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Phosphoinositide 3-Kinase Accelerates Calpain-Dependent Proteolysis of Fodrin during Hypoxic Cell Death[1]

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We have shown recently that phosphoinositide 3-kinase (PI 3-kinase) accelerates the hypoxia-induced necrotic cell death of H9c2, derived from rat cardiomyocytes, by enhancing metabolic acidosis. Here we show the downstream events of acidosis that cause hypoxic cell death. Hypoxia induces the proteolysis of fodrin, a substrate of calpain. Intracellular Ca^{2+} chelation by BAPTA, and the addition of SJA6017, a specific peptide inhibitor of calpain, also reduces cell death and fodrin proteolysis, indicating that Ca^{2+} influx and calpain activation might be involved in these events. The overexpression of wild type PI 3-kinase accelerates fodrin proteolysis, while dominant-negative PI 3-kinase reduces it. Both (*N*-ethyl-*N*-isopropyl)amiloride (EIPA), an inhibitor of the Na^+/H^+ exchanger, and KB-R7943, an inhibitor of the Na^+/Ca^{2+} exchanger, reduce hypoxic cell death and fodrin proteolysis. The depletion of intracellular Ca^{2+} stores by thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, also reduces cell death and fodrin proteolysis, indicating that Ca^{2+} release from intracellular Ca^{2+} stores might be also involved. These results indicate that PI 3-kinase might accelerate hypoxic cell death by enhancing the calpain-dependent proteolysis of fodrin.

Key words: calpain, fodrin, H9c2, hypoxia, PI 3-kinase.

The development of acidosis during hypoxia/reoxygenation or ischemia/reperfusion is thought to play essential roles in cell injury (*1*). There are several ion exchangers, including a Na^+/H^+ exchanger (*2*) and Na^+-HCO_3^- cotransporter (*3*), that are involved in recovery from acidosis. The Na^+/H^+ exchanger is the primary exchanger by which protons leave cells during ischemia/reperfusion (*4, 5*). The Na^+/H^+ exchanger is an integral membrane protein expressed ubiquitously in mammalian tissues (*6*). Proton extrusion on the Na^+/H^+ exchanger results in Na^+ influx, which in turn leads to Ca^{2+} influx through the Na^+/Ca^{2+} exchanger (*7–9*). These reports indicate that the Na^+/H^+ exchanger and the Na^+/Ca^{2+} ex-changer synergistically induce the influx of extracellular Ca^{2+} in response to acidosis. Endoplasmic reticulum (ER) Ca^{2+}-ATPase is also involved in Ca^{2+} influx during hypoxia. Walters *et al.* showed that pretreatment of cells with thapsigargin, a selective inhibitor of ER Ca^{2+}-ATPase (*10, 11*), protects against hypoxia-induced cell death (*12*).

Many lines of evidence show that Ca^{2+} influx during ischemia/reperfusion or hypoxia/reoxygenation induces cell injuries through the activation of Ca^{2+}-dependent neutral proteases, calpains (*13, 14*). Two major types of calpains, which require millimolar and micromolar concentrations of Ca^{2+} for their activation, respectively, are expressed ubiquitously in mammalian tissues. Calpain has been shown to be involved in the breakdown of many proteins (*13, 14*). Fodrin, a membrane-cytoskeletal protein that forms a two-dimensional meshwork beneath the plasma membrane, is the most well-known substrate for calpain. We have shown the importance of the calpain-dependent proteolysis of fodrin in injury resulting from ischemia or reperfusion in the heart (*15–17*), brain (*18*), and testis (*19*).

Phosphoinositide 3-kinase (PI 3-kinase) is a lipid kinase that phosphorylates phosphoinositides at the D-3 position and mediates various cellular functions including glucose transport, vesicular transport, and cell survival (*20, 21*). PI 3-kinase inhibits the apoptotic cell death of cardiomyocytes during hypoxia (*22*). In contrast, we found that PI 3-kinase stimulates necrotic cell death in a cardiomyocyte-derived cell line, H9c2, in the presence of glucose during hypoxia through the activation of glucose transport and glycolysis (*23*). The latter finding prompted us to investigate whether the Na^+/H^+ and Na^+/Ca^{2+} exchangers, ER Ca^{2+}-ATPase, and calpain are involved in hypoxic cell death downstream of PI 3-kinase.

In this study we demonstrate that acidosis-induced Ca^{2+} influx, which might be mediated by the Na^+/H^+ and Na^+/Ca^{2+} exchangers, might cause calpain activation and fodrin proteolysis. Ca^{2+} influx from intracellular Ca^{2+} stores through ER Ca^{2+}-ATPase might be also involved in fodrin proteolysis. PI 3-kinase stimulates the calpain-dependent proteolysis of fodrin and subsequent cell death.

MATERIALS AND METHODS

Materials—(*N*-ethyl-*N*-isopropyl)amiloride was obtained

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Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; EIPA, (*N*-ethyl-*N*-isopropyl)amiloride; ER, endoplasmic reticulum.

from Molecular Probes (Eugene, OR, USA). Anti-m- and μ-calpain antibodies were kindly provided by Dr. M. Kunimatsu (Nagoya City University of Medicine, Nagoya). Anti-α fodrin antibody was obtained from Locus Genex (Helsink, Finland). Anti-phospho-Akt (serine-473) and anti-Akt antibodies were from New England Biolabs (Beverly, MA, USA). 2-Deoxy glucose was purchased from Wako Pure Chemicals (Osaka). Peroxidase-conjugated anti-rabbit and mouse IgG antibodies were obtained from Promega (Madison, WI, USA). LY294002, thapsigargin, BAPTA-AM, and purified μ-calpain (porcine erythrocyte) were obtained from Calbiochem (San Diego, CA, USA). Purified m-calpain (rabbit skeletal muscle) was obtained from Sigma (St. Louis, MO, USA). KB-R7943 was obtained from Tocris (Ellisville, MO, USA). SJA6017 was kindly provided by Senjyu Pharmaceuticals (Kobe). All other reagents were commercially available.

Cell Culture and Hypoxia—Embryonic rat heart cardiomyocyte–derived cells, H9c2, were plated at a density of approx. 1.5×10^5/3.5 cm diameter dish and grown to confluence in DMEM (containing 4.5 g/liter glucose and no HEPES buffer) supplemented with 10% fetal bovine serum. Then, the cells were subjected to hypoxia ($O_2/N_2/CO_2$, 1:94:5) for the indicated times. Cell viability was determined by dye exclusion assay using erythrosin B.

Adenovirus-Mediated Gene Transfer—The construction of recombinant adenoviruses (kindly provided by Dr. Y. Oka, Tohoku University, Graduate School of Medicine) encoding either the wild type p110 catalytic subunit cDNA (PI 3-K-WT) or the truncated p85 regulatory subunit cDNA (PI 3-K-DN) of PI 3-kinase, were described previously (*24, 25*). Recombinant adenoviruses were applied to cells at a multiplicity of infection of 20–30 plaque-forming units/cell. One day after infection, cells were subjected to hypoxia.

Western Blot Analysis—The cells were lysed in 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-mercaptoetanol, 150 nM pepstatin, 20 μM leupeptin). Protein concentrations of the cell extracts were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The cellular extracts were subjected to SDS–polyacrylamide gel electrophoresis (6.5% gels for fodrin, 7.5% gels for m- and μ-calpains, and 12.5% gels for Akt) by the method of Laemmli (*26*) and transferred to nitrocellulose membranes (Hybond ECL, Amersham-Pharmacia Biotech, Buckinghamshire) by the method of Towbin *et al.* (*27*). 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). Band intensities were quantified using an image analyzer (Densitograph AE-6900M, Atto, Tokyo).

In Vitro Proteolysis of Fodrin by Calpain—Cell lysates were incubated with either μ-calpain or m-calpain in reaction buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1 mM, EDTA, 10 mM 2-mercaptoethanol, 3 mM $CaCl_2$) at 30°C for 0, 3, 10, 30, or 90 min.

RESULTS

Viability, Proteolysis of Fodrin, Induction of μ- and m-Calpains, and Phosphorylation of Akt during Hypoxia—We first examined the viabilities of cells during hypoxia. Hypoxia reduced viability in a time-dependent manner (Fig. 1A). We next examined the proteolysis of fodrin by immunoblotting. Proteolytic 145/150 kDa fragments of fodrin appeared during hypoxia, although a concomitant loss of intact fodrin was not observed (Fig. 1B). This could be because cell lysates from dead cells might be membrane-rich fractions, in which fodrin resides, because the cytoplasmic contents were released to the medium. Alternatively, the whole amount of fodrin might be up-regulated during hypoxia. Since the 145/150 kDa fragments of fodrin are known to be generated by calpain-dependent proteolysis (*13, 14*), we examined whether calpain is activated during hypoxia. As calpain is activated by limited-autolysis (*13, 14*), we measured the amount of the inactive pro-form during hypoxia. Both pro-m-calpain and pro-μ-calpain were up-regulated during hypoxia, with a peak at 24 h, followed by down-regulation up to 48 h of hypoxia (Fig. 1, C and D). These data show that hypoxia up-regulates and activates

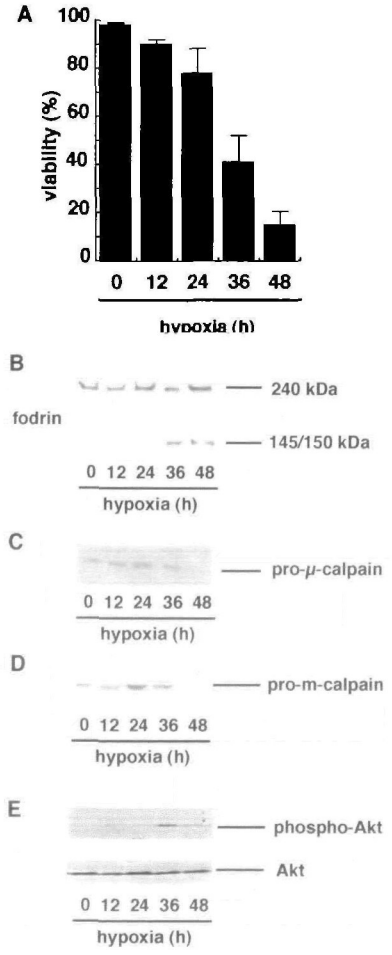

Fig. 1. **Time course of viability, fodrin proteolysis, calpain induction, and Akt phosphorylation during hypoxia.** (A) Cell viability during hypoxia was determined as described in "MATERIALS AND METHODS." Data represent the means and SD of four samples. Similar results were obtained in a separate experiment. (B–E) Cell extracts (40 μg protein) were prepared from H9c2 cells subjected to hypoxia for the indicated time periods. Proteolysis of fodrin, pro-m- and μ-calpains, and phosphorylation of Akt were analyzed by immunoblotting. The figures show representative immunoblots obtained from at least three experiments.

m- and μ-calpains, as reported by Zhang *et al.* (*28*), and that this might cause fodrin proteolysis. The time course of phosphorylation of Akt, a downstream effector of PI 3-kinase, correlates well with that of fodrin proteolysis (Fig. 1E), indicating the relationship between them.

In Vitro Proteolysis of Fodrin by Calpain—To confirm that calpain proteolyzes fodrin, we performed *in vitro* experiments using purified μ- or m-calpain. The 145/150 kDa fragments are minimally generated in the absence of exogenous calpain, whereas the addition of either μ- or m-calpain greatly increases the amount of the 145/150 kDa fragments (Fig. 2). These fragments are the same molecular sizes as in hypoxic cells (data not shown). These results indicate that the formation of the proteolytic 145/150 kDa fragments of fodrin during hypoxia might be calpain-dependent.

Involvement of Ca^{2+} Influx and Calpain Activation in Fodrin Proteolysis and Hypoxic Cell Death—To evaluate

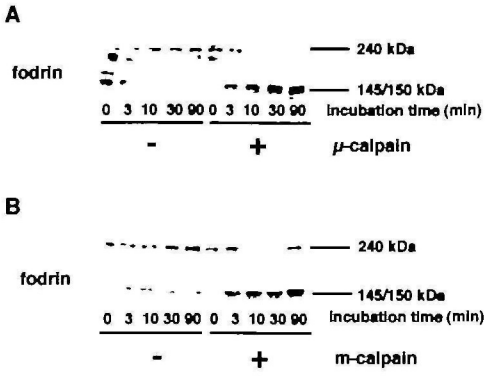

Fig. 2. ***In vitro* proteolysis of fodrin by calpain.** Whole cell lysates (4 mg/ml of proteins) of H9c2 cells were incubated with μ- (A) or m- (B) calpain (0.01 U/ml) for the indicated time periods as described in "MATERIALS AND METHODS." The reactions were stopped by boiling and fodrin proteolysis was analyzed by immunoblotting.

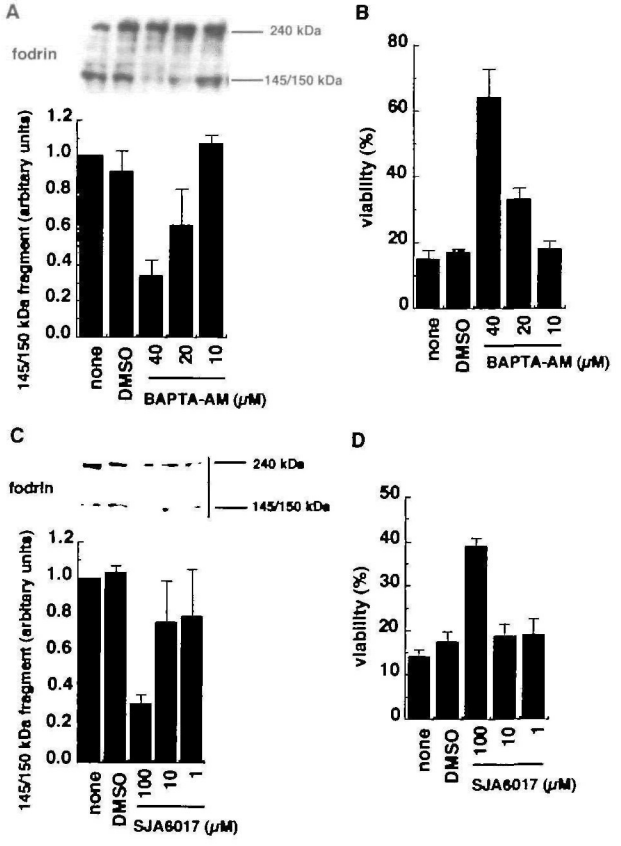

Fig. 3. **Effect of BAPTA-AM and SJA6017 on cell viability and fodrin proteolysis during hypoxia.** H9c2 cells were preincubated with the indicated amounts of BAPTA-AM or SJA6017 for 5 min, then subjected to hypoxia for 48 h. The proteolysis of fodrin was analyzed by immunoblotting (A and C) and cell viability was determined (B and D) as described in "MATERIALS AND METHODS." The data show representative immunoblots; (A and C) the quantified data represent the means and SD of three samples (A–D). Similar results were obtained in a separate experiment.

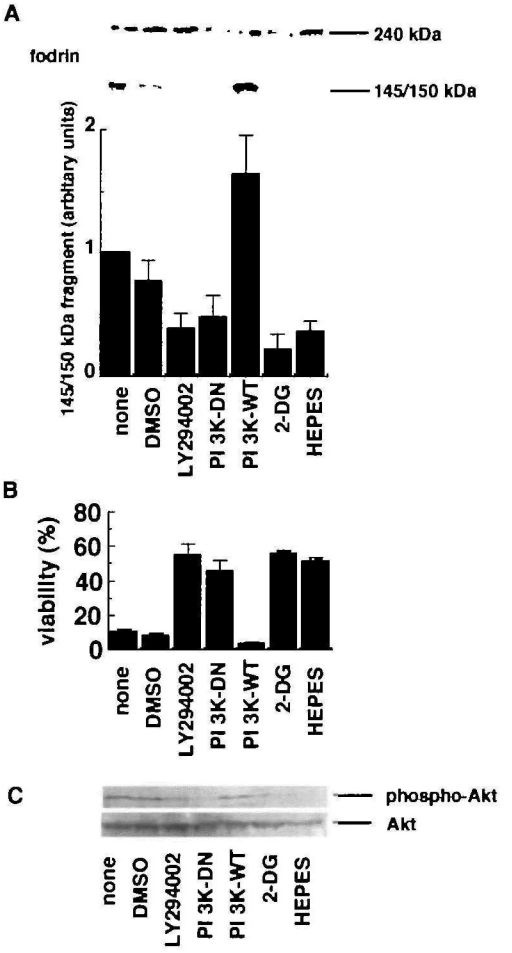

Fig. 4. **Effect of LY294002, 2-deoxy glucose, and HEPES, or the overexpression of wild type or dominant negative phosphoinositide 3-kinase, on fodrin proteolysis, viabilities, and phosphorylation of Akt during hypoxia.** H9c2 cells were preincubated with 10 μM LY294002, 5.5 mM 2-deoxy glucose (2-DG) or 25 mM HEPES for 5 min, or preinfected with adenoviruses expressing truncated p85 (PI 3-K-DN) or wild type p110 (PI 3-K-WT) for 24 h, then subjected to hypoxia for 48 h (A and B) or 12 h (C). The proteolysis of fodrin and the phosphorylation of Akt were analyzed by immunoblotting (A and C) and cell viability was determined (B) as described in "MATERIALS AND METHODS." The data show representative immunoblots (A and C) and the quantified data represent the means and SD of three samples (A and B). Similar results were obtained in a separate experiment.

the involvement of Ca^{2+} influx and subsequent calpain activation on fodrin proteolysis and hypoxic cell death, we first examined the effect of a Ca^{2+} chelating reagent, BAPTA, on fodrin proteolysis and cell death during hypoxia. The addition of BAPTA-AM, a cell permeable derivative (acetoxymethylester) of BAPTA, also reduced hypoxic cell death (Fig. 3, A and B). We next examined the involvement of calpain. SJA6017, a peptide inhibitor of calpain (*29*), reduced cell death as well as fodrin proteolysis (Fig. 3, C and D), confirming that fodrin proteolysis during hypoxia is calpain-dependent.

Acceleration of Fodrin Proteolysis by PI 3-Kinase—We examined the effect of PI 3-kinase activity on fodrin proteolysis during hypoxia. As shown in Fig. 4A, the proteolysis of fodrin is reduced in the presence of 10 μM LY294002, an inhibitor of PI 3-kinase (*30*). The adenovirus-mediated overexpression of dominant negative PI 3-kinase also reduced fodrin proteolysis, while wild type PI 3-kinase enhanced proteolysis. Moreover, the inhibition of metabolic acidosis by 2-deoxy glucose, an inhibitor of glycolysis, or by HEPES buffer also reduced fodrin proteolysis. The degradation of fodrin correlated well with the pH of the medium (data not shown), cell viabilitiy (Fig. 4B), and the phosphorylation of Akt (Fig. 4C), as described in our previous report (*23*). These results imply a relationship between acidosis and Ca^{2+} influx.

Involvement of the Na^+/H^+ Exchanger and Na^+/Ca^{2+} Exchanger in Fodrin Proteolysis during Hypoxia—To evaluate the correlation between acidosis and calcium influx, we examined the involvement of the Na^+/H^+ exchanger in fodrin proteolysis and hypoxic cell death using its inhibitor, (*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (*31*). Both fodrin proteolysis and hypoxic cell death were reduced by EIPA in a dose-dependent manner (Fig. 5, A and B). We next examined the effect of KB-R7943, an inhibitor of the Na^+/Ca^{2+} exchanger (*30*). KB-R7943 is known to cause the selective inhibition of the reverse (calcium influx) mode of exchange at concentrations less than 5 μM (*32*). Both of fodrin proteolysis and hypoxic cell death were also reduced by concentrations of KB-R7943 below 5 μM (Fig. 5, C and D). These results indicate that these exchangers might be involved in Ca^{2+} influx, fodrin proteolysis, and cell death during hypoxia.

Involvement of ER-Ca^{2+}-ATPase in Fodrin Proteolysis during Hypoxia—Finally, we examined the effect of the inhibition of ER Ca^{2+}-ATPase on fodrin proteolysis and hypoxic cell death by using its inhibitor, thapsigargin (*10,*

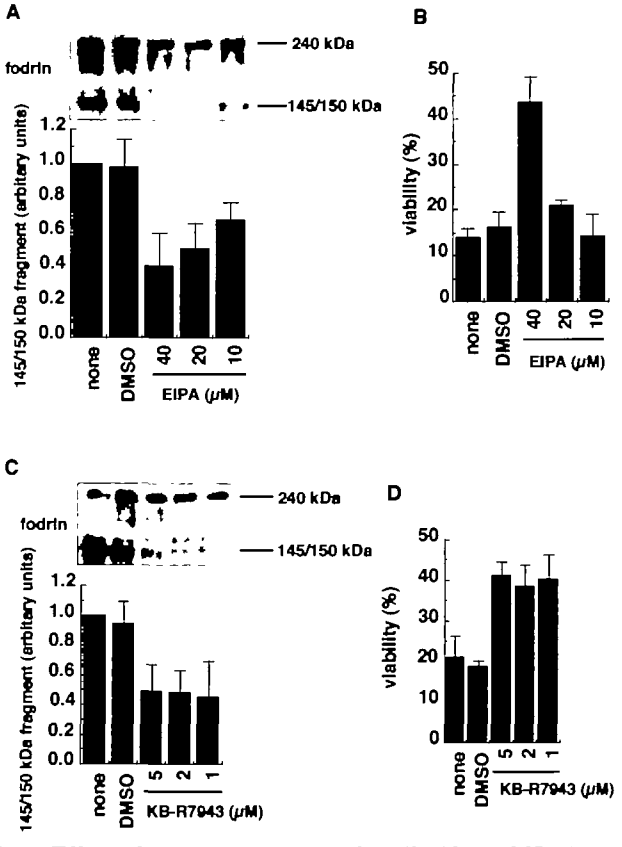

Fig. 5. **Effect of (*N*-ethyl-*N*-isopropyl)amiloride and KB-R 7943 on cell viability and fodrin proteolysis during hypoxia.** H9c2 cells were preincubated with the indicated amounts of (*N*-ethyl-*N*-isopropyl)amiloride (EIPA) or KB-R7943 for 5 min, then subjected to hypoxia for 48 h. The proteolysis of fodrin was analyzed by immunoblotting (A and C) and cell viability was determined (B and D) as described in "MATERIALS AND METHODS." The data show representative immunoblots (A and C) and the quantified data represent the means and SD of three samples (A–D). Similar results were obtained in a separate experiment.

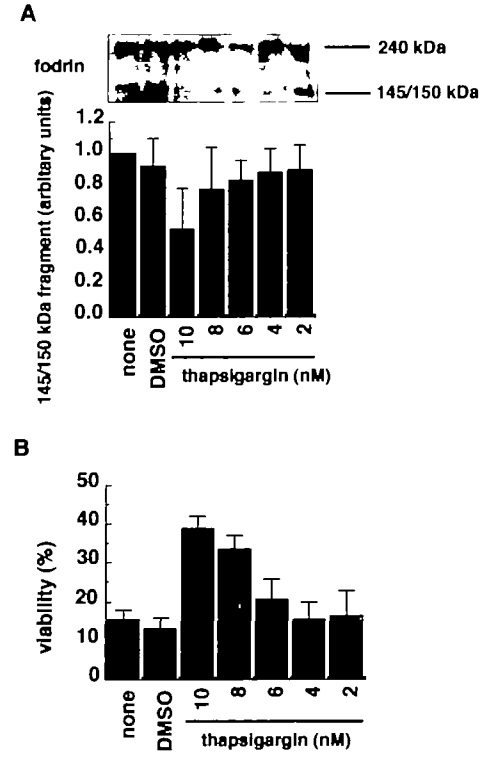

Fig. 6. **Effect of thapsigargin on cell viability and fodrin proteolysis during hypoxia.** H9c2 cells were preincubated with the indicated amounts of thapsigargin for 5 min, then subjected to hypoxia for 48 h. The proteolysis of fodrin was analyzed by immunoblotting (A) and cell viability was determined (B) as described in "MATERIALS AND METHODS." The data show representative immunoblots (A) and the quantified data represent the means and SD of three samples (A and B). Similar results were obtained in a separate experiment.

11). The depletion of intracellular Ca^{2+} stores by thapsigargin reduced fodrin proteolysis and hypoxic cell death in a dose-dependent manner (Fig. 6, A and B). These results indicate that Ca^{2+} influx from intracellular Ca^{2+} stores, as well as extracellular Ca^{2+}, might also be involved in fodrin proteolysis and cell death during hypoxia.

DISCUSSION

We have found for the first time that PI 3-kinase might promote the necrosis of cardiomyocyte-derived H9c2 cells through Ca^{2+} overloading and fodrin proteolysis by up-regulated m- and/or μ-calpains during hypoxia. This is in contrast with the generally accepted protective role of PI 3-kinase against apoptotic cell death (*21*). This study clarifies one of the downstream pathways of PI 3-kinase–activated glycolysis and metabolic acidosis during hypoxia with glucose supplementation. We found that either synergistic Ca^{2+} influx through the Na^+/H^+ and Na^+/Ca^{2+} exchangers or Ca^{2+} influx from intracellular Ca^{2+} stores might activate calpain.

PI 3-kinase was shown to enhance fodrin proteolysis in hypoxic H9c2 cells from the observations that the introduction of wild type PI 3-kinase promotes the proteolysis while the specific inhibitor LY294002 or the introduction of dominant negative PI 3-kinase reduces proteolysis (Fig. 4A). Because 2-deoxy glucose and HEPES, which we previously showed to inhibit hypoxic cell death by reducing metabolic acidosis (*23*), also reduced the phosphorylation of Akt, we think that the effect of PI 3-kinase on fodrin proteolysis is mediated not only by metabolic acidosis but by some other mechanisms.

The Na^+/Ca^{2+} exchanger mediates both the influx (reverse mode) and efflux (forward mode) of Ca^{2+} from cells, but it should operate only in the reverse mode to mediate acidosis-induced Ca^{2+} influx during hypoxia. Levels as low as 1–5 μM KB-R7943 specifically inhibit the Na^+/Ca^{2+} exchanger in the reverse mode (*32*), clearly indicating that Ca^{2+} influx on the Na^+/Ca^{2+} exchanger plays crucial roles in hypoxic cell death and fodrin proteolysis. The protective effect of KB-R7943 on ischemia/reperfusion injuries has also been reported for ischemic heart (*33*). Taken together, our results provide further evidence of the involvement of the Na^+/H^+ and Na^+/Ca^{2+} exchangers in pathological processes that occur during hypoxia/ischemia. Additionally, thapsigargin also inhibited both fodrin proteolysis and cell death after hypoxia (Fig. 6) to an extent similar to that of EIPA or KB-R7943 (Fig. 5). This indicates that Ca^{2+} released from intracellular Ca^{2+} stores through Ca^{2+}-ATPase might also be involved in calpain-mediated fodrin proteolysis. It should be remembered that Ca^{2+} influx triggers Ca^{2+} release from intracellular storage sites (*34*). Recently, Macrez *et al.* demonstrated that the L-type Ca^{2+} channel is activated by PI 3-kinase (*35*). Ménard *et al.* reported that H9c2 cells express the L-type Ca^{2+} channel (*36*). Moreover, we found that nifedipine, an inhibitor of the L-type Ca^{2+} channel, also inhibits hypoxic cell death and fodrin proteolysis (T. Aki and T. Fujimiya, unpublished observations). Therefore, in addition to the indirect pathways demonstrated in the present study, PI 3-kinase might stimulate Ca^{2+} influx directly by activating L-type Ca^{2+} channels.

In conclusion, we have demonstrated that metabolic acidosis might induce Ca^{2+} influx, which in turn activates calpain and induces subsequent fodrin proteolysis. PI 3-kinase accelerates metabolic acidosis and subsequent fodrin proteolysis during hypoxia. These results reveal one of the pathways by which PI 3-kinase accelerates hypoxic cell death.

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REFERENCES

1. Dennis, S.C., Gevers, W., and Opie, L.H. (1991) Protons in ischemia: Where do they come from; where do they go? *J. Mol. Cell. Cardiol.* **23**, 1077–1086
2. Lazdunski, M., Frelin, C., and Vigne, P. (1985) The sodium/hydrogen exchange system in cardiac cells; its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. *J. Mol. Cell. Cardiol.* **17**, 1029–1042
3. Lagadic-Gossmann, D., Buckler, K.J., and Vaughan-Jones, R.D. (1986) Role of bicarbonate in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. *J. Physiol. (London)* **458**, 361–384
4. Park, C-O., Xiao, X-H., and Allen, D.G. (1999) Changes in intracellular Na^+ and pH in rat heart during ischemia: role of Na^+/H^+ exchanger. *Am. J. Physiol.* **276**, H1581–H1590
5. Xiao, X-H., and Allen, D.G. (1999) Role of Na^+/H^+ exchanger during ischemia and preconditioning in the isolated rat heart. *Circ. Res.* **85**, 723–730
6. Orlowski, J. and Grinstein, S. (1997) Na^+/H^+ exchangers of mammalian cells. *J. Biol. Chem.* **272**, 22373–22376
7. Tani, M. and Neely, J.R. (1989) Role of intracellular Na^+ in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H^+-Na^+ and Na^+-Ca^{2+} exchange. *Circ. Res.* **65**, 1045–1056
8. Tani, M. (1990) Mechanism of Ca^{2+} overload in reperfused ischemic myocardium. *Annu. Rev. Physiol.* **52**, 543–559
9. Lee, J.A. and Allen, D.G. (1991) Mechanism of acute ischemic contractile failure of the heart: role of intracellular calcium. *J. Clin. Invest.* **88**, 361–367
10. Sagara, Y. and Inesi, G. (1991) Inhibition of the sarcoplasmic reticulum Ca^{2+} transport ATPase by thapsigargin at subnanomolar concentrations. *J. Biol. Chem.* **266**, 13503–13506
11. Thastrup, O., Cullen, P., Drobak, B.K., Hanley, M.R., and Dawson, A.P. (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA* **87**, 2466–2470
12. Walters, S.L., Wong, J.K., and Schnellmann, R.G. (1997) Depletion of endoplasmic calcium stores protects against hypoxia- and mitochondrial inhibitor-induced cellular injury and death. *Biochem. Biophys. Res. Commun.* **240**, 57–60
13. Saido, T.C., Sorimachi, H., and Suzuki, K. (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *FASEB J.* **8**, 814–822
14. Sorimachi, H., Ishiura, S., and Suzuki, K. (1997) Structure and physiological function of calpain. *Biochem. J.* **328**, 721–732
15. Yoshida, K., Inui, M., Harada, K., Saido, T.C., Sorimachi, Y., Ishihara, T., Kawashima, S., and Sobue, K. (1995) Reperfusion of rat heart after brief ischemia induces proteolysis of calspectin (Nonerythroid spectrin or fodrin) by calpain. *Circ. Res.* **77**, 603–610
16. Yoshida, K., Sorimachi, Y., Fujiwara, M., and Hironaka, K. (1995) Calpain is implicated in rat myocardial injury after ischemia or reperfusion. *Jpn. Circ. J.* **59**, 40–48
17. Yoshida, K., Yamasaki, Y., and Kawashima, S. (1995) Calpain activity alters in rat myocardial subfractions after ischemia and reperfusion. *Biochem. Biophys. Acta* **1182**, 215–220

18. Fukada, S., Harada, K., Kunimatsu, M., Sakabe, T., and Yoshida, K. (1998) Postischemic reperfusion induces α-fodrin proteolysis by m-calpain in the synaptosome and nucleus in rat brain. *J. Neurochem.* **70**, 2526–2532
19. Shiraishi, K., Naito, K., and Yoshida, K. (2000) Inhibition of calpain but not caspase protects the testis against injury after experimental torsion of rat. *Biol. Reprod.* **63**, 1538–1548
20. Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katsuo, R., Driscoll, P.C., Woscholski, R., Parker, P.J., and Waterfield, M.D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70**, 535–602
21. Fanke, T.F., Kaplan, D.R., and Cantley, L.C. (1997) PI3K: Downstream AKTion blocks apoptosis. *Cell* **88**, 435–437
22. Matsui, T., Li, L., del Monte, F., Fukui, Y., Franke, T.F., Hajjar, R.I., and Rosenzweig, A. (1999) Adenoviral gene transfer of activated phosphatidylinositol 3′-kinase and Akt inhibits apoptosis of hypoxic cardiomyocyte in vitro. *Circulation* **100**, 2373–2379
23. Aki, T., Mizukami, Y., Oka, Y., Yamaguchi, K., Uemura, K., Fujimiya, T., and Yoshida, K. (2001) Phosphoinositide 3-kinase accelerates necrotic cell death during hypoxia. *Biochem. J.* **358**, 481–487
24. Katagiri, H., Asano, T., Ishihara, H., Inukai, K., Shibasaki, Y., Kikuchi, M., Yazaki, Y., and Oka, Y. (1996) Overexpression of catalytic subunits of p110α of phosphatidylinositol 3-kinase increases glucose transporters in 3T3-L1 adipocytes. *J. Biol. Chem.* **271**, 16987–16990
25. Katagiri, H., Asano, T., Inukai, K., Ogihara, T., Ishihara, H., Shibasaki, Y., Murata, T., Terasaki, J., Kikuchi, M., Yazaki, Y., and Oka, Y. (1997) Role of PI 3-kinase and Ras on insulin stimulated glucose transport in 3T3-L1 adipocyte. *Am. J. Physiol.* **272**, E326–E331
26. Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
27. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354
28. Zhang, J., Patel, J.M., and Block, E.R. (1998) Hypoxia-specific upregulation of calpain activity and gene expression in pulmonary artery endothelial cells. *Am. J. Physiol.* **275**, L461–L468
29. Fukiage, C., Azuma, M., Nakamura, Y., Tamada, Y., Nakamura, M , and Shearer, T.R. (1997) SJA6017, a newly synthesized peptide inhibitor of calpain: amelioration of cataract in cultured rat lenses. *Biochem. Biophys. Acta* **1361**, 304–312
30 Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269**, 5241–5248
31. Vigne, P., Frelin, C., Cragoe, Jr. E.J., and Lazdunski, M. (1983) Ethylisopropyl-amiloride: A new and highly potent derivative of amiloride for the inhibition of the Na^+/H^+ exchange system in various cell types. *Biochem. Biophys. Res. Commun.* **116**, 86–90
32. Iwamoto, T., Watano, T., and Shigekawa, M. (1996) A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cell expressing NCX1. *J. Biol. Chem.* **271**, 22391–22397
33. Ladilov, Y., Haffer, S., Balser-Schäfer, C., Maxeiner, H., and Piper, H.M. (1999) Cardioprotective effects of KB-R7943: a novel inhibitor of reverse mode of Na^+/Ca^{2+} exchanger. *Am. J. Physiol.* **276**, H1868–H1876
34. Endo, M., Tanaka, M., and Ogawa, Y. (1970) Calcium induced release of calcium from the sarcoplasmic reticulum of skinned muscle fibres. *Nature (London)* **228**, 34–36
35. Macrez, N., Mironneau, C., Carricaburu, V., Quignard, J-F., Babich, A., Czupalla, C., Nürunberg, B., and Mironneau, J. (2001) Phosphoinositide 3-kinase isoforms selectively couple receptors to vascular L-type Ca^{2+} channels. *Circ. Res.* **89**, 692–699
36. Ménard, C., Pupier, S., Mornet, D., Kitzmann, M., Nargeot, J., and Lory, P. (1999) Modulation of L-type calcium channel expression during retinoic acid-induced differentiation of H9C2 cardiac cells. *J. Biol. Chem.* **274**, 29063–29070