Hypoxia Induces the Activation of the Phosphatidylinositol 3-Kinase/Akt Cell Survival Pathway in PC12 Cells

PROTECTIVE ROLE IN APOPTOSIS*

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Hypoxia is a common environmental stress that influences signaling pathways and cell function. Several cell types, including neuroendocrine chromaffin cells, have evolved to sense oxygen levels and initiate specific adaptive responses to hypoxia. Here we report that under hypoxic conditions, rat pheochromocytoma PC12 cells are resistant to apoptosis induced by serum withdrawal and chemotherapeutic treatment. This effect is also observed after treatment with deferoxamine, a compound that mimics many of the effects of hypoxia. The hypoxia-dependent protection from apoptosis correlates with the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is detected after 3–4 h of hypoxic or deferoxamine treatment and is sustained while hypoxic conditions are maintained. Hypoxia-induced Akt activation can be prevented by treatment with cycloheximide or actinomycin D, suggesting that de novo protein synthesis is required. Finally, inhibition of PI3K impairs both the protection against apoptosis and the activation of Akt in response to hypoxia, suggesting a functional link between these two phenomena. Thus, reduced oxygen tension regulates apoptosis in PC12 cells through activation of the PI3K/Akt survival pathway.

Mammalian cell function is critically dependent on a continuous supply of oxygen. Organisms respond to changes in oxygen tension with specific local and systemic adaptations aimed to restore a normal oxygen supply. Several tissues and cell types are responsible for the detection of blood O2 and the induction of specific adaptive responses; among them, the chromaffin cells of the adrenal medulla play a critical role by releasing catecholamines in response to hypoxia (1). PC12, a rat pheochromocytoma cell line derived from a tumor of adrenal medulla chromaffin tissue, is an oxygen-sensitive cell type that provides a useful system to study the effects of hypoxia on catecholamine gene expression (2). PC12 cells are exquisitely sensitive to hypoxia, because very small reductions in atmospheric oxygen dramatically induce tyrosine hydroxylase gene expression and mRNA stability (3). In response to hypoxia several transcription factors are activated in PC12 cells. These include the cAMP response element-binding protein, the hypoxia-inducible factor (HIF-2 or EPAS)1 and c-Fos (2, 4, 5). PC12 cells also express hypoxia-regulated ion channels, as shown by the finding that PC12 cells depolarize under hypoxia via an oxygen-regulated K+ current; as a consequence of depolarization, they secrete dopamine and noradrenaline.

In addition, PC12 cells have been used extensively as a model to study programmed cell death. Programmed cell death, or apoptosis, is an evolutionary conserved mechanism of cellular demise developed by animals to delete damaged, misplaced, or redundant cells during development and tissue homeostasis. Apoptosis was first described as cell death with specific morphologic features (6). In addition to the characteristic morphologic changes observed during apoptosis, caspase processing also leads to the activation of specific nucleases, which in turn cleave genomic DNA, giving rise to a characteristic pattern of DNA degradation that is considered a hallmark of apoptosis (7).

Cell fate is largely dependent upon extracellular survival signals that prevent the activation of the apoptotic machinery (8). Studies on the survival effect of nerve growth factor (NGF) on PC12 cells provided the first evidence that activation of the enzyme PI3K was critical for its protective effect (9). Upon activation, PI3K phosphorylates membrane phosphoinositides at the D-3 position. These 3'-phosphorylated phospholipids act as second messengers that mediate the diverse cellular functions of PI3K. One of the targets of these lipid second messengers is the serine/threonine kinase Akt/protein kinase B (10). The amino terminus of Akt contains a pleckstrin homology domain that is thought to directly bind the phospholipid products of PI3K activation. This binding recruits Akt to the plasma

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1 The abbreviations used are: EPAS, endothelial PAS (Per-Arnt-Sim); NGF, nerve growth factor; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase.

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membrane and induces a conformational change that allows the phosphorylation of Akt by the phosphoinositide-dependent kinases I and II at the residues Thr-308 and Ser-473, respectively (11). Phosphorylation of Akt results in the full activation of its kinase activity and the subsequent regulation of multiple cellular processes, including the transmission of growth factor-dependent survival signals. The effects of PI3K are controlled by the product of the tumor suppressor gene pten, which encodes a phosphatase that dephosphorylates 3′-phosphorylated phosphoinositides (12).

The PI3K/Akt pathway is activated in response to a large number of stimuli (13). In addition to many different agonists, it has been described that Akt is also activated in response to several types of stress including oxidative stress (14). Importantly, the activation of this pathway, even by stress signals, results in an antiapoptotic effect. Finally, it has recently been shown that hydroxy activates Akt in pten-deficient glioma cells (15).

In the present study we show that hydroxy results in the activation of the PI3K/Akt pathway in PC12 cells by a novel mechanism that involves de novo protein synthesis. The activation of this pathway by hydroxy results in the protection against apoptosis induced by different stimuli.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Undifferentiated PC12 cells were maintained in RPMI 1640 medium with GLUTAMAX-I (Life Technologies, Inc.) supplemented with 10% horse serum (Life Technologies, Inc.), 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. Hydroxy (1% O₂) was induced by culture of cells inside an air-tight chamber with inflow and outflow valves that was infilled with a mixture of 1% O₂, 5% CO₂, 94% N₂ (S.E. Carburos Metalicos S.A.. Madrid, Spain); in those experiments a 20 mM phosphate-buffered saline (PBS) and harvested in 70% or 200 mM EDTA in PBS) and collected together with those floating (detached) cells were pretreated during 1 h with kinase inhibitors, cells were pretreated during 1 h with 50% confluence (typically 24–48 h). Medium was then replaced with medium and normoxic (21% O₂) or hypoxic conditions (1% O₂) for 18–24 h. After treatments (Sigma) diluted in PBS was added to the culture medium to a final concentration of 1.5 mM/ml and all samples were incubated at 37 °C for 2–4 h under normoxic conditions. Finally, medium was removed, and precipitated MTT was solubilized in 0.04 N HCl diluted in isopropanol. Product formation was monitored by reading absorbance at 550 nm using a microplate reader.

**Kinase Assays**—Cells were plated in 100-mm culture dishes and grown to the indicated confluence. After treatments, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested in 70% or 200 mM EDTA in PBS and collected together with those floating (detached) cells that were contained in gates 1 and 2 were plotted in a histogram representing the number of events (cells) containing a specific PI intensity-area (e.g., specific amount of DNA). Apoptosis was measured as the percentage of cells with a sub G₁/G₀ DNA content in the PI intensity-area histogram plot (16).

**Viability Assay**—Viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, bromide) (MTS) (Promega) method as described (17). Briefly, cells were plated on poly-L-lysine pre-coated 96-well plates and grown in complete medium until they reached 30–50% confluence (usually 12–24 h). Then medium was replaced with complete or serum-free medium, and cells were placed under normoxic (21% O₂) or hypoxic conditions (1% O₂) for 12 h. Where indicated, cells were stimulated with 10 μM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) diluted in PBS and added to the culture medium to a final concentration of 1.5 mM/ml and all samples were incubated at 37 °C for 2–4 h under normoxic conditions. Finally, medium was removed, and precipitated MTT was solubilized in 0.04 N HCl diluted in isopropanol. Product formation was monitored by reading absorbance at 550 nm using a microplate reader.

**RESULTS**

**Hydroxy Protects PC12 Cells from Apoptosis**—To assess the effect of hydroxy on apoptosis, PC12 cells were treated with different combinations of either complete or serum-free medium and normoxic (21% O₂) or hypoxic conditions (1% O₂) for various periods of time (Fig. 1A). After treatments, apoptosis was measured as the percentage of cells with a content of DNA lower than G₀/G₁ by analysis of the cell cycle by flow cytometry (16). Fig. 1A shows that hydroxy itself can induce apoptosis in PC12 cells, as previously reported (20), but its importance is only significant after longer than 36 h in low oxygen conditions (Fig. 1A, inset). In contrast, serum withdrawal, which is a potent apoptotic signal for PC12 cells (21), results in a significant percentage of cells undergoing apoptosis as early as 9 h (Fig. 1A). Interestingly, the induction of apoptosis upon serum withdrawal was greatly reduced when cells were cultured under hypoxic conditions (Fig. 1A). The reduction of apoptosis was statistically significant at 12 h (p < 0.01), 24 h (p < 0.01), and 36 h (p < 0.01) after serum removal (57, 62, and 62% inhibition, respectively). The results show the cell cycle profile of PC12 cells 24 h after the indicated treatments in a representative experiment. The effect of hydroxy on cellular viability was confirmed using the MTT assay (17, 22). Whereas hydroxy (1% O₂, for 20 h) itself had little effect on cell viability (14% reduction in viability as compared with cells in normoxia), serum starvation for 20 h
reduced viability to 53% as compared with control cells (Fig. 1C). In agreement with flow cytometry data, the reduction of cell viability caused by serum withdrawal was decreased under hypoxic conditions (21% reduction of cell viability as compared with cells in normoxia) (Fig. 1C). The cellular responses to hypoxia can be observed at higher concentrations of oxygen, although with less efficiency. Consistently, a significant reduction in apoptosis, triggered by serum withdrawal, was also observed at 5% O$_2$ (Fig. 2A), which corresponds to a pO$_2$ (36 torr) within the physiological range found in venous blood in vivo. Moreover, treatment with deferoxamine or cobalt chloride (CoCl$_2$)$_2$ two compounds widely used to mimic many of the effects of hypoxia (23), resulted in a reduction of apoptosis, triggered by serum withdrawal, was also observed at 5% O$_2$ (Fig. 2A), which corresponds to a pO$_2$ (36 torr) within the physiological range found in venous blood in vivo. Moreover, treatment with deferoxamine or cobalt chloride (CoCl$_2$)$_2$ two compounds widely used to mimic many of the effects of hypoxia (23), resulted in a reduction of apoptosis.

We next tested whether hypoxia was able to protect against apoptotic stimuli other than serum withdrawal. Treatment of PC12 cells with the anti-cancer drugs taxol or fluorouracil (5-fluoro-2,4(1H,3H)-pyrimidinedione) for 12–18 h induced a low, but significant, percentage of apoptosis (Fig. 2B). As seen for serum withdrawal, under hypoxic conditions (1% O$_2$) PC12 cells were significantly protected against apoptosis (p < 0.05 for taxol and p < 0.001 for fluorouracil) induced by the chemotherapeutic drugs (Fig. 2B). Thus, hypoxic conditions induced by low oxygen or the drug deferoxamine are able to prevent apoptosis induced by different stimuli in PC12 cells.

Hypoxia Protects PC12 Cells from Apoptosis—To identify the mechanism responsible for the pro-survival effect of hypoxia, we decided to study the effect of hypoxia on the protooncogenic serine/threonine kinase Akt, a molecule involved in the transduction of antiapoptotic signals (13, 24). Activation of Akt can be easily detected by Western blotting with antibodies that specifically recognize Akt molecules phosphorylated at serine 473, because this phosphorylation correlates with Akt activity (13).

Hypoxia treatment resulted in phosphorylation of Akt on serine 473 (Fig. 3A). The hypoxia-induced phosphorylation of Akt was observed regardless of the presence or absence of serum or the type of culture substrate used (Fig. 3A). Phosphorylation of Akt on serine 473 reflects its activation as demonstrated by increased kinase activity of immunoprecipitated Akt from hypoxia-treated cells (Fig. 3B).

Interestingly, the activation of PI3K/Akt does not seem to be a general cellular response to hypoxia, because Akt phosphorylation was not observed in other cell lines tested including human vascular endothelial cells, neuroblastoma-69 cells, human embryo kidney 293 cells, HeLa cells, Chinese hamster ovary cells, COS cells, and Hepa cells. However, it seems to be a response restricted to specific cell types, because, in addition to the PC12 cell line, Akt phosphorylation was observed in primary chromaffin cells from cow adrenal gland and neuro-2A cells under hypoxic conditions. Akt phosphorylation was also observed in PC12 cells that were differentiated to a neuronal phenotype by treatment with NGF for 3–6 days, and it correlated with protection against apoptosis.

Kinetic studies showed that Akt phosphorylation was detected 4–6 h after initiation of hypoxic treatment (Fig. 4A). The time course of Akt activation in response to hypoxia was dis-
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Fig. 2. Hypoxia protects from apoptosis. A, PC12 cells were plated in 6-well plates and grown until they reached 40–50% confluency. Thereafter, the culture medium was replaced with serum-free (-) or complete culture medium (+), and cells were incubated at the indicated concentrations of oxygen. Where indicated, deferoxamine was included in the culture medium at a final concentration of 75 μM (150DFX) or 150 μM (300DFX), and cells were incubated in 21% O2. Apoptosis was measured by flow cytometry 18–24 h after treatments. B, PC12 cells were grown as in A. Thereafter, the culture medium was replaced with fresh complete culture medium supplemented, where indicated, with 1 μg/ml taxol or 50 μg/ml fluorouracil (5FU). Cells were incubated for 12–15 h under normoxic (21% O2, N) or hypoxic conditions (1% O2, H), and apoptosis was measured by flow cytometry. Results from five independent experiments are shown. Each symbol represents the percentage of apoptosis in samples from a single experiment (Exp.). The horizontal bars (Aver.) represent the means of all the experiments shown. The statistical significance of the results (see "Experimental Procedures") is shown.

Hypoxia-induced Akt Activation Requires Protein Synthesis

Fig. 3. Hypoxia induces phosphorylation of Akt at serine 473. A, PC12 cells were plated in 6-well plates, some of which were pre-coated with poly-l-lysine (PLL) and grown until they reached 40–50% confluency. Thereafter, the culture medium was replaced with fresh complete ( + ) or serum-free medium ( – ), and cells were transferred to a normoxic (N, 21% O2) or hypoxic (H, 1% O2) atmosphere for 7 h. Cells were collected and lysed, and proteins were detected by Western blotting as described. The experiment was repeated once with similar results. B, PC12 cells were plated in 10-cm culture dishes and grown until they reached ~50–70% confluence. Then the medium was replaced with serum-free medium, and cells were incubated under normoxic (21% O2, N) or hypoxic (1% O2, H) conditions for 17 h. Where indicated, cells were stimulated with 150 ng/ml NGF for 10 min. Akt was immunoprecipitated from cells, and its activity was assessed in vitro using H2B as substrate (Kinase React.). The total amount of immunoprecipitated Akt is shown (Immunop.). Phosphorylation of Akt at serine 473 is also shown (Lyssate). WB, Western blot.

Hypoxia-induced Akt Activation Requires Protein Synthesis and PI3K Activity—Next, we studied the molecular mechanism by which hypoxia induced Akt activation. Because Akt activation, in most instances, is dependent on PI3K activation (13), we first analyzed whether PI3K activity was affected by hypoxia. As shown in Fig. 5A, PI3K was activated by hypoxia in PC12 cells. Interestingly, the PI3K activity shown in Fig. 5A was detected in anti-phosphotyrosine immunoprecipitates from hypoxic cells, suggesting that hypoxia resulted in the activation of a tyrosine kinase upstream of PI3K/Akt. To confirm the involvement of PI3K, we tested the effect of two structurally nonrelated PI3K inhibitors, LY294002 and wortmannin. Both inhibitors, completely prevented Akt phosphorylation induced by hypoxia (Fig. 5B).

The delayed time course of Akt activation (Fig. 4), together with the observation that it occurred after the induction of hypoxia-responsive transcription factors such as EPAS (Fig. 4), suggested that Akt activation by hypoxia required gene expression. Thus, treatment of cells with inhibitors of RNA (actinomycin D) or protein (cycloheximide) synthesis com-
For comparison, cells were cultured under normoxic (21% O₂, modulin signaling (W12 and W132; calmidazolium) had no effect on Akt phosphorylation. The calmodulin-regulated and kinase activator, and phospho-tyrosine-associated PI3K activity was measured. The experiment was repeated once, with similar results. B, PC12 cells were plated in 6-well plates and grown until they reached 40–50% confluence prior to treatments. After treatments, cell lysates were processed for Western blotting using the indicated antibodies. A, the culture medium was replaced with fresh complete medium, and cells were cultured for the indicated periods of time (h) in a 21% O₂ (Nₓ) or 1% O₂ (Hypoxia) atmosphere. Similar results were obtained in at least two more experiments. B, cells were exposed to 1% O₂ (Hypoxia), 100 ng/ml NGF, or 380 µM deferoxamine (DFX) for the indicated periods of time. The NGF and deferoxamine treatments were performed under normoxic conditions (21% O₂). Similar results were obtained in an independent experiment. C, culture medium in wells was replaced with fresh medium alone or containing the indicated amounts of deferoxamine (µM) or 100 µM CoCl₂ for 9 h under normoxic conditions. For comparison, cells were cultured under normoxic (21% O₂, N) or hypoxic (1% O₂, H) conditions for 9 h. WB, Western blot.

**FIG. 4.** Akt is phosphorylated in response to hypoxia and deferoxamine at late times. In all cases PC12 cells were plated in 6-well plates and grown until they reached 40–50% confluence prior to treatments. After treatments, cell lysates were processed for Western blotting using the indicated antibodies. A, the culture medium was replaced with fresh complete medium, and cells were cultured for the indicated periods of time (h) in a 21% O₂ (Nₓ) or 1% O₂ (Hypoxia) atmosphere. Similar results were obtained in at least two more experiments. B, cells were exposed to 1% O₂ (Hypoxia), 100 ng/ml NGF, or 380 µM deferoxamine (DFX) for the indicated periods of time. The NGF and deferoxamine treatments were performed under normoxic conditions (21% O₂). Similar results were obtained in an independent experiment. C, culture medium in wells was replaced with fresh medium alone or containing the indicated amounts of deferoxamine (µM) or 100 µM CoCl₂ for 9 h under normoxic conditions. For comparison, cells were cultured under normoxic (21% O₂, N) or hypoxic (1% O₂, H) conditions for 9 h. WB, Western blot.

**DISCUSSION**

Reduction of oxygen supply has deleterious effects on many tissues. Neurons and cardiac muscle cells are particularly sensitive because they suffer both necrotic and apoptotic cell death when deprived of oxygen, which occurs during ischemia (29, 30). However, hypoxia is not always lethal. Faced with this
stress, some cell types undergo cell cycle arrest but remain viable (31), and others remain unaltered. Moreover, the induction of apoptosis observed after long periods of hypoxia could be due to acidosis rather than to a direct effect of hypoxia (31). In addition, organisms are able to respond to both acute and chronic reductions in oxygen tension with specific adaptive responses aimed to restore appropriate oxygen supply. Some of these responses, such as induction of angiogenesis, are local, whereas others are systemic. The systemic responses include hyperventilation, increase in heart output, increase in erythropoiesis, pulmonary vasconstriction, and carotid body hypertrophy, among others. The induction of these responses is under the control of specific cell types that have evolved to sense oxygen levels. These cell types include the glomus cells of the carotid body, the cells of the neuroepithelial bodies in the lung, and the chromaffin cells of the medulla of the adrenal gland, among others. In particular, the chromaffin cells are critical players in the response to oxygen deprivation in fetuses and neonates (1). Thus, it is feasible that these cell types have developed specific mechanisms ensuring their viability and functionality during hypoxia.

In this study, we have shown that under hypoxic conditions PC12 cells are protected against apoptosis triggered by different stimuli. The protection against apoptosis is observed at pO₂ levels within the physiopathological range, and it can be induced by pharmacological agents that mimic hypoxia. This effect is paralleled by activation of the pro-survival PI3K/Akt pathway through a mechanism that requires de novo protein synthesis. Moreover, inhibition of PI3K not only prevents Akt activation but also the antiapoptotic effect of hypoxia. Thus, it is likely that the activation of the PI3K/Akt pathway by hypoxia is the mechanism responsible for its antiapoptotic effect.

To our knowledge, this is the first report showing that, at least in specific cell types, hypoxia is able to promote survival. Our data concur with a previous report describing defereroxamine treatment as being able to prevent apoptosis (32). Whether the phenomenon of ischemic preconditioning (33), the partial resistance to ischemia-induced damage found after a previous episode of moderate ischemia, is due to a mechanism similar to the one described here will require further work. However, it is intriguing that preconditioning, similar to activation of Akt, requires de novo protein synthesis (29). In addition, we have shown that hypoxia renders cells resistant to apoptosis induced by the chemotherapeutic drugs taxol and fluorouracil, an effect that might contribute to the partial resistance to therapy observed in the hypoxic regions of tumors as compared with the normoxic areas of the same tumors (34). Nevertheless, this is not the only example in which proapoptotic stimuli induce an antiapoptotic response. Exposure of cells to H₂O₂ results in the activation of the PI3K/Akt pathway and resistance to induction of apoptosis (14). Hence, activation of the PI3K/Akt survival pathway could be a general cellular response to cell and tissue injury.

The activation of Akt by hypoxia in cells derived from pten−/− tumors has recently been described (15). Here we provide evidence that the activation of the PI3K/Akt pathway by hypoxia also occurs in some specific cells that are apparently normal for pten (35), including PC12 cells and primary chromaffin cells from cow adrenal gland medulla. This seems to be a specific response, restricted to at least cells of chromaffin lineage, rather than a general effect of hypoxia, because we did not detect any significant phosphorylation of Akt in other cell types tested. The lack of Akt activation in these cell lines was not the result of defective responses to hypoxia, because in all cases hypoxia-inducible factors were stabilized in response to hypoxia.²

In addition, the mechanism, involving de novo protein synthesis, and the sustained activation of Akt have not been described previously. One remaining question about this novel mechanism of Akt activation is the nature of the protein whose synthesis is required for the activation of Akt. Most likely it is not a soluble factor, at least not a stable one, released by hypoxic cells, because conditioned medium from hypoxia-treated cells failed to induce Akt activation in PC12 cells grown in normoxia.² It is still possible that the effect is mediated by the induction of a nonsecreted membrane-bound ligand or by the induction of both a soluble factor and its receptor. One further possibility is that the synthesized molecule acts in a cell-autonomous manner by direct activation of the PI3K/Akt pathway from inside the cell. Further work will be required to differentiate among these possibilities. The identification of such a molecule could explain why the effect of hypoxia on Akt is restricted to some cell types.

Hypoxia treatment results in changes in gene expression that are mediated by the activation of different transcription factors. Hypoxia-inducible factors are the best characterized;
others include cAMP response element-binding protein (4), NF-κB (36), and c-Fos (2). Although we cannot formally rule out that hypoxia-inducible transcription factors are involved in Akt activation, indirect evidence suggests that it is not the case, because both calmodulin antagonists and the MEK inhibitor PD98059 prevent EPAS activity in PC12 cells (25) without affecting hypoxia-induced Akt activation.

We suggest that the activation of survival pathways by hypoxia, at least in chromaffin cells, ensures that the cells remain viable and able to trigger the responses required for the adaptation to varying oxygen tension. Finally, activation of Akt has many other effects, in addition to promoting survival. Among these effects are changes in gene expression, induction of cell proliferation, and increasing glucose uptake (13). It is thus possible that many other effects of hypoxia in cell biology, in this cell type, could be mediated by activation of the PI3K/Akt pathway.

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