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Induction of the glucose-6-phosphate dehydrogenase gene expression by chronic hypoxia in PC12 cells

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Abstract We studied the regulation of glucose-6-phosphate dehydrogenase (G6PD) gene expression by chronic hypoxia. G6PD mRNA level and activity were increased in PC12 cells by hypoxia in a dose- and time-dependent manner. Cobalt chloride and dimethyloxalylglycine, which can mimic hypoxia, also activated G6PD gene expression. Interestingly, hypoxia-induced G6PD expression followed a time course much slower than that of phosphoglycerate kinase 1 (PGK1), a hypoxia-inducible factor (HIF)-dependent glycolytic enzyme. Hypoxic-G6PD induction was almost negligible in non-excitable Buffalo rat liver cells, although in these cells PGK1 was strongly upregulated by low PO_2. Furthermore, G6PD but not PGK1 induction was blocked by the antioxidants glutathione and *N*-acetylcysteine. These results suggest the dependence of G6PD gene expression on HIF and intracellular redox status and the differential hypoxic regulation of glucose-metabolizing enzymes.

Keywords: Glucose-6-phosphate dehydrogenase; Phosphoglycerate kinase 1; Chronic hypoxia; Hypoxia-inducible factor; Glutathione

1. Introduction

Exposure of eukaryotic cells to chronic hypoxia stimulates the expression of glucose transporters and enzymes that accelerate glucose utilization to compensate for the reduced activity of the mitochondrial electron transport chain [1]. The shift from aerobic respiration to glycolysis is essential for cell survival in hypoxic conditions, since it provides sufficient ATP to maintain the energy-dependent cellular functions. Some of the glycolytic enzymes upregulated by hypoxia are phosphoglycerate kinase (PGK1), aldolase A, pyruvate kinase M, and 6-phosphofructo-2-kinase [1–3]. Chronic hypoxia induces gene expression through activation of the transcriptional activator hypoxia-inducible factor 1 (HIF-1) and isoforms. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β. Whereas HIF-1β is constitutively expressed, the level of HIF-1α increases dramatically at low oxygen tension. Although the level of reactive oxygen species (ROS) can modulate HIF-1α activity and thus contribute to chronic oxygen sensing [4,5], recent work has demonstrated that the regulation of HIF-1α by hypoxia mainly depends on O_2-sensitive prolyl hydroxylases [6,7]. In normoxia, hydroxylation of a specific proline residue of the HIF-1α molecule facilitates its rapid ubiquitylation and degradation by the proteasome. During hypoxia, this degradation pathway is greatly suppressed, thus resulting in stabilization of HIF-1α, its translocation to the nucleus, and upregulation of the HIF-dependent gene expression.

Activation of glycolysis by hypoxia must be coordinated with other metabolic pathways that also use glucose. Glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme in the pentose phosphate pathway, is necessary for the synthesis of pentoses. G6PD is also involved in antioxidant defense by providing NADPH, a major intracellular reductant. NADPH is a cofactor in the glutathione (GSH) reductase-catalyzed reaction to regenerate antioxidant GSH and it is also required in maintaining the antioxidant enzyme catalase in its active form. Mouse embryonic stem cells with deleted G6PD gene fail to induce NADPH production in response to oxidative stress, and this results in decreased GSH/GSH disulfide ratio and cell death [8]. Even mild G6PD deficiency produces a distortion of redox control and oxidative mutagenesis in mouse brain [9]. On the other hand, overexpression of G6PD suppresses hydrogen peroxide-induced cell death and the protection is mediated through the induction of GSH production [10,11]. Previous studies have found that G6PD gene expression is regulated by intracellular redox status. ROS/reactive nitrogen species (RNS), such as hydrogen peroxide and peroxynitrite, activate G6PD as part of a cellular response against oxidative damage [12,13]. On the contrary, the regulation of G6PD gene expression by low oxygen tension is still not clear and controversial. Anoxia triggers an elevation of G6PD activity in the brain of gold fish [14]. Intermittent hypoxia increases G6PD activity in the right but not in the left ventricle in rat [15]. Chronic hypoxia has been found to either have no effect on G6PD activity in rat intestine and mouse lung [16,17], or cause a decrease in rat liver [18].

Therefore, in the present study it was investigated whether chronic hypoxia can regulate G6PD gene expression. It was also examined if G6PD, an enzyme metabolizing glucose through a non-energy-producing pathway (pentose phosphate pathway), was controlled by changes in O_2 tension (PO_2) in the

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Abbreviations: BRL, Buffalo rat liver; $CoCl_2$, cobalt chloride; DMEM, Dulbecco's modified Eagle's medium; DMOG, dimethyloxalylglycine; G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; HIF, hypoxia-inducible factor; NAC, *N*-acetylcysteine; PGK1, phosphoglycerate kinase 1; PO_2, O_2 tension; RNS, reactive nitrogen species; ROS, reactive oxygen species; S.E.M., standard error of the mean; TH, tyrosine hydroxylase

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same way as glycolytic enzymes. We have found in rat pheochromocytoma PC12 cells, a classic O_2-sensitive excitable cell [19,20], that G6PD gene expression and activity are induced by chronic hypoxia in a dose- and time-dependent manner. However, this regulation has a different time course than that of PGK1, a well-studied HIF-dependent glycolytic enzyme. Interestingly, hypoxia strongly upregulates PGK1 gene induction in non-excitable Buffalo rat liver (BRL) cells but has almost no effect on the G6PD gene. Hypoxic induction of G6PD in PC12 cells seems to be mediated by ROS, whereas PGK1 induction is ROS-independent. These results demonstrate that cellular adaptive responses (anaerobic ATP production and antioxidant defense) against damage caused by changes in PO_2 vary in distinct cell types and are dependent on the differential hypoxic regulation of glucose-metabolizing enzymes.

2. Materials and methods

2.1. Cell culture and treatments

Rat pheochromocytoma PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 10% horse serum, and 1% penicillin/streptomycin. Cells were routinely cultured with 10% CO_2 and ambient air (normoxia, 21% O_2) at 37 °C. BRL cells were cultured in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. In these cells, the normoxic environment had 21% O_2 and 5% CO_2. For hypoxic treatments, cells were incubated for variable periods of time in a hypoxic incubator (ThermoForma, Marietta, OH), where different levels of oxygen were achieved by balancing ambient air with nitrogen. A batch of cells, maintained during the same time periods in normoxia, were used as control in all cases. Some cells were treated with either 2 mM GSH ethyl ester or 2 mM *N*-acetylcysteine (NAC) during the hypoxic treatments. To mimic the hypoxic effect, cells maintained in normoxic PO_2 were treated with either 100 μM cobalt chloride ($CoCl_2$), a stabilizer of HIF [21], or 1 mM dimethyloxalylglycine (DMOG), a competitive inhibitor of prolyl hydroxylases [6].

2.2. Analysis of glucose-6-phosphate dehydrogenase activity

Cells were homogenized in extraction buffer containing 20 mM Tris–HCl, 1 mM EDTA, 3 mM $MgCl_2$, 1 mM ε-amino-*n*-caproic acid, and 0.02% (w/v) β-mercaptoethanol, pH 8.0. After centrifugation, the supernatant was collected to measure G6PD activity. G6PD activity was determined by measuring the rate of NADPH production spectrophotometrically, as previously described [22]. The activity was then normalized to the amount of protein determined with the Bradford method (BioRad Protein Assay, BioRad).

2.3. RNA isolation and Northern blot analysis

Total RNA was isolated from either PC12 or BRL cells by the method of Cathala et al. [23]. Equal amount of RNA (10 g) from each sample was used to perform the Northern blot analysis. [^{32}P]UTP-labeled RNA antisense probes were generated from the following linearized templates: pCRII-G6PD (G6PD), pCRII-TH (tyrosine hydroxylase (TH)), and p1B15 (cyclophilin). RNA probes were transcribed with either T7 (pCRII-G6PD and pCRII-TH) or SP6 (p1B15) RNA polymerases. PGK1 DNA probe was generated by reverse transcription and PCR amplification followed by labeling with [^{32}P]dCTP. The primers used to amplify the PGK1 fragment were: forward 5′-GGC TGG ATG GGC TTG GAC TG-3′ and reverse 5′-ATC AAC CTC CCA AAC CTA CA-3′. Blots were hybridized with each probe and the radioactive bands were visualized by autoradiography.

2.4. Reverse transcription and real-time quantitative PCR

The first-strand cDNA was synthesized from 3 μg of total RNA using the Superscript™ first-strand cDNA synthesis system with random primers according to the manufacturer's instruction (Invitrogen). Real-time quantitative PCR was performed in an ABI Prism 7000 Sequence Detection System using SYBR Green PCR Master Mix and the thermocycler conditions recommended by the manufacturer (Applied-Biosystems). PCRs were performed in duplicates in a total volume of 30 μl containing 0.2 μl of the reverse transcription reaction mixture. Primers used to amplify a fragment of each gene were: G6PF1 (5′-GCC TTC TAC CCG AAG ACA CCT T-3′) and G6PR1 (5′-CTG TTT GCG GAT GTC ATC CA-3′) for G6PD, LG9 (5′-GCA CTG GTG GCA AGT CCA T-3′) and LG10 (5′-GCC AGG ACC TGT ATG CTT CAG-3′) for cyclophilin, LG31 (5′-TCG GAA GCT GAT TGC AGA GA-3′) and LG32 (5′-TTC CGC TGT GTA TTC CAC ATG-3′) for TH, and LG33 (5′-AGA GCC CAC AGT TCC ATG GT-3′) and LG34 (5′-GCA AAG TAG TTC AGC TCC TTC TTC A-3′) for PGK1. Each sample was normalized with cyclophilin to perform the relative quantification. Dissociation curve analysis showed a single sharp peak with the expected melting temperature for all samples.

2.5. Statistical analysis

Data were presented as means ± standard error of the mean (S.E.M.), and were analyzed with either Student's *t*-test or one-way analysis of variance followed by Tukey test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Differential time-dependent induction of G6PD and PGK1 genes by hypoxia

PC12 cells were treated with chronic hypoxia (3% O_2) for up to 72 h. The mRNA levels of TH, a classic hypoxia-inducible gene [19], as well as of PGK1 and G6PD increased upon treatment, as determined by Northern blot analysis and real-time quantitative PCR (Fig. 1, panels A and B, respectively). A rapid elevation in mRNA levels of TH and PGK1 (about 2.5-fold, $P < 0.05$) was observed after 7 and 24 h of treatments, respectively, and this increase was maintained for 72 h, as examined in the present study. G6PD mRNA level also increased in hypoxia, although it was not appreciable until 48 h of treatment and only reached a 2-fold induction at 72 h (Fig. 1, panels A and B). Therefore, chronic hypoxia-induced G6PD gene expression at the transcript level but the effect was slower than that for TH and PGK1.

To determine the dose dependence of G6PD gene induction by chronic hypoxia, PC12 cells were incubated at different levels of oxygen and the mRNA was collected after 72 h of treatment. 10% O_2 caused a moderate but significant increase in G6PD mRNA level (~1.5-fold) and the increase was enhanced to a maximum of about 2-fold with the treatment of 3% O_2 (Fig. 1, panel C). 1% O_2, tested in only one experiment, caused a similar induction as 3% O_2. These results indicated that even mild hypoxia could activate G6PD gene expression and that the induction was dose-dependent. The TH and PGK1 gene induction followed the same pattern as G6PD in term of dose, although the level of increase was higher than for G6PD (data not shown).

The enzymatic activity of G6PD was then measured to assess whether the increase in G6PD transcript upon chronic hypoxic treatment resulted in an enhancement of its physiological function. Indeed, 3% O_2 caused a 30% and 50% increase in G6PD activity after 48 and 72 h of treatments, respectively ($P < 0.05$, Fig. 1, panel D). This increase in activity followed the same time course as mRNA induction (see Fig. 1, panel B). Therefore, these results demonstrated that chronic hypoxia-induced G6PD gene expression in PC12 cells, which resulted in an increase in its functional activity.

As chronic hypoxia has been shown to have variable effects on G6PD activity in different tissues [16–18], we examined whether the G6PD upregulation described above was also present in BRL cells, used as an example of non-excitable cells.

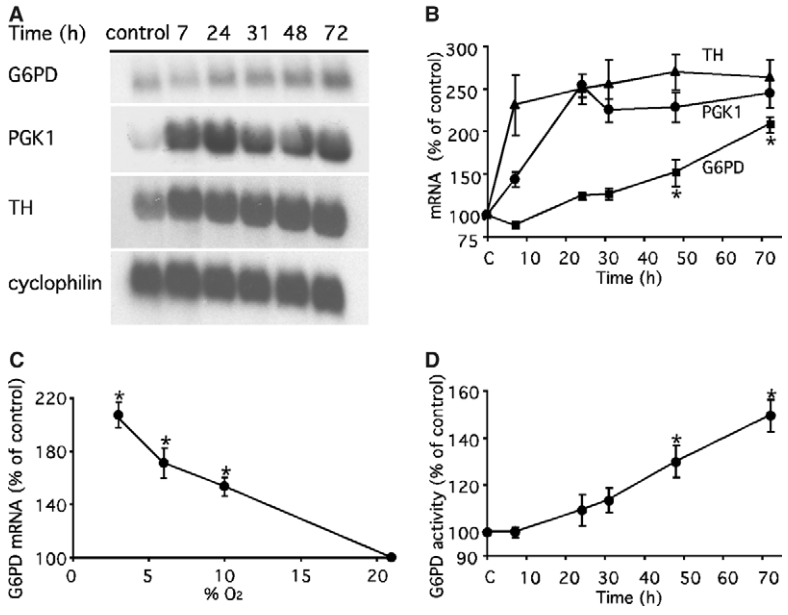

Fig. 1. Differential time-dependent induction of G6PD and PGK1 genes by chronic hypoxia in PC12 cells. Total RNA and protein were isolated from PC12 cells treated with 3% O_2 for different periods of time. A batch of cells, maintained during the same time periods in normoxia, were used as control (c) in all cases. The mRNA level of each gene was determined by Northern blot analysis (panel A) and real-time quantitative PCR analysis (panel B). (C) PC12 cells treated with different levels of oxygen for 72 h followed by RNA isolation and real-time quantitative PCR. (D) G6PD activity in cells treated with 3% O_2 for different periods of time. Data are presented as means ± S.E.M. from at least four independent experiments. In the case of G6PD, statistically significant values ($P < 0.05$) are indicated by asterisks.

As in PC12 cells, BRL cells treated with chronic hypoxia (3% O_2) for up to 72 h demonstrated a fast, time-dependent, up-regulation of PGK1 mRNA. Although the hypoxic induction of PGK1 in BRL cells (about 6-fold after 24 h; Fig. 2, panel A) was much more potent than in PC12 cells (see Fig. 1, panel B), lowering PO_2 had almost no effect on either G6PD mRNA level (Fig. 2, panel A) or activity (Fig. 2, panel B). These data indicate that quantitatively the differential regulation of G6PD and PGK1 by hypoxia can change among the various cell types.

3.2. *G6PD is induced by the hypoxia mimics $CoCl_2$ and DMOG*

It was further investigated whether G6PD gene expression in PC12 cells is upregulated by $CoCl_2$ and the membrane-permeable prolyl hydroxylase inhibitor DMOG. These compounds have been used to mimic the HIF-dependent hypoxic effect under normoxic conditions [6,21,24]. As shown in Fig. 3, both compounds caused a 2-fold increase in mRNA (panel A) and a 70–80% elevation in G6PD activity (panel B) after 72 h of treatments, which was similar to the effect caused by 3% O_2 (see Fig. 1). These hypoxia mimics induced G6PD with a time course similar to that of low PO_2. An iron chelator desferrioxamine, which has also been used to mimic the HIF-dependent hypoxic effect [6,24], had a smaller effect on the G6PD mRNA level but also caused a 50% increase in G6PD activity (data not shown). Therefore, these data suggested that the hypoxia-induced G6PD gene expression was mediated, at least in part, by HIF.

3.3. *Differential effect of intracellular redox buffers on the hypoxic regulation of the G6PD and PGK1 genes*

ROS have been suggested to be involved in the cellular response to hypoxia [4,5]. Therefore, it was examined whether the G6PD induction was mediated by changes in intracellular

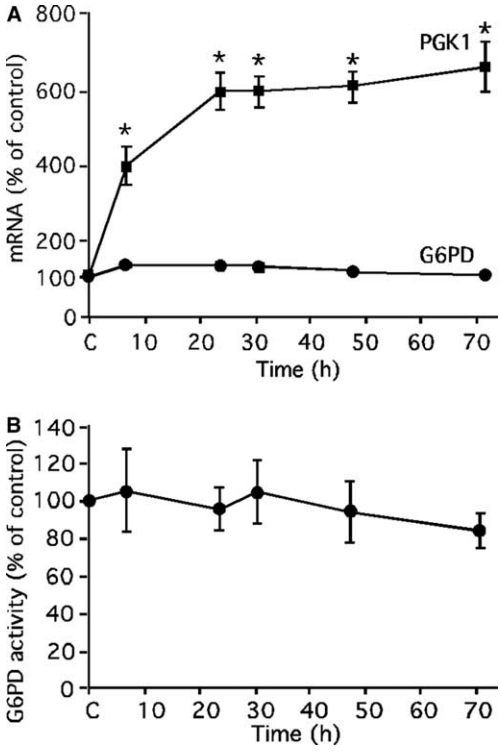

Fig. 2. Differential induction of PGK1 and G6PD genes by chronic hypoxia in BRL cells. Total RNA and protein were isolated from BRL cells treated with 3% O_2 for different periods of time. The mRNA level of each gene was determined by real-time quantitative PCR analysis (panel A). (B) G6PD activity in cells treated with 3% O_2 for different periods of time. Data are presented as means ± S.E.M. from at least four independent experiments. Statistically significant values ($P < 0.05$) are indicated by asterisks.

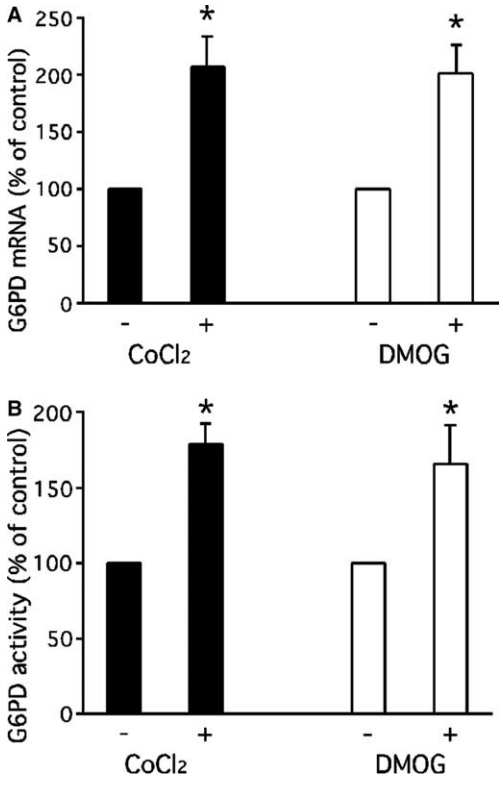

Fig. 3. $CoCl_2$ and DMOG mimicked the hypoxic effect on G6PD induction. PC12 cells were treated with 100 μM $CoCl_2$ or 1 mM DMOG for 72 h under normoxia. The mRNA level of G6PD was measured by real-time quantitative PCR (panel A) and the enzymatic activity was determined spectrophotometrically (panel B). Data were presented as means ± S.E.M. from at least three independent experiments. $^*P < 0.05$ compared to control.

redox status. GSH ethyl ester (2 mM), a membrane-permeable GSH donor, was used to inhibit oxidative stress. As shown in Fig. 4, GSH ethyl ester itself had no effect on the mRNA levels of G6PD and PGK1 in PC12 cells under normoxic condition (21% O_2). However, the increase in G6PD transcript, which was induced by 3% O_2 for 72 h, was inhibited almost completely by 2 mM GSH ethyl ester (Fig. 4, panel A). GSH ethyl ester was unable to prevent G6PD induction by cobalt (Fig. 4, panel A) or to suppress the PGK1 induction after either 24 or 72 h of hypoxic treatment (Fig. 4, panel B). Qualitatively, similar effects were seen with NAC, another widely used antioxidant. At 2 mM, NAC completely suppressed hypoxia-induced G6PD gene expression in PC12 cells but was without effect on PGK1 induction ($n = 4$ experiments). These data indicated that buffering of ROS production with GSH ethyl ester had no effect on cobalt-induced G6PD gene induction. Whereas the hypoxia-induced G6PD gene expression was ROS-dependent, the hypoxic PGK1 induction was not.

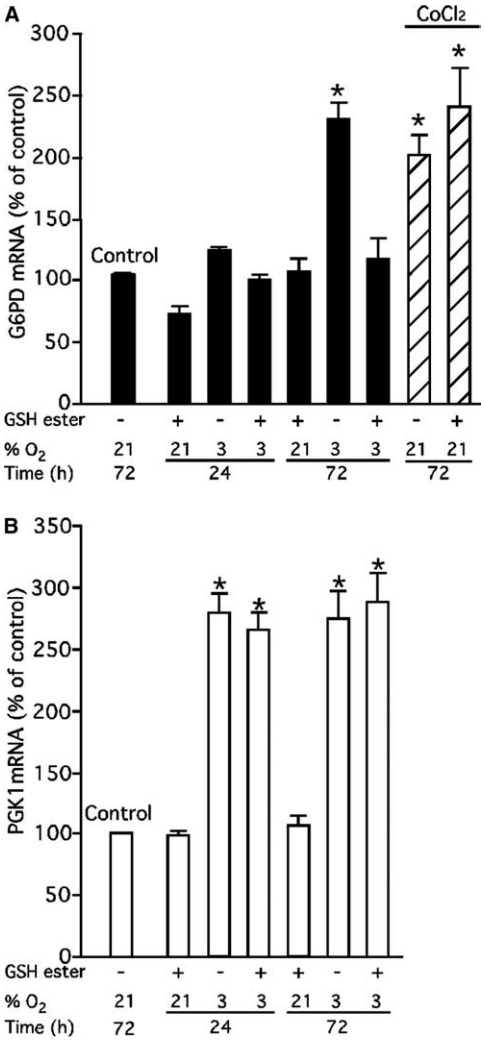

Fig. 4. Differential effect of intracellular redox buffers on the hypoxic regulation of the G6PD and PGK1 genes. PC12 cells were treated with 2 mM GSH ethyl ester followed by immediate treatment of 3% O_2 for 24 or 72 h, or 100 μM $CoCl_2$ for 72 h. Total RNA was isolated and the mRNA levels of G6PD (panel A) and PGK1 (panel B) were determined by real-time quantitative PCR. Data were presented as means ± S.E.M. ($n = 4$). $^*P < 0.05$ compared to control.

4. Discussion

In the present study, we have shown that chronic hypoxia can induce G6PD gene expression and functional activity in PC12 cells, a classic excitable O_2-sensitive cell type [19,20]. In addition, we report that the hypoxic induction of G6PD differs with respect to that of PGK1, another glucose-metabolizing enzyme, in terms of time course and dependence on oxidation. In the case of PGK1, which is a typical HIF-inducible enzyme [1,3], the hypoxic induction occurred within a few hours and was independent of ROS production. On the other hand, the induction of G6PD occurred relatively late and the antioxidants GSH or NAC completely abolished the effect. Induction of PGK1 gene by low PO_2 was also observed even with higher potency in non-excitable BRL cells, although in this preparation the effect of hypoxia on G6PD was almost negligible.

Although HIF-dependent induction of gene expression by chronic hypoxia is maintained in the absence of a functional mitochondrial electron transport chain [25], numerous studies have suggested that an elevation of ROS production, such as mitochondria-derived superoxide and subsequently hydrogen peroxide, can influence HIF-1 activation in hypoxia-induced gene expression [4,26–30]. Indeed, it has been reported that HIF-1 can be activated by changes in intracellular redox status. Buthionine-sulfoximine, nitrofurantoin, and phorone, which can induce oxidative stress, have been reported to increase HIF-1 protein in the nuclear extracts of rat liver [31],

and pretreatment with arsenite, which can induce ROS production, results in accumulation of HIF-1α protein and induction of the vascular endothelial growth factor gene [32]. Reducing agents, such as GSH and NAC, block hypoxia-induced PC12 cell death [33]; however, a decrease of ROS production during hypoxia-induced erythropoietin gene expression has also been reported [34].

Our results support the view that induction by hypoxia of some HIF-dependent genes, i.e., PGK1, appears to be completely independent of ROS formation in excitable PC12 cells, whereas in other genes, as in the case of G6PD, hypoxic regulation depends both on HIF and ROS accumulation. We have observed clear differences in the potency of hypoxia to induce G6PD in PC12 and BRL cells, which could be due to a higher production of ROS in response to hypoxia in excitable (PC12) cells. The involvement of HIF in hypoxic G6PD-induction is suggested because the same increase of G6PD mRNA is produced in normoxic conditions and independently of ROS by $CoCl_2$ and DMOG (Figs. 3 and 4). In fact, there is a putative HIF-1 binding site (TACGTG) [24,35] located in the promoter region of rat G6PD gene, which is about 290 bases upstream of the transcription initiation site (GenBank Accession No. X69768). Desferrioxamine, an iron chelator that also mimics the HIF-dependent hypoxic effect [6,24], induced a smaller increase of G6PD mRNA than $CoCl_2$ or DMOG. Nevertheless, this result is expected since desferrioxamine activates HIF and mimics hypoxia but also diminishes the iron-dependent ROS formation [36] and thus reduces G6PD expression.

In conclusion, our data show that glucose-metabolizing enzymes are regulated differentially by hypoxia through separate mechanisms. Fast hypoxic induction is observed for PGK1, a gene highly sensitive to HIF activation. In contrast, a much slower induction is produced in the case of G6PD, possibly because it is less sensitive to HIF, and more importantly, requires ROS accumulation and a prolonged exposure to low PO_2. The regulation of glycolytic enzymes (PGK1 among others) and G6PD by hypoxia with different time courses could have an important adaptive role for cell survival. In the early stages of hypoxia, prior to the accumulation of ROS, induction of glycolytic enzymes is surely of critical need to accelerate the anaerobic synthesis of ATP. During long hypoxic exposures, however, the antioxidant and protective effect of G6PD due to NADPH synthesis [10,11] could be more necessary to avoid ROS accumulation and cell death. There are differences in the potency of hypoxia to induce G6PD in PC12 and BRL cells, which could be a consequence of a higher and faster accumulation of ROS in response to hypoxia in excitable cells. It would be interesting to investigate in future experiments whether the ROS-dependent hypoxic regulation of the G6PD gene reported here occurs in other O_2-sensitive tissues and thus represents a more general adaptive mechanism to fight against oxidative damage in excitable cells.

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