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Hypoxia up-regulates glyceraldehyde-3-phosphate dehydrogenase in mouse brain capillary endothelial cells: involvement of Na^+/Ca^{2+} exchanger☆

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Abstract

The molecular regulatory mechanisms and the characterization of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in hypoxia were studied in a mouse brain capillary endothelial cell line, MBEC4. Activation of GAPDH gene expression by hypoxia was suppressed by an intracellular Ca^{2+} chelator and inhibited by a non-selective cation channel blocker or a Na^+/Ca^{2+} exchanger (NCX) blocker. Sequencing of reverse transcription-PCR products demonstrated that MBEC4 expressed an mRNA encoding NCX3, which functions even under cellular ATP-depleted conditions, in addition to mRNAs encoding NCX1 and NCX2. The inhibition of Ca^{2+}/calmodulin-dependent protein kinases or c-Jun/AP-1 activation caused a significant decrease in the activation of GAPDH mRNA by hypoxia. These results suggest that hypoxia stimulates Ca^{2+} influx through non-selective cation channels and causes the reverse operation of the three NCX isoforms, and consequently, increased intracellular Ca^{2+} up-regulates GAPDH gene expression through an AP-1-dependent pathway. Furthermore, subcellular fractionation experiments showed that hypoxia increased GAPDH proteins not only in the cytosolic fraction, but also in the nuclear and particulate fractions, in which GAPDH should play no roles in glycolysis. However, the GAPDH activity did not rise in proportion to the increase of GAPDH protein by hypoxia even in the cytosolic fraction. These results suggest that not all hypoxia-induced GAPDH molecules contribute to glycolysis.

Keywords: Hypoxia; Glyceraldehyde-3-phosphate dehydrogenase; Ca^{2+}; Na^+/Ca^{2+} exchanger; AP-1; Brain capillary endothelial cell

1. Introduction

Molecular oxygen is an essential factor for survival in mammals because it is available as an electron acceptor for ATP production in oxidative phosphorylation. Mammals possess genetic programs that respond to alterations in intracellular oxygen tension in a tissue-specific fashion. Insufficient supply of oxygen to body tissues stimulates both erythropoiesis and angiogenesis by up-regulating the production of erythropoietin and vascular endothelial growth factor (VEGF) [1,2]. Furthermore, mammalian cells enhance the rate of glucose utilization by up-regulating the expression of genes involved in glycolysis and glucose transport to compensate for a decrease in ATP production when deprived of oxygen [3–6]. Most of these hypoxia-induced genes are transactivated by hypoxia-inducible factor-1 (HIF-1), which interacts with the enhancer sequence termed hypoxia-response element [7,8].

Endothelial cells are located inside blood vessels, which play a pivotal role in transporting oxygen to all tissues, and

☆ The nucleotide sequence data of the cDNA fragments coding the three NCX isoforms cloned here have been deposited with the DDBJ, EMBL and GenBank nucleotide sequence databases and are available under accession numbers AB080744 (NCX1), AB080745 (NCX2) and AB080746 (NCX3).

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initially sense an alteration in blood oxygen tension. Because vascular endothelial cells can remain viable during prolonged hypoxic exposure much better than nonendothelial cells such as fibroblasts, smooth muscle cells, and renal tubular cells [9], endothelial cells are presumed to be one of the mammalian cell types with a higher hypoxia tolerance. Chinese hamster ovary cells, and myocardiac and brain tissues express heat shock proteins in response to hypoxia [10–12]. In contrast, endothelial cells up-regulate some hypoxia-specific and unique proteins, termed hypoxia-associated proteins (HAPs), which are different from heat shock proteins [13,14]. Furthermore, exposure of endothelial cells to sodium cyanide, which irreversibly blocks oxidative phosphorylation and is used as a form of chemical hypoxia, causes no induction of any specific proteins [9], indicating that environmental hypoxia is necessary to up-regulate the expression of HAPs in endothelial cells. Graven et al. [15] found that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a HAP. GAPDH is one of the glycolytic enzymes for energy production in the cytosolic fraction and is composed of a tetramer of identical 36 kDa subunits. It catalyzes the reversible oxidation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. In addition, GAPDH has a variety of diverse biological properties such as DNA repair and replication, translational regulation and endocytosis in the nuclear and membrane fractions [16–19].

HIF-1 binding is necessary, but not sufficient, for the induction of GAPDH gene expression in endothelial cells [20]. Previous studies have shown that hypoxia causes an increase in intracellular Ca^{2+} concentration. Some studies have represented that the increase is due to Ca^{2+} release from the internal store [21–23]. The others have demonstrated that the source of increased Ca^{2+} is derived from the extracellular space [24–27]. The release of Ca^{2+} from the internal store is needed to activate the transcription of VEGF gene in 293 cells (an adenovirus-transformed human fetal kidney cell line) exposed to hypoxia [22]. In contrast, hypoxia accelerates Ca^{2+} influx from the extracellular space through L-type Ca^{2+} channels in PC12 cells (a rat pheochromocytoma cell line), followed by the up-regulation of tyrosine hydroxylase gene expression [24]. Here we report that the level of hypoxia-induced GAPDH mRNA is inhibited by chelation of intracellular Ca^{2+} in MBEC4 cells, a mouse brain capillary endothelial cell line [27], and analyze Ca^{2+} channels and transcriptional factors involved in hypoxia-stimulated GAPDH gene expression. Furthermore, we determined the protein and activity levels of GAPDH in subcellular fractions under normoxic and hypoxic conditions.

2. Materials and methods

2.1. Materials

The following chemicals and reagents were used: AlkPhos Direct with CDP-star and Hybond-N^+ membrane from Amersham Pharmacia Biotech, RNAzol from Tel-Test (TX, USA), oligotex-dT30 ⟨super⟩ kit from Roche Diagnostics, Dulbecco's modified Eagle's medium from Nissui Pharmaceutical (Tokyo, Japan), fetal calf serum, penicillin/streptomycin and reverse transcriptase (Superscript II RNase H^-) from GIBCO, 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM) from Dojindo (Kumamoto, Japan), KN-93 from Calbiochem (Darmstadt, Germany), bepridil hydrochloride, curcumin and $LaCl_3$ from Nacalai Tesque (Kyoto, Japan), anti-lamin B_1 antibody from Zymed Laboratories (CA, USA), and nifedipine and verapamil hydrochloride from Wako Pure Chemical Industries (Osaka, Japan). Stock solutions of BAPTA-AM, KN-93 and nifedipine were made in dimethyl sulphoxide and curcumin was dissolved in ethanol.

2.2. Cell culture and viability

MBEC4 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, as described previously [28] and maintained at 37°C in a 5% CO_2/95% air atmosphere at 100% humidity unless otherwise indicated. Cell viability was based on the ability of cells to exclude trypan blue.

2.3. Exposure to pharmacological reagents

MBEC4 cells that had reached approximately 80% confluence were exposed to normoxia (5% CO_2/95% air atmosphere) or hypoxia (5% CO_2 and 2% O_2 balanced with N_2: ESPEC, Model BNP-110 M) for 6 h in the presence or absence of BAPTA-AM, $LaCl_3$, nifedipine, verapamil, bepridil, KN-93 or curcumin. The medium was renewed 24 h before the hypoxic exposure. BAPTA-AM, $LaCl_3$, bepridil, nifedipine, verapamil, KN-93 or curcumin was added to the medium 1 h before the exposure.

2.4. Northern blot analysis

Total RNA was prepared from MBEC4 cells using RNAzol, and 20 μg of the RNA was separated by electrophoresis through 1% agarose gel containing 2.2 M formaldehyde, and transferred to a Hybond-N^+ membrane in 20 × SSC (1 × : 15 mM sodium citrate and 150 mM sodium chloride, pH 7.0). The membrane was cross-linked with UV light. GPDN5 plasmid containing rat GAPDH cDNA was kindly donated by Dr. Fort (Université Montpellier I-Université Montpellier II, France) and pHr14E3 containing human 28S ribosomal DNA was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). *Kpn*I-digested GPDN5 and *Bam*HI-digested pHr14E were labeled with alkaline phosphatase using AlkPhos Direct according to the manufacturer's instructions, and used as probes for detection of GAPDH and 28S rRNA, respectively. Hybrid-

ization was performed at 55°C, and chemiluminescence signals derived from hybridized probes were detected by LAS-1000 plus (Fujifilm, Tokyo, Japan).

2.5. Reverse transcription-PCR

MBEC4 cells were exposed to normoxia or hypoxia for 6 h. Total RNA (20 μg) was prepared as described above and mRNA was purified from total RNA using oligotex-dT30 ⟨super⟩ kit according to the manufacturer's instructions. The mRNA was denatured at 70°C for 5 min, chilled on ice, and added to 50 U RNase inhibitor from human placenta, 0.5 μg oligo(dT) primer, 10 mM dithiothreitol, 1 mM dNTPs, 200 U reverse transcriptase and 1 × reverse transcription (RT) buffer (provided with the enzyme) in a volume of 25 μl, followed by incubation at 50°C for 60 min. The cDNA fragments for the three isoforms of NCX were amplified by nested PCR. The first PCR was performed using the synthesized first-strand cDNA (1 μl) as a template, 200 μM dNTPs and 500 nM each of the following primers combination in a volume of 50 μl: (a) primers F11 (5′-CTTCTTCTTTCCCATCTGCGTT-3′; position 808–829 [29]) and R11 (5′-GACTCTGACATTGCTAAGGTGC-3′; position 1591–1570 [29]) for NCX1, (b) primers F21 (5′-GCCGTAAGATCAAGCACTTAAGGG-3′; position 566–589 [30]) and R21 (5′-TGCGGTAATCCACGTAGAAGGT-3′; position 1297–1276 [30]) for NCX2, and (c) F31 (5′-AAGAAGACCGCCAGCATGAGTGA-3′; position 1965–1987 [31]) and R31 (5′-CACCCGATGTCCTCAAAACCTT-3′; position 2505–2484 [31]) for NCX3. Furthermore, the second PCR was performed using the first PCR products (1 μl) as a template, 200 μM dNTPs and 500 nM each of the following primers combination in a volume of 50 μl: (a) primers F12 (5′-ACATGAAGGAGACAGACCAGCTTC-3′; position 922–945 [29]) and R12 (5′-CTCTGATTTCCTTCTGGGTCTC-3′; position 1520–1499 [29]) for NCX1, (b) primers F22 (5′-ACTGCTCACGCTGGTCTTCTTC-3′; position 687–708 [30]) and R22 (5′-TGGGCTCAAAGAAGATGCGGCT-3′; position 1201–1180 [30]) for NCX2, and (c) primers F32 (5′-TTTTGACCCATGCTCTTACCAG-3′; position 2030–2051 [31]) and R32 (5′-CTCTTCTACACGGACATTGCTC-3′; position 2306–2285 [31]) for NCX3. All PCRs were performed using recombinant *Taq* DNA polymerase (Toyobo, Osaka, Japan) under the following conditions: 1 min at 94°C followed by 35 cycles of 1 min at 94°C, 1.5 min at 55°C and 1.5 min at 72°C, followed by a single extension of 10 min at 72°C. The PCR products were cloned into pCR2.1-TOPO vector according to the manufacturer's instructions (Invitrogen) and sequenced.

2.6. Preparation of anti-GAPDH and anti-TPI antibodies

Recombinant rat GAPDH and mouse triosephosphate isomerase (TPI) were used as antigens to prepare anti-GAPDH and anti-TPI antibodies. Briefly, the expression vectors of recombinant rat GAPDH and mouse TPI were constructed using a pET-30 Ek/LIC vector according to the manufacturer's instructions (Novagen), and were named pET/rGAPDH and pET/mTPI. Recombinant rat GAPDH and mouse TPI, which had been expressed in pET/rGAPDH- and pET/mTPI-transformed *Escherichia coli* BL21(DE3) in the presence of 1 mM isopropyl 1-thio-β-D-galactoside, respectively, were purified using Ni-NTA agarose according to the manufacturer's instructions (Qiagen). Anti-rat GAPDH and anti-mouse TPI antibodies were raised in rabbits by using each of the affinity-purified proteins, which had been analyzed to be a single band by SDS-PAGE.

2.7. Subcellular fractionation and Western blotting

For analysis of GAPDH in the total fraction, MBEC4 cells were exposed to hypoxia for 6 or 12 h. Cells were washed twice with ice-cold phosphate-buffered saline, pH 7.4, scrapped and harvested by centrifugation at 270 × *g* for 2 min. These cells were resuspended in buffer A (20 mM Hepes–NaOH, pH 7.5, containing 250 mM sucrose, 1 mM EDTA, 2 mM dithiothreitol, 1 mM AEBSF, 10 μg/ml leupeptin and 1 μg/ml aprotinin) and sonicated. The sonicated cell lysates were used for Western blotting analysis and for determination of the activity of GAPDH. On the other hand, to analyze GAPDH in the subcellular fractions, cells that had been exposed to hypoxia for 12 h were harvested and resuspended in buffer A as described above, followed by homogenization with 30 strokes of a glass cell homogenizer. The homogenate was centrifuged at 270 × *g* for 2 min and the supernatant was transferred to a fresh tube. The pellet was resuspended in buffer A, homogenized with the homogenizer and centrifuged at 270 × *g* for 2 min. The supernatant obtained was mixed with the first supernatant and centrifuged at 750 × *g* for 10 min to precipitate the nuclei. The post-nuclear fraction was further centrifuged at 105,000 × *g* for 1 h. The final supernatant and precipitate are referred to as the cytosolic fraction and particulate fraction, respectively. Nuclei were sonicated in buffer A, followed by centrifugation at 15,000 × *g* for 10 min. The supernatant obtained is referred to as the nuclear fraction. Total, cytosolic, nuclear and particulate fractions (10 μg of protein) were subjected to SDS-PAGE in 12.5% gel and transferred to a poly(vinylidene difluoride) membrane. The membrane was incubated with anti-GAPDH, anti-TPI or anti-lamin B_1 antibodies. Immunoreactive GAPDH or TPI, and lamin B1 were further incubated with goat anti-rabbit IgG and goat anti-mouse IgG, respectively, conjugated to horseradish peroxidase, and detected by LAS-1000 plus using Super Signal Chemiluminescent substrate (Pierce), followed by quantification.

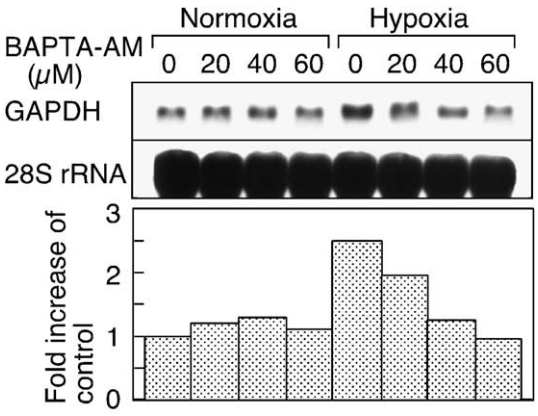

Fig. 1. Inhibitory effect of BAPTA-AM on induction of GAPDH mRNA by hypoxia. MBEC4 cells were exposed to hypoxia for 6 h in the presence of 0, 20, 40 or 60 μM BAPTA-AM, which had been added to the medium 1 h before the exposure. Total RNA was extracted and Northern blot analysis was performed using probes against GAPDH and 28S rRNA. Similar data were obtained in three independent experiments.

2.8. GAPDH activity

Enzyme activity was measured by monitoring the change in absorbance at 340 nm according to the method of Molina y Vedia et al. [32].

3. Results

3.1. Inhibition of hypoxia-induced GAPDH mRNA by BAPTA-AM

Exposure of MBEC4 cells to hypoxia for 6 h substantially increased the level of GAPDH mRNA (Fig. 1). To assess whether intracellular Ca^{2+} is involved in the up-regulation of GAPDH gene expression by hypoxia, MBEC4 cells were exposed to hypoxia in the presence of BAPTA-AM, an intracellular Ca^{2+} chelator. The up-regulation of GAPDH mRNA by hypoxia was inhibited in a dose-dependent manner by BAPTA-AM, while under normoxic conditions, BAPTA-AM had no effects on GAPDH gene expression (Fig. 1). In addition, neither hypoxia nor BAPTA-AM had any significant effects on the level of 28S rRNA that was used as control. These results suggest that intracellular Ca^{2+} contributed to the activation of GAPDH gene expression in hypoxia.

3.2. Participation of Ca^{2+} channels in the up-regulation of GAPDH mRNA by hypoxia

To determine whether Ca^{2+} channels are involved in hypoxia-induced GAPDH mRNA expression, MBEC4 cells were exposed to hypoxia in the presence of each of the Ca^{2+}

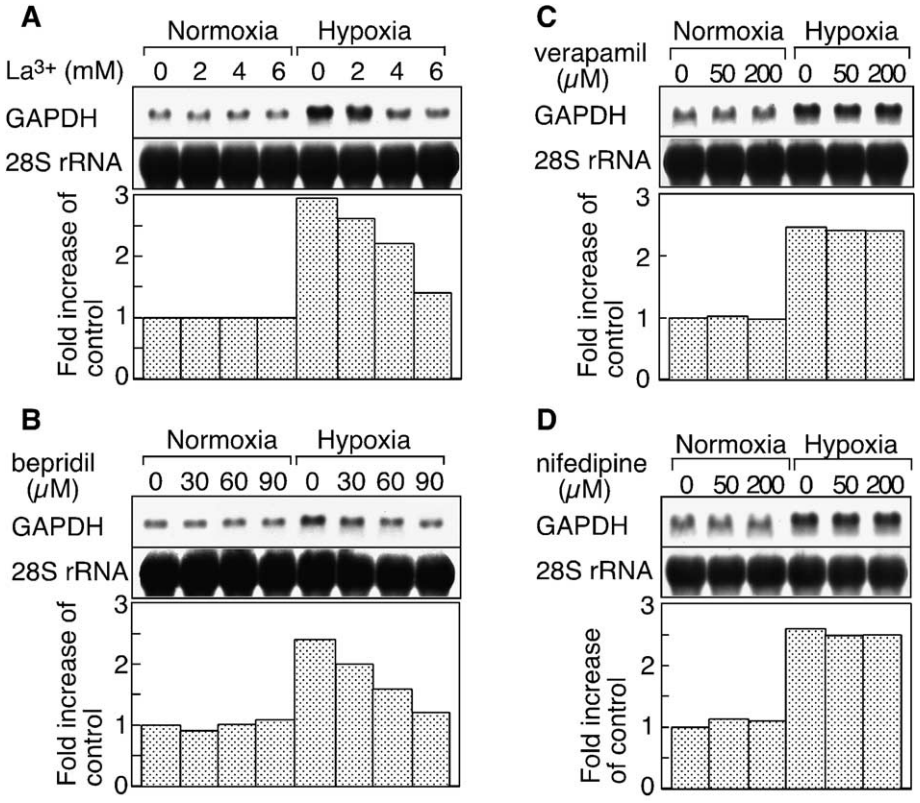

Fig. 2. Effects of Ca^{2+} channel blockers on up-regulation of GAPDH mRNA by hypoxia. MBEC4 cells were exposed to hypoxia for 6 h in the presence of $LaCl_3$ (A), bepridil (B), verapamil (C), or nifedipine (D) at a concentration cited. Ca^{2+} blockers were added to the medium 1 h before the exposure. Northern blot analysis of total RNA was performed by GAPDH cDNA and 28S rRNA probes. Data were similar in three experiments.

channel blockers. When La^{3+}, a blocker of non-selective cation channels, was tested, the induction of expression of GAPDH mRNA by hypoxia was inhibited (Fig. 2A). The inhibitory effect was dose dependent. Furthermore, bepridil, a blocker of the Na^+/Ca^{2+} exchanger (NCX), dose-dependently inhibited the hypoxia-induced increase in the GAPDH mRNA level (Fig. 2B). On the other hand, verapamil and nifedipine, two L-type Ca^{2+} channel blockers, had no significant inhibitory effect on the up-regulation of GAPDH mRNA by hypoxia (Fig. 2C and D). These results suggest that the participation of Ca^{2+} in hypoxia-induced GAPDH gene expression is through non-selective cation channels and NCX.

3.3. Existence of mRNAs encoding three isoforms of NCX

Transcripts encoding three NCX isoforms, NCX1, NCX2, and NCX3, are thought to have unique tissue-specific expression patterns. To analyze the MBEC4 cell-specific expression of the NCX isoforms, RT-PCR was performed using specific primers for each of the NCX isoforms and mRNA from normoxic or hypoxic cells. As shown in Fig. 3, agarose gel electrophoresis of the product from RT-PCR using NCX1-specific primers detected a single band of 599 bp, which is the exact size predicted from mouse NCX1 [29], in the cells under both normoxic and hypoxic conditions. Similarly, the RT-PCRs using NCX2- and NCX3-specific primers combination resulted in single bands of 515 and 277 bp, which are the sizes predicted from rat NCX2 [30] and rat NCX3 [31], respectively, in either the normoxic or hypoxic cells. Each RT-PCR product was cloned, sequenced, and confirmed to be the expected NCX isoform. Sequence analyses showed that the molecular size of each NCX isoform was the same as the predicted size as described above. These results indicate that MBEC4 cells expressed mRNAs for the three NCX isoforms in both normoxia and hypoxia.

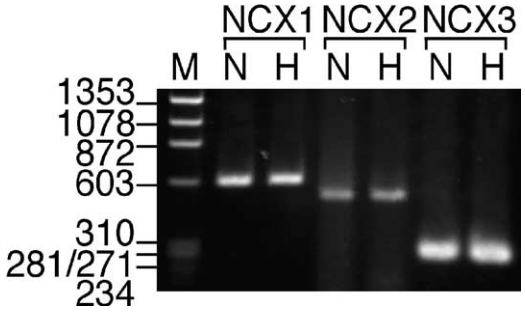

Fig. 3. Expression of NCX isoform mRNAs in MBEC4 cells. MBEC4 cells were exposed to normoxia (N) or hypoxia (H) for 6 h. The RT reaction was performed using mRNAs. The RT products were amplified by nested PCRs using NCX isoform-specific primers. The PCR products (10 μl) were subjected to agarose gel electrophoresis, followed by detection using ethidium bromide. For estimation of molecular mass, ϕX174 fragments digested with *Hae*III were used as a DNA marker (M).

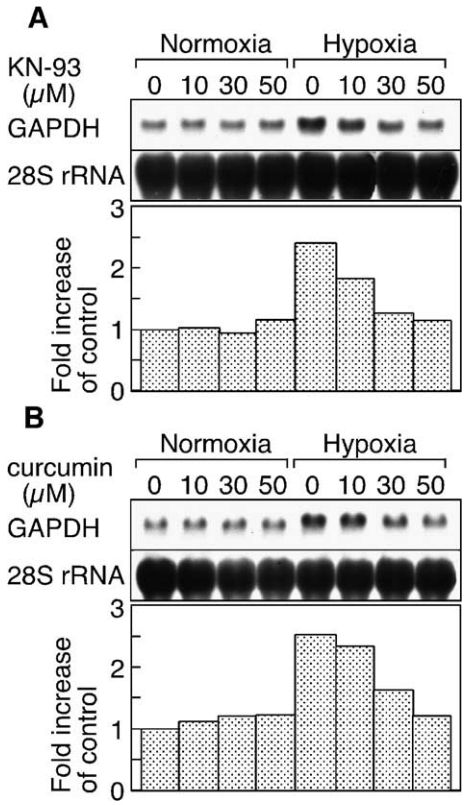

Fig. 4. Inhibitory effect of KN-93 or curcumin on hypoxia-induced GAPDH mRNA expression. MBEC4 cells were exposed to hypoxia for 6 h in the presence of 0, 10, 30 or 50 μM KN-93, or 0, 10, 30 or 50 μM curcumin, which had been added to the medium 1 h before the exposure. Total RNA was extracted and analyzed by Northern blot using probes against GAPDH and 28S rRNA. Similar data were observed in three experiments.

3.4. Repression of hypoxia-induced GAPDH mRNA expression by KN-93 or curcumin

Calmodulin, which is a Ca^{2+}-binding protein, is a messenger that carries the Ca^{2+} signal into the nucleus through the activation of Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) [33]. To determine whether CaMKs participate in the mechanism by which hypoxia increases GAPDH mRNA, cells were exposed to hypoxia in the presence of KN-93, a CaMKs inhibitor. As shown in Fig. 4A, KN-93 abolished the activation of GAPDH gene expression by hypoxia. The inhibition by KN-93 was dose dependent. CaMKs have been reported to contribute to the activation of the c-*fos* promoter by hypoxia [34]. A protein complex composed of c-Fos and c-Jun, which are the translational products of the protooncogenes c-*fos* and c-*jun*, respectively, binds to the AP-1 element [35]. To assess whether c-*jun* gene expression controls the up-regulation of GAPDH gene expression in hypoxia, cells were exposed to hypoxia in the presence of curcumin, an inhibitor of c-*jun* gene expression. As shown in Fig. 4B, curcumin suppressed the activation of GAPDH gene expression by hypoxia in a

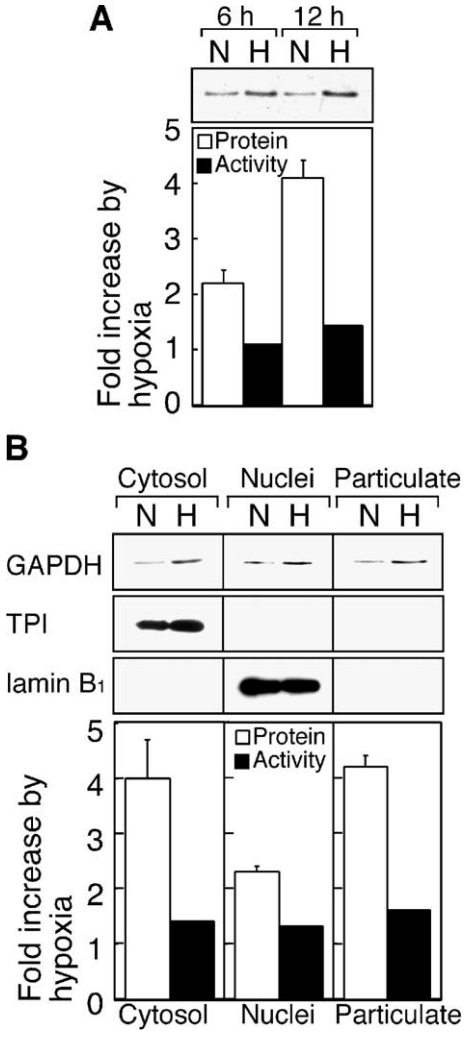

Fig. 5. GAPDH proteins and activities in subcellular fractions in hypoxia. (A) MBEC4 cells were exposed to normoxia (N) or hypoxia (H) for 6 or 12 h and harvested. The sonicated cell lysates were prepared as total fraction, and samples (10 μg protein) were subjected to SDS-PAGE and immunoreacted on Western blot with anti-GAPDH antibody, followed by detection with chemiluminescent substrate and quantification using densitometry. The amount of GAPDH protein and the level of GAPDH activity in the hypoxic cells were expressed as multiples of the values in the normoxic cells (mean ± S.E.). GAPDH activities determined in the 6- and 12-h normoxic cells were 966 ± 10 and 985 ± 21 nmol/min/mg protein, respectively. (B) Cells were exposed to hypoxia for 12 h and harvested, followed by homogenization. The homogenates were fractionated by centrifugation and subcellular fractions were prepared. Equal amounts of protein (10 μg) from each subcellular fraction were subjected to SDS-PAGE, followed by Western blotting with anti-GAPDH, anti-TPI or anti-lamin B_1 antibody. Immunoreactive bands were detected using chemiluminescent substrate, followed by quantification using densitometry. GAPDH protein and GAPDH activity in each fraction of the hypoxic cells were expressed as described above. GAPDH activities in the cytosolic, nuclear and particulate fractions of the normoxic cells were 2350 ± 80, 281 ± 21.2 and 154 ± 8.0 nmol/min/mg protein, respectively. Experiments were replicated three times.

dose-dependent manner. These results suggest that hypoxia up-regulated GAPDH gene expression through the AP-1 element.

3.5. GAPDH proteins and activities in subcellular fractions under hypoxic conditions

MBEC4 cells were exposed to hypoxia for 6 or 12 h, and the cell lysates were subjected to SDS-PAGE, followed by Western blotting with anti-GAPDH antibodies. As shown in Fig. 5A, 6 and 12 h of hypoxia resulted in 2.2- and 4-fold increases in the levels of GAPDH protein in the total fraction, respectively, compared with normoxia. However, the activities of GAPDH under hypoxic conditions did not increase in the same proportions as GAPDH protein. To determine the intracellular distribution of hypoxia-induced GAPDH, cells were exposed to hypoxia for 12 h and homogenized, and the cytosolic, nuclear and particulate fractions were separated by differential centrifugation. Proteins were subjected to SDS-PAGE and transferred to a membrane. Western blot analyses with anti-GAPDH antibodies indicated that GAPDH protein levels in the cytosolic, nuclear and particulate fractions of hypoxic cells were elevated 4.0-, 2.3- and 4.2-fold, respectively, compared with those in normoxic cells (Fig. 5B). TPI, a cytosolic marker protein, and lamin B_1, a nuclear marker protein, were detected only in each corresponding fraction. Glycolytic GAPDH activities were detected in the cytosolic, nuclear and particulate fractions. On the other hand, the relative GAPDH activities of hypoxic cells to normoxic cells in these fractions were 1.4-, 1.3-, and 1.6-fold, respectively. This indicates that the increase of GAPDH proteins was lower than the increase of GAPDH activities, even in the hypoxic cytosolic fraction.

4. Discussion

Changes in the cellular environment allow mammalian cells to express specific stress proteins to maintain homeostasis. As a universal cellular response mechanism to hypoxia, cells induce the expression of glycolytic enzymes and shift the primary biosynthetic pathway of ATP from oxidative phosphorylation to glycolysis [3–5]. Furthermore, hypoxia increases intracellular Ca^{2+} concentration by increasing Ca^{2+} influx from extracellular space or by releasing Ca^{2+} from intracellular Ca^{2+} stores [21–26]. The rise of intracellular Ca^{2+} concentration can influence a variety of biochemical processes including transcriptional regulation of gene expression [36]. Although GAPDH gene has been thought to be one of the housekeeping genes, recent evidence demonstrates that GAPDH gene expression is up-regulated by hypoxia in certain types of cells such as endothelial cells and alveolar epithelial cells [15,37]. In the present study, the involvement of intracellular Ca^{2+} in the pathway transmitting the hypoxic stimulus to GAPDH gene

activation in MBEC4 cells was estimated by using pharmacological reagents.

Data obtained by using BAPTA-AM suggest that intracellular Ca^{2+} contributes to the increase in the transcriptional level of GAPDH in MBEC4 cells exposed to hypoxia (Fig. 1). In addition, the hypoxia-induced up-regulation of GAPDH expression was inhibited by a blocker for non-selective cation channels or NCX (Fig. 2). In the normal state, non-selective cation channels and NCX regulate the primary Ca^{2+} influx and efflux mechanisms, respectively; however, the net direction in which NCX transports Ca^{2+} can be reversed in response to changes in physiological conditions [38]. It is thus reasonable to postulate that NCX, in addition to non-selective cation channels, participates in the influx of Ca^{2+} from the extracellular space to increase the intracellular Ca^{2+} concentration when MBEC4 cells are exposed to hypoxia, and the increase in the Ca^{2+} concentration is crucial for the hypoxia-induced transcriptional activation of the GAPDH gene.

NCX has been found to have three isoforms, NCX1, NCX2 and NCX3 [29–31,39]. The mRNAs for the three NCX isoforms are thought to have unique tissue-specific expression patterns. NCX1 and NCX2 mRNAs are ubiquitously expressed in many tissues, suggesting that NCX1 and NCX2 function in a broader range of tissues. On the other hand, NCX3 mRNA is distributed in a narrower range of tissues, in particular, brain and skeletal muscle. Rat thoracic aortic endothelial cells express NCX1 and NCX2 mRNAs, but not NCX3 mRNA [39], whereas MBEC4 cells expressed not only NCX1 and NCX2 mRNAs, but also NCX3 mRNA (Fig. 3). Although the overall functional properties of the three NCX isoforms are fundamentally similar, depletion of cellular ATP inhibits NCX1 and NCX2 activities, but not NCX3 activity [40]. In MBEC4 cells under hypoxic conditions, in which cellular ATP production is decreased, NCX3 is expected to play an important role in the regulation of cytosolic Ca^{2+}. These results suggest that hypoxia accelerates Ca^{2+} influx through non-selective cation channels and causes the three isoforms of NCX to operate in reverse.

It has been reported that hypoxia stimulates Ca^{2+} influx from the extracellular space through L-type Ca^{2+} channels in PC12 cells, leading to the up-regulation of tyrosine hydroxylase and c-*fos* mRNAs [24,34]. However, in MBEC4 cells, L-type Ca^{2+} channel blockers (nifedipine and verapamil) did not affect the hypoxia-induced increase in GAPDH mRNA (Fig. 2). A similar result was reported in bovine aortic and pulmonary artery endothelial cells [41]. L-type Ca^{2+} channels do not seem to be necessary for regulating intracellular Ca^{2+} concentration in response to hypoxia in endothelial cells.

We used KN-93 and curcumin to analyze downstream pathways activated by the rise in intracellular Ca^{2+} concentration in response to hypoxia. Both KN-93 and curcumin abolished the induction of GAPDH gene expression by hypoxia (Fig. 4). Ca^{2+} regulates c-*fos* gene expression in response to various stimuli through the activation of CaMKs [34], and hypoxia results in the induction of c-*jun* gene expression in endothelial cells [42]. A heterodimer of c-Fos and c-Jun makes up the AP-1 complex [35], implying that AP-1 participates in the transcriptional activation of the GAPDH gene by hypoxia. Furthermore, the human GAPDH gene possesses a HIF-1 binding site, which requires for the flanking sequence to be functionally active [20]. The transcription of VEGF and tyrosine hydroxylase genes is up-regulated by the binding of both HIF-1 and AP-1 to each consensus enhancer sequence in hypoxia [43,44]. This suggests that both AP-1 and HIF-1 participate in the hypoxia-activated gene expression in GAPDH as well as in VEGF and tyrosine hydroxylase. There are some candidates for AP-1 element on the 5′ -flanking region of the human GAPDH gene (-2474 to -2467, -1845 to -1839, -1592 to -1586 and -1062 to -1056). Our preliminary studies suggest that the region located at -1091 to -1053 is not involved in the induction of human GAPDH gene expression by hypoxia (data not shown). We are now studying the involvement of other candidates.

In bovine aortic endothelial cells, hypoxia primarily induces the cytosolic GAPDH and only slightly induces the nuclear GAPDH [15]. In MBEC4 cells, the hypoxia-induced increase of GAPDH protein was observed in the particulate fraction, in addition to the cytosolic and nuclear fractions, and the extent of the increase in the particulate fraction was comparable to that in the cytosolic fraction (Fig. 5). However, even in the cytosol, the increased GAPDH activity did not correlate with the increase in GAPDH protein. In the nuclear and particulate fractions, GAPDH is not needed for glycolysis. However, in these fractions, GAPDH has been found to possess several non-glycolytic functions such as DNA repair, DNA replication, nuclear tRNA export, tubulin polymerization, endocytosis, apoptosis, translational regulation of gene expression and vesicular transport in the early secretory pathway [16–19]. Our present data taken together with the results of previous reports suggest that, in MBEC4 cells, GAPDH is induced by hypoxia not only to compensate for a reduced ATP by accelerating turnover rate of cytosolic glycolysis, but also to participate in certain functions unassociated with glycolytic activity in the nuclear and particulate fractions, and probably even in the cytosolic fraction.

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References

[1] M.A. Goldberg, S.P. Dunning, H.F. Bunn, Science 242 (1988) 1412–1415.

[2] A.P. Levy, N.S. Levy, S. Wegner, M.A. Goldberg, J. Biol. Chem. 270 (1995) 13333–13340.
[3] G.L. Semenza, Curr. Opin. Genet. Dev. 8 (1998) 588–594.
[4] G.L. Semenza, P.H. Roth, H.-M. Fang, G.L. Wang, J. Biol. Chem. 269 (1994) 23757–23763.
[5] G.L. Semenza, B.-H. Jiang, S.W. Leung, R. Passantino, J.-P. Concordet, P. Maire, A. Giallongo, J. Biol. Chem. 271 (1996) 32529–32537.
[6] B.L. Ebert, J.D. Firth, P.J. Ratcliffe, J. Biol. Chem. 270 (1995) 29083–29089.
[7] R.H. Wenger, M. Gassmann, Biol. Chem. 378 (1997) 609–616.
[8] B.-H. Jiang, E. Rue, G.L. Wang, R. Roe, G.L. Semenza, J. Biol. Chem. 271 (1996) 17771–17778.
[9] K.K. Graven, L.H. Zimmerman, E.W. Dickson, G.L. Weinhouse, H.W. Farber, J. Cell. Physiol. 157 (1993) 544–554.
[10] J.J. Sciandra, J.R. Subjeck, C.S. Hughes, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 4843–4847.
[11] B.E. Dwyer, R.N. Nishimura, I.R. Brown, Exp. Neurol. 104 (1989) 28–31.
[12] A.A. Knowlton, P. Brecher, C.S. Apstein, J. Clin. Invest. 87 (1991) 139–147.
[13] L.H. Zimmerman, R.A. Levine, H.W. Farber, J. Clin. Invest. 87 (1991) 908–914.
[14] M. Tucci, R.J. McDonald, R. Aaronson, K.K. Graven, H.W. Farber, Am. J. Physiol. 271 (1996) L341–L348.
[15] K.K. Graven, R.F. Troxler, H. Kornfeld, M.V. Panchenko, H.W. Farber, J. Biol. Chem. 269 (1994) 24446–24453.
[16] M.A. Sirover, J. Cell. Biochem. 66 (1997) 133–140.
[17] M.A. Sirover, Biochim. Biophys. Acta 1432 (1999) 159–184.
[18] M.D. Berry, A.A. Boulton, J. Neurosci. Res. 60 (2000) 150–154.
[19] E.J. Tisdale, J. Biol. Chem. 276 (2001) 2480–2486.
[20] K.K. Graven, Q. Yu, D. Pan, J.S. Roncarati, H.W. Farber, Biochim. Biophys. Acta 1447 (1999) 208–218.
[21] C.G. Salvaterra, W.F. Goldman, Am. J. Physiol. 264 (1993) L323–L328.
[22] D. Mukhopadhyay, H.I. Akbarali, Biochem. Biophys. Res. Commun. 229 (1996) 733–738.
[23] M. Dipp, P.C. Nye, A.M. Evans, Am. J. Physiol. 281 (2001) L318–L325.
[24] R. Raymond, D. Millhorn, Kidney Int. 51 (1997) 536–541.
[25] Y. Aono, H. Ariyoshi, M. Sakon, A. Ueda, Y. Tsuji, T. Kawasaki, M. Monden, J. Cell. Biochem. 78 (2000) 458–464.
[26] L.A. Shimoda, J.S.K. Sham, T.H. Shimoda, J.T. Sylvester, Am. J. Physiol., Lung Cell. Mol. Physiol. 279 (2000) L884–L894.
[27] T. Tatsuta, M. Naito, T. Oh-hara, I. Sugawara, T. Tsuruo, J. Biol. Chem. 267 (1992) 20383–20391.
[28] R. Yamaji, T. Okada, M. Moriya, M. Naito, T. Tsuruo, K. Miyatake, Y. Nakano, Eur. J. Biochem. 239 (1996) 494–500.
[29] I. Kim, C.O. Lee, Ann. N.Y. Acad. Sci. 779 (1996) 126–128.
[30] Z. Li, S. Matsuoka, L.V. Hryshko, D.A. Nicoll, M.M. Bersohn, E.P. Burke, R.P. Lifton, K.D. Philipson, J. Biol. Chem. 269 (1994) 17434–17439.
[31] D.A. Nicoll, B.D. Quednau, Z. Qui, Y.R. Xia, A.J. Lusis, K.D. Philipson, J. Biol. Chem. 271 (1996) 24914–24921.
[32] L. Molina y Vedia, B. McDonald, B. Reep, B. Brune, M. Di Silvio, T.R. Billiar, E.G. Lapetina, J. Biol. Chem. 267 (1992) 24929–24932.
[33] A.N. Malviya, P.J. Rogue, Cell 92 (1998) 17–23.
[34] D.R.D. Premkumar, R.R. Mishra, J.L. Overholt, M.S. Simonson, N.S. Cherniack, N.R. Prabhakar, J. Appl. Physiol. 88 (2000) 1898–1906.
[35] P. Angel, M. Karin, Biochim. Biophys. Acta 1072 (1991) 129–157.
[36] D.D. Ginty, Neuron 18 (1997) 183–186.
[37] B. Escoubet, C. Planès, C. Clerici, Biochem. Biophys. Res. Commun. 266 (1999) 156–161.
[38] K.D. Philipson, D.A. Nicoll, Annu. Rev. Physiol. 62 (2000) 111–133.
[39] B.D. Quednau, D.A. Nicoll, K.D. Philipson, Am. J. Physiol. 272 (1997) C1250–C1261.
[40] B. Linck, Z. Qiu, Z. He, Q. Tong, D.W. Hilgemann, K.D. Philipson, Am. J. Physiol. 274 (1998) C415–C423.
[41] K.K. Graven, R.J. McDonald, H.W. Farber, Am. J. Physiol. 274 (1998) C347–C355.
[42] R.S. Bandyopadhyay, M. Phelan, D.V. Faller, Biochim. Biophys. Acta 1264 (1995) 72–78.
[43] A. Damert, E. Ikeda, W. Risau, Biochem. J. 327 (1997) 419–423.
[44] M.L. Norris, D.E. Millhorn, J. Biol. Chem. 270 (1995) 23774–23779.