Phosphoinositide 3-kinase accelerates necrotic cell death during hypoxia

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Using H9c2 cells derived from rat cardiomyocytes, we investigated the mechanism of cell death during hypoxia in the presence of serum and glucose. Hypoxic cell death is by necrosis and is accompanied by metabolic acidosis. Moreover, hypoxic cell death is inhibited by Hepes buffer as well as by 2-deoxyglucose, an inhibitor of glycolysis, indicating that metabolic acidosis should play an essential role in hypoxic injury. The involvement of phosphoinositide 3-kinase (PI 3-kinase), which is known to activate glucose metabolism, was examined using its inhibitor, LY290042, or adenovirus-mediated gene transfer. Hypoxic cell death was inhibited by LY294002 in a dose-dependent manner. Overexpression of dominant negative PI 3-kinase was found to reduce cell death, whereas wild-type PI 3-kinase enhanced it.

Dominant negative PI 3-kinase also reduced glucose consumption and acidosis, but this was stimulated by wild-type PI 3-kinase. The data indicate that PI 3-kinase stimulates cell death by enhancing metabolic acidosis. LY294002 significantly reduced glucose uptake, showing that PI 3-kinase regulates glycolysis at the step of glucose transport. These findings indicate the pivotal role of glucose metabolism in hypoxic cell death, and reveal a novel death-promoting effect of PI 3-kinase during hypoxia, despite this enzyme being considered to be a survival-promoting factor.

Key words: acidosis, glycolysis, necrosis.

INTRODUCTION

Investigations of the pathway responsible for cell death during hypoxia have attracted much concern because this pathway plays crucial roles in many diseases, including cardiac infarction, asthma and tumorigenicity [1]. It has been demonstrated that many signalling molecules involved in the decision between cell survival and death are activated or inactivated during hypoxia. For example, an increase in Fas antigen, and its involvement in apoptosis, have been reported in hypoxic cardiomyocytes [2]. Hypoxia also upregulates p53, an inducer of apoptosis, in cardiomyocytes [3]. A decrease in hypoxia-induced apoptosis was observed in the hypoxia-inducible factor-1 α knockout mouse, indicating the importance of the induction of hypoxia-inducible factor-1 α for protection against hypoxic injury [4]. A protective effect of bcl-2 on hypoxic death was reported by Shimizu et al. [5] and Jacobson et al. [6] in PC12 cells. Although many studies have shown the importance of these signalling molecules in hypoxic cell death, the effect of metabolism on hypoxic injury remains largely unknown. One well-known effect of hypoxia on metabolism is the 'Pasteur effect', which is characterized by increased glucose consumption, so that cells maintain their intracellular ATP levels under reduced oxygen concentrations. A reduction in oxygen concentration also inhibits the oxidation of pyruvate in the citric acid cycle; therefore pyruvate should be converted into lactate by lactate dehydrogenase [7].

Other than hypoxia, insulin is known to increase glucose metabolism through phosphoinositide 3-kinase (PI 3-kinase) [8]. PI 3-kinase, a heterodimeric enzyme consisting of a p85 regulatory subunit and a p110 catalytic subunit, phosphorylates phosphoinositides at the D-3 position [9]. The phospholipid product of PI 3-kinase, in turn, activates various downstream effectors,

including Akt/protein kinase B [10] and protein kinase C ζ [11]. The most important role of PI 3-kinase in insulin signalling is mediation of the translocation of the facilitative glucose transporter from cytoplasmic vesicle pools to the plasma membrane, thereby enhancing glucose transport activity [12]. PI 3-kinase is stimulated not only by insulin, but also by platelet-derived growth factor [13] and epidermal growth factor [14], suggesting that PI 3-kinase is involved in the signalling of various growth factors. PI 3-kinase is also known to have an anti-apoptotic effect. For example, the apoptotic death of PC12 cells due to withdrawal of nerve growth factor is inhibited by the activation of PI 3-kinase [15] or Akt [16], indicating that the PI 3-kinase/Akt pathway is a critical mediator of a survival signal by a growth factor. Although many studies support the anti-apoptotic effect of PI 3-kinase, its effect on necrosis has not been evaluated.

In the present study, we examined the mechanism of cell death in H9c2 cells exposed to hypoxia in the presence of serum and glucose, and found that hypoxia stimulates glucose metabolism, leading to necrotic cell death. Furthermore, we show a novel aspect of the relationship between PI 3-kinase and cell death through glucose metabolism.

MATERIALS AND METHODS

Materials

Cycloheximide and 2-deoxyglucose were purchased from Wako Pure Chemicals (Osaka, Japan). Benzyloxycarbonyl-Val-Ala-D,L-Asp-fluoromethylketone (Z-VAD-FMK), an antibody against poly(ADP-ribose) polymerase (PARP) p85 fragment and peroxidase-conjugated anti-rabbit IgG antibody were obtained from

Abbreviations used: PI 3-kinase, phosphoinositide 3-kinase; PARP, poly(ADP-ribose) polymerase; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-D,L-Asp-fluoromethylketone.

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Figure 1 Effect of Z-VAD-FMK on viability during hypoxia or cycloheximide treatment (A), Western-blot analysis of the cleavage of PARP, caspase 3 and lamin B during hypoxia or cycloheximide treatment (B–D), and morphological analysis of hypoxic cell death by electron microscopy (E)

(A) H9c2 cells were preincubated with or without 50 μ M Z-VAD-FMK, then exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 48 h. Cycloheximide (CHX) was added at a concentration of 25 μ g/ml and the cells were incubated for 48 h under normoxic conditions. Cell viability was measured as described in the Materials and methods section. Data represent the means $\pm$ S.D. of three samples. Similar results were obtained in another experiment. (B–D) Cell extracts (40 μ g of protein) were prepared from H9c2 cells subjected to either normoxia or hypoxia, or treated with 25 μ g/ml CHX for 48 h. The Figures show representative immunoblots obtained in three separate experiments. (E) Ultrastructure of H9c2 cells after 48 h of normoxia or hypoxia. Magnification, $\times 1040$.

Promega, peroxidase-conjugated anti-goat IgG antibody was obtained from Sigma, and LY294002 was obtained from Calbiochem. Anti-caspase 3 and anti-lamin B antibodies and Protein A/G-agarose were obtained from Santa Cruz Biotechnology. Anti-phosphotyrosine (PY20) antibody and an antibody against the p85 subunit of PI 3-kinase were from Transduction Laboratories (Kentucky, CA, U.S.A.). Anti-phospho-Akt (serine-473) and anti-Akt antibodies were from New England Biolabs (Beverly, MA, U.S.A.). All other reagents were commercially available.

Cell culture and hypoxia

Cells of the embryonic rat heart cardiomyocyte-derived cell line H9c2 were grown to confluence (approx. 1.5×10^5 /3.5 cm diameter dish) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were then subjected to hypoxia ($O_2/N_2/CO_2$, 1:94:5) for the indicated time periods. The viability of the cells was determined by dye-exclusion assay using erythrosin B.

Electron microscopy

Culture cells on plastic dishes were fixed with a 2% glutaraldehyde/2% paraformaldehyde mixture in 0.1 M cacodylate buffer (pH 7.4) for 30 min after 2% osmic acid for 1 h. The cells were dehydrated with graded alcohol and embedded in Epon 812 resin. Ultrathin sections were cut on an ultramicrotome (Reichert–Jung, Vienna, Austria) using a diamond knife (Diatome, Biel, Switzerland). The sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEOL200CX; Jeol, Tokyo, Japan).

Adenovirus-mediated gene transfer

The construction of recombinant adenoviruses encoding either the p110 cDNA (wild-type p110) or truncated p85 cDNA (Δ p85) of PI 3-kinase was described previously [17,18]. Recombinant adenoviruses were applied to cells at a multiplicity of infection of 20–30 plaque-forming units/cell. At 1 day after infection, the cells were subjected to hypoxia.

Measurement of glucose and lactate

Glucose and lactate concentrations in the medium were determined by the glucose B test (Wako Pure Chemicals) and the lactate assay kit (Sigma) respectively.

Western-blot and immunoprecipitation analysis

Cells were lysed with lysis buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 mM 2-mercaptoethanol, 150 nM pepstatin, 20 μ M leupeptin and 1 mM Na_3VO_4). Protein concentrations of the cell extract were determined using a Bio-Rad protein assay kit. The cellular extracts were subjected to SDS/PAGE (7.5% gels for p85 fragment of PARP, 10% gels for p85 PI 3-kinase and Akt, and 12.5% gels for caspase 3 and lamin B) by the method of Laemmli [19], and transferred on to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech) by the method of Towbin et al. [20]. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20, then incubated with the antibodies. The bands were visualized by an ECL[®] detection system (Amersham Pharmacia Biotech). For immunoprecipitation of tyrosine-phosphorylated proteins, cells were washed with PBS and lysed in immunoprecipitation buffer

Figure 2 Time course of glucose concentration remaining and pH of the medium during hypoxia or normoxia

The glucose concentration remaining (**B**) and pH (**A**) of the medium were determined as described in the Materials and methods section in cells subjected to hypoxia or normoxia for the indicated time periods. Data represent the means $\pm$ S.D. of three samples. Similar results were obtained in another experiment.

(20 mM Tris/HCl, pH 7.4, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Nonidet P40, 5% glycerol, 1 mM PMSF and 1 mM Na_3VO_4). Equal amounts of proteins were incubated with anti-phosphotyrosine antibody and Protein A/G-agarose. Immunocomplexes were washed three times in immunoprecipitation buffer, then subjected to immunoblotting.

Measurement of glucose uptake

Glucose uptake was measured as described previously [21]. Briefly, cells were glucose-starved for 30 min in Krebs–Ringer phosphate buffer (10 mM sodium phosphate, pH 7.4, 137 mM NaCl, 4.7 mM KCl, 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$), and then incubated with 0.1 mM 2-deoxy-D- $[^3H]$ glucose (Amersham Pharmacia Biotech) for 15 min. Incorporated radioactivity in the cells was measured with a liquid-scintillation counter (Aloka, Tokyo, Japan).

RESULTS

Hypoxic cell death in the presence of glucose is necrosis

We first examined whether hypoxic cell death is by necrosis or apoptosis. Apoptosis is characterized by the sequential activation of a number of cysteine proteases called caspases [22]. Apoptosis is inhibited by Z-VAD-FMK, a broad-spectrum inhibitor of caspases. Although cell death induced by cycloheximide, an inhibitor of protein synthesis known to induce apoptosis, was inhibited by 50 μ M Z-VAD-FMK, hypoxic cell death was not affected (Figure 1A). Immunoblotting experiments showed that both the proteolysis of PARP, a target of caspase 3 [23], and the disappearance of caspase 3, due to proteolysis of its pro-form, were induced by cycloheximide, but not during hypoxia (Figures 1B and 1C). These results suggest that hypoxia induces cell death without PARP or caspase 3 cleavage, which is typically associated with apoptosis. We next examined the proteolysis of lamin B, a nuclear cytoskeletal protein known to be degraded by caspase 6, since integrity of the nuclear membrane is maintained in necrosis

Figure 3 Effects of 2-deoxyglucose or Hepes (**A–C**), LY294002 (**D**), and the overexpression of $\Delta p85$ or wild-type p110 (**E–G**) on cell death, glucose concentration and pH of the medium during hypoxia

(**A–C**) H9c2 cells were preincubated with or without 5.5 mM 2-deoxyglucose (2-DG) or 25 mM Hepes, then subjected to normoxia or hypoxia for 48 h for measurement of viability (**A**) or for 24 h for measurement of pH (**B**) and glucose (**C**). The glucose concentration remaining and pH of the medium were determined as described in the Materials and methods section. Data represent the means $\pm$ S.D. of three samples (* $P < 0.05$ versus 'none'). Similar results were obtained in five additional experiments. (**D**) The viability of cells subjected to hypoxia or normoxia for 48 h in the presence or absence of the indicated concentrations of LY294002 was determined by dye-exclusion assay. Data represent the means $\pm$ S.D. of three samples. Similar results were obtained in four additional experiments. (**E–G**) H9c2 cells were preinfected with or without adenoviruses that express $\Delta p85$ (PI 3K-DN) or wild-type p110 (PI 3K-WT) for 24 h, then subjected to hypoxia for 36 h (**E**; for measurement of viability) or 24 h (**F**, **G**; for measurement of glucose and pH). The glucose concentration remaining and pH of the medium were determined as described in the Materials and methods section. Data represent the means $\pm$ S.D. of three samples. Similar results were obtained in five additional experiments.

Figure 4 Hypoxia-induced changes in H9c2 cell morphology

Shown are phase-contrast views ($\times 400$) of H9c2 cells incubated under normoxia (a) or hypoxia (b–f). Abbreviations are defined in the legend to Figure 3.

but lost in apoptosis [24]. Lamin B was not degraded during hypoxia, but was degraded during treatment with cycloheximide (Figure 1D). The loss of viability without degradation of nuclear cytoskeletal proteins further supports necrosis. To confirm that this hypoxic cell death is necrosis, we used electron microscopy to analyse the morphological features of H9c2 cells incubated under normoxic or hypoxic conditions. Ultrastructural analysis revealed that hypoxic cell death is characterized by degenerating chromatin with large cytosolic vacuoles and disrupted membrane integrity (Figure 1E), characteristics of necrosis [25–27].

Metabolic acidosis causes cell death

We next examined the pH of the medium during hypoxia because acidosis might play a crucial role in hypoxic cell death [28]. After 48 h of hypoxia, the pH dropped to approx. 6.3, whereas it was approx. 6.8 after 48 h of normoxia, indicating that hypoxia accelerates acidosis (Figure 2A). Since it has been demonstrated that increased glycolysis occurs in oxygen-starved cells [7], we examined whether glucose consumption is increased during hypoxia by measuring the glucose concentration remaining in the medium. An approx. 2-fold increase in the rate of glucose

consumption was observed during hypoxia, as compared with normoxia (Figure 2B). The close correlation between glucose consumption and acidosis strongly suggests that hypoxia causes metabolic acidosis. To evaluate the relationship between metabolic acidosis and cell death, we examined the effect of 2-deoxyglucose, an inhibitor of hexokinase, and Hepes buffer. The addition of 5.5 mM 2-deoxyglucose to the medium inhibited cell death as well as glucose consumption and acidosis (Figures 3A–3C and Figure 4). The addition of 25 mM Hepes to the medium also reduced cell death by inhibiting acidosis, whereas glucose consumption was somewhat stimulated, indicating that Hepes does not inhibit glycolysis but inhibits the subsequent acidosis (Figures 3A–3C and Figure 4). The effects of 2-deoxyglucose and Hepes were relatively small, although we observed the same tendency in more than five experiments (results not shown). These results strongly indicate that acidosis is a result of enhanced glycolysis and decreased oxidative phosphorylation during hypoxia, and seems to be a critical event in hypoxic cell death.

PI 3-kinase promotes hypoxic cell death

Given the evidence that PI 3-kinase is an essential regulator of glucose metabolism [8], we examined the involvement of PI 3-kinase in hypoxic cell death. We examined the effect of LY294002, an inhibitor of PI 3-kinase [29], on cell death during 48 h of hypoxia. LY294002 inhibited hypoxic cell death at a concentration of 10 μ M, which is close to the minimum concentration required for blocking PI 3-kinase activity completely (Figure 3D). To confirm the involvement of PI 3-kinase, we next examined the effect of the overexpression of Δ p85, a truncated form of the regulatory subunit of PI 3-kinase, on cell death during 36 h of hypoxia using adenovirus-mediated gene transfer. Δ p85 lacks the binding site for the catalytic subunit (p110) of PI 3-kinase and cannot mediate the upstream signalling of PI 3-kinase activation. Overexpression of Δ p85 also reduced hypoxic cell death (Figure 3E and Figure 4), confirming the effect of PI 3-kinase inhibitors. Δ p85 also inhibited glucose consumption and acidosis (Figures 3F and 3G). In contrast, overexpression of wild-type p110, which possesses constitutively active PI 3-kinase activity, enhanced cell death, glucose consumption and acidosis (Figures 3F–3G and Figure 4). These results clearly indicate that PI 3-kinase accelerates cell death during hypoxia by enhancing glucose metabolism and subsequent acidosis.

Presence of activated PI 3-kinase and its involvement in serum-stimulated glucose uptake

The presence of activated PI 3-kinase was investigated after immunoprecipitation of whole cell lysates with anti-phosphotyrosine antibodies and immunoblot analysis with an antibody against the p85 subunit of PI 3-kinase. In anti-phosphotyrosine immunoprecipitates, immunoreactive p85 subunit of PI 3-kinase was observed and slightly upregulated during hypoxia, although the amounts of immunoreactive p85 in total cellular lysates were unchanged (Figures 5A and 5B). We next examined the presence of activated Akt, a downstream effector of PI 3-kinase. Serine-473-phosphorylated Akt was also observed and upregulated during hypoxia, although total levels of Akt were unchanged during hypoxia (Figure 5C). These results indicate the presence of activated PI 3-kinase and its upregulation during hypoxia. We next examined the mechanism by which PI 3-kinase activates glucose consumption. Since the regulation of glucose

Figure 5 Effect of hypoxia on phosphorylation of PI 3-kinase and its downstream effector, Akt

Anti-phosphotyrosine immunoprecipitates (**A**; αPY) from whole cell lysates or total cellular proteins (**B** and **C**) were electrophoresed, transferred and immunoblotted with the indicated antibodies.

transport is a major function of PI 3-kinase [12], we measured glucose transport activity. Although hypoxia activates glucose uptake by approx. 2-fold, glucose uptake was inhibited by LY294002 in both hypoxic and normoxic cells in the presence of serum (Figure 6A). In contrast, in the absence of serum, the inhibition of PI 3-kinase by LY294002 caused a very slight inhibition of glucose uptake in both hypoxic and normoxic cells (Figure 6A), indicating that PI 3-kinase is involved in the stimulation of glucose uptake by serum, but not by hypoxia. These results are consistent with previous reports [30,31] showing that PI 3-kinase is not involved in the activation of glucose uptake by mitochondrial uncoupling or stress. The most likely candidate that causes metabolic acidosis during hypoxia is lactate. Therefore we measured the lactate levels in the medium. Although, even under normoxic conditions, lactate was accumulated in the medium (Figure 6B), suggesting that H9c2 cells may be highly glycolytic, hypoxia enhanced the accumulation of lactate. Overexpression of Δp85 significantly reduced lactate accumulation, whereas wild-type p110 stimulated it. The addition of 2-deoxyglucose also reduced lactate accumulation, but Hepes did not (Figure 6B), indicating that Hepes does not reduce lactate accumulation but prevents subsequent acidosis. Moreover, the exposure of normoxic cells to lactate could inhibit survival (Figure 6C). These results indicate that the cytotoxic effect of metabolic acidosis is mediated, at least in part, by lactate accumulation through the glycolytic pathway downstream of PI 3-kinase.

DISCUSSION

The present study shows that, in the presence of glucose in the medium, hypoxia induces necrosis of cardiomyocyte-derived H9c2 cells through glycolysis-induced acidosis. Both apoptosis and necrosis have been observed in the ischaemic heart [32],

Figure 6 2-Deoxyglucose uptake (**A**), lactate accumulation in the medium (**B**) during hypoxia, and effect of lactate on viability during normoxia (**C**)

(**A**) The uptake of 2-deoxyglucose after incubation for 24 h under normoxic or hypoxic conditions in the presence or absence of serum was assayed as described in the Materials and methods section. Serum starvation was performed for 48 h prior to subjecting the cells to hypoxia (H) or normoxia (N). Values represent the means $\pm$ S.D. of four samples. (**B**) H9c2 cells were preinfected with adenoviruses that express Δp85 (PI 3K-DN) or wild-type p110 (PI 3K-WT) for 24 h, or preincubated with 5.5 mM 2-deoxyglucose (2-DG) or 25 mM Hepes, then subjected to hypoxia for 24 h. Lactate concentration in the medium was determined as described in the Materials and methods section. Data represent the means $\pm$ S.D. of three samples (* P < 0.05 versus 'none'). Similar results were obtained in another experiment. (**C**) The indicated concentrations of lactate were added to H9c2 cells and the cells were incubated for 48 h under normoxic conditions. Cell viability was measured as described in the Materials and methods section. Data represent the means $\pm$ S.D. of three samples.

although other reports [33] indicate that apoptosis is predominant after reperfusion, whereas prolonged ischaemia causes necrotic cell death. Hypoxia is a milder stress than ischaemia because nutrients such as glucose allow cells to produce energy, even under conditions where oxidative phosphorylation is inhibited. In fact, Malhotra and Brosius [34] have demonstrated that hypoxia-

induced apoptosis of isolated cardiomyocytes is blocked by the addition of glucose to the medium, suggesting that glucose metabolism inhibits the apoptotic process. In contrast, it has been reported [35] that the injury due to cerebral ischaemia is reduced by dietary restriction or by 2-deoxyglucose, indicating the cytotoxic effect of glucose metabolism. Consistent with the latter findings, we demonstrated that metabolic acidosis through glycolysis induces necrosis during hypoxia. Taken together, it is likely that glucose metabolism inhibits hypoxia-induced apoptosis, but instead stimulates necrosis by inducing metabolic acidosis.

A major finding of the present study is that PI-3 kinase promotes cell death, in contrast with the well-known inhibitory effect of PI 3-kinase on cell death [36]. For example, the apoptotic cell death of cardiomyocytes upon hypoxia is blocked by the insulin-like growth factor-I-activated PI 3-kinase/Akt pathway [37]. Moreover, apoptosis of the ischaemic/reperfused heart is inhibited by insulin-like growth factor-I [38]. The discrepancy can be explained by the fact that these studies were carried out under glucose-deficient conditions. In contrast, we found that PI 3-kinase has a death-promoting effect on hypoxic cell death in the presence of glucose. This condition of hypoxia without glucose deprivation is representative of respiratory failure due to pulmonary diseases without coronary diseases, rather than of ischaemic diseases. It is likely that under these conditions, PI 3-kinase promotes necrosis rather than suppressing apoptosis. Schmaltz et al. [28] demonstrated the crucial role of acidosis in hypoxic cell death of rat embryonic fibroblasts. They observed that the addition of Hepes to the medium inhibited hypoxic cell death [28], which is consistent with the results in the present study. Sauvage et al. [39] have reported that PI 3-kinase stimulates the Na^+/H^+ exchanger, which is considered to be the central regulator of pH homeostasis. It is possible, therefore, that PI 3-kinase synergistically promotes acidosis by stimulating metabolic acidosis and activating the Na^+/H^+ exchanger. In accordance with this idea, we observed that amiloride, an inhibitor of the Na^+/H^+ exchanger, can inhibit hypoxic cell death (T. Aki, K. Yoshida and T. Fujimiya, unpublished work). We showed that up-regulation of activated PI 3-kinase during hypoxia is not involved in hypoxia-induced glucose uptake (Figure 6A), but it might be involved in another process including Na^+/H^+ exchange.

In conclusion, we have demonstrated that metabolic acidosis plays a central role in hypoxic cell death in the presence of glucose. PI 3-kinase enhances metabolic acidosis and subsequent cell death by activating glucose uptake and glycolysis. The activation of glucose uptake is a well-known function of PI 3-kinase, whereas the stimulation of cell death has not previously been attributed to PI 3-kinase. The present study provides new insights into the role of PI 3-kinase in cell death.

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