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

PROTECTIVE ROLE IN APOPTOSIS*

Received for publication, December 26, 2000, and in revised form, April 6, 2001
Published, JBC Papers in Press, April 9, 2001, DOI 10.1074/jbc.M011688200


From the ‡Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, 28006 Madrid, Spain and the ¶Department of Immunology and Oncology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Cantoblanco, 28049 Madrid, Spain

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 chemotherapy 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 pO2 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, secretes dopamine and norepinephrine in response to hypoxia (1). 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 norepinephrine. 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, apoptotic cells present specific biochemical alterations including exposure of phosphatidylserine to the extracellular side of the plasma membrane and activation of specific proteases (caspases). Upon activation, caspases cleave several intracellular molecules, leading to the functional and morphological 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

* This work was supported in part by grants from Ministerio de Educación y Cultura (PM 98/1328 and PM 97/0132), from Fondo de Investigaciones Sanitarias (FIS 98/1328), and from Comunidad Autónoma de Madrid (CAM 08.3/0016/99). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a postdoctoral fellowship from the Comunidad de Madrid.
‡ Supported by a fellowship from Universidad Autónoma de Madrid.
§§ Both senior authors contributed equally to this work.
‡‡ Recipient of a Contrato de Investigación from Fondo de Investigaciones Sanitarias. To whom correspondence should be addressed. Tel.: 34-91-520-2371; Fax: 34-91-520-2374; E-mail: lpeso@hlpq.insalud.es.

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-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase.

This paper is available on line at http://www.jbc.org
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 hypoxia activates Akt in pten-deficient glioma cells (15).

In the present study we show that hypoxia 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 hypoxia 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.), 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C. Hypoxia (1% O2) was induced by culture of cells inside an air-tight chamber with inflow and outflow valves that was mixed with a mixture of 1% O2, 5% CO2, 94% N2 (S.E. Carburos Metalicos S.A., Madrid, Spain); in those experiments, the different inhibitors or vehicle and then placed in hypoxia chambers. In experiments in which drugs were used, the compounds were added to the hypoxia chamber, the medium was removed from the plates, cells were washed, and fresh (complete or serum-free) medium was added to the plates. In experiments in which drugs were used, the compounds were added immediately prior to transfer to hypoxia/normoxia conditions. For experiments using kinase inhibitors, cells were pretreated during 1 h with the different inhibitors or vehicle and then placed in hypoxia.

**Western Blotting**—After treatments, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested in 70 or 200 μl (P35 or P60 mm dishes, respectively) of a lysis buffer containing 2% SDS, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 20 mM NaF, 1 mM Na3VO4, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (for PI3K assays 1 mM CaCl2 was included). About 10% of each lysate was saved to assay for proteins in the lysate, and the remaining was immunoprecipitated with 5 μg of anti-Akt antibody (Cell Signaling; number 9272) or 2.5 μg of anti-phosphoarginine monoclonal antibody (4G10, Upstate Biotechnology, Lake Placid, NY; number 05–321) for 2 h at 4 °C. Immunoprecipitates were washed, and immunocomplexes were subjected to an in vitro kinase assay using histone H2B (Akt assay) or phosphatidylinositol 4,5-bisphosphate (PI3K assay) as substrates (17, 18).

**Viability Assay**—Viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 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 ~80% confluence. At later than 36 h under hypoxic conditions. Finally, medium was removed, and precipitated MTT dye 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 ~70% confluence. When they reached ~80% confluence, after, culture medium was replaced with 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 100–150 ng/ml NGF for 10 min. After treatments, cells were lysed with 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 10% glycerol, 37 mM NaCl, 20 mM Tris-HCl, pH 7.5, 20 mM NaF, 1 mM Na3VO4, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for PI3K assays 1 mM CaCl2 was included. About 10% of each lysate was saved to assay for proteins in the lysate, and the remaining was immunoprecipitated with 5 μg of anti-Akt antibody (Cell Signaling; number 9272) or 2.5 μg of anti-phosphoarginine monoclonal antibody (4G10, Upstate Biotechnology, Lake Placid, NY; number 05–321) for 2 h at 4 °C. Immunoprecipitates were washed, and immunocomplexes were subjected to an in vitro kinase assay using histone H2B (Akt assay) or phosphatidylinositol 4,5-bisphosphate (PI3K assay) as substrates (18, 19).

**Statistical Analysis**—Where indicated, experimental data were analyzed using the Prism3® GraphPad (version 2.0) software. Data were analyzed by the analysis of variance (ANOVA) test followed by the Newman-Keuls test for multiple comparisons or the Dunnett test for multiple comparisons against one control. The p values obtained in this analysis are indicated in the text and figures. Similar results (statistical significance) were obtained when using the Tukey or Bonferroni test after ANOVA or when comparing relevant pairs of data by the Student’s t test.

**RESULTS**

**Hypoxia Protects PC12 Cells from Apoptosis**—To assess the effect of hypoxia 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 hypoxia 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 (p < 0.01), 24 (p < 0.01), and 36 (p < 0.05) h after serum withdrawal (57, 68, and 62% reduced, respectively). Fig. 1B shows the cell cycle profile of PC12 cells 24 h after the indicated treatments in a representative experiment. The effect of hypoxia on cellular viability was confirmed using the MTT assay (17, 22). Whereas hypoxia (1% O2, 20 h) itself had little effect on cell viability (14% reduction in viability as compared with cells in normoxia), serum starvation for 20 h
Hypoxia protects PC12 cells from apoptosis induced by serum withdrawal. A, PC12 cells were plated in 6-well plates and grown until they reached 40–50% confluence. Thereafter, the culture medium was replaced with serum-free (−S) or complete culture medium (+S), and cells were incubated in a 21% (Nx) or 1% (Hx) oxygen atmosphere (see “Experimental Procedures”) for the indicated times. Apoptosis was measured by flow cytometry. Results represent the mean ± S.E. (except for t = 72 h, where it is represented as the mean ± range) of several independent experiments (n = 3 at 3 h, n = 3 at 9 h, n = 12 at 12 h, n = 5 at 24 h, n = 3 at 36 h, and n = 2 at 72 h). The asterisk indicates that the difference in the percentage of apoptosis in samples without serum under normoxia and hypoxia is statistically significant (p < 0.01, see “Experimental Procedures”). B, flow cytometry cell cycle profile analysis of samples from one of the experiments shown in A (24 h after treatments). C, PC12 cells were plated in 96-well plates pre-coated with poly-L-lysine. The culture medium was replaced with serum-free (cytometry cell cycle profile analysis of samples from one of the experiments shown in A of apoptosis in samples without serum under normoxia and hypoxia is statistically significant (p < 0.01, see “Experimental Procedures”). Moreover, treatment with deferoxamine or cobalt chloride (CoCl2), two compounds widely used to mimic many of the effects of hypoxia (23), resulted in a reduction of apoptosis, although with less efficiency. Consistently, a significant reduction in apoptosis induced 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% O2 (Fig. 2A), which corresponds to a pO2 (36 torr) within the physiological range found in venous blood in vivo. Moreover, treatment with deferoxamine or cobalt chloride (CoCl2), two compounds widely used to mimic many of the effects of hypoxia (23), resulted in a reduction of apoptosis (Fig. 2A).

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% O2), 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 Induces Akt Activation in PC12 Cells—To identify the mechanism responsible for the pro-survival effect of hypoxia, we decided to study the effect of hypoxia on the proto-oncogenic 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 tested2 including human vascular endothelial cells, neuroblastoma-69 cells, human embryonic 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.2 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.2

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-

---

2 M. Álvarez-Tejado, M. O. Landázuri, and L. del Peso, unpublished observations.
Hypoxia Protects PC12 Cells from Apoptosis

Hypoxia-induced Akt Activation Requires Protein Synthesis—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) or hypoxic (1% O₂) conditions for 9 h. Similar results were obtained in at least two more experiments. We have shown that hypoxia prevents apoptosis in response to several stimuli and that this effect correlates with the activation of the PI3K/Akt pathway. To investigate whether there was a functional link between these two phenomena, we investigated the effect of PI3K inhibitors on the protective effect of hypoxia. As shown in Fig. 6, the reduction of apoptosis, upon serum removal, observed under hypoxia was completely prevented by treatment of cells with the PI3K inhibitors LY294002 or wortmannin. These results indicate that the protection against apoptosis induced by hypoxia depends on the activation of the PI3K/Akt survival pathway. In addition to the PI3K/Akt pathway, the MAPK cascade has an important role in protection against apoptosis induced by hypoxia and deferoxamine at late times.

Inhibition of Apoptosis by Hypoxia Requires PI3K Activity

We have shown that hypoxia prevents apoptosis in response to several stimuli and that this effect correlates with the activation of the PI3K/Akt pathway. To investigate whether there was a functional link between these two phenomena, we investigated the effect of PI3K inhibitors on the protective effect of hypoxia. As shown in Fig. 6, the reduction of apoptosis, upon serum removal, observed under hypoxia was completely prevented by treatment of cells with the PI3K inhibitors LY294002 or wortmannin. These results indicate that the protection against apoptosis induced by hypoxia depends on the activation of the PI3K/Akt survival pathway. In addition to the PI3K/Akt pathway, the MAPK cascade has an important role in survival of PC12 cells (28). However, the MEK inhibitor (PD98059) had no effect on the protective effect of hypoxia (Fig. 6), despite increasing the percentage of cells undergoing apoptosis upon serum withdrawal under normoxic conditions (Fig. 6). In agreement with these results, the protective effects of deferoxamine and CoCl₂ treatment on apoptosis upon serum withdrawal (Fig. 2A) were also abrogated by inhibition of PI3K.

**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 condition, cells can adapt and survive by activating survival mechanisms. In PC12 cells, hypoxia protects against apoptosis through the activation of the PI3K/Akt pathway. This protective effect was completely prevented by treatment with PI3K inhibitors, indicating that the activation of PI3K is essential for the survival of PC12 cells under hypoxic conditions. Furthermore, the protective effect of hypoxia was not due to the synthesis of a soluble factor, as the conditioned medium from hypoxia-treated PC12 cells was unable to induce Akt activation in normoxic cells, suggesting that the effect of hypoxia was due to the synthesis of a soluble factor able to activate the PI3K/Akt pathway in an autocrine or paracrine fashion.

**Inhibition of Apoptosis by Hypoxia Requires PI3K Activity—**We have shown that hypoxia prevents apoptosis in response to several stimuli and that this effect correlates with the activation of the PI3K/Akt pathway. To investigate whether there was a functional link between these two phenomena, we investigated the effect of PI3K inhibitors on the protective effect of hypoxia. As shown in Fig. 6, the reduction of apoptosis, upon serum removal, observed under hypoxia was completely prevented by treatment of cells with the PI3K inhibitors LY294002 or wortmannin. These results indicate that the protection against apoptosis induced by hypoxia depends on the activation of the PI3K/Akt survival pathway. In addition to the PI3K/Akt pathway, the MAPK cascade has an important role in survival of PC12 cells (28). However, the MEK inhibitor (PD98059) had no effect on the protective effect of hypoxia (Fig. 6), despite increasing the percentage of cells undergoing apoptosis upon serum withdrawal under normoxic conditions (Fig. 6). In agreement with these results, the protective effects of deferoxamine and CoCl₂ treatment on apoptosis upon serum withdrawal (Fig. 2A) were also abrogated by inhibition of PI3K.

**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 condition, cells can adapt and survive by activating survival mechanisms. In PC12 cells, hypoxia protects against apoptosis through the activation of the PI3K/Akt pathway. This protective effect was completely prevented by treatment with PI3K inhibitors, indicating that the activation of PI3K is essential for the survival of PC12 cells under hypoxic conditions. Furthermore, the protective effect of hypoxia was not due to the synthesis of a soluble factor, as the conditioned medium from hypoxia-treated PC12 cells was unable to induce Akt activation in normoxic cells, suggesting that the effect of hypoxia was due to the synthesis of a soluble factor able to activate the PI3K/Akt pathway in an autocrine or paracrine fashion.

**Inhibition of Apoptosis by Hypoxia Requires PI3K Activity—**We have shown that hypoxia prevents apoptosis in response to several stimuli and that this effect correlates with the activation of the PI3K/Akt pathway. To investigate whether there was a functional link between these two phenomena, we investigated the effect of PI3K inhibitors on the protective effect of hypoxia. As shown in Fig. 6, the reduction of apoptosis, upon serum removal, observed under hypoxia was completely prevented by treatment of cells with the PI3K inhibitors LY294002 or wortmannin. These results indicate that the protection against apoptosis induced by hypoxia depends on the activation of the PI3K/Akt survival pathway. In addition to the PI3K/Akt pathway, the MAPK cascade has an important role in survival of PC12 cells (28). However, the MEK inhibitor (PD98059) had no effect on the protective effect of hypoxia (Fig. 6), despite increasing the percentage of cells undergoing apoptosis upon serum withdrawal under normoxic conditions (Fig. 6). In agreement with these results, the protective effects of deferoxamine and CoCl₂ treatment on apoptosis upon serum withdrawal (Fig. 2A) were also abrogated by inhibition of PI3K.
Hypoxia Protects PC12 Cells from Apoptosis

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 dereroxamine 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.

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. 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;
Hypoxia Protects PC12 Cells from Apoptosis

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.

Acknowledgments—We thank M. Vitón for valuable assistance with flow cytometry, M. C. Castellanos for help with the calmodulin inhibitors, A. Vara for valuable technical assistance, A. Alfranca, J. Aragones, E. Temes, S. Garcia, F. Vidal, and A. Garcia for critically reviewing the manuscript, and V. Alvarez for suggestions. We also thank D. Jones for valuable help.

REFERENCES