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Secretion of phosphoglycerate kinase from tumour cells is controlled by oxygen-sensing hydroxylases

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Abstract

Solid tumour cells employ glycolytic enzymes including phosphoglycerate kinase (PGK) to make ATP when their supply of oxygen is limiting. PGK is also secreted by tumour cells and facilitates cleavage of disulfide bonds in plasmin, which triggers proteolytic release of the angiogenesis inhibitor, angiostatin. Although PGK production by tumour cells was enhanced by hypoxia, its secretion was inhibited. Inhibition of secretion correlated with decrease in angiostatin formation by the tumour cells. In contrast, hypoxia did not inhibit the secretion of the angiogenesis activator, vascular endothelial cell growth factor (VEGF). PGK secretion was reversed by normoxia and was under control of the oxygen-sensing protein hydroxylases, as inhibitors of this class of enzymes mimicked the effect of hypoxia on PGK secretion. Direct hydroxylation of PGK was not the mechanism by which the protein hydroxylases controlled its secretion. These findings show that production and secretion of PGK are regulated separately and indicate that oxygen and the protein hydroxylases can control not only gene expression but also protein secretion.

Keywords: Phosphoglycerate kinase; Angiostatin; Hypoxia; Protein hydroxylase; Secretion

1. Introduction

Tumour expansion and metastasis is dependent on angiogenesis, which is controlled by several protein activators and inhibitors [1]. One such inhibitor is angiostatin [2], which is an internal fragment of the serine proteinase, plasmin. Plasmin contains five consecutive triple-disulfide linked domains, called kringle domains, followed by a serine proteinase module. Angiostatin formation by tumour cells is a three-step process. Plasminogen is first converted to plasmin and then at least two disulfide bonds in the fifth kringle domain are cleaved in a process that is facilitated by tumour-derived phosphoglycerate kinase (PGK) [3]. The modified plasmin is finally proteolysed by serine- and metallo-proteinases to produce kringle $1-4\frac{1}{2}$, 1–4 or 1–3 (reviewed in Ref. [4]).

PGK was named for its role in glycolysis, where it catalyses phosphoryl transfer from 1,3-bisphosphoglycerate to ADP. When tumour cells become hypoxic, they make PGK to facilitate anaerobic production of ATP and angiogenesis activators such as vascular endothelial cell growth factor (VEGF) that trigger new blood vessel formation and enable the tumour to grow (reviewed in Ref. [5]). Production of angiostatin by tumour cells in response to hypoxia would be counterproductive.

We show herein that although PGK production by different tumour cells is enhanced by hypoxia, its secretion is inhibited. The hypoxia-mediated inhibition of PGK secretion was reversible by normoxia and was mimicked by inhibitors of the oxygen-sensing protein hydroxylases. Direct hydroxylation of PGK was not the mechanism by which the protein hydroxylases controlled its secretion.

2. Materials and methods

2.1. Hypoxic cell culture and collection of conditioned medium and cell lysate

The medium of cells at ~ 80% confluence in T75 flasks (~ 7×10^6 cells) was replaced with 10 ml of Hanks balanced

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salt solution (HBSS; Gibco, Gaithersburg, MD), pH 7.2 and cultured for 12 or 24 h under normoxic (20% O_2) or hypoxic (< 1% O_2) conditions at 37 °C. On some occasions the hypoxia mimetics, desferrioxamine (DFO; Sigma, St. Louis, MO) or dimethyloxalylglycine (DMOG; gift from Dr. Murray Whitelaw) was added to the HBSS. The hypoxic chamber was a 2.5-l airtight container. A sachet of activated ascorbic acid (Oxoid, Hampshire, England) was used to remove the oxygen, which was confirmed with an anaerobic indicator (Oxoid). The conditioned mediums were collected, centrifuged for 10 min at 3000 × *g* to remove cell debris and stored at −20 °C until analysis. Cell lysates were collected by scraping the cells into 0.5 ml of ice cold 50 mM Tris, pH 8 buffer containing 0.5 M NaCl, 1% Triton X-100, 10 μM leupeptin (Sigma), 100 μM 4-2-(aminoethyl)-benzene sulfonyl fluoride (Sigma), 5 mM EDTA and 10 μM aprotinin (Bayer, Sydney, Australia).

2.2. Production of anti-PGK antibodies

Balb/C mice were immunised with recombinant human PGK produced in *E. coli* [3] and splenocytes from the immunised mice were fused with the non-immunoglobulin-secreting mouse myeloma cell line SP2/0. Hydridomas were screened, cloned and isotyped and antibody was produced in mouse ascites. Monoclonal anti-PGK IgM antibodies were purified by SP-Sepharose ion-exchange chromatography from ascites fluid. Polyclonal anti-PGK antibodies were produced in sheep and affinity purified on a column of the antigen linked to Sepharose CL-4B (Amersham, Uppsala, Sweden).

2.3. ELISAs for PGK and VEGF

The anti-PGK murine IgM antibodies (100 μl of 5 μg ml^{-1} in 0.1 M $NaHCO_3$, pH 8.3 buffer containing 0.02% NaN_3,) were adsorbed to Nunc MaxiSorp 96-well plates overnight at 4 °C in a humid environment. Nonspecific binding sites were blocked with 2% BSA and conditioned medium (100 μl) or cell lysate (1:10 dilution with binding buffer; 20 mM HEPES, pH 7.4 buffer containing 0.14 M NaCl, 1 mM EDTA, and 0.05% Tween-20) was added to the antibody-coated wells and incubated for 30 min at room temperature with orbital shaking. The wells were then washed three times with binding buffer and 100 μl of 20 μg ml^{-1} of affinity purified anti-PGK sheep polyclonal antibodies in binding buffer was added and incubated for 30 min at room temperature with orbital shaking. The wells were then washed three times with binding buffer, before the addition of 100 μl of a 1:5000 dilution of horseradish peroxidase-conjugated rabbit anti-sheep IgG antibodies (Dako, Glostrup, Denmark) in binding buffer. After incubation for 30 min at room temperature with orbital shaking, the wells were washed three times with binding buffer. The colour was then developed with 100 μl of 0.003% H_2O_2, 1 mg ml^{-1} 2,2′ -azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) in 50 mM citrate, pH 4.5 buffer. There was a linear relationship between absorbance at 405 nm and PGK concentration up to 250 ng ml^{-1}.

VEGF levels in conditioned mediums were measured using a VEGF accucyte EIA kit from Oncogene Research Products (CN Biosciences, San Diego, CA).

2.4. Angiostatin assay

Conditioned mediums (0.5 ml) were incubated with plasmin (2 μg ml^{-1}) for 30 min at 37 °C and the angiostatin fragments were labeled with a biotin-linked maleimide, collected on lysine-Sepharose beads and detected using peroxidase-conjugated avidin as described previously [6].

2.5. Detection of PGK mRNA

Following incubation in hypoxic or normoxic conditions, HT1080 cells (7×10^6) were lysed in TRIZOL buffer (Gibco) and total RNA extracted as described by the manufacturer. The RNA (20 μg) was blotted onto Nylon Hybond transfer membrane (Amersham) and probed with [^{32}P] dCTP-labelled riboprobes complementary to PGK or ribosomal protein S14 mRNA.

2.6. Peptide mass fingerprinting of secreted PGK

Ten litres of conditioned medium of HT1080 cells was prepared using Nunc Cell Factories and the secreted PGK was purified by Cibachron Blue-Sepharose and Heparin-Sepharose affinity chromatography [3,6]. Fractions from the Heparin-Sepharose affinity chromatography containing PGK were concentrated, resolved on 4–20% gradient iGels (Gradipore, Sydney, Australia) and stained with Coomassie brilliant blue (Sigma). Gel slices containing PGK were reduced, carbamidomethylated and digested with trypsin (20 μg ml^{-1}; Roche Diagnostics, Basel, Switzerland) or GluC (20 μg ml^{-1}; Roche Diagnostics) overnight at 37 °C. Digested peptides were resolved on a C18 RP silica column (PEPMAP, 75 μm × 15 cm, LC-Packings) and analysed using an API QStar Pulsar i hybrid tandem mass spectrometer (Applied Biosystems, Foster City, CA). A TOF MS survey scan was acquired (*m*/*z* 350–1700, 0.5 s) and the two largest multiply charged ions (counts>10) sequentially selected by Q1 for MS-MS analysis. A processing script generated data suitable for submission to the database search programs (Mascot, Matrix Science or SEQUEST).

3. Results

3.1. Hypoxia stimulated PGK gene expression but inhibited PGK secretion and angiostatin formation

Human fibrosarcoma HT1080 cells were exposed to normoxia or hypoxia for 24 h and analysed for PGK gene

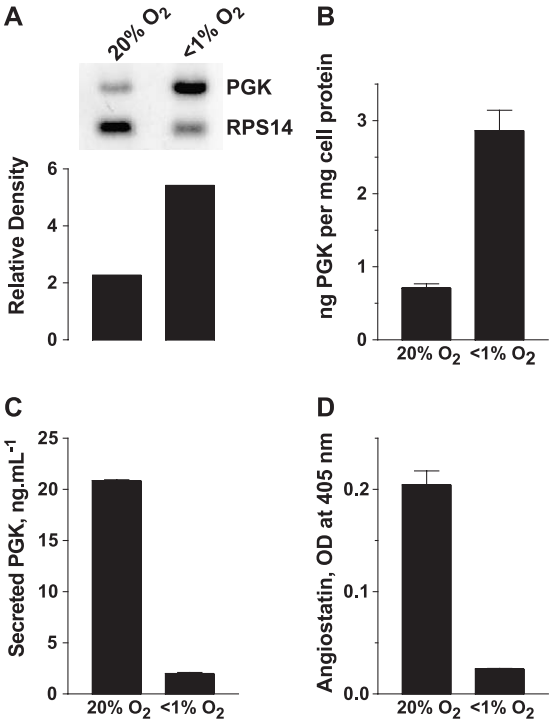

Fig. 1. Hypoxia stimulated PGK gene expression but inhibited PGK secretion and angiostatin formation. HT1080 cells were exposed to normoxia or hypoxia for 24 h and analysed for PGK gene expression (A), PGK cellular protein (B), secreted PGK (C), and angiostatin formation (D) in the conditioned medium. RPS14 mRNA controlled for PGK mRNA in (A). The bars and errors represent the mean ± S.E. of triplicate measurements. The result is representative of three experiments.

expression, PGK cellular protein, and secreted PGK and angiostatin formation in the conditioned medium (Fig. 1). In accordance with previous studies [7], hypoxia stimulated PGK gene expression. PGK mRNA and cellular protein increased 2.4- and 4.0-fold, respectively. In contrast, PGK secretion was inhibited by hypoxia. The concentration of PGK in the conditioned medium of cells exposed to hypoxia was 10% of that for cells exposed to normoxia. Secreted PGK levels correlated with the angiostatin-generating activity. The angiostatin-generating activity of the conditioned medium of cells exposed to hypoxia was 12% of that for cells exposed to normoxia.

The effect of hypoxia on PGK secretion was not a secondary effect of change in pH of the culture medium during the 24-h incubation. This was confirmed by buffering the culture medium (HBSS) with 50 mM HEPES, pH 7.2. PGK secretion was similarly inhibited following exposure to hypoxia and there was no detectable change in the pH of the culture medium.

3.2. Comparison of PGK secretion and angiostatin formation by tumour and non-tumour cell lines and effect of hypoxia

Tumour cells secrete much more angiostatin-generating activity than other cells [8] and plasma of mice bearing HT1080 tumours contained several-fold more PGK than mice without tumours [3]. These findings implied that relatively small amounts of PGK were secreted from untransformed cells.

The effect of hypoxia on PGK secretion by different tumour cell lines and non-tumour cells was measured. Hypoxia inhibited secretion of PGK from human pancreatic (SU.86.86) and mammary carcinoma (MDA-231) cell lines, similarly to that observed for HT1080 cells (Fig. 2A). The concentration of PGK in the conditioned medium of SU.86.86 or MDA-231 cells exposed to hypoxia was 30% and 40%, respectively, of that for cells exposed to normoxia. Bovine endothelial (BAE) cells secreted small but detectable levels of PGK, which was slightly enhanced by hypoxia, while human fibroblasts (HFF) did not secrete detectable levels of PGK either under normoxic or hypoxic conditions (Fig. 2A).

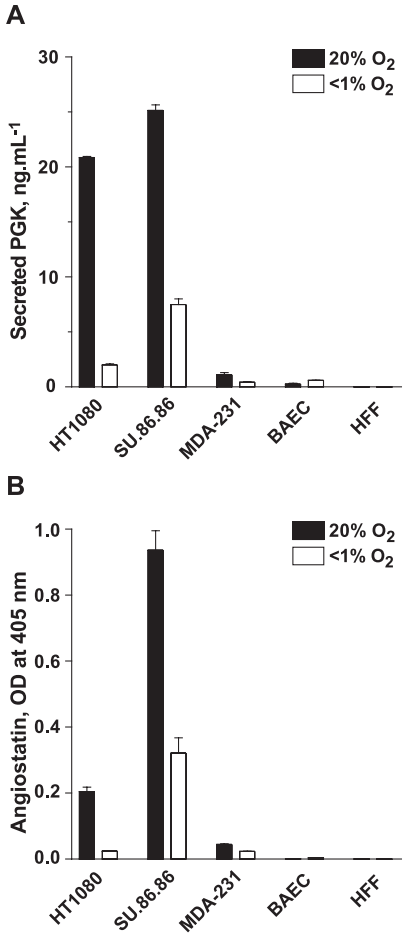

Fig. 2. Comparison of PGK secretion and angiostatin formation by tumour and non-tumour cell lines and effect of hypoxia. Tumour (HT1080, SU.86.86 and MDA-231) or non-tumour (BAEC and HFF) cells were exposed to normoxia (closed bars) or hypoxia (open bars) for 24 h and the conditioned mediums analysed for secreted PGK (A) and angiostatin formation (B). The bars and errors represent the mean ± S.E. of triplicate measurements. The result is representative of two to six experiments.

PGK secretion correlated with the angiostatin-generating activity of the different cell types (Fig. 2B). The angiostatin-generating activity of the conditioned medium of SU.86.86 or MDA-231 cells exposed to hypoxia was 34% and 52%, respectively, of that for cells exposed to normoxia.

The results of Figs. 1 and 2 indicate that carcinoma cells secrete significant amounts of PGK and that the level of secretion is controlled by the oxygen concentration of the culture medium.

3.3. Hypoxia inhibited PGK, but not VEGF, secretion from tumour cells

Expression of the VEGF gene, like the PGK gene, is controlled by oxygen. In contrast to secretion of PGK (Fig. 3A), hypoxia did not inhibit secretion of VEGF from HT1080 cells (Fig. 3B). Secretion of VEGF was enhanced by hypoxia, which is in accordance with enhanced expression of the protein. This result indicated that not all oxygen-responsive proteins are also regulated at the level of secretion.

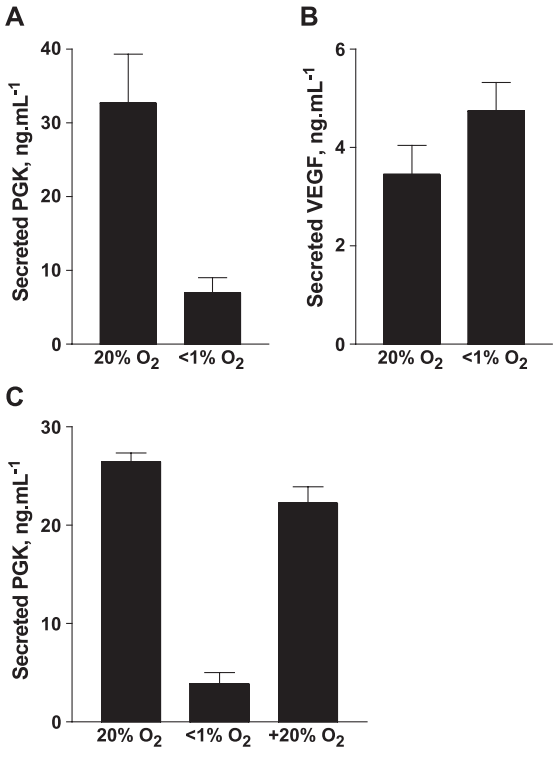

Fig. 3. Hypoxia inhibited PGK, but not VEGF, secretion from tumour cells. HT1080 cells were exposed to normoxia or hypoxia for 24 h and the conditioned mediums analysed for secreted PGK (A) and VEGF (B). The result is representative of six experiments. (C) shows that the effect of hypoxia on PGK secretion is reversible by normoxia. HT1080 cells were exposed to normoxia or hypoxia for 12 h. The medium was replaced in both flasks, which were then exposed to normoxia for a further 12 h (+20% O_2). The conditioned mediums were analysed for secreted PGK. The bars and errors represent the mean ± S.E. of triplicate measurements. The result is representative of two experiments.

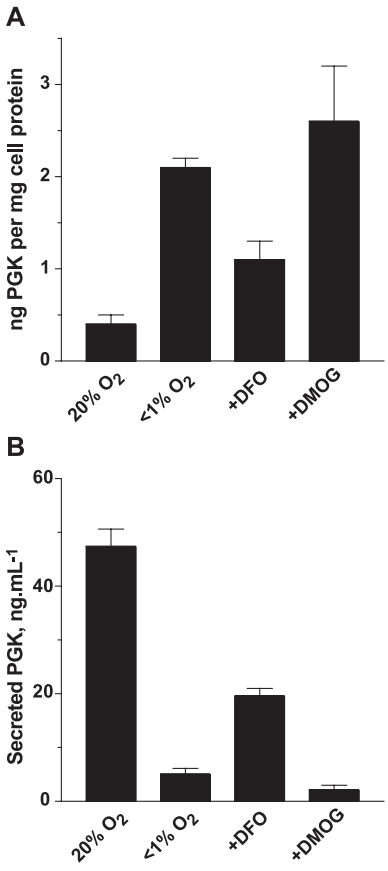

Fig. 4. Inhibitors of dioxygenases mimicked the effect of hypoxia on PGK secretion. HT1080 cells were exposed to normoxia, hypoxia, or normoxia in the presence of 100 μM DFO or 1 mM DMOG for 24 h and the cells analysed for PGK protein and the conditioned mediums for secreted PGK. The bars and errors represent the mean ± S.E. of triplicate measurements. The result is representative of three experiments.

3.4. Inhibition of PGK secretion by hypoxia was reversible

Inhibition of secretion of PGK by hypoxia was reversed by exposure to normoxic conditions. HT1080 cells were exposed to normoxia or hypoxia for 12 h, the medium was replaced and then both flasks of cells were exposed to normoxia for a further 12 h. The level of secreted PGK in the second 12 h of normoxic exposure was the same in both flasks (Fig. 3C). This result implied that the hypoxic-dependent events that mediated inhibition of PGK secretion were reversed within hours by normoxic conditions.

3.5. Inhibitors of dioxygenases mimicked the effect of hypoxia on PGK secretion

It seemed likely that the hypoxia-mediated inhibition of PGK secretion was regulated by the same oxygen sensors that mediate oxygen-dependent gene expression, the protein hydroxylases. This theory was tested be examining the effect of protein hydroxylase inhibitors on PGK secretion by tumour cells.

The prolyl hydroxylases contain iron, which is co-ordinated by histidine and aspartic acid residues. Oxygen binds to the ferrous state of the iron and one oxygen atom is transferred onto proline or asparagine residues while the other reacts with 2-oxoglutarate yielding succinate and carbon dioxide. Chelation of the iron with DFO inactivates the hydroxylases. DMOG is a competitive substrate analogue that also inactivates the hydroxylases.

DFO and DMOG mimicked the effect of hypoxia on PGK gene expression and protein secretion (Fig. 4). For example, treatment with 1 mM DMOG increased cellular PGK levels 6.5-fold, while secretion of PGK was 4% of control. The result directly implicates the protein hydroxylases in oxygen-dependent control of PGK secretion.

3.6. Secreted PGK was not hydroxylated

It was possible that direct hydroxylation of PGK by the protein hydroxylases was targeting it for secretion. This notion was tested by examining the secreted protein for hydroxylated Asn or Pro residues. In-gel reduction and carbamidomethylation of Cys residues followed by digestion with trypsin or GluC and MS/MS spectrometry identified peptides covering the entire sequence of PGK, suggesting that no posttranslational modifications had occurred prior to secretion. In addition, allowing hydroxylations (mass addition +16 on Asn and Pro) while searching the obtained MS/MS data identified no modified peptides (data not shown). Taken together, these data show that the secreted PGK was not hydroxylated.

4. Discussion

The correct and efficient localization of nascent cytosolic polypeptides is mediated by specific sorting or targeting sequences. Most proteins destined for secretion contain a recognisable N-terminal signal sequence that mediates their translocation across the endoplasmic reticulum (ER) membrane, which commits them to the exocytotic pathway [9]. PGK is a new member of a growing family of proteins that are secreted despite not having an identifiable signal sequence [10].

Several proteins that lack a signal sequence have been found to have a defined extracellular function [11]. These include thioredoxin, annexin 1, platelet-derived growth factor, fibroblast growth factors 1 and 2, interleukin-1, transglutaminase, and coagulation factor XIIIα, among others [12]. These proteins and PGK [13] share some biochemical features such as lack of N-linked glycosylation at potential N-X-S/T sites and presence of cysteines but lack of disulfide bonds. The fact that glycosylation and disulfide bond formation take place in the ER suggests that these proteins exit the cell by a means other than the classical ER–Golgi route.

Increase in expression usually translates to increased secretion for proteins that exit via the ER–Golgi route [9]. Secretion of signal-less proteins on the other hand is often associated with cellular stress and may not reflect expression levels of the protein [12,14]. PGK is the first example of oxygen-dependent control of protein secretion, including proteins that either contain or lack a secretory signal sequence.

Many factors come into play to ensure that the right amount of oxygen reaches cells throughout the body. Specific prolyl and asparaginyl hydroxylases [15,16] and the transcription factor HIF-1 [17–20] underpin many of the biological responses to oxygen deprivation in mammalian cells, including angiogenesis, red cell production and cellular metabolism (including PGK expression) (reviewed in Ref. [5]). As oxygen is a co-substrate for the hydroxylases, its concentration controls their activity. HIF-1 is a heterodimer composed of α and β subunits. Hydroxylation of two proline residues (Pro^{402} and Pro^{564}) in the oxygen-dependent degradation domain of HIF-1α confers rapid degradation, while hydroxylation of two asparagine residues (Asn^{803} and Asn^{851}) in the C-terminal *trans*-activation region regulates HIF-1α activity.

PGK secretion was under the control of the protein hydroxylases, as inhibitors of these enzymes mimicked the effects of hypoxia on PGK secretion. Direct hydroxylation of PGK, however, was not the mechanism by which the protein hydroxylases controlled its secretion.

It is possible that a component of the secretory pathway used by PGK was activated by hydroxylation, which enabled PGK secretion under normoxic conditions. Under hypoxic conditions the secretory component was not hydroxylated and therefore PGK secretion was turned off. This model is analogous to the control of HIF-1 degradation and activity by protein hydroxylation. Another possibility is that the product of one or more HIF-1α regulated genes may negatively regulate PGK secretion. HIF-1α controls the expression of at least 30 genes when oxygen levels are low [21]. None of the genes identified so far have been implicated in protein export, however.

Oxygen-dependent control of PGK secretion may be a useful handle to probe the nature of the illusory nonclassical secretory pathway. More generally, these observations indicate that oxygen and the protein hydroxylases can control not only gene expression but also protein secretion. Elucidation of the mechanism by which the hydroxylases can control protein secretion may provide an important insight into tumour biology.

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