg/ml ± 0.7 in normoxia). Moreover, IL-6 secretion was increased from 0.1 ±0.02 ng/ml in normoxia up to 2.1 ± 0.28 ng/ml after 16 h of hypoxia. In contrast, IL-1β protein level was not modified. Shorter incubations did not allow us to detect IL-6 or IL-1β expression (data not shown).

Then, we investigated whether the inhibition of insulin signaling by hypoxia could be mimicked by conditioned media. 3T3-L1 adipocytes were incubated under hypoxia or normoxia for 16 h, the conditioned medium was collected and incubated with 3T3-L1 adipocytes for 16 h before stimulation with insulin (Figure 5C). The effect of conditioned media was compared to the effect of hypoxia. As previously shown, hypoxia inhibited insulin-induced insulin receptor tyrosine phosphorylation and PKB phosphorylation compared to normoxic conditions. In presence of conditioned media from hypoxic adipocytes, we did not observe a significant decrease in the insulin-induced phosphorylation of insulin receptor or PKB.

These results suggest that although adipokines secretion is dysregulated during hypoxia, they cannot be responsible for the inhibition of insulin signaling induced by hypoxia.

**The effect of hypoxia on inhibition of insulin signaling pathway is mimicked by CoCl2 and HIFα expression.** Hypoxia allows the stabilization of HIFα subunits leading to the activation of HIF transcription factor. CoCl2 is a hypoxia mimicking agent, which promotes HIFα stabilization, HIF activation and expression of hypoxia-induced genes (22). We investigated whether CoCl2 could mimic the effect of hypoxia on insulin signaling pathway. 3T3-L1 adipocytes were treated with CoCl2 for 16 h followed by insulin stimulation. As shown in Figure 6A, CoCl2 induced HIF-2α expression. Concomitantly, CoCl2 decreased the ability of insulin to stimulate insulin receptor and IRS tyrosine phosphorylation without affecting their respective protein levels. In parallel, CoCl2 inhibited the phosphorylation of S6K in response to insulin.

Similar results were obtained when the expression of HIF-1α and HIF-2α was induced by transfection of HIF-1α and HIF-2α into HEK-293 cells before insulin stimulation. Ectopic expression of HIF-1α or HIF-2α subunits led to a decrease in the ability of insulin to stimulate its receptor autophosphorylation compared to control cells (Figure 6B).

All together, these results show that enhanced HIF expression inhibits insulin signaling. **Inhibition of HIF activity restores insulin stimulation of insulin receptor.** Since we showed that HIF expression was sufficient to induce insulin resistance, we investigate if expression of these proteins was necessary for the inhibition of insulin signaling. HIF-1α or HIF-2α expression was inhibited by siRNA transfection in 3T3-L1 adipocytes (Figure 7). The siRNAs were able to partly inhibit (57% ± 3) HIF-1α and HIF-2α expression under hypoxia. This was accompanied by a partial restoration of the ability of
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insulin to stimulate the tyrosine phosphorylation of its receptor.

The use of echinomycin, an inhibitor which has been characterized to inhibit HIF activity without modulating HIFα subunits protein level (23), led also to restore partially the insulin-induced tyrosine phosphorylation of insulin receptor in hypoxia (data not shown). Alltogether, these results show that hypoxia inhibited insulin receptor and IRS tyrosine phosphorylation through mechanisms which are dependent of the HIF transcription factors.

Discussion

Obesity is associated with insulin resistance and type 2 diabetes. Massive development of the adipose tissue leads to the formation of hypoxic area. As adipose tissue expands, some adipocytes become too distant from the vasculature to be correctly oxygenated. Indeed, development of hypoxia in the adipose tissue has been described in several genetic models of obesity in rodents. Partial pressure of O₂ in the adipose tissue decreases from 47.9 mm Hg in lean mice to 15.2 in ob/ob mice (2; 3). In human, existence of hypoxia in the adipose tissue of obese patient is supported by the observation that, although obese patients have more adipose tissue than lean ones, the cardiac output and blood flow directed to adipose tissue is not increased during obesity (24). Moreover, hypoxia-inducible factor-1 (HIF-1), and HIF-1 target genes are over-expressed in the adipose tissue of obese individuals and decreased after weight loss (25). Finally, obesity is associated with hypertrophic adipocytes which size prohibits a correct diffusion of O₂ within the tissue.

In the present study, we propose that this hypoxic status within the adipose tissue could contribute to the development of insulin resistance directly through the inhibition of insulin signaling pathway in adipocytes. In rodent adipocytes, hypoxia induces a rapid and robust inhibition of insulin signaling, visualized by a 50% inhibition of insulin receptor autophosphorylation and nearly complete inhibition of insulin-stimulated glucose transport. Since hypoxia affects the earliest intracellular step of insulin signaling, i.e. insulin receptor autophosphorylation, all subsequent steps such as IRS and PKB phosphorylation and glucose transport were affected. The mechanisms used by hypoxia to inhibit insulin signaling pathway is not fully understood. Using experiments of gain and loss of function, we have shown that the inhibition of insulin signaling by hypoxia is dependent upon HIF-1 and HIF-2 transcription factors. HIF-1 and HIF-2 regulate more than 100 genes implicated in a broad range of cellular responses. HIF-1α and HIF-2α are activated at different oxygen concentration, HIF-1α being more sensitive to oxygen concentration. Although HIF-1 and HIF-2 share common target genes, some specificity in gene expression has been unravelled (5; 7). As downregulation of HIF-1α or HIF-2α counteracts hypoxia-induced insulin resistance, it is likely that proteins involved are common target genes. These target genes are predicted to have a short half life, or to be rapidly inhibited. Indeed, hypoxia-induced insulin resistance is rapidly reversible. Reoxygenation restores insulin response after 45 min as observed by an increase in insulin stimulated insulin receptor
phosphorylation and glucose transport. Interestingly, HIF proteins are not only induced during hypoxia, but have also been reported to be induced by growth factors and cytokines. Since several of these circulating molecules have been shown to interact with insulin signaling, the HIF-mediated inhibition of insulin signalling described here could not be restricted to hypoxia. Moreover, we cannot exclude that HIF proteins mediate their effect independently of their action on transcription.

What could be the molecular mechanisms by which hypoxia mediates insulin resistance? PTPases, which could dephosphorylate the insulin receptor, seem not to be regulated by hypoxia. We show that hypoxia induces the production of ROS, which have been linked to impaired insulin signaling (26), through increase in serine phosphorylation of IRS. We have not detected any increase in the phosphorylation status of IRS-1 serine phosphorylation which has been associated with insulin resistance (Ser307, 789 and 632). Moreover, phosphorylation of these sites has not been reported to affect insulin receptor kinase activity. Hypoxia decreased IRS-1 serine phosphorylation on Ser632 residue, which is a target for mTOR and S6K (19). Under hypoxia, energy expenditure is decreased leading to the inhibition of high energy consuming events such as protein translation leading to the inhibition of mTOR and S6K (27). However, one cannot exclude that ROS could affect insulin receptor tyrosine kinase activity directly or not. Unfortunately, this is particularly difficult to investigate since ROS inhibitors by themselves directly affect insulin receptor tyrosine kinase activity (data not shown and (28)).

In the adipose tissue, hypoxia dysregulates the expression of some adipokines and pro-inflamatory cytokines (2-4; 24; 29; 30) such as leptin, adiponectin, IL-1β and IL6 expression although TNFα expression seems to be insensitive to hypoxia in human adipocytes (29). These cytokines are known to be involved in the downregulation of insulin signaling pathway, and to induce local and systemic insulin resistance (2; 15). Analysis of conditioned media revealed that IL-6 protein secretion was increased by hypoxia, but at concentrations inferior to the one described to inhibit insulin signaling pathway in adipocytes (2 vs 20 ng/ml) (31). Thus, incubation of conditioned media from hypoxic adipocytes for 16 h did not mimic the effect of hypoxia on the inhibition of insulin signaling pathway. This suggests that hypoxia-induced cytokine production by adipocyte is not the mechanism responsible for the inhibition of insulin signaling that we describe here. This is consistent with the observation that during hypoxia and obesity, cytokines are mainly produced by macrophages that invade the adipose tissue, and not by adipocytes per se. Our in vitro system allows to show the direct effect of hypoxia on adipocytes.

The role of hypoxia in insulin resistance could be envisioned as a multi-steps process. In a first and early series of events, hypoxia induces a local insulin resistance inside the adipose tissue, by inhibiting the insulin signaling pathway. In a second and longer process, hypoxia attracts macrophages within the adipose tissue leading to dysregulation of
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adipokines expression (3; 32), which will be involved in the development of local, but also systemic insulin resistance. Third, by creating a niche, hypoxia could increase mesenchymal stem cells self renewal while inhibiting adipocyte differentiation in restricted part of the tissue (6; 33). Then, through angiogenesis, hypoxia could create new vessels that would lead to re-oxygenation of these niches leading to the development of new adipocytes. As obesity progresses this mechanisms could create a vicious circle aggravating the syndrome.

In conclusion, we show here that hypoxia inhibits insulin signaling in adipocytes through HIF proteins. This mechanism could be involved in the establishment of an insulin resistant state during obesity.

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Figure 1: Hypoxia inhibited insulin-induced insulin receptor tyrosine phosphorylation in human and 3T3-L1 adipocytes

(A) 3T3-L1 adipocytes were incubated for 16 h at 37°C in normoxia (21% O₂) or hypoxia (1% O₂). Cell lysates were analyzed by immunoblots with the indicated antibodies. (B) 3T3-L1 adipocytes were incubated for 16 h at 37°C in normoxia (21% O₂) or hypoxia (1% O₂) before being stimulated with insulin (100 nM) for 5 min. (C) 3T3-L1 adipocytes were incubated for 16 h at 37°C in normoxia (21% O₂) or hypoxia (1% O₂) before being stimulated with decreasing concentrations of insulin ranging from 100 to 0.01 nM. (D) Cell lysates were subjected to immunoprecipitation using antibodies to IRS-1 or IRS-2 followed by immunoblots using indicated antibodies. (E) Human adipocytes were obtained after differentiation of preadipocytes, and were incubated for 24 h at 37°C in normoxia (21% O₂) or hypoxia (1% O₂). After insulin stimulation (100 nM) for 5 min, cell lysates were analyzed by immunoblots with indicated antibodies. Representative experiments of at least three independent experiments done in duplicate or triplicate are shown.
Figure 2: The effect of hypoxia on insulin signaling pathway is rapid and reversible

(A) Prior hypoxic incubation, the medium was gazed with N₂ within an anaerobic glove chamber to remove oxygen from the medium. 3T3-L1 adipocytes were incubated in normoxia (21% O₂) or hypoxia (1% O₂) for 1, 4, 8 and 16 h at 37°C. Adipocytes were stimulated with insulin (100 nM) for 5 min, and cell lysates were analyzed by immunoblots with anti-phosphotyrosine and insulin receptor antibodies. Quantification of insulin receptor tyrosine phosphorylation compared to the amount of insulin receptor of four to five independent experiments done in duplicate is shown. (B) 3T3-L1 adipocytes were incubated in normoxia or hypoxia (1% O₂) for 16 h before being reoxygenated for 15 and 45 min at 37°C. Normoxic, hypoxic and reoxygenated adipocytes were stimulated with insulin (100 nM) for 5 min. Cell lysates were analyzed by immunoblots with indicated antibodies. A representative experiment of three independent experiments done in duplicate is shown.
Figure 3: Hypoxia inhibits insulin-induced glucose transport
(A) 3T3-L1 adipocytes were incubated for 16 h in normoxia or in hypoxia before insulin-stimulated glucose transport was measured as described in methods section. Data are means ± SE of three independent experiments done in triplicate. (B) Cell lysates were analyzed by immunoblots using the indicated antibodies.
Figure 4: Hypoxia increased ROS generation but did not activate protein tyrosine phosphatases

(A) 3T3-L1 adipocytes were incubated with DCFH-DA for 30 min before being incubated for 16 h in normoxia or in hypoxia, in absence or in presence of NAC (10 mM). Adipocytes were lysed and fluorescence was measured. Results are expressed as fold of stimulation compared to controls. Data are means ± SE of three independent experiments done in triplicate. (B) 3T3-L1 adipocytes were incubated in normoxia (21% O₂) or hypoxia (1% O₂) for 16 h before being stimulated for 5 min with insulin (100 nM). Total phosphatase activity was determined by the dephosphorylation of synthetic substrate pNPP (paranitrophenylphosphate) compared to the activity of CIP (calf intestine phosphatase). Data are means ± SE of three independent experiments done in triplicate. (C) 3T3-L1 adipocytes were incubated in normoxia or hypoxia for 16 h, in absence or in presence of vanadate 2 mM. Adipocytes were stimulated with insulin (100 nM) for 5 min, and cell lysates were analyzed by immunoblots with indicated antibodies. A representative experiment of three independent experiments is shown.
**Figure 5: Effect of conditioned medium on insulin receptor phosphorylation**

3T3-L1 adipocytes were incubated for 16 h in normoxia or in hypoxia. Conditioned media were collected and secretion of glycerol (A), IL-1β and IL-6 (B) was measured as described in methods section. Data are means ± of four independent experiments done in triplicate. (C) 3T3-L1 adipocytes incubated for 16 h in normoxia, in hypoxia or treated with conditioned medium from normoxic adipocytes (Normoxic CM) or hypoxic adipocytes (Hypoxic CM) were stimulated with insulin 100 nM for 5 min. Cell lysates were analyzed with indicated antibodies. A representative experiment of four independent experiments done in duplicate is shown. (D) Quantification of tyrosine phosphorylation of insulin receptor compared to insulin receptor protein of four independent experiments is shown.
Figure 6: The effect of hypoxia on inhibition of insulin signaling pathway is mimicked by CoCl$_2$ and HIF$_{\alpha}$ expression

(A): 3T3-L1 adipocytes were incubated for 16 h with CoCl$_2$ (200 $\mu$M) and stimulated with insulin (100 nM) for 5 min. (B): HEK-293 cells were transfected with indicated plasmids and stimulated with insulin for 5 min. Cell lysates were analyzed by immunoblots with indicated antibodies. A representative experiment of three independent experiments done in duplicate is shown.
Figure 7: Inhibition of HIF-1α or HIF-2α expression restores insulin stimulation of insulin receptor in hypoxia

3T3-L1 adipocytes were transfected with siRNA for HIF-1α (A) or HIF-2α (B). 48 h after transfection, adipocytes are incubated for 16 h in normoxia or in hypoxia for 16 h and stimulated with insulin (100 nM) for 5 min. Cell lysates were analyzed by immunoblots with indicated antibodies. A representative experiment of three independent experiments done in duplicate is shown. (C) Quantification of insulin receptor tyrosine phosphorylation compared to insulin receptor amount. Data are means ± SE of three independent experiments done in duplicate. *p<0.05
References

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