

Hypoxia Inhibits Expression of Prolactin and Secretion of Cathepsin-D by the GH4C1 Pituitary Adenoma Cell Line

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SUMMARY: Diminished oxygen concentration within growing tumors may stimulate neovascularization by inducing both up-regulation of angiogenic factors and down-regulation of antiangiogenic agents. A potentially important molecule in the growth of pituitary adenomas is prolactin (PRL), which can be cleaved by cathepsin-D to yield a 16-kDa form (16K-PRL) with potent antiangiogenic effects. We examined the expression of PRL in cultured GH4C1 pituitary adenoma cells after exposure to hypoxia (0.1% oxygen) for periods of 12 to 36 hours. In contrast to increased expression of the angiogenic factor vascular endothelial growth factor in hypoxic cells, PRL mRNA and levels of intracellular and secreted PRL were significantly reduced under hypoxia. The reduction was not attributable to a general suppression of either transcription or protein synthesis. Although 16K-PRL was not evident in conditioned medium at physiologic pH, lowering the pH to mimic the acidic tumor microenvironment resulted in generation of 16K-PRL, which was sharply reduced in medium drawn from hypoxic cells. Production of 16K-PRL was blocked by the cathepsin-D inhibitor pepstatin-A, and the reduced 16K-PRL formation in hypoxic-conditioned medium correlated with a decrease in secretion of cathepsin-D and its precursor, procathepsin-D. Thus, hypoxia acts upon GH4C1 cells to increase vascular endothelial growth factor expression, decrease PRL synthesis, and suppress conversion of PRL to 16K-PRL via inhibition of cathepsin-D proteolysis. These mechanisms may act in concert to stimulate angiogenesis in prolactinomas. (*Lab Invest* 2003, 83:1627–1636).

The growth of pituitary adenomas, as of all tumors, depends on adequate vascularization once tumors have grown beyond a few millimeters in size (Folkman, 1990). However, in contrast to other tumors, pituitary adenomas are less vascularized than the normal tissue (Jugenburg et al, 1995; Schechter, 1972; Turner et al, 2000a). Because an adequate blood supply enables tumor cells to proliferate further or metastasize to another organ, the reduced vascularization of pituitary adenomas suggests that inhibition of angiogenesis in these tumors plays a role in their characteristic slow growth and failure to reach the carcinoma stage (Jugenburg et al, 1995; Turner et al, 2000a, 2000b; Vidal et al, 2000a). Among pituitary tumors, those secreting prolactin (PRL) occur most frequently. They range from microprolactinomas,

which rarely progress in size, to large extracellular macroprolactinomas that may invade local tissues (Cunnah and Besser, 1991). These differences fit with the observation that the vascular density of macroprolactinomas is significantly greater than that of microprolactinomas (Turner et al, 2000a) and further suggest that inhibitors of angiogenesis might influence the phenotype and behavior of these tumors.

One putative inhibitor of angiogenesis in the pituitary gland is a fragment of PRL. The full-length 23-kDa PRL molecule can be posttranslationally processed by proteolytic cleavage to a 16-kDa form (16K-PRL) that has unique antiangiogenic actions not shared with the full-length molecule (Clapp et al, 1993; Corbacho et al, 2002). 16K-PRL can be generated in the anterior pituitary gland of rats (Shah and Hymer, 1989; Torner et al, 1995), mice (Sinha and Gilligan, 1984), and humans (Pellegrini et al, 1988; Sinha et al, 1985; Warner et al, 1993) and acts as a potent inhibitor of angiogenesis in vivo and in vitro, inhibiting endothelial cell proliferation (Clapp et al, 1993) and stimulating type 1 plasminogen activator inhibitor expression (Lee et al, 1998) and endothelial cell apoptosis (Martini et al, 2000). Inhibition of angiogenesis by 16K-PRL would be critically influenced by the availability of both the parent molecule (PRL) and the protease responsible for processing PRL. Various lines of evidence suggest that the protease is cathepsin-D, a ubiquitous acidic-aspartyl endopro-

DOI: 10.1097/01.LAB.0000098429.59348.36

Received August 27, 2003.

This work was supported by Grants 36041-N and 34309-N from the National Council of Science and Technology of Mexico (CONACYT), Grants PUIS, IN227502, and IN221702 from the National Autonomous University of Mexico, and Grant 55000595 from the Howard Hughes Medical Institute.

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teinase in lysosomes (Baldocchi et al, 1993; Corbacho et al, 2002). Cathepsin-D is secreted by tumor cells (Morikawa et al, 2000; Rochefort et al, 1990) and is thought to be associated with tumor progression (Berchem et al, 2002; Rochefort et al, 2000).

Hypoxia, a common feature of solid tumors, results from insufficient blood perfusion and oxygen consumption rates that exceed oxygen supply (Hockel and Vaupel, 2001). Hypoxic conditions affect the expression of a well-characterized battery of genes that help cells cope with low oxygen concentrations, including the up-regulation of several genes encoding angiogenic factors and the down-regulation of angiogenesis inhibitors (Richard et al, 1999; Tenan et al, 2000). Because 16K-PRL is antiangiogenic, we speculated that a hypoxic milieu could reduce the generation of 16K-PRL in pituitary tumor cells, either by affecting expression of the parent PRL form or by modulating the availability of the protease responsible for processing PRL. Lowering 16K-PRL may be one of the mechanisms determining the increased vascularization of macroprolactinomas versus microprolactinomas and other pituitary adenomas. In the present study, we examined the effects of hypoxia on PRL gene expression and cathepsin-D levels in the rat GH4C1 pituitary adenoma cell line.

Results

The experimental protocol used to study hypoxia involved exposure of GH4C1 cells to 0.1% oxygen for periods of up to 36 hours. GH4C1 cells were found to be relatively resistant to hypoxia-induced reduction of cell viability. No morphologic change was seen in GH4C1 cells at any of the time points (not shown), and the number of adherent cells was constant for up to 24 hours of hypoxia (Fig. 1A). By 36 hours there was a significant ($p < 0.05$) reduction in the number of adherent cells; however, for the cells that remained attached, hypoxic treatment for up to 36 hours induced no discernible loss of viability (Fig. 1B).

Because low concentrations of oxygen have previously been reported to stimulate the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) in tumor cells (Minchenko et al, 1994; Shweiki et al, 1992) and because the GH3 cell line, parental to GH4C1 cells, expresses VEGF (Lohrer et al, 2001; Ochoa et al, 2000; Vidal et al, 2000b), we used RT-PCR to assay transcription of several forms of VEGF in GH4C1 cells in response to hypoxia. Transcription of both the 164 and 120 amino acid variants of VEGF was elevated in GH4C1 cells after hypoxic treatment for 12 hours (Fig. 2A), and this increase persisted for at least 24 hours. At each time point, the increase in the mRNA encoding for VEGF164 was about 2-fold and that of VEGF120 was about 1.5-fold (Fig. 2B). No band corresponding to a VEGF188 transcript was detected.

In contrast to VEGF, PRL mRNA levels were significantly decreased under the same conditions. Northern blots showed that PRL mRNA was reduced in GH4C1 cells after 12 hours of exposure to hypoxia.

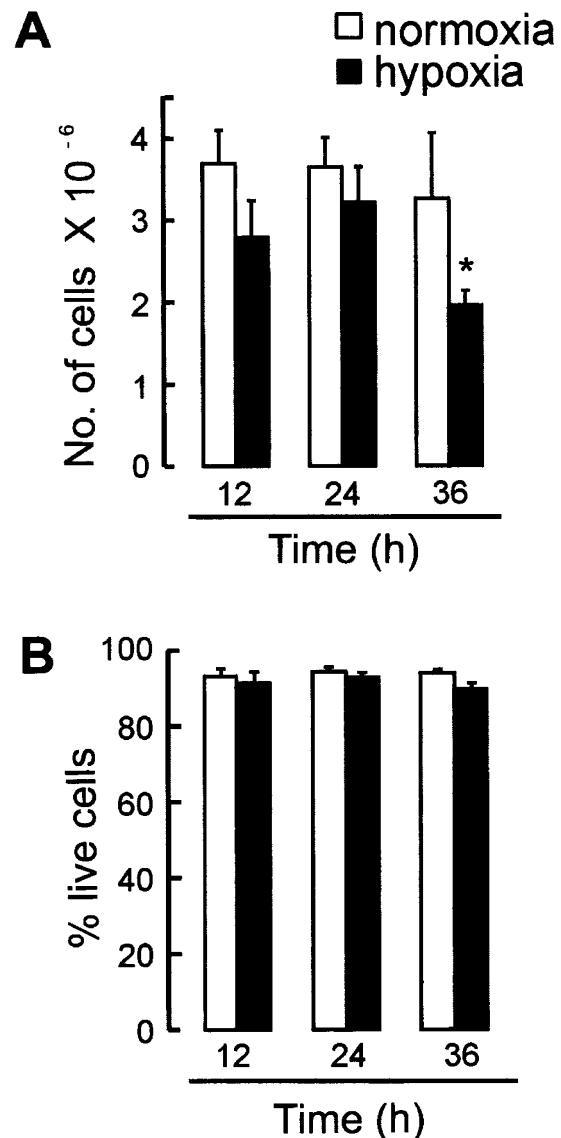


Figure 1.

Effect of hypoxia on cell number (A) and viability (B). Confluent GH4C1 cells were cultured in normoxic or hypoxic conditions for 12 to 36 hours. Adherent cells were lifted by trypsin treatment and counted by hemocytometer, and the percentage of viable cells was determined by trypan blue exclusion. Data are mean values \pm SEM of three independent experiments. * $p < 0.05$ vs corresponding normoxic value.

The decrease was more apparent with longer treatments, because the PRL transcript accumulated in cells under normoxic conditions but declined in hypoxic cells (Fig. 3A). After 36 hours of hypoxia, PRL mRNA was reduced by approximately 80% (Fig. 3B). Reduction of transcription was not a general phenomenon in hypoxic GH4C1 cells, because rehybridizing with a probe for β -actin showed no alteration of expression of this constitutive gene under hypoxia (Fig. 3A).

The decrease in transcription of the PRL gene was accompanied by a reduction in levels of the protein. Western blotting of GH4C1 homogenates showed decreased PRL levels after 12 to 36 hours of hypoxic treatment (Fig. 4A). As with mRNA levels, intracellular

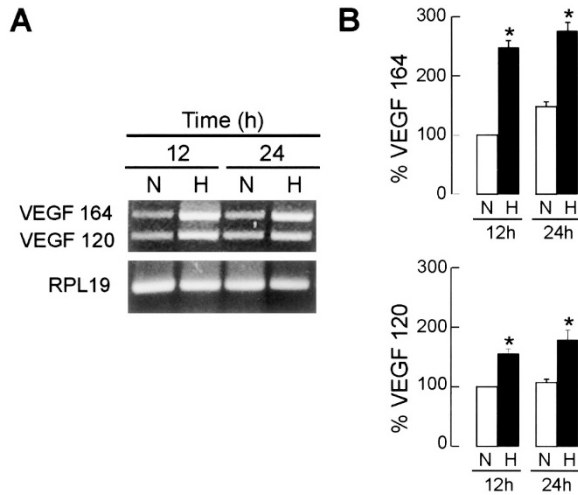


Figure 2.

Hypoxia-induced up-regulation of vascular endothelial growth factor (VEGF) expression. A, VEGF mRNAs encoding isoforms 164 (563 bp) and 120 (431 bp) were analyzed by RT-PCR performed on total RNA extracted from GH4C1 cells cultured for 12 or 24 hours under normoxic (N) or hypoxic (H) conditions. Amplification of the housekeeping gene ribosomal protein L19 was used as a control. B, VEGF mRNA values were evaluated densitometrically and normalized with respect to the 12-hour normoxia value. Data are mean \pm SEM of three independent experiments. * $p < 0.05$ with respect to the corresponding normoxic value.

PRL continued to accumulate when oxygen concentration was normal but declined under hypoxia. In contrast, levels of β -actin remained unaltered by the oxygen status of the culture. Secreted PRL was also examined and found to rise under normoxic conditions, increasing more than 2-fold between 12 and 36 hours of incubation (Fig. 4B). In comparison, hypoxic cells secreted less PRL after 12 hours of treatment, and PRL levels at subsequent time points were consistently lower than in normoxic cells. Furthermore, the rate of accumulation of secreted PRL was lower under hypoxia than under normoxia. These findings were confirmed by ELISA determinations showing a significant ($p < 0.05$) 2-fold reduction of secreted PRL under hypoxia (83.5 ± 6.1 vs 43.3 ± 6.6 $\mu\text{g}/10^6$ cells for 24 hours of normoxia vs hypoxia, respectively). To examine whether reduction of PRL secretion could be a general phenomenon for all proteins secreted by GH4C1 cells, we compared levels of secreted proteins under hypoxia and normoxia by silver staining (Fig. 4C). Levels of the majority of secreted proteins were not altered under hypoxia.

Although the polyclonal antibody used in these experiments binds to several forms of PRL, including the 23-kDa and 16-kDa variants, no product other than 23K-PRL was detected in either GH4C1 cell homogenates or conditioned media. Because cathepsin-D, the presumed PRL-processing enzyme, acts at acidic pH and because the extracellular pH of tumors is acidic (Stubbs et al, 2000), we speculated that 16K-PRL could be generated extracellularly via cathepsin-D secreted by tumor cells into an acidic microenvironment. To determine whether the proteolytic activity responsible for generation of 16K-PRL was present in the conditioned medium of GH4C1

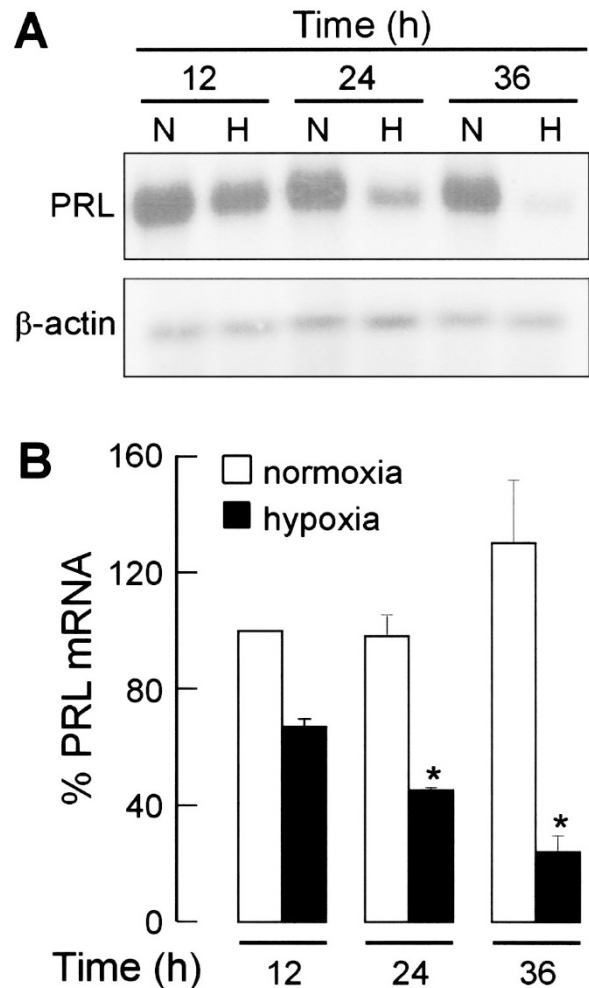


Figure 3.

Hypoxia-induced down-regulation of prolactin (PRL) mRNA. A, Northern blot for PRL mRNA and β -actin in GH4C1 cells exposed for 12 to 36 hours to normoxia (N) or hypoxia (H). B, PRL mRNA values were quantified densitometrically and normalized with respect to the 12-hour normoxia value. Data are mean \pm SEM of three independent experiments. * $p < 0.05$ with respect to the corresponding normoxic value.

cells, medium drawn from cells after 24 hours of normoxia or hypoxia was incubated at pH 5.6 for 24 hours at 37°C, and proteolysis of PRL in the medium was evaluated by Western blots. When conditioned medium was exposed to low pH, a second immunoreactive product was seen in addition to PRL that corresponded in size to 16K-PRL (Fig. 5A). The concentration of the 16-kDa product relative to that of 23K-PRL was 4-fold lower in medium drawn from hypoxic cells (Fig. 5B). Generation of the 16-kDa product seemed to be attributable to a protease released by the cells, because no 16K-PRL was evident when the conditioned medium was heat inactivated (not shown) or when a 23K-PRL standard was incubated at a low pH in nonconditioned medium (Fig. 5A).

In support of cathepsin-D being the responsible protease, the generation of the 16K-PRL at acidic pH was inhibited by the cathepsin-D inhibitor pepstatin-A (Fig. 5A). We examined the acidified medium from

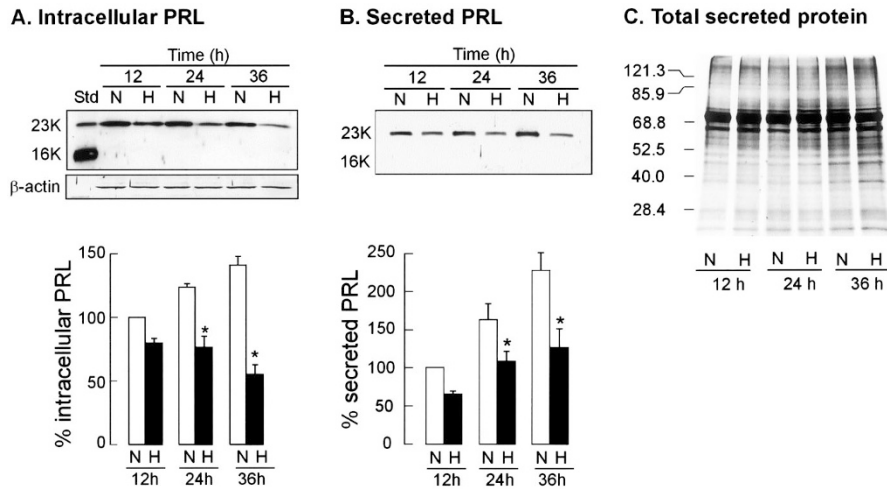


Figure 4.

Hypoxia-induced inhibition of intracellular and secreted PRL. PRL was analyzed by Western blots in homogenates (A) and conditioned media (B) of GH4C1 cells exposed to normoxia (N) or hypoxia (H) for 12 to 36 hours. Blots from homogenates were reblotted with anti- β -actin antibodies (A). PRL bands were evaluated densitometrically and normalized with respect to the 12-hour normoxia value. Data are mean \pm SEM from three independent experiments. * $p < 0.05$ with respect to the corresponding normoxic value. C. Silver staining of secreted proteins from GH4C1 cells exposed to normoxia (N) or hypoxia (H) for 12 to 36 hours. Proteins from conditioned medium drawn from 6.5×10^4 cells were precipitated with trichloroacetic acid and loaded. The major protein band of 70 kDa is a medium constituent, and none of the other proteins were detected in nonconditioned medium processed in the same way as GH4C1-conditioned medium.

GH4C1 cells by immunoblotting to detect cathepsin-D isoforms and determined whether their levels decreased under hypoxic conditions. Procathepsin-D, the 52-kDa precursor of cathepsin-D, and mature cathepsin-D (33 kDa) were detected in GH4C1 conditioned medium (Fig. 6A) but not in nonconditioned medium (not shown). The concentration of both forms of the enzyme increased with time but was lower in hypoxic cells at the two times measured (Fig. 6B).

Discussion

Cells exposed to low oxygen levels adapt by altering the transcription of numerous genes involved either in decreasing cellular oxygen consumption or in increasing oxygen availability to the tissues (Bunn and Poyton, 1996). Angiogenesis is one major mechanism that increases oxygen availability, and hypoxia up-regulates angiogenesis activators and down-regulates inhibitors. Because 16K-PRL, a potent inhibitor of angiogenesis, is derived from PRL by proteolysis, we reasoned that hypoxia might down-regulate PRL gene expression in pituitary lactotrophs. Here, we report that PRL production, as well as its cathepsin-D-mediated conversion to 16K-PRL, are decreased by exposure of a pituitary tumor cell line to low oxygen.

We demonstrate that hypoxia reduces the expression of PRL mRNA and protein in the GH4C1 lactotroph cell line. In contrast to PRL, VEGF164 and VEGF120 mRNA were up-regulated by hypoxia, demonstrating the ability of GH4C1 cells to respond to low oxygen tension via the transcription of hypoxia-inducible genes. VEGF164 and 120 are secreted isoforms of VEGF that are active in angiogenesis (Enholm et al, 1997), and their expression is increased in several tumor cell lines in response to hypoxia (Minchenko et al, 1994; Shweiki et al, 1992). Thus, the

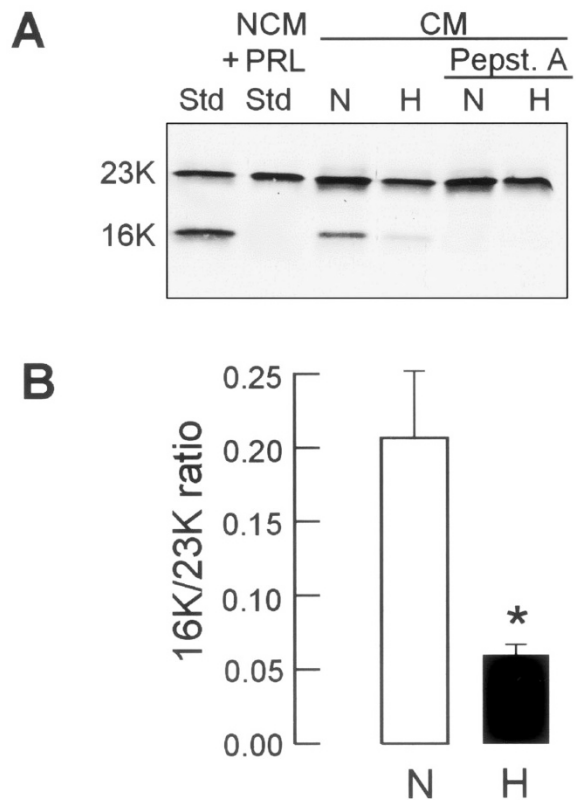


Figure 5.

Hypoxia-induced inhibition of PRL-cleaving activity. A, The conditioned medium (CM) of GH4C1 cells exposed to normoxia (N) or hypoxia (H) for 24 hours was acidified (pH 5.6), incubated at 37°C for 24 hours in the absence or presence of pepstatin-A (Pepst.A, 1 μ M), and analyzed by Western blot. Acidified nonconditioned medium (NCM) was incubated with a purified 23K-PRL standard under the same conditions as CM. B, PRL bands were evaluated densitometrically and expressed as the ratio of 16K-PRL to 23K-PRL. Ratios are mean \pm SEM from three independent experiments. * $p < 0.05$ with respect to the corresponding normoxic value.

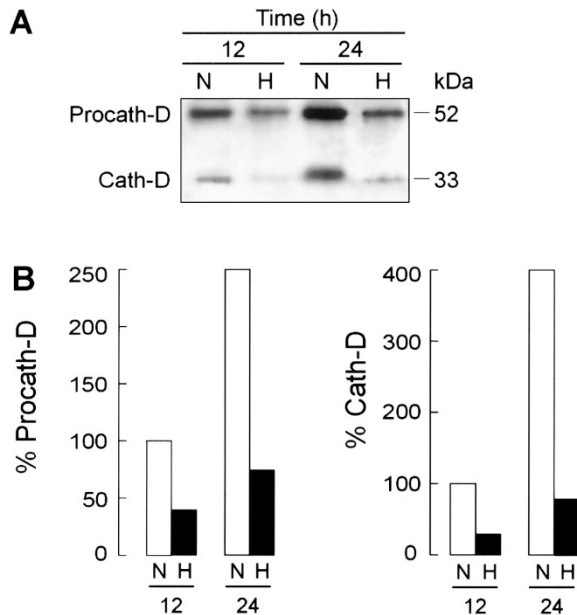


Figure 6.

Hypoxia-induced inhibition of secretion of cathepsin-D isoforms. A, Western blot analysis of pro-cathepsin-D (*Procath-D*) and cathepsin-D (*Cath-D*) in acidified (pH 5.6) conditioned medium from GH4C1 cultures exposed to normoxia (N) or hypoxia (H) for 12 or 24 hours. B, Protein bands were evaluated densitometrically and normalized with respect to the 12-hour value under normoxia.

up-regulation of VEGF by hypoxia in GH4C1 cells supports the notion that hypoxia is an important factor for the progression of PRL-secreting adenomas. In this regard, VEGF levels increase in the pituitary gland during the development of estrogen-induced prolactinomas (Banerjee et al, 1997), and both VEGF164 and VEGF120 are up-regulated by estrogens in the pituitary gland and in the GH3 lactotroph cell line (Ochoa et al, 2000).

Hypoxia did not inhibit PRL mRNA and protein through toxic effects on GH4C1 cells. Morphologically, the cells appeared unaffected by the extreme levels of oxygen deprivation used in this study, which recreate near-zero tissue oxygen levels measured in experimental models of wound repair (Niinikoski et al, 1972) or in areas within solid tumors (Hockel and Vaupel, 2001). Functionally, there was no difference in the number of GH4C1 cells for up to 24 hours of hypoxia, and the viability of attached cells remained constant at all times. Prolonged exposure to hypoxia can induce programmed cell death in some mammalian cells (Graeber et al, 1996) but not in others (Berk et al, 1999; Lou et al, 1997). Resistance to hypoxia-induced apoptosis seems particularly important among tumor cells, in which some studies claim that by inducing apoptosis, hypoxia may select for cells with defective apoptotic regulators such as p53 (Graeber et al, 1996) and a more aggressive phenotype (Brizel et al, 1996).

Likewise, hypoxia did not reduce PRL levels in GH4C1 cells by nonspecific suppression of protein synthesis and secretion. The mRNA levels of a constitutive protein (β -actin) and the levels of the majority of

secreted proteins remained unaltered under hypoxic conditions. Hypoxia exposure for 12 to 24 hours exerts no effect on total protein levels in some cell types (Tenan et al, 2000), although prolonged exposures generally decrease the rate of total protein synthesis (Hochachka et al, 1996).

The functional implication of hypoxia-induced inhibition of PRL expression is unclear. Reduction in tissue oxygen concentration is usually associated with numerous cellular, local, and systemic adaptive responses. Reduction in PRL production by pituitary cells could represent a systemic adaptive response to hypoxia. In fact, exposure to hypoxia does affect systemic PRL concentrations in rats (Zhang and Du, 2000) and humans (Bouissou et al, 1987). Placing male rats for 2 hours in hypobaric chambers simulating an altitude of 7 km (7.2% oxygen) reduced the content of PRL in the pituitary gland and in the circulation (Zhang and Du, 2000). Similarly, acute exposure to hypoxia (14.5% oxygen) inhibits the increase in blood PRL induced by maximal exercise in human subjects (Bouissou et al, 1987). At the whole body level, the inhibitory effect of acute hypoxia upon PRL is not entirely unexpected. Hypoxia increases expression of the gene encoding tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine, which is the main inhibitor of pituitary PRL synthesis and secretion (Czyzyk-krzeska et al, 1994). Furthermore, the effects of blood oxygen partial pressure on PRL levels may be part of a stress-induced response. Hypoxia can activate the hypothalamo-pituitary-adrenocorticoid axis (Chen and Du, 1996), inhibit immune function (Bai et al, 1997), and suppress body growth and reproduction (Du and Li, 1982; Moromisato et al, 1999; Yang et al, 1995).

Nevertheless, here we show that hypoxia acts directly on pituitary lactotrophs to inhibit PRL expression. Although further studies are underway to localize hypoxia-responsive motifs repressing PRL promoter function, our current results suggest that inhibition of PRL expression by a reduction in oxygen supply could have an impact at the local pituitary level, perhaps by promoting neovascularization. Inhibition of PRL expression would limit the production of 16K-PRL, a potent antiangiogenic factor. However, the experiments conducted in GH4C1 cells failed to detect 16K-PRL beyond trace levels. These findings contrast with those detecting 16K-PRL by immunoblotting in the pituitary gland (Shah and Hymer, 1989; Torner et al, 1995) and in pituitary lactotrophs in primary culture (Andries et al, 1992). The reason for this difference is unclear but may relate to the fact that unlike pituitary lactotrophs, which possess dense secretory granules, GH4C1 cells have minimal storage capacity and release PRL rapidly after synthesis (Dannies et al, 1984; Ho et al, 1989). Secretory granules are a possible site for the cleavage of PRL to 16K-PRL, because the putative PRL-protease, cathepsin-D, is active at acidic pH values (<5.5) and PRL granules are acidic (Lorenson et al, 1984). Likewise, because of the acidic pH requirement, proteolysis of PRL may not take place extracellularly in normal tissues but can occur in the extracellular milieu of tumors. Low interstitial pH is a

well-established pathophysiologic characteristic of solid tumors (Helmlinger et al, 1997; Tannock and Rotin, 1989). On average, tumor pH ranges from 6.6 to 7.2 (Helmlinger et al, 2002), although the extracellular space within malignant tissues has been measured to be as low as pH 5.8 (Wike-Hooley et al, 1984a, 1984b). In addition, cancerous tissues and cell lines secrete abnormally high levels of the inactive cathepsin-D precursor (procathepsin-D) (Rocheffort et al, 2000), and the tumor environment allows for acid-dependent autoactivation of procathepsin-D to yield cathepsin-D and for the catalytic action of the mature enzyme (Conner, 1989; Morikawa et al, 2000). In fact, cathepsin-D secreted by prostate carcinoma cells is responsible for the generation of angiostatin, a potent endogenous inhibitor of angiogenesis that is produced by tumor-mediated proteolysis of plasminogen (Morikawa et al, 2000).

Consistent with the extracellular generation of 16K-PRL by PRL-secreting tumor cells, acidification of conditioned medium from GH4C1 cells resulted in the proteolytic processing of the secreted hormone to a 16-kDa product. Because this conversion was inhibited by pepstatin-A, and because both procathepsin-D and mature cathepsin-D were detected in acidified GH4C1 conditioned medium, these findings indicate that the cells secrete active cathepsin-D able to generate 16K-PRL under acidic conditions. Interestingly, the degree of conversion of PRL to 16K-PRL was significantly decreased in medium drawn from hypoxic cells, indicating a reduction in secreted cathepsin-D isoforms. Lower levels of both the precursor and the mature protease in hypoxic medium were directly shown by Western blot. When coupled with the decrease in expression of the parent PRL molecule, the suppression of cathepsin-D activity suggests a substantial reduction of 16K-PRL under hypoxic conditions.

In conclusion, our results demonstrate that hypoxia acts in GH4C1 cells to up-regulate VEGF expression and to down-regulate PRL production and cathepsin-D-mediated proteolysis. GH4C1 cells are a clonal strain of rat pituitary tumor cells that have been used extensively to explore mechanisms controlling PRL synthesis and secretion. It would be particularly convenient to extend the present studies in GH4C1 cells to primary cultures of human PRL tumor cells as a better model of the human neoplastic condition, particularly because there are species differences with respect to PRL that have weakened extrapolations from the rat to the human pituitary. For example rat pituitaries can be exquisitely sensitive to induction of prolactinomas by estrogens, whereas a similar role for estrogens in the etiology of human prolactinomas has not been demonstrated (Horvath and Kovacs, 1994).

Although we hesitate to extend results with a cell line to interpretations concerning tumors, it is useful to consider whether these hypoxia-induced mechanisms might play a role in the development of the human disease. Because pituitary adenomas have limited angiogenic capacity (Jugenburg et al, 1995; Turner et al, 2000a, 2000b; Vidal et al, 2000a), it is conceivable

that inhibition of angiogenesis plays a significant role in controlling the dynamics of their progression. Hence, we hypothesize that endocrinologically hyperactive PRL-tumor cells (such as GH3 cells) would generate more 16K-PRL in the extracellular space due to their high release of PRL and cathepsin D into an acidic microenvironment. The increase in 16K-PRL could be important for maintaining reduced vascularization of prolactinomas. Eventually, such reduction would result in hypoxic areas able to counteract 16K-PRL inhibition by stimulating the expression of VEGF and by decreasing the generation of 16K-PRL via reduced expression of PRL and lower availability of mature cathepsin-D. Although these mechanisms are consistent with the observation that macroprolactinomas are the most vascularized of pituitary adenomas (Turner et al, 2000a), these tumors continue to be less vascularized than the normal tissue (Turner et al, 2000a), indicating that inhibition of angiogenesis is still important and may reflect the action of remaining antiangiogenic factors, including 16K-PRL.

It should be emphasized that tumor angiogenesis depends on the interplay of multiple regulatory factors. Here, we are proposing the interaction of two that are important in the pituitary gland, but it is clear that much more information is needed to form the complete picture of this interaction. For example, 16K-PRL is known to inhibit the angiogenic action of VEGF (Clapp et al, 1993); however, its potency relative to that of VEGF has not been determined, nor its concentration in the pituitary gland adequately measured.

In addition to promoting angiogenesis, hypoxia-induced down-regulation of PRL and cathepsin-D activity can affect other mechanisms limiting the progression of prolactinomas. PRL can act as an autocrine growth factor within the pituitary itself, because it stimulates the proliferation of GH3 cells (Krown et al, 1992), and both GH3 cells and normal lactotrophs express PRL receptors (Krown et al, 1992; Morel et al, 1994). In addition, cathepsin-D can promote tumor invasiveness by stimulating cell growth (Glondou et al, 2001; Liaudet et al, 1995), by reducing contact inhibition (Liaudet et al, 1995; Rocheffort and Liaudet-Coopman, 1999; Rocheffort et al, 2000), and by digestion of extracellular matrix components (Briozzo et al, 1988; Heylen et al, 2002). Thus, our findings suggest new pathways contributing to the slow growth and limited invasiveness of prolactinomas and warrant further investigation.

Materials and Methods

Cell Culture

GH4C1 cells are a subclone of the cell line GH3 isolated in 1966 from pituitary tumors that had been induced by x-irradiation and found to produce both growth hormone and PRL (Tashjian et al, 1970). The stock line was maintained between passages 3 and 10. GH4C1 cells were cultured in F10 medium (Sigma Chemicals, St. Louis, Missouri) supplemented with 15% heat-inactivated horse serum (GIBCO, Invitrogen

Corporation, Carlsbad, California), 2.5% heat-inactivated fetal bovine serum (GIBCO, Invitrogen Corporation), and penicillin-streptomycin at 37° C in 5% CO₂. All hypoxia experiments were performed using a standard Plexiglas chamber (Bellco Glass, Vineland, New Jersey) under standard tissue culture conditions for pressure, humidity, and temperature. The chamber was deoxygenated by positive infusion of a 5% CO₂-95% nitrogen gas mixture. During the experiment, O₂% saturation was maintained at 0.1% and was continuously monitored with an ambient oxygen sensor (BW Technologies, Arlington, Texas). Subconfluent GH4C1 cell cultures (2 × 10⁶ cells/100 mm plates) were plated and incubated in supplemented F10 medium for 12 hours, then incubated in deoxygenated low-serum Opti-MEM I medium (GIBCO, Invitrogen Corporation) supplemented with antibiotics for 12 hours before exposure to hypoxia for 12 to 36 hours. The medium was changed to fresh deoxygenated Opti-MEM I at the beginning of the experiment. Cells under normoxic conditions were treated identically, except that Opti-MEM and the chamber were maintained at normal oxygen concentrations (20%). At the end of incubation, the number of viable cells was estimated by the dye exclusion method (Harlow and Lane, 1988).

RT-PCR

Total RNA was isolated using the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). RNA (5 µg) was reverse transcribed using M-MLV Reverse Transcriptase (Promega, Madison, Wisconsin) and random hexamers (Roche Applied Science, Indianapolis, Indiana). All PCR reactions and primers used for VEGF and the internal control, ribosomal protein L19, were done as described previously (Ochoa et al, 2000). Briefly, PCR reactions were performed using the following primers for VEGF: antisense primer 5'-GCTCTCTTGGGTGCACTGGA-3' and sense primer 5'-CACCGCCTTGGCTGTGACA-3' (Nakamura et al, 1996), with expected sizes of 635, 563, and 431 bp for amplification of VEGF188, VEGF164, and VEGF120, respectively. Primers for the internal control, ribosomal protein L19, were antisense primer 5'-CGAAATCGCCAATGCCAACTC-3' and sense primer 5'-TCGTCCATGAGAATCCGCTTG-3', with an expected size of 333 bp. The PCR conditions were as follows: denaturation at 95° C for 30 seconds, annealing at 65° C for 45 seconds, and extension at 72° C for 45 seconds, for 28 cycles. PCR products were separated on a 2% agarose gel, visualized using ethidium bromide, photographed, and analyzed by scanning densitometry.

Northern Blot

Total RNA was prepared as previously described (Chomczynski and Sacchi, 1987), and 10 µg was analyzed by Northern blot as described elsewhere (Sambrook and Russell, 2001). RNA subjected to electrophoresis was transferred to Hybond-N nylon

membranes (Millipore, Billerica, Massachusetts), cross-linked, prehybridized at 63° C (1 hour), and hybridized (8–12 hours) at 63° C with rat PRL or β -actin probes. The complete cDNA for rat PRL was used as a probe (generously supplied by R. Maurer, Oregon Health Sciences University, Portland, Oregon). The β -actin probe was prepared by RT-PCR from rat fibroblasts. The β -actin primers were the sense primer 5'- ATGGTGGGAATGGGTCAGAA-3' and the antisense primer 5'-TCGTA CTCTGCTTGCTGA-3'. The probes were labeled with [α -³²P]dCTP using the random primer procedure (Feinberg and Vogelstein, 1983). Autoradiography followed posthybridization washes. PRL and β -actin mRNA expression was quantified using scanning densitometry.

GH4 Lysates and Conditioned Media

After hypoxic exposure, conditioned media were collected, clarified by centrifugation (1200 ×g/10 minutes), concentrated 10 times (Centricon 3; Amicon Millipore, Billerica, Massachusetts), and stored at -70° C. Cells were lysed in 1% Nonidet P-40, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 1 µg/ml aprotinin, and 100 µg/ml phenylmethylsulfonyl fluoride (Sigma).

Western Blot Analysis

Eleven micrograms of total protein from GH4C1 cell lysates or 170 ng of protein from conditioned media was mixed with electrophoresis sample buffer containing β -mercaptoethanol, boiled, and resolved in a 15% SDS-polyacrylamide gel for PRL analysis and β -actin, and in a 12% SDS-polyacrylamide gel for cathepsin-D analysis. Gels were blotted onto nitrocellulose membranes and probed with a 1:1000 dilution of a locally produced anti-PRL antiserum or with 0.8 µg/µl of anti-cathepsin-D polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California). β -actin was detected by using a 1:250 dilution of a monoclonal anti-actin (mouse ascites fluid, clone AC-40; Sigma). The proteins were detected by using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, Illinois) or the alkaline phosphatase second antibody kit (Bio-Rad Laboratories, Hercules, California). Optical density values were determined using 1D image analysis software v3–5 (Eastman Kodak Company, Rochester, New York).

Silver Staining

Serum-free conditioned medium drawn from 6.5 × 10⁴ GH4C1 cells grown under normoxia or hypoxia was precipitated with trichloroacetic acid. Proteins were subjected to electrophoresis on a reducing 10% SDS-PAGE. Silver staining was performed according to the method described (Bollag et al, 1996).

ELISA

ELISA was performed as described elsewhere (Dueñas et al, 1999), using wells coated with 10 ng of

PRL, a 1:4000 dilution of locally raised anti-PRL anti-serum, and a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania). Bound horseradish peroxidase-conjugated antibodies were revealed by reaction with o-phenylenediamine dihydrochloride in the presence of hydrogen peroxide. Optical density was measured at 490 nm.

PRL Cleavage Analyses

The activity of the enzymes that cleave PRL to 16K-PRL was assayed as described previously (Clapp, 1987) with the following modifications: briefly, 650 ng (5 μ l) of conditioned media was mixed with 5 μ l of 0.1 M Tris-HCl (pH 7.4) and 10 μ l of reaction buffer (0.1 M citrate-phosphate, 0.15 M NaCl, pH 5.0) for 24 hours at 37°C. The final pH of the reaction mixture was 5.6. The PRL cleavage products were determined by reducing SDS-PAGE and Western blot analyses. Immunoreactive bands were quantified using scanning densitometry.

Statistics

Results are expressed as mean \pm SE of at least three replicates. Means were compared using either Student's *t* test or ANOVA followed by Fisher's test for post-hoc comparisons. All PCR and blotting experiments included a control gene or protein that was used to verify identical initial conditions. Because of nonlinearity in signal strength between the experimental and control bands, in some instances the 12-hour normoxia group was used as a baseline to normalize the data. In these instances, the 12-hour normoxia group was not included in the ANOVA, and post-hoc comparisons to this group were made with respect to the normalized mean.

Acknowledgements

We thank G. Nava, A. Prado, D. Mondragón, M. García, and P. Galarza for their expert technical assistance, and D.D. Pless for editing the manuscript.

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