The insulin and insulin-like growth factor-1 (IGF-1) receptors mediate signaling for energy uptake and growth through insulin receptor substrates (IRSs), which interact with these receptors as well as with downstream effectors. Oxygen is essential not only for ATP production through oxidative phosphorylation but also for many cellular processes, particularly those involved in energy homeostasis. The oxygen tension in vivo is significantly lower than that in the air and can vary widely depending on the tissue as well as on perfusion and oxygen consumption. How oxygen tension affects IRSs and their functions is poorly understood. Our findings indicate that transient hypoxia (1% oxygen) leads to caspase-mediated cleavage of IRS-1 without inducing cell death. The IRS-1 protein level rebounds rapidly upon return to normoxia. Protein tyrosine phosphatases (PTPs) appear to be important for the IRS-1 cleavage because tyrosine phosphorylation of the insulin receptor was decreased in hypoxia and IRS-1 cleavage could be blocked either with H2O2 or with vanadate, each of which inhibits PTPs. Activity of Akt, a downstream effector of insulin and IGF-1 signaling that is known to suppress caspase activation, was suppressed in hypoxia. Overexpression of dominant-negative Akt led to IRS-1 cleavage even in normoxia, and overexpression of constitutively active Akt partially suppressed IRS-1 cleavage in hypoxia, suggesting that hypoxia-mediated suppression of Akt may induce caspase-mediated IRS-1 cleavage. In conclusion, our study elucidates a mechanism by which insulin and IGF-1 signaling can be matched to the oxygen level that is available to support growth and energy metabolism.

The insulin receptor substrate (IRS)3 family of proteins is essential for the intracellular signaling of both the insulin receptor (IR) (1–3) and the IGF-1 receptor (IGF-1R) (4–6). When ligands such as insulin or IGF-1 bind to their receptors, the tyrosine kinase activity of the receptor is activated, triggering autophosphorylation (7–9) as well as tyrosine phosphorylation of IRSs (1–6). Once phosphorylated, IRSs bind and activate phosphatidylinositol 3-kinase (PI3K) (10, 11), which produces phosphatidylinositol trisphosphate on the membrane leading to activation of effector kinases such as PKB (12) and its downstream substrate, Akt (13). In a parallel pathway, interaction with Grb-2 triggers insulin-dependent activation of mitogen-activated protein kinase (see Fig. 7e) (14). IRS proteins have an N-terminal pleckstrin homology domain (15), which directs IRS proteins to IR/IGF-1R by interacting with membrane lipids or with membrane bound proteins. Located adjacent to the pleckstrin homology domain of IRSs, the phosphotyrosine-binding domain (16) interacts with tyrosine-phosphorylated residues in both the IR and the IGF-1R. The C-terminal domain of IRS-1 contains at least 21 tyrosine residues (2, 17, 18) that can be phosphorylated by the tyrosine kinases residing in IR and IGF-1R. IRS proteins also contain a number of serine residues including Ser-302 (19), Ser-307 (20), Ser-612 (21), Ser-636 (22), Ser-639 (22), Ser-731 (23) and Ser-789 (24, 25), which can be phosphorylated by Ser/Thr kinases such as Akt (26), JNK (20, 27), mTOR (22, 28), AMPK (24), MAPK (29), PKC (21, 30–33), IKK (34), and GSK-3b (35, 36). Phosphorylation of these serine residues has an attenuating effect on insulin/IGF-1 signaling by suppressing IR-IRS interaction and IRS tyrosine phosphorylation after prolonged exposure to insulin or to inflammatory signals.

Cellular oxygen tension is governed by a number of factors including the oxygen tension of the blood, the rate of perfusion, the distance from the nearest capillary, and the rate of oxygen consumption by neighboring cells (37). Although the partial pressure of oxygen in the air at sea level is 149 mm Hg (20%), it is considerably lower in vivo. The oxygen tension in arterioles ranges from 10 to 80 mm Hg depending on the diameter of the arteriole and the location. In capillaries, the tension ranges from 10 to 70 mm Hg and in veins from 10 to 40 mm Hg. The oxygen tension in the tissues decreases exponentially as a function of the distance from the blood vessels and varies depending on the tissue. The mean oxygen tension of the spleen, thymus, and myocardium are ~20, 10, and 5–15 mm Hg, respectively (38). The tissue oxygen tension can be further diminished in pathological conditions. For example, the oxygen tension can be severely reduced in tissues distal to an obstructed blood vessel. These types of severe hypoxia can lead to cell death and to tissue or organ damage. A more subtle fluctuation of cellular oxygen tension can occur in the setting of capillary recruitment and derecruitment. In endothelial cells, Akt phosphorylates and activates endothelial nitric-
oxide synthase (eNOS), increasing the production of the potent vasodilator nitric oxide, which in turn recruits capillaries and increases the delivery of oxygen and nutrients to cells (39, 40). Individuals suffering from sleep apnea develop hypoxemia and are insulin-resistant (41). Although individuals suffering from sleep apnea tend to be obese, hypoxemia is an independent risk factor for insulin resistance (42).

MATERIALS AND METHODS

Transfection and Hypoxic Incubation—Plasmids were transfected into 293T cells by using Effectene. After 40 h, the confluent transfected cells were placed into a hypoxic chamber (Hypoxia Work Station, Biotrace Inc.) for 6 h or left in the tissue culture incubator. Cells were then harvested by scraping into radioimmune precipitation assay buffer containing protease and phosphatase inhibitors. Hypoxic samples were harvested in the hypoxic chamber. Proteins were analyzed by SDS-PAGE followed by immunoblotting. The hypoxic samples from mouse embryonic fibroblast (MEF), 3T3L1, and MCF7 cells were produced in the same manner.

Antibody and Immunoblotting—β-Actin, phospho-Akt, and phospho-IRS-1 (Ser-302, Ser-612) antibodies were purchased from Cell Signaling. IRS-1 antibody, phosphotyrosine antibody (4G10), and PTP1B antibody was purchased from Oncogene Research. Horseradish peroxidase-conjugated anti-rabbit and antimouse secondary antibodies were purchased from Amersham Biosciences and were used at dilutions of 1:5000. ECL-Plus reagent was purchased from Amersham Biosciences.

Degradation of IRS-1 in Vitro by Caspases—293T cells were transfected with a human IRS-1 expression vector. After 48 h, IRS-1 was immunoprecipitated using anti-IRS-1 antibody (Upstate catalog no. 06-248) and protein A/G beads (Pierce). The beads were washed three times with phosphate-buffered saline and once with 50 mM Hepes, pH 7.2, and divided equally into nine tubes. Digestion with caspases (Calbiochem) was performed according to the manufacturer’s directions using 1 unit of each caspase at 37 °C for 1 h in a final volume of 20 μl. Digested samples were analyzed by electrophoresis using 4–12% acrylamide gels followed by immunoblotting.

Treatment of Cells with Caspase 3/7 Inhibitor—293T cells transfected with plasmid expressing human IRS-1 were treated with 20 μM Z-DEVD-fmk (Calbiochem, stock, 2 mM in Me2SO) 30 min prior to hypoxic treatment (2 h). HeLa cells were treated with 17 μM Z-DEVD-fmk beginning 30 min prior to hypoxic treatment (3 h).

DNA Fragmentation Assay—Confluent HeLa or 293T cells transfected with human IRS-1 were treated or not with 2 μM staurosporine overnight or for the indicated times and harvested by scraping into phosphate-buffered saline. The samples were divided in two so that both protein and genomic DNA could be extracted. Genomic DNA was harvested by using a DNA ladder kit (Biovision) and was analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide. Simultaneously, confluent HeLa or 293T cells transfected with human IRS-1, which had been incubated in normoxia or 1% oxygen for 5 or 2 h, respectively, as well as untreated controls were scraped into phosphate-buffered saline. Each sample was divided in two and analyzed as above.

Soluble and Insoluble Fractionation—293T cells were ~98% confluent. Following normoxia or hypoxia (6 h), cells were scraped into radioimmune precipitation assay buffer. They were centrifuged for 20 min. The supernatant was the soluble fraction. The pellet was washed three times with radioimmune precipitation assay buffer and then extracted with urea buffer (20 mM Tris-HCl, pH 8, 8 M urea) to obtain the insoluble fraction.

Plasmid Constructs—IRS-1 deletion mutants were prepared from a human IRS-1 expression vector (from M. Quon) by using a QuikChange mutagenesis kit (Stratagene) and the following oligonucleotides: P1213 (del 1), 5'-CAATGGCCACCGTGCAACCCTTCCGACTGCTACTAGC-3; P1212 (del 2), 5'-CCTTGCAAGGTGACTAGCAGTACTGAG-3'; P1217 (del 3), 5'-GCTGTAGCATTTGGCAAGGCCAGGACCCGTACG-3'; P1195 (del 4), 5'-GAGCGATGCGTGCTGGACCCCCCTTTGAGAGCTGCTTCTC-3'; P1196 (del 5), 5'-GCCGCCAACACCGGCACAGCGGAG-3'; P1189 (del 6), 5'-CGTGGCCAGGACGGACAGGATCGGCGGGCTTTGGAGAGCTGCTTCTC-3'; P1197 (del 7), 5'-CATCAGGCAGGACAGGATCGGCGGGCTTTGGAGAGCTGCTTCTC-3'; P1198 (del 8), 5'-GAGCGATGCGTGCTGGACCCCCCTTTGAGAGCTGCTTCTC-3'.

FIGURE 1. The protein level of IRS-1, but not IRS-2, is decreased in hypoxia. a, IRS-1 and IRS-2 levels in cells exposed to hypoxia. Confluent MEF (wild-type), 3T3-L1, and MCF7 cell lines in serum-free media were exposed to normoxia (N; atmospheric oxygen) or hypoxia (H; 1% oxygen) in a hypoxic chamber for the indicated lengths of time. Cell extracts from the hypoxic cells were prepared in the hypoxic chamber. After the extracts were subjected to SDS-PAGE and transferred to a filter, they were immunoblotted with antibodies specific for IRS-1, IRS-2, or actin. b, IRS-1 level in MEFs exposed to 5 and 1% oxygen for the indicated lengths of time.
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TCCGACTGCTACTACGGC-3′; P1198 (del 8), 5′-CCTTGCACAGGTGACTACCCAGGCAGTCTAGTAG-3′.

pG716 is an expression vector consisting of rat IRS-1 in plasmid pM. pG674 is an expression vector consisting of mouse IRS-2 cDNA in plasmid pCis-2 (from M. Quon). p1206 was generated from plasmid pG716 by destruction of the AflII site and cloning into AflII/NotI-digested p1206; P1207 (ch3), 5′-AGCCCTTAAGCCACTGTGGC-3′. IRS-1-IRS-2 fusion proteins were generated by PCR of mouse IRS-2, restriction digest, and exchange with the corresponding rat IRS-1 fragment in p1206. The primers used were as follows: P1209 (ch1), 5′-AATTACCAAGCTTTTAGGTCTAGCCGGCCTCTGAGCTGGG-3′ and 5′-GTGGCTTAAAGGCTATCCATGTC-3′ (PCR products were digested with HindIII and AflII and cloned into HindIII/AflII-digested p1206); P1208 (ch2), 5′-AGACCCCTTAAGCCACTGTGGC-3′ and 5′-AATTTCGAGTTGAGCTCCTGCTCTG-3′ (PCR products were digested with AflII and NotI and cloned into EcoR1 and NotI-digested p1198, 5′-AATTTGCGGCCGCAACTCTT TCACGAC-3′. The PCR products were digested with EcoRI and NotI and cloned into EcoR1-NotI-digested p1206. Insulin and IGF-1 Treatment—Unless indicated otherwise, serum-starved cells were treated with 10−5 units/ml insulin (65 pm) or 125 pm IGF-1 for 5 min immediately before harvesting. Cells were treated with 60 nm insulin for 5 min immediately before harvesting (see Fig. 7c).

RESULTS

Although the functions and properties of IRSs have been extensively characterized, virtually nothing is known about how oxygen tension affects the IRS proteins. To study the effect of mild hypoxia on the IRS proteins, we incubated a confluent culture of MEFs in serum-free media overnight, after which they were incubated either in a hypoxic chamber containing 1% oxygen or in a tissue culture incubator in room air (normoxia). To ensure that the oxygen tension did not change during the experiment, the lysate preparation after hypoxia treatment was also performed inside the hypoxic chamber. We discovered that within 1–4 h of hypoxia, the IRS-1 protein level was drastically reduced (Fig. 1a). The protein level of IRS-2 and of the insulin receptor did not change significantly with hypoxia. The IRS-1 level was also drastically reduced in other cell types including 3T3-L1 and MCF7 cells (Fig. 1a). Similar results were seen in other cell lines including NIH3T3, HeLa, and 293T cells (see below), suggesting that hypoxia-induced reduction in IRS-1 levels may be a general phenomenon. However, it was possible that the oxygen tension did not change significantly with hypoxia. The IRS-1 mRNA level with real-time PCR and detected no significant decrease with hypoxia (data not shown), suggesting that the hypoxia-induced reduction in IRS-1 levels occurred at the level of translation or post-translation.

It has been shown previously that prolonged treatment with insulin also leads to degradation of IRS-1 protein (43–45). Ser/Thr hyperphosphorylation of IRS-1, which is induced by prolonged insulin treatment, is required for degradation (46), and inhibition of PI3K blocks IRS-1 degradation (44, 45, 47, 48). Ser/Thr hyperphosphorylation of IRS-1 and -2 can be induced with the phosphatase inhibitor okadaic acid in the absence of insulin treatment, indicating that there is basal Ser phosphorylation. Treatment of cells with okadaic acid resulted in an electrophoretic mobility shift of...
IRS-1, a consequence of Ser/Thr hyperphosphorylation. Indeed, okadaic acid treatment dramatically increased the phosphorylation of Ser-302 and Ser-612 in IRS-1 (Fig. 2a). If reductions of the IRS-1 level by hypoxia and by chronic insulin treatment were mediated by the same biochemical pathway, phosphorylation of Ser-302 and Ser-612 and gel mobility shift should occur prior to the reduction of IRS-1 level by hypoxia. However, phosphorylation of Ser-302 and Ser-612 and gel mobility shift were not induced by hypoxia. It is possible that Ser-302 and Ser-612 were not phosphorylated because the cells were not exposed to hypoxia long enough. However, this is unlikely to be the case because the presence of okadaic acid during exposure (4 h) to hypoxia prevented reduction of IRS-1 levels (Fig. 2b). Taken together, these results indicate that the reduction of IRS-1 level by hypoxia and by chronic insulin treatment occurs through different pathways.

The reduction in IRS-1 level after prolonged insulin (45) or IGF-1 (44) treatment is mediated by proteasome-dependent degradation. To determine whether proteasome-dependent degradation decreases IRS-1 levels in hypoxia, hypoxic cells were treated with the proteasome inhibitor MG132. As shown in Fig. 2c, MG132 did not prevent IRS-1 degradation in hypoxia. Taken together, these findings indicate that the hypoxia-induced decrease in IRS-1 levels is not mediated by Ser/Thr hyperphosphorylation and proteasome-mediated degradation and therefore is mechanistically different from the insulin-induced degradation of IRS-1.

We then asked whether the caspase pathway (49), which is induced by apoptotic signals but suppressed by survival signals, might be responsible for IRS-1 degradation. First, we examined whether transient hypoxia affected caspase 3 activation. As shown in Fig. 3a, cleavage of procaspase 3 to active caspase 3 increased with hypoxia. Then, we tested a panel of recombinant caspases to determine whether caspases are capable of degrading IRS-1 directly (Fig. 3b). IRS-1 was immunoprecipitated from cells with IRS-1-specific antibody and incubated with recombinant caspases 1–10. Caspases 3 and 7 had very potent IRS-1-degrading activity, and caspase 10 had significant but weaker IRS-1-degrading activity, and caspase 10 had significant but weaker IRS-1-degrading activity. To determine whether caspases 3 and 7 mediated hypoxia-induced cleavage of IRS-1 in cells, we incubated hypoxic cells with Z-DEVD-fmk, an inhibitor of caspases 3 and 7 (50). As shown in Fig. 3c, inhibition of caspases 3 and 7 prevented hypoxia-induced IRS-1 degradation.

Because caspases are normally involved in executing apoptosis, we isolated genomic DNA from HeLa cells exposed to hypoxia for 4 h and electrophoresed it on an agarose gel (Fig. 4a). As a positive control, we isolated genomic DNA from HeLa...
cells that had been treated with staurosporine to induce apoptosis. Although there was DNA laddering in staurosporine-treated cells, there was no DNA laddering in hypoxic cells, indicating that apoptosis was not induced during 4 h of hypoxia. Consistent with this, cells that were previously exposed to hypoxia for 4 h had a normal proliferation rate when returned to atmospheric oxygen. For example, it is possible that IRS-1 moved from the cytosol to the cell compartments that form the insoluble fraction, which is discarded during lysate preparation, but returned to the cytosol with reoxygenation. The insoluble fraction, which had been discarded in past experiments, was solubilized and immunoblotted with IRS-1 antibody after electrophoresis (Fig. 4d). With or without hypoxia, there was no IRS-1 in the insoluble fraction, suggesting that the fluctuation in IRS-1 level with hypoxia and reoxygenation was not a result of compartmental shift.

Because IRS-1, but not IRS-2, was cleaved in hypoxia, we sought to determine the site of regulation of IRS-1 cleavage (Fig. 5a). 293T cells were transiently transfected with IRS-1 expression vectors containing various deletions and exposed to hypoxia for 4 h. As shown in Fig. 5a, no single deletion abrogated hypoxia-induced IRS-1 degradation. We transiently transfected expression vectors for IRS-1/2 chimeric proteins in which different regions of IRS-1 were replaced with the corresponding regions from IRS-2. All three chimeric proteins were degraded with hypoxia. Thus, it appears that multiple regions in IRS-1 can regulate or mediate caspase-dependent degradation. This finding is in contrast to prolonged insulin-induced degradation of IRS-1, which requires the N-terminal region of IRS-1 (51).

To determine whether the IRS-dependent pathway was affected by hypoxia, we examined insulin-induced tyrosine phosphorylation of the IR (β-subunit) and of IRS-1 in NIH3T3 cells exposed to hypoxia for only 1 h. Although there was not enough time for the IRS-1 level to decline, tyrosine phosphorylation of IR and IRS-1 was decreased (Fig. 6a). Consistent with this finding, the amount of the p85 subunit of PI3K (18) coimmunoprecipitated with IRS-1 was decreased in hypoxic H929 cells exposed to hypoxia for 4 h. As shown in Fig. 6b, coimmunoprecipitation of IRS-1, which requires the N-terminal region of IRS-1 (51), was modestly, but significantly, reduced compared with normoxic cells. Previously, Akt had been shown to suppress caspase activity (52). To determine whether suppression of Akt could affect cleavage of IRS-1, we coexpressed dominant-negative (kinase-dead) Akt or constitutively active Akt with IRS-1. Expression of dominant-negative Akt resulted in IRS-1 degradation even in normoxia, whereas expression of constitutively active Akt suppressed IRS-1 degradation modestly (Fig. 6c). Taken together, these results suggest that Akt, although downstream of IRS, may also regulate IRS-1 cleavage in hypoxia.

Protein tyrosine phosphatases (PTPs), which negatively regulate insulin and IGF-1 signaling by dephosphorylating...
tyrosine residues on IR, IGF-1, and IRS proteins, are transiently suppressed by the PI3K pathway (see Fig. 7e) (53). It is thought that phosphatidylinositol trisphosphate recruits guanine nucleotide exchange factors to the cell membrane for the small GTP-binding protein Rac1. Activated Rac1 in turn stimulates NADPH oxidase, converting oxygen to superoxide, which upon dismutation is converted to H$_2$O$_2$ (54–56). PTPs such as PTP1B (57, 58), which reverses the tyrosine phosphorylation stimulated by insulin, contain an active site cysteine residue that is susceptible to oxidation by oxidants such as H$_2$O$_2$. Once oxidized, PTP is inactivated, and the balance shifts toward tyrosine phosphorylation until H$_2$O$_2$ is degraded and PTP is reactivated by electron donors. This negative control of PTP is necessary to prevent a futile phosphorylation-dephosphorylation cycle during insulin and IGF-1 signaling. We show in Fig. 6a that tyrosine phosphorylation of IR and IRS-1 is reduced in hypoxia. It is possible that reduced production of H$_2$O$_2$ in hypoxia may have increased the PTP activity, which in turn led to suppression of Akt and its ability to suppress caspases. If this were the case, PTPs would be important for hypoxia-induced degradation of IRS-1. To test this possibility, we treated hypoxic cells with the PTP inhibitor vanadate (Fig. 7a). The IRS-1 level was partially restored with vanadate treatment in hypoxia. Treatment with H$_2$O$_2$ also restored the IRS-1 level in hypoxia (Fig. 7b). Moreover, strong stimulation of the PI3K-Akt pathway with a higher concentration of insulin (60 nM) also inhibited IRS-1 degradation in hypoxia (Fig. 7c). Taken together, these findings are consistent with the notion that hypoxia-induced activation of PTPs, which suppresses Akt and its ability to suppress caspases, is important for IRS-1 degradation in hypoxia (Fig. 7e). In an attempt to identify the PTP important for IRS-1 cleavage, we examined IRS-1 cleavage in PTP1B$^{-/-}$ and PTP1B$^{+/+}$ MEFs. Previous work had shown that PTP1B is a major inhibitor of insulin signaling (57, 58). However, IRS-1 cleavage occurred in both hypoxic PTP1B$^{-/-}$ and PTP1B$^{+/+}$ MEFs, suggesting that PTP1B is either not important for hypoxia-induced IRS-1 cleavage or that in the absence of PTP1B, other PTPs can take its place. We cannot distinguish between these two possibilities at this point.

**DISCUSSION**

The oxygen tension in the microenvironment of a cell *in vivo* is significantly different from that generally used in most *in vitro* experiments. Depending on the tissue, the oxygen tension can vary widely, but it is significantly lower than the atmospheric oxygen tension. The intracellular oxygen tension is controlled by a number of factors including the efficiency of oxygenation in the lungs, the blood flow in the arteries, arterioles, and capillaries that supply the tissue, the rate of oxygen consumption, and the distance from the nearest capillaries. Oxygen is essential for growth and for energy generation as well as for many other cellular processes, especially those involved in energy homeostasis. In this report, we have examined the effect of mild hypoxia on IRS proteins. Our findings indicate that IRS-1 is cleaved by caspases during transient hypoxia. Although caspases were involved in IRS-1 cleavage, transient exposure to hypoxia did not cause apoptosis, and IRS-1 cleavage was reversed within a few minutes of reoxygenation. PTPs may be important for the hypoxic effect because tyrosine phosphorylation of the IR is decreased in hypoxia and also because inhibition of PTPs with H$_2$O$_2$ or vanadate blocks IRS-1 cleavage. It is difficult to prove directly that PTPs are activated in hypoxia, because the redox state of the PTPs is largely determined by the redox potential of the buffers used in the PTP reaction. Although NADPH oxidase is the best known candidate to be the oxygen sensor in the regulation of IRS-1 degradation, we have not been able to demonstrate that NADPH oxidase is important for IRS-1 degradation. However, based on the current knowledge of the role of H$_2$O$_2$ in PTP inhibition, our find-
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Oxygen supply, thereby allowing for more efficient energy signal and supply of nutrients to the cells that have sufficient IGF-1 signaling to oxygen levels is that it restricts the growth homeostasis by matching insulin and IGF-1 signaling with the oxygen supply, thereby allowing for more efficient energy signal and supply of nutrients to the cells that have sufficient IGF-1 signaling to oxygen levels is that it restricts the growth homeostasis by matching insulin and IGF-1 signaling with the oxygen availability. The net result of matching insulin and IGF-1 signaling to oxygen levels is that it restricts the growth signal and supply of nutrients to the cells that have sufficient oxygen supply, thereby allowing for more efficient energy distribution.

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