Hypoxia and Nitric Oxide Treatment Confer Tolerance to Glucose Starvation in a 5′-AMP-activated Protein Kinase-dependent Manner*

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Hypoxia is a critical event for higher organisms, and cells and tissues react by increasing the oxygen supply by vasodilatation, angiogenesis, and erythropoiesis and maintaining cellular energy by increasing glycolysis and inhibiting anabolic pathways. Stimulation of glycolysis has been regarded as the main response that increases energy production during hypoxia; however, there is an obvious conflict during ischemia, because both the oxygen and glucose supply are insufficient. In this study, we found that exposure of HepG2 cells and normal fibroblasts to hypoxia induces cellular tolerance to glucose starvation. The tolerance induced by hypoxia is dependent on several amino acids, indicating a switch from glucose to amino acids as the energy source. When antisense RNA expression vector for 5′-AMP-activated protein kinase or protein kinase B/Akt was transfected into HepG2 cells, the induction of tolerance to glucose was greatly inhibited, indicating that the tolerance was dependent on 5′-AMP-activated protein kinase and protein kinase B/Akt. Similar tolerance was induced by nitric oxide exposure. The tolerance induced was observed in various cells and may represent a previously unknown physiological response related to hypoxia-preconditioning and tumor progression/austerity.

The oxygen supply to cells and tissues is pivotal in maintaining their function and integrity, and cellular reactions to hypoxia have been studied extensively (1, 2). Basically, the reactions of tissues are classified into two categories: 1) improvement of the oxygen supply and 2) adaptation to anaerobic conditions. Reactions that improve the oxygen supply include vasodilatation, erythropoiesis, and angiogenesis, and adaptation reactions to anaerobic conditions include a metabolic switch to anaerobic glycolysis, repression of anabolism, and cell cycle arrest. These reactions are governed by various reactions, both physiological and biochemical.

The hypoxia response is very important to understanding tumor tissue biology. Tumor cells are continuously exposed to hypoxia, because the oxygen demand of tumors always exceeds their supply due to their unregulated growth caused by genetic and epigenetic alterations (3, 4). That is why angiogenesis is so critical for tumor progression and why anti-angiogenesis is regarded as such a promising novel strategy for cancer therapy (5). Although the results of studies of the hypoxia response thus far provide a good explanation for the survival strategy of tissues during pure oxygen deficiency caused by high altitude and pulmonary diseases, it is still difficult to explain fully the adaptive reactions of tumor tissue. This is particularly true of hypovascular tumors, such as pancreatic cancer, in which even the liver metastases are hypovascular (6). Because of the chronic oxygen deficiency due to increased demand as a result of cancer growth or insufficient angiogenesis, the supply of both oxygen and nutrients may be insufficient. The only known response of energy metabolism to hypoxia is a switch to anaerobic glycolysis (7), but what is the source of the glucose? Most is supplied by the bloodstream, and utilization of glycogen stores and gluconeogenesis are alternatives. Glycogen storage in tumor tissue is generally very limited, and it is unimaginable that glycogen could be the main source of glucose in tumors during chronic hypoxia. Glutamine is suspected of being the main energy source in tumors (8), but it is most efficiently used for energy production under aerobic conditions (9). Thus cells and tissues, such as those of tumors, are confronted by an insufficiency of both oxygen and nutrients, and the switch to glycolysis seems inadequate to explain the adaptation. Repeated brief exposure of organs to ischemia has been found to confer strong tolerance to subsequent ischemia and is referred to as “ischemic preconditioning” (10). We therefore suspected that hypoxia might induce other reactions that confer tolerance to glucose insufficiency. Our primary purpose in the present study was to determine how cells respond to insufficient supplies of oxygen and nutrients.

Nitric oxide (NO)† has been found to be a mediator of vasodilatation (11). It also blocks apoptosis under certain conditions (12), stimulates vascular endothelial growth factor gene expression (13), and inhibits cytochrome oxidase activity (14), resulting in destruction of mitochondria without cell death (15). Based on these findings, we speculated that NO might activate some unknown pathway that generates energy without con-

* This work was supported in part by a grant for the Second Term Comprehensive Strategy for Cancer Control from the Ministry of Health and Welfare and a grant-in-aid for Cancer Research from the Ministry of Education, Science, Sports, and Culture, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. § To whom correspondence should be addressed: National Cancer Center Research Institute East, 6-5-1, Kashiwanoha, Kashiwa, Chiba 277-8577 Japan. Fax: 81-471-34-6859; E-mail: hesumi@east.ncc.go.jp.

† The abbreviations used are: NO, nitric oxide; PKB/Akt, protein kinase B/Akt; AMPK, 5′-AMP-activated protein kinase; FCS, fetal calf serum; DMEM, Dulbecco’s modified Eagle’s medium; HIF-1, hypoxia-inducible factor-1; SNAP, N-nitroso-N-acetyl-L-phenylisocyanate; HepG2/a1A, HepG2 cell transfected with AMPKα1 antisense RNA expression vector; HepG2/α2A, HepG2 cell transfected with AMPKα2 antisense RNA expression vector; HepG2/αCR, HepG2 cell transfected with pCR3.0; PI3-kinase, phosphatidylinositol 3-kinase.
ssuming oxygen, and therefore might be a mediator of cellular responses to an insufficient blood supply.

In the present study, we examined the effects of hypoxia and NO on the cellular response to nutrient deprivation to determine whether there are any unknown physiological adaptations of cells to overcome deprivation of both oxygen and nutrients.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture**—HepG2 cells were obtained from Japanese Collection of Research Bioresources, and WI-38 cells were purchased from ATCC. All cells were maintained under the standard culture conditions recommended by the suppliers. When nutrient starvation had been achieved, cells were seeded into 12-well dishes (Falcon). After 24 h, the cells were washed with phosphate-buffered saline, and the medium was changed to either DMEM (Invitrogen) supplemented with 10% dialyzed FCS (Sigma), minimum Eagle’s medium nonessential amino acid mixture (Invitrogen), glutamine and antibiotic mixture (Invitrogen), or DMEM-base with the same supplements as above. S-Nitroso-N-acetyl-DL-penicillamine (SNAP) was dissolved in dimethyl sulfoxide (Me2SO4) to create a 1 M stock solution, and it was frozen at −20 °C until used. The same volume of Me2SO4 was added to control cultures. After detaching the cells from each well by trypsinization, they were counted with a hemocytometer, and viable cells were identified by dye-exclusion with trypan blue (Invitrogen). Hypoxia was achieved by culturing cells under 1% O2, 5% CO2, and 94% N2.

**Analysis of Organic Acids**—Organic acids in the culture medium were analyzed after a 24-h culture of cells in 10-cm plastic dishes with DMEM supplemented with 10% FCS with or without addition of SNAP. The organic acid fraction was prepared by addition of perchloric acid to a final concentration of 1%. The precipitate was removed by centrifugation and filtration through a filter having a 0.45-μm pore size. The filtrate was then subjected to HPLC analysis on a Shodex column KC-811 (YMC Co. Ltd., Kyoto, Japan). In some experiments, lactate was determined with a kit from Sigma. The 1% O2 culture was conducted in a humidified incubator under 1% O2, 5% CO2, and 94% N2.

**Antisense RNA Expression Vectors and Transfection**—The antisense RNA expression vectors for Akt1 and Akt2 were constructed as described previously (16). Briefly, the cDNAs for Akt1 and Akt2 were ligated into pcDNA3 (Invitrogen) in an antisense orientation. Similarly, cDNA for AMPKβ1 and the β2 subunit was cloned in the antisense orientation into the XhoI/EcoRV site of pcDNA3.1 vector and the EcoRI site of pCR3.0 vector, respectively. All expression vectors were verified by sequencing. The cDNA for AMPKα1 and α2 was obtained by reverse transcription-coupled PCR using the following primers and the mRNA fraction of human fetal brain (CLONTECH). The primers for the α1 subunit were 5'-agtctgagctggaagaaaagaagga-

| Table I: Organic acid accumulation in HepG2 cell medium |
|---------------------------------------------------------|
| All the values are means of at least two independent experiments. |
| Organic acids, μmol/24 h/1×10⁶ cell | Lactate | Acetate |
|--------------------------------------|--------|--------|
| 21% O2 | 8.5 | 1.41 |
| 0.5 mM SNAP | 14.5* | 1.95* |
| 1% O2 | 15.0* | 2.63* |

* Statistical significance at p < 0.01 from 21% O2 culture.

**FIG. 1.** Cell survival during glucose starvation. Each cell count represents the mean of at least three independent wells. Statistically significant differences from the respective control in response to hypoxia or SNAP are marked * (p < 0.05) or ** (p < 0.01). a, time course of cell death caused by glucose starvation under atmospheric oxygen and 1% oxygen. □, 1% oxygen, DMEM-base; ◊, atmospheric oxygen, DMEM-base. The number of cells at the start of glucose starvation was set equal to 100%. b, cell survival without glucose under atmospheric oxygen, with or without SNAP. •, without SNAP; □, with 0.25 mM SNAP; ○, with 0.5 mM SNAP. c, effect of oxygen concentration, NaCN, and SNAP on cell survival during glucose starvation. Cell viability was assessed 36 h after the start of glucose starvation. The final concentrations are as follows: NaCN, 4 mM; SNAP, 0.5 mM. d, effect of SNAP concentration on cell survival under oxygen concentrations of 21% and 1%. Cells were counted after 36 h of starvation.
AMPK Activity Determination—AMPK activity was assayed essentially based on the method of Davies et al. (17) using SAMS peptide as the substrate, with modifications. The details of the modifications will be published elsewhere, but briefly are as follows. Glutathione S-transf erase-SAMS fusion protein expressed in E. coli was used as the substrate instead of synthetic SAMS peptide. GST-SAMS fusion protein was purified by preparing an expression vector having a cDNA coding for SAMS peptide at the 5′-end of the glutathione S-transferase of pGEX vector and transfecting it into E. coli. The fusion protein was purified by glutathione-Sepharose (Amersham Biosciences) column chromatography after extraction. After the AMPK enzyme reaction, GST-SAMS fusion protein was recovered by binding to glutathione Sepharose and precipitation by centrifugation. Radioactivity was determined with a liquid scintillation counter (Beckman Instruments).

Electromobility Shift Assay of HIF-1—The DNA binding activity of HIF-1 was assessed by electromobility shift assay, essentially based on the method described previously (15). HepG2 cells exposed to 30 μM CoCl2 or 200 μM desferrioxamine in DMEM at atmospheric oxygen tension or cultured at 1% oxygen for 12 h were used or HepG2 cells cultured in DMEM or DMEM-base either at atmospheric or 1% oxygen tension for 12 h.

Statistical Analysis—All of the data were analyzed by unpaired Student’s t test where possible, and differences with a p value of <0.05 were considered significant.

Reagents—S-Nitroso-N-acetyl-l-penicillamine (SNAP) and N-acetyl-l-penicillamine were purchased from Dojindo, Kumamoto, Japan. D-Glucose, 2-deoxy-D-glucose, D-glucosamine, and N-acetyl-D-glucosamine were purchased from Sigma and were more than 99% pure.

RESULTS

Exposure to Hypoxia and NO Confer Tolerance to Glucose Starvation—As shown in Fig. 1a, when human hepatoma HepG2 cells were cultured in glucose-free Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal calf serum at the atmospheric oxygen concentration with 5% CO2, most of the cells underwent necrotic cell death within 24 h. However, under 1% O2, 5% CO2 and 94% N2, the cells survived much longer (Fig. 1a), and the surviving cells grew normally upon addition of glucose. There was a significant increase in cell survival when 0.5 mM or 0.25 mM SNAP, an NO generator, was included in the culture medium (Fig. 1b). Because NO strongly inhibits cytochrome c oxidase and hypoxia limits oxygen utilization, cells were exposed to various concentrations of NaCN, a specific inhibitor of cytochrome c oxidase, to assess its effect on survival (Fig. 1c); however, it did not affect cell survival up to 4 mM, at which concentration oxygen consumption was completely blocked, the same as with 0.5 mM SNAP (data not shown). SNAP is an NO generator but also produces N-acetyl-l- penicillamine as a by-product and nitrite as an oxidized product of NO; however, these products alone had negligible effects on cell survival (data not shown). NaCN did not alter cell survival, but 0.5 mM SNAP enhanced cell survival even at low oxygen concentrations (1%). The half-life of NO and the release of NO from SNAP are greatly influenced by the oxygen concentration (19). When the effect of NO on cell survival under 1% O2 was examined at various SNAP concentrations, SNAP was found to enhance cell survival at much lower concentrations (Fig. 1d), and a clear additive effect of SNAP and hypoxia was seen under these conditions. By contrast, the high concentration of SNAP was cytotoxic at both atmospheric and hypoxic oxygen tension (Fig. 1d).

Glycolysis has long been considered the major pathway of energy production under hypoxic conditions, and consistent with this, when glucose was removed accumulation of both glycolytic products lactate and acetate was greatly increased under 1% O2, and/or in the presence of SNAP (Table 1). As shown in Fig. 2, NO again prolonged the survival of cells exposed to 5 μM iodoacetate, a strong inhibitor of glycolysis (Fig. 2a). This enhanced cell survival was reproduced by hypoxia but not by NaCN. Similar prolonged cell survival was also observed with

**Fig. 2. Effect of SNAP on cell death induced by iodoacetate.** α, time course of cell death caused by iodoacetate. ○, cells cultured in DMEM plus 5 μM iodoacetate; ■, cells cultured in DMEM plus 5 μM iodoacetate and 0.5 mM SNAP. Statistically significant differences from the respective control are marked ** (p < 0.01); b, effect of SNAP and NaCN on iodoacetate-induced cell death. α, HepG2 cells in DMEM; β, HepG2 cells in DMEM with 5 μM iodoacetate; γ, HepG2 cells in DMEM with 5 μM iodoacetate and 4 mM NaCN; δ, HepG2 cells in DMEM with 0.5 mM SNAP; ε, HepG2 cells in DMEM with 5 μM iodoacetate and 0.5 mM SNAP; δ, HepG2 cells in DMEM with 5 μM iodoacetate, 4 mM NaCN, and 0.5 mM SNAP; γ, WI-38 cells in DMEM; η, WI-38 cells in DMEM with 5 μM iodoacetate; ι, WI-38 cells in DMEM with 5 μM iodoacetate and 0.5 mM SNAP. These photographs were taken at 12 h.

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cag-3' and 5'-ccgaatcttagaagagctgagaacttcc-3', covering ~11 to 942 nucleotides, and for α2 they were 5'-gaagatggtctgaagcag3-3' and 5'-atactagagaagacaqg-3', covering the entire coding sequence (~4 to 1672 nucleotides). HepG2 cells were transfected with the above expression vectors by the calcium phosphate co-precipitation method, and selection was achieved by adding G418 (Invitrogen) at 640 μg/ml for at least 2 weeks.

Western Blot Analysis of Akt Protein—The total amounts of Akt protein and phosphorylated Akt were determined by Western blot analysis as described previously (16) using Akt-specific and Ser-473-phosphorylated Akt-specific antisera (New England Biolabs). Antiserum against AMPK α1 and α2 subunits were prepared by injecting denatured α1 and α2 subunits into rabbits. AMPK α1 and α2 subunits were purified from Escherichia coli transfected with expression vector containing the 11–942 nucleotides of α1 subunit cDNA or the entire coding sequence (~4 to 1672 nucleotides) of α2 subunit cDNA constructed in a PET 19b vector (Novagen) having the His tag. His-tagged subunits were purified by nickel-nitrilotriacetic acid column chromatography and preparative SDS-PAGE. The cross-reactivity of both sera was minimized by absorbing them with another antigen. The details of the antibody preparation will be published elsewhere.
WI-38 human fibroblasts (Fig. 2b). WI-38 cells exposed to 5 μM iodoacetate alone formed blebs within 6 h and died shortly thereafter. NaCN did not alter this cell death, but cells treated with both iodoacetate and SNAP maintained their morphology and viability until 24 h and died only after 48 h. These results clearly indicate that the glycogen utilization is not the mechanism of the enhanced survival.

**Induction of Tolerance to Glucose Starvation Is Dependent on Amino Acids**—Another possible mechanism of the improved cell survival is enhanced utilization of amino acids or fatty acids for energy production. To test this possibility, we conducted a series of experiments in which amino acids, fatty acids, or both were withdrawn from the cell culture. When all amino acids were present, there was clear hypoxia-induced tolerance (Fig. 3a). The same observations were made in HLE human hepatoma cells (data not shown) and showed that amino acids are essential for induction of tolerance. Accordingly, experiments were conducted with medium supplemented with only one amino acid and no serum. When cells were cultured at atmospheric oxygen tension, they all died, whereas significant numbers of cells survived during hypoxia in the presence of certain amino acids, including glutamine, arginine, serine, cysteine, proline, glutamic acid, and aspartic acid (Fig. 3b). When a fatty acid mixture was included in the medium, cell survival was slightly enhanced under normoxic conditions but not under hypoxic conditions (data not shown).

**HIF-1 Activation Might Not be Involved in the Induction of Tolerance**—Both hypoxia and exposure to NO activate the hypoxia-response transcription factor, HIF-1. HIF-1 activates a series of genes encoding glycolytic enzymes and the glucose transporter, and probably also modulates a series of genes whose products shift energy metabolism away from glycolysis. Cobalt chloride and desferrioxamine are known to activate HIF-1, but neither compound induced tolerance (Fig. 4a) despite clearly activating HIF-1, as shown in Fig. 4b. HIF-1 was also clearly activated by exposure to NO or hypoxia in the presence of glucose, but relatively weakly activated in the absence of glucose (Fig. 4c). All these findings suggest that HIF-1 activation is insufficient for induction of tolerance, and it does not seem to be involved in tolerance.

**AMPK Is Involved in the Induction of Tolerance**—AMPK was examined for possible involvement in tolerance because it is a heterotrimeric serine-threonine protein kinase that is activated under various stress conditions under which the cellular ATP level decreases. The AMPK activity of parental HepG2 cells and HepG2 cells transfected with the control vector pCR3.0 slightly decreased after glucose starvation but increased significantly in response to hypoxia or SNAP even after glucose starvation (Table II). When an antisense RNA expression vector for the α1 or α2 subunit of AMPK was transfected into HepG2 cells (HepG2/α1A cells and HepG2/α2A cells, respectively), the cells grew normally in ordinary DMEM supplemented with 10% FCS but started to die within 24 h in glucose-free medium under normoxia, the same as the parental HepG2 cells (data not shown). As mentioned previously, however, HepG2 cells survive much longer when cultured under 1% O₂. 
By contrast, HepG2/H92511A and HepG2/H92512A cells died much sooner than HepG2 cells transfected with control vector pCR3.0 (HepG2/pCR), although the AMPK a2 antisense vector was less effective than /H92511 (Fig. 5a). Similar reduction of cell survival was observed in HepG2/H92511A and HepG2/H92512A cells during SNAP-induced tolerance to glucose starvation (data not shown). The basal level of expression of each AMPK /H9251 subunit in the respective transfectants was examined by Western blot analysis (Fig. 5b), and the expression of each of the respective isoforms in transfectants was found to decrease slightly but significantly. Because it is currently impossible to assay differential AMPK isotype activity, total AMPK activity after glucose starvation at 1% oxygen and during SNAP exposure was determined. Basal activity at the beginning of starvation was slightly lower in HepG2/H92511A cells than in HepG2/pCR cells, but after 6 h of glucose starvation, its activity in HepG2/a1A and HepG2/a2A cells was higher than in the HepG2/pCR cells (Table II). When cells were exposed to either hypoxia or SNAP during glucose starvation, AMPK activity was found to be markedly increased in HepG2/pCR and HepG2/a1A cells but to a lesser extent in HepG2/a2A cells during 6 h of starvation. These complicated changes in AMPK activity indicated that AMPK activity assessed by SAMS peptide alone does not explain the mechanisms of prolonged cell survival under hypoxia or SNAP treatment.

**Table II**

AMPK activity of HepG2 cells and AMPK a1 and a2 antisense RNA-transfected cells under various conditions

Activity is expressed as nmol of phosphate incorporated per mg of protein/min. All values are means ± S.D. of at least four experiments. Glc-, glucose-free medium; hypoxia, 1% oxygen tension; SNAP, 0.5 mM SNAP included; a1A, HepG2 cells stably transfected with AMPK a1 antisense RNA expression vector; a2A, HepG2 cells stably transfected with AMPK a2 antisense RNA expression vector. The results of the statistical analyses are shown only for comparisons among HepG2 cells under various conditions and comparisons among three cell lines under the same conditions.

| Conditions    | HepG2    | a1A       | a2A       |
|--------------|----------|-----------|-----------|
| Control      | 0.090 ± 0.015 | 0.076 ± 0.011<sup>a</sup> | 0.082 ± 0.022 |
| Hypoxia      | 0.073 ± 0.0099 | 0.063 ± 0.0066<sup>a</sup> | 0.062 ± 0.0040<sup>a</sup> |
| SNAP         | 0.059 ± 0.0054<sup>a</sup> | 0.050 ± 0.011 | 0.054 ± 0.0053 |
| Glc-hypoxia  | 0.063 ± 0.0048<sup>a</sup> | 0.085 ± 0.011<sup>a</sup> | 0.170 ± 0.059<sup>a</sup> |
| Glc/SNAP     | 0.110 ± 0.0035<sup>a</sup> | 0.133 ± 0.0066<sup>a</sup> | 0.132 ± 0.015<sup>a</sup> |
| Glc/SNAP     | 0.170 ± 0.018<sup>a</sup> | 0.183 ± 0.014 | 0.109 ± 0.016<sup>a</sup> |

*Statistically significant difference from HepG2 cells cultured in DMEM under normoxia.

Conditions. By contrast, HepG2/a1A and HepG2/a2A cells died much sooner than HepG2 cells transfected with control vector pCR3.0 (HepG2/pCR), although the AMPK a2 antisense vector was less effective than a1 (Fig. 5a). Similar reduction of cell survival was observed in HepG2/a1A and HepG2/a2A cells during SNAP-induced tolerance to glucose starvation (data not shown). The basal level of expression of each AMPK a subunit in the respective transfectants was examined by Western blot analysis (Fig. 5b), and the expression of each of the respective isoforms in transfectants was found to decrease slightly but significantly. Because it is currently impossible to assay differential AMPK isotype activity, total AMPK activity after glucose starvation at 1% oxygen and during SNAP exposure was determined. Basal activity at the beginning of starvation was slightly lower in HepG2/a1A cells than in HepG2/pCR cells, but after 6 h of glucose starvation, its activity in HepG2/a1A and HepG2/a2A cells was higher than in the HepG2/pCR cells (Table II). When cells were exposed to either hypoxia or SNAP during glucose starvation, AMPK activity was found to be markedly increased in HepG2/pCR and HepG2/a1A cells but to a lesser extent in HepG2/a2A cells during 6 h of starvation. These complicated changes in AMPK activity indicated that AMPK activity assessed by SAMS peptide alone does not explain the mechanisms of prolonged cell survival under hypoxia or SNAP treatment.
PKB/Akt is also involved in the tolerance mechanism—PKB/Akt is known to be involved in the cell survival reaction under various stress conditions, and we previously discovered (16) that PKB/Akt is involved in the tolerance of pancreatic cancer cells to nutrient starvation. In this study we investigated the possibility that PKB/Akt is also involved in the mechanisms of tolerance. PKB/Akt was found to be phosphorylated strongly during glucose starvation, both under normoxic and hypoxic conditions, and the PKB/Akt phosphorylation was inhibited by the PI3-kinase inhibitor wortmannin but not by the mTOR inhibitor rapamycin or the AMPK inhibitor adenosine arabinoside (Fig. 6a). These findings strongly suggest that PKB/Akt might be involved in the sensing mechanisms in a PI3-kinase-dependent fashion. Based on the above findings, antisense expression vectors for Akt1 and Akt2 were transfected into HepG2 cells, and as expected, induction of tolerance to glucose starvation was markedly inhibited in the HepG2 cells transfected with the Akt1 antisense expression vector but not in the cells transfected with Akt2 antisense expression vector (Fig. 6b). The amount of total Akt protein expression in both transfectants and the parental HepG2 cells was examined by Western blot analysis, but the amounts had not changed significantly (Fig. 6c). When wild-type sense expression vectors for Akt1 and the constitutive active fragment of AMPK a1 were transfected into HepG2 cells, cell survival during normoxia and glucose starvation was significantly higher, but the effects were only minimal and transient (data not shown).

DISCUSSION

The present work clearly showed that exposure to NO and/or hypoxia clearly conferred tolerance to glucose starvation in both normal and cancer cell lines. This result is paradoxical because it has long been believed that glycolysis is the main mechanism for energy production when oxygen consumption is restricted. Restriction of oxygen consumption can also be achieved with cyanide, which strongly inhibits cytochrome c oxidase. However, cyanide did not confer any protective effect on cell survival during glucose starvation. These findings strongly suggest that hypoxia and/or NO might induce a previously known biological response to an insufficient oxygen and nutrient supply (famine response).

HIF-1 is a key molecule in the hypoxia response, and both hypoxia and NO activate HIF-1 (18). It therefore seemed reasonable to hypothesize that HIF-1 is also a key molecule in the induction of tolerance. However, HIF-1 activation is insufficient to induce tolerance, because CoCl2 and desferrioxamine clearly activated HIF-1 but did not induce tolerance to glucose starvation, and it was not activated effectively during glucose starvation. By contrast, the AMPK activator 5-aminimidazole-4-carboxamide-1-beta-D-ribofuranoside strongly induced tolerance to glucose starvation, but it did not activate HIF-1 at all (20). Moreover, whereas methylene blue and LY83583 inhibited HIF-1 activation by NO, but not by hypoxia, they inhibited the induction of tolerance to glucose starvation by both stimuli. These findings support the notion that induction of tolerance is independent of HIF-1 activation, although its dependence on HIF-1 activation cannot be completely ruled out until definitive results are obtained, such as in an experiment using HIF-1 knockout cells.

A transforming growth factor beta-like signaling pathway through PI3-kinase and PKB/Akt regulates Dauer larva formation in nematodes (21). Dauer larva formation is an adaptive response to harsh conditions that is achieved by changing both the energy metabolism of the organism and the cell cycle program (21, 22). The PI3-kinase-PKB/Akt pathway is also a well-known tumor survival pathway (29). When activation of PKB/Akt was examined, it was found to be activated by glucose starvation, and similar activation was found when amino acids were withdrawn (16). These findings suggest that the PKB/Akt pathway might be involved in the mechanism that senses the state of nutrient availability. Although wortmannin, an inhibitor of PI3-kinase, had no effect on cell survival during glucose starvation, the antisense RNA expression vector for Akt1 partially but significantly inhibited tolerance induction, suggesting that Akt may modulate the induction of tolerance of Akt partial instead of being involved in it directly. The reason the total amount of Akt did not change significantly after transfecting antisense RNA expression vectors remains unknown, but it may be due to the inability to differentiate two isoforms, as shown by Western blot. Interaction between the regulation of Akt1 and Akt2 is another possibility.

The results of this study clearly show that AMPK is involved in the induction of tolerance to glucose starvation, but how it is involved is not yet clear. Because AMPK is activated by various metabolic stresses, the AMPK pathway may be the primary

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sensor of hypoxia and glucose starvation and mediate subsequent responses (24). Actually, the AMPK activator 5-aminomidazole-4-carboxamide-1-beta-D-ribofuranoside has been found to strongly induce tolerance to glucose starvation in an AMPK alpha2-dependent manner (20). There was an obvious contradiction. When AMPK activity was examined with GST-SAMS fusion protein as a substrate, it was found to be decreased in HepG2/a1A cells but not in HepG2/a2A cells, although the amount of AMPK alpha subunit protein was decreased in each of the respective antisense-transfected cells. At present, there is no method for differentially determining the enzymatic activity of each isozyme composed of the alpha and alpha2 and beta and gamma isoforms, and this limitation might be the source of the apparent contradiction. There may also be interactions between alpha1 and alpha2 expression under various stress conditions. A new member of the AMPK family, SNARK, that has kinase activity against SAMS peptide has recently been identified, and its activity may be another reason for the apparent contradiction (25). It is also not known whether there is interaction or cross-talk between the Akt and AMPK pathways, but both share targets, including glucose synthetase, glucose transporter, and amino acid transporters (6). In the present study we tested the possibility that AMPK might be responsible for the phosphorylation of Akt with an AMPK inhibitor, adenosine arabinoside, but it had no effect on Akt phosphorylation, suggesting that AMPK is not upstream of Akt activation during nutrient starvation.

The present study showed that the tolerance to glucose starvation was dependent on amino acids. Some tumors are known to utilize glutamine for energy production better than glucose (8), and this seems to be consistent with our findings. However, because the biochemical mechanisms for energy production from amino acids were analyzed mostly during normoxia, how energy is produced from amino acids under hypoxia remains to be elucidated. Aspartic acid was reported recently (26) to be protective of renal tubules subjected to ischemia, and the biochemical mechanisms for this effect was suggested to be anaerobic respiration in which fumarate is reduced to succinate. Our own findings may be related to this observation, but further biochemical investigation is needed. Another important question involves the source of the amino acids when the blood supply is restricted in vivo. Autophagy is one of the best known biological responses to starvation, and many organisms survive nutrient starvation by degrading their own constituents (27). We recently found that exposing cells to NO or nutrient starvation up-regulates some matrix metalloproteinases. The notion that degradation of the extracellular matrix supplies amino acids to cancer cells for energy production is an interesting hypothesis.

The present findings are still preliminary, but they are quite important from both a biological and medical viewpoint. Once the mechanisms for induction of tolerance (austerity) are elucidated, they will open a new field of research in regard to the prevention and treatment of myocardial infarction, stroke, and cancer. Tolerance to nutrient starvation might be part of the biological response to an insufficient blood supply, and in the future it might be possible to include it in the so-called hypoxia response, but to avoid confusion it might be wise to call it a “famine (extreme poverty) response” for the time being. It is also possible that the tolerance to nutrient starvation is not

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reduced to glucose and that the tolerance can be referred to as “austerity,” which might cover energy production, energy consumption, differentiation, apoptosis, and cell proliferation.

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