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Review

A unique pathway of cardiac myocyte death caused by hypoxia–acidosis

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Summary

Chronic hypoxia in the presence of high glucose leads to progressive acidosis of cardiac myocytes in culture. The condition parallels myocardial ischemia *in vivo*, where ischemic tissue becomes rapidly hypoxic and acidotic. Cardiac myocytes are resistant to chronic hypoxia at neutral pH but undergo extensive death when the extracellular pH (pH[o]) drops below 6.5. A microarray analysis of 20 000 genes (cDNAs and expressed sequence tags) screened with cDNAs from aerobic and hypoxic cardiac myocytes identified >100 genes that were induced by >2-fold and ~20 genes that were induced by >5-fold. One of the most strongly induced transcripts was identified as the gene encoding the pro-apoptotic Bcl-2 family member BNIP3. Northern and western blot analyses confirmed that BNIP3 was induced by 12-fold (mRNA) and 6-fold (protein) during 24 h of hypoxia. BNIP3 protein, but not the mRNA, accumulated 3.5-fold more rapidly under hypoxia–acidosis. Cell fractionation experiments indicated that BNIP3 was loosely bound to mitochondria under conditions of neutral hypoxia but was translocated into the membrane when the myocytes were acidotic. Translocation of BNIP3 coincided with opening of the mitochondrial permeability pore (MPTP). Paradoxically, mitochondrial pore opening did not promote caspase activation, and broad-range caspase inhibitors do not block this cell death pathway. The pathway was blocked by antisense BNIP3 oligonucleotides and MPTP inhibitors. Therefore, cardiac myocyte death during hypoxia–acidosis involves two distinct steps: (1) hypoxia activates transcription of the death-promoting *BNIP3* gene through a hypoxia-inducible factor-1 (HIF-1) site in the promoter and (2) acidosis activates BNIP3 by promoting membrane translocation. This is an atypical programmed death pathway involving a combination of the features of apoptosis and necrosis. In this article, we will review the evidence for this unique pathway of cell death and discuss its relevance to ischemic heart disease. The article also contains new evidence that chronic hypoxia at neutral pH does not promote apoptosis or activate caspases in neonatal cardiac myocytes.

Key words: cardiac myocyte, heart, apoptosis, ischemia, pH, BNIP3, mitochondria, necrosis.

Ischemic heart disease

Coronary artery disease

High blood pressure and elevated cholesterol are risk factors for coronary artery disease, promoting the formation of fatty streaks and lesions in the coronary vasculature that are the precursors of plaque. Progressively expanding atherosclerotic plaque ultimately restricts blood flow through coronary arteries and provides a substrate for occlusive thrombus formation. Reduced blood flow produces hypoxia in the tissues downstream of the lesion. In the most extreme cases of coronary artery disease, a complete occlusion occurs, eliminating blood flow and presenting an acute threat to the viability of the effected tissue (acute coronary event or heart attack). When the myocardial tissue is reperfused by removal of the plaque (thrombolysis), downstream cells are subjected to further damage involving oxidative stress, and a region of permanent injury containing dead and dying cells, known as an infarct, develops (Jennings et al., 1995). Blood supply is interrupted inside the developing infarct and in the bordering tissue, and hypoxia may persist in these regions for days or weeks, exacerbating the injury (Narula et al., 1998; Vanoverschelde et al., 1997; Zuurbier et al., 1999). Hypoxia causes a switch from oxidative to glycolytic energy generation, with increased glucose consumption, lactic acid production and lower intracellular pH (Dennis et al., 1991; Neely and Grotyohann, 1984; Webster et al., 2000). Increased blood lactate levels reflect this metabolic shift and are diagnostic of infarction in ischemic heart disease. The extent of tissue loss to infarction is determined by the severity and duration of the

ischemic period and is known to involve both necrotic and apoptotic cell death pathways (Anversa and Kajstura, 1998; Gottlieb et al., 1994; Kajstura et al., 1996). Oxidative stress caused by reperfusion may account for up to 60% of the tissue damage during early infarction (Fliss and Gattinger, 1996; Horwitz et al., 1994; Yoshida et al., 1996). Multiple additional factors contribute to cell death as the infarcted area expands and more border cells die. These include collateral damage from necrosis and infiltrating macrophages (Williams et al., 1994), additional necrotic and apoptotic death resulting from energy (glucose and ATP) depletion (Buja et al., 1993; Hochachka et al., 1996; Majno and Joris, 1995; Reimer and Ideker, 1987; Mani and Kitsis, 2003), and changes associated with hypoxia. The pathways of ischemic injury are the subjects of intensive study because descriptions of these pathways may lead to new methods of reducing tissue loss and minimizing the damage caused by infarction and heart attack.

Ischemia

Numerous reports have documented the extensive cell death and tissue loss that accompanies myocardial ischemia (Anversa and Kajstura, 1998; Narula et al., 1996; Chen et al., 1997; Fiss and Gattinger, 1996). The contribution of hypoxia to cell death is controversial and different groups have reported markedly different results (Tanaka et al., 1994; Long et al., 1997; Matsui et al., 1999; Kang et al., 2000; Regula et al., 2002; Kubasiak et al., 2002). The discrepancies are almost certainly due to differences in the models, especially related to glucose levels and pH. Our results indicate that it is the secondary consequences of hypoxia rather than hypoxia *per se* that causes cell death (Webster and Bishopric, 1992; Webster et al., 1999). Although hypoxia is an obligatory consequence of ischemia, it rarely exists alone. Hibernating myocardium may represent a condition of chronic hypoxia (Heusch and Schulz, 1996) but in most other conditions of moderate to severe ischemia, hypoxia is accompanied by or precedes energy depletion, acidosis and/or reoxygenation. Each of these latter conditions involves cell and tissue loss, and the pathways of cell death in each case are unique. Reperfusion damage has been the subject of numerous articles and reviews (Gottlieb et al., 2003; Kumar and Jugdutt, 2003; Valen, 2003) and will not be addressed in detail here. The damage caused by reperfusion is widely believed to be initiated by surges of oxygen free radicals that initiate stress responses and culminate in cell death. Our studies showed that the exposure of cardiac myocytes to cycles of hypoxia–reoxygenation results in ~30% cell death in each cycle (Webster et al., 1999; Dougherty et al., 2002). The response of cardiac myocytes to ischemia–reperfusion (I/R) is complex, and the survival/death pathways have not been fully described. During early ischemia, ATP levels may be maintained by increased glycolysis but at the expense of the limited reserves of glucose and glycogen. Glucose utilization increases by >10-fold in ischemic/hypoxic cardiac myocytes with corresponding lactate accumulation (Webster et al., 1993, 1994, 1999). Acidosis is further exacerbated when ATP levels begin to decline (Allen et al., 1989; Allen and Orchard, 1987; Neely and Grotyohann, 1984). If ATP is depleted during ischemia, necrosis will occur because of the passive loss of transmembrane ion gradients, followed by cell swelling and loss of membrane integrity (Buja et al., 1993; Hochachka et al., 1996; Majno and Joris, 1995; Reimer and Ideker, 1987). In the latter condition, apoptosis as well as necrosis probably occurs, and both may be ‘programmed’ at least in the early stages (Bishopric et al., 2001). Programmed death is an active, energy-consuming process requiring ATP; as ATP levels fall at late time points, the programmed pathways may fail and be replaced by a more classical necrotic death (Kajstura et al., 1996; Ohno et al., 1998; Buja and Entman, 1998).

Regulation of pH during ischemia

It has been recognized for some time that proton pumps and pH regulation may play a role in apoptosis signaling (Anversa and Kajstura, 1998; Gottlieb et al., 1996; Karwatowska-Prokopczuk et al., 1998; Long et al., 1998). Ischemic cardiac myocytes generate excess H^+ through increased anaerobic metabolism, net hydrolysis of ATP and CO_2 retention (Dennis et al., 1991). These protons are extruded from the myoplasm to the interstitial space by the combined action of three major ion-specific membrane transporters, including the Na^+/H^+ exchanger, the Na^+/HCO_3^- cotransporter and the vacuolar proton-ATPase (Karwatowska-Prokopczuk et al., 1998; Lagadic-Gossmann et al., 1992; Lazdunski et al., 1985). Increased activity of the Na^+/H^+ exchanger can cause Ca^{2+} overload because the elevated intracellular Na^+ is subsequently exchanged for Ca^{2+} *via* the Na^+/Ca^{2+} exchanger (Pierce and Czubryt, 1995). Inhibition of Na^+/H^+ exchange has been shown to protect against ischemic injury, possibly by preventing this increase in Ca^{2+} (Bond et al., 1993; Shimada et al., 1996). Conversely, inhibition of the vacuolar ATPase promotes apoptosis, in part by shifting the proton load towards the Na^+/H^+ transporter, thereby increasing Ca^{2+} uptake, and in part by reducing the myocyte capacity to control intracellular pH (Gottlieb et al., 1996; Karwatowska-Prokopczuk et al., 1998; Long et al., 1998). Acidosis has been shown to correlate with apoptosis in a number of other systems where it may promote the activation of caspases (Gottlieb et al., 1995; Li and Eastman, 1995; Perez-Sala et al., 1998).

Bcl-2 and BNIP3

The Bcl-2 gene family encodes a group of proteins with the ability to promote or repress programmed cell death in response to a wide variety of stimuli (Boise et al., 1995; Hockenbery, 1995; Korsmeyer, 1995). Additional genes in this family include those encoding Bcl-Xl, Mcl-1, A1, Bcl-W and CED-9, which are anti-apoptotic, and Bak, Bax, Bcl-xs, Diva and Mtd/Bok, which are pro-apoptotic. These proteins are usually associated with the cell membranes, particularly the mitochondria, endoplasmic reticulum (ER) and nuclear envelope, where they are anchored by a C-terminal domain. Individual family members may remain in the cytosol or be loosely membrane bound and translocate after a death signal

is received (Adams and Cory, 2001; Regula et al., 2002; Vande Velde et al., 2000). The physical location and activity of each Bcl-2 family protein is determined partly by its binding to other Bcl-2-related proteins in the cell cytosol. This in turn is determined by the relative concentrations of each protein, and the balance of pro- and anti-apoptotic members is an important feature of the regulation. Bcl-2 has been attributed antioxidant and proton translocating properties (Hockenbery et al., 1993; Shimizu et al., 1998), and one major function of the family as a whole is to determine the on/off state of the mitochondrial permeability transition pore (MPTP; reviewed in Green and Reed, 1998; Earshaw et al., 1999; Crompton, 2000). In some animal ischemia models of coronary occlusion, myocardial levels of Bcl-2 proteins have been shown to decline while pro-apoptotic Bax increases (Kajstura et al., 1996; Hockenbery, 1995). Over-expression of Bcl-2 reduces apoptosis in some models of neuronal ischemia (Kane et al., 1993; Lawrence et al., 1996).

BNIP3 is a member of the so-called BH3-only subfamily of Bcl-2 family proteins that antagonize the activity of pro-survival proteins and promote apoptosis (Ray et al., 2000; Vande Velde et al., 2000). These proteins do not possess the same protein binding domains (BH1 and BH2) as the other Bcl-2 family members but instead bind through a common BH3 domain. Related members of this group include Bik, Blk, Hrk, BimL, Bad, Bid and Nix. BNIP3 was originally identified as a Bcl-XL or E1B 19K-binding protein (Zhang et al., 2003). The BH3 domain of BNIP3 may not be required for the death-promoting functions, but the C-terminal transmembrane domain is required, indicating membrane targeting as essential for pro-apoptosis function. BNIP3 is expressed below detectable levels in most organs including the heart under normal (non-ischemic) conditions (Bruick, 2000; Vande Velde et al., 2000). Overexpression of BNIP3 protein by transfection of the cDNA into some cultured cell lines results in membrane translocation and initiation of cell death (Vande Velde et al., 2000). The death pathway has characteristics of both apoptosis and necrosis, including DNA fragmentation and MPTP opening, but early loss of plasma membrane integrity and no caspase activation. Expression of the BNIP3-encoding gene is induced by hypoxia through a 5′ promoter hypoxia-inducible factor-1 (HIF-1)-binding site (Bruick, 2000).

Hypoxia and cardiac death

In the next sections, we will review the evidence, including new data, for a unique pathway of death caused by hypoxia with acidosis. These data are a compilation of results reported by our groups over the past decade and include, in particular, the following references: Webster and Bishopric, 1992; Webster et al., 1999, 2000; Dougherty et al., 2002, 2004; Kubasiak et al., 2002.

As discussed above, the role of hypoxia in ischemia-mediated cell death is controversial. We previously reported that rapidly contracting cardiac myocytes remained fully viable and contractile during culture under severe hypoxia for up to 5 days (Webster and Bishopric, 1992). Glycolysis was induced by 10-fold within 1 h, and there was no evidence of major cell loss; ATP remained high and the only significant change was a reduced level of contractility that correlated with lower intracellular cAMP in the hypoxic cultures. These results contrast with other reports of significant cardiac myocyte cell loss by apoptosis during 24–72 h of exposure to an equivalent degree of hypoxia (Tanaka et al., 1994; Long et al., 1997; Matsui et al., 1999; Regula et al., 2002). To resolve this apparent controversy, we subjected cardiac myocytes to severe hypoxia for a week under conditions where the glucose and extracellular pH ([pH]o) were constantly monitored and maintained within the physiological range, and we measured both caspase activity and cell death at intervals. As indicated in Fig. 1A, there was no significant change in the number of

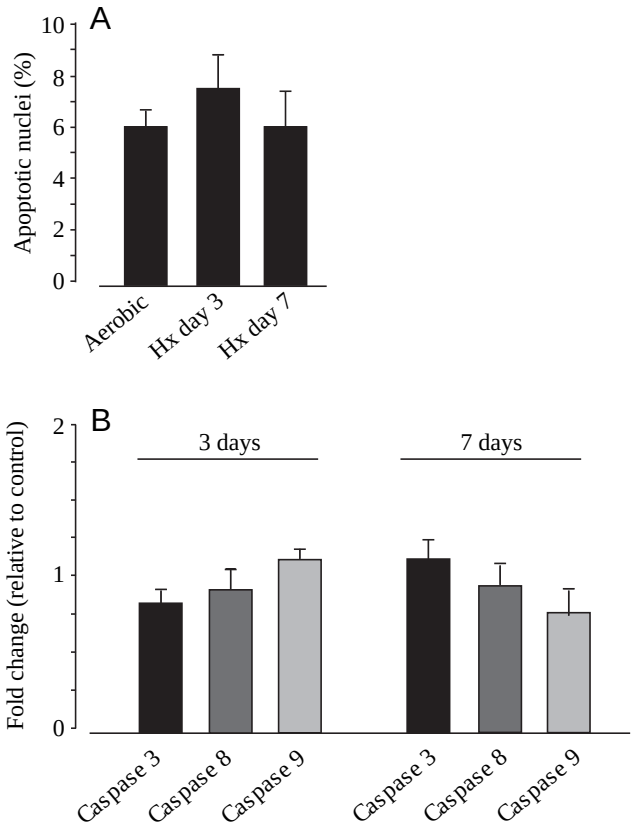

Fig. 1. Nuclear condensation and caspase activity during chronic hypoxia. (A) Rapidly contracting neonatal cardiac myocytes were exposed to hypoxia (P_{O_2} <8 mmHg) in minimal essential medium (MEM) with 5% fetal calf serum (FCS) as described previously (Webster and Bishopric, 1992). The medium was replaced every 12 h with fresh hypoxic medium so that the pH was maintained at >7.2, glucose was not depleted and ATP level was maintained at >70% control. Nuclei were stained with Hoechst 33342 and examined microscopically as described previously (Dougherty et al., 2002; Webster et al., 1999). (B) Cultures were maintained as in A, harvested as indicated, and the cells lysed for caspase assays using a kit from Ambion Inc. (Austin, TX, USA) as described previously (Dougherty et al., 2002). Differences were not significant at P=0.30 (N=6). Values are means ± S.E.M.

apoptotic nuclei at either 3 days or 7 days compared with aerobic cultures. Under the same conditions of hypoxia, endothelial cells died within 3 days (data not shown). Caspase activities are shown in Fig. 1B; there were no significant changes in the activities of caspases 3, 8 or 9 at any time during this time course (staurosporine-treated plates incubated in parallel generated a maximal 2.7-fold activation of caspase 3 and 1.8-fold activation of caspase 9; not shown). These results confirm our previous reports that hypoxia alone does not activate programmed cell death in neonatal cardiac myocytes. This conclusion cannot necessarily be extrapolated to other models or to the intact heart. To remain viable under hypoxia, the cells must be able to maintain glycolysis at a level that is sufficient to sustain ATP, a condition that requires a continuous supply of glucose. In addition, the cell must be able to clear excess acid produced by anaerobic glycolysis. If these conditions can not be fully accommodated, the cell will die. Todor et al. (2002) recently reported that cardiac myocytes from failing hearts, but not those from normal hearts, were susceptible to hypoxia-mediated death. It is possible that the failing myocytes were compromised in their ability to activate adaptations to hypoxia. It should also be noted that oxygen tension may be an important determinant in the cellular response including the mechanism of oxygen sensing. Budinger et al. (1998) described a model of myocardial ischemia where myocytes were subjected to 3% O_2. There was a small decrease in contractility under hypoxia coincident with a similar small loss of ATP but no loss of cell viability. The authors concluded that oxygen sensing, and the positive adaptation, was probably initiated by reactive oxygen species (ROS) generated in the mitochondria under these conditions because of partial inhibition of cytochrome oxidase. Under some conditions, mitochondrial ROS may play a driving role in cell death during hypoxia. Yermolaieva et al. (2004) described a stroke model where death of PC12 cells subjected to a brief period of hypoxia followed by reoxygenation correlated with ROS. In our studies, oxygen tension is maintained at less than 0.5%, mitochondrial functions are severely impaired but we did not detect any increase of ROS as measured by the lucigenin reagent (*N*-methyl-acridinium nitrate; Dougherty et al., 2004).

Hypoxia–acidosis and cardiac death

To investigate the impact of pH on hypoxic myocytes, we exposed parallel cultures of myocytes to hypoxia at neutral pH or hypoxia with coincident acidosis and measured the levels of apoptosis by DNA ladders and Hoechst staining (for experimental details, see Webster et al., 1999). For hypoxia–neutral exposure, the cultures were maintained continuously under hypoxia, and the medium was replaced twice daily with fresh hypoxic medium to prevent the build-up of waste metabolites. For hypoxia–acidosis, the cultures were maintained in parallel but the medium was not replaced. The

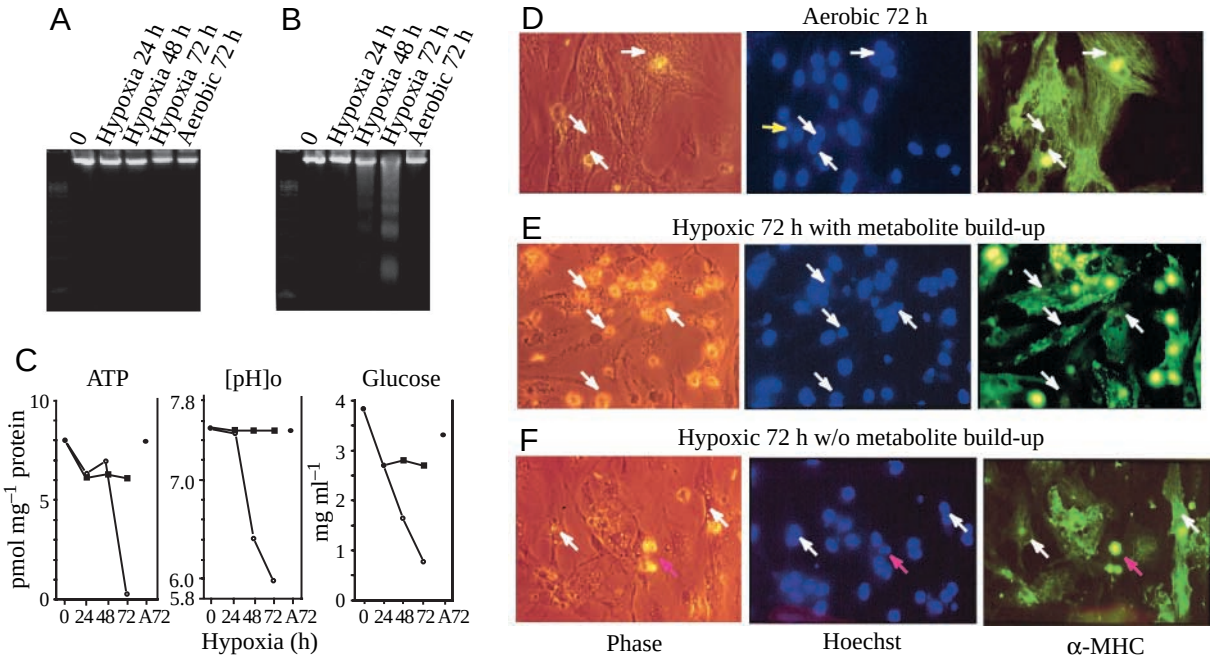

Fig. 2. Activation of programmed death by waste metabolites under chronic hypoxia. (A,B) Parallel cultures of cardiac myocytes were exposed to hypoxia. In A, the media was replaced with fresh hypoxic medium every 6 h. In B, the media was not replaced. Cultures were harvested at the indicated times and processed for DNA fragmentation. (C) Intracellular ATP, medium glucose and [pH]o were measured in parallel cultures (Webster et al., 1999); results are means of three separate experiments; squares represent results from cultures with media replacement; open circles show results without media replacement; filled circles represent the 72 h aerobic control. (D–F) Typical fields of myocytes stained with Hoechst 33342 and anti-α-MHC antibody (Webster et al., 1999). 1G Quantitations of Hoechst-stained condensed nuclei were as described in Webster et al. (1999. Results are representative of at least three experiments.

cultures were monitored for ATP, glucose and [pH]o. Results from these experiments are shown in Fig. 2A,B. In agreement with the results shown in Fig. 1, there was no evidence of DNA fragmentation under the first set of conditions (Fig. 2A), but cultures subjected to hypoxia without daily media change showed significant DNA laddering after 48 h and extensive laddering after 72 h (Fig. 2B). In cultures with medium replacement, ATP levels dropped to ~75% of aerobic control levels after 24 h and remained stable thereafter; [pH]o did not change, and glucose levels remained high (Fig. 2C). Under these conditions, the myocytes continued to contract for the duration of the experiment, as previously reported (Webster and Bishopric, 1992). When the medium was not replaced, intracellular ATP levels were sustained for up to 48 h, and the dramatic loss at 72 h was coincident with cell death. Glucose declined but was not depleted, and [pH]o declined steadily to a final value of ~6.0 (Fig. 2C). Under these conditions, contractions ceased before 48 h (data not shown).

In Fig. 2D–F, the effects of coincident hypoxia and acidosis on myocyte structure and integrity are shown by staining with Hoechst 33342 and anti-α-MHC antibody (see Dougherty et al., 2002; Webster et al., 1999). In cultures grown aerobically, abundant myofilaments with clear cross-striations are evident with the α-MHC stain (Fig. 2D). The white arrows indicate smooth, oval-shaped nuclei with sparse evidence of condensation or internal fragmentation. In this field, 29 nuclei were scored normal and one was condensed. At 72 h, the hypoxic–acidotic cultures (Fig. 2E) still stained strongly with α-MHC antibody but there was clear deterioration of the myofilaments, and cross-striations were no longer clearly visible. In the field shown, 22 nuclei were scored condensed (examples are indicated by the arrows), and 14 were normal. By contrast, hypoxic–neutral cells still exhibited myofilaments with intact cross-striations after 72 h (Fig. 2F, see arrow at far right), and most of the nuclei were normal. Control aerobic cultures contained 5–7% apoptotic cells; this increased to 44% after 48 h of hypoxia with metabolite build-up and to 60% after 72 h of hypoxia.

These results demonstrate that cardiac myocyte death under hypoxia occurs only if the media is not frequently changed, indicating either that proapoptotic factors accumulate in the media during hypoxia or vital components are depleted. To test these possibilities, medium from 48 h hypoxic cultures that were just beginning to show signs of DNA laddering was transferred to fresh cardiac myocytes and the cells were incubated for an additional 24–48 h under either hypoxic or aerobic conditions. Apoptosis was again monitored by DNA fragmentation. Control plates received medium from hypoxic cells that underwent daily medium replacement. Results are shown in Fig. 3. Significant apoptosis was apparent in both aerobic and hypoxic cultures 24 h after exposure to the spent medium, but significantly more DNA fragmentation appeared in the sample from the 24 h hypoxic plate correlating with the lower [pH]o. These results implicate a death factor(s) associated with deteriorating pH control.

As a second approach to identify the death factors, we collected the spent medium from 48 h hypoxic cultures and readjusted the pH to 7.6 before transferring to fresh cardiac myocytes. The effect of neutralized spent medium is shown in Fig. 4. Acidic spent medium again caused extensive apoptosis of fresh myocytes but the neutralized medium caused minimal DNA fragmentation (Fig. 4A). This suggests that acidosis is necessary to induce apoptosis of hypoxic cultures. To confirm this, parallel cardiac myocyte cultures were again exposed to hypoxia; in one set, acid accumulated in the medium and in the other the acid was neutralized every 12 h to maintain [pH]o

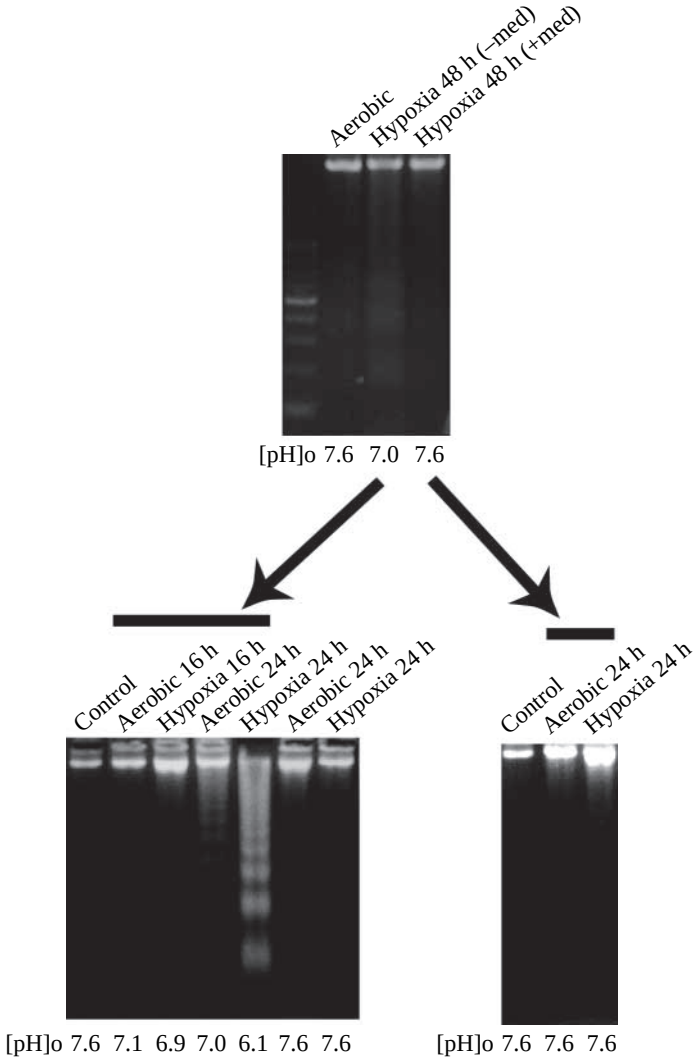

Fig. 3. Induction of apoptosis by conditioned medium. Cultures were grown under hypoxia with or without medium change. After 48 h, the medium was removed, and the cells were analyzed for DNA fragmentation (top panel). The conditioned media was centrifuged at 800 ***g*** for 5 min to pellet cells and debris, added directly to a second set of plates, and these were incubated in air or under hypoxia as indicated. After 24 h, these cells were also harvested and analyzed for DNA fragmentation. [pH]o was measured in all cases immediately before harvesting the cells. Note that the control samples shown in the last two lanes of the bottom left panel did not receive conditioned medium. Results are representative of three separate experiments.

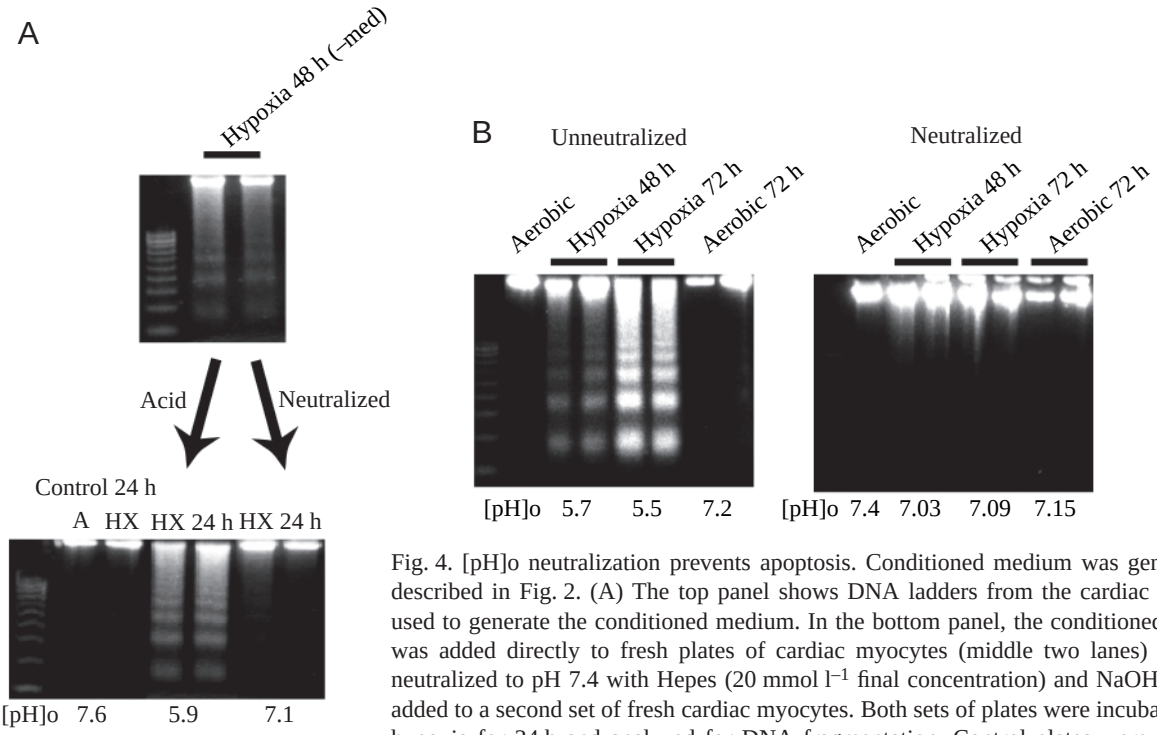

Fig. 4. [pH]o neutralization prevents apoptosis. Conditioned medium was generated as described in Fig. 2. (A) The top panel shows DNA ladders from the cardiac myocytes used to generate the conditioned medium. In the bottom panel, the conditioned medium was added directly to fresh plates of cardiac myocytes (middle two lanes) or it was neutralized to pH 7.4 with Hepes (20 mmol l^{-1} final concentration) and NaOH and then added to a second set of fresh cardiac myocytes. Both sets of plates were incubated under hypoxia for 24 h and analyzed for DNA fragmentation. Control plates were incubated under aerobic or hypoxic conditions in parallel. (B) Parallel sets of cardiac myocytes were exposed to hypoxia without medium change; in the first set (left panel), the acid was allowed to accumulate exactly as described in Fig. 2B; in the second set (right panel), experimentally predetermined aliquots of Hepes and NaOH were added every 12 h to maintain a [pH]o of ~7.2.

above 7.0 during hypoxic incubation. These results are shown in Fig. 4B. In the absence of additional buffer, [pH]o dropped to 5.7 at 48 h, and there was extensive apoptosis. In the pH-neutral cultures, there was no visible DNA fragmentation. Hoechst 33342 and anti-α-MHC antibody stains again revealed >60% apoptosis of acidotic cardiac myocytes and <10% when the pH was neutralized.

BNIP3 and the hypoxia–acidosis death pathway

Results described above indicate an essential contribution of acidosis to promote death under hypoxia when glucose and ATP levels are sustained. In further experiments, we sought to investigate the specific components of this death pathway. As a first step, we used a microarray screen to determine which gene groups responded to hypoxia. Cardiac myocytes were subjected to normoxic incubation or 24 h hypoxia, and mRNA was reverse-transcribed and hybridized to an array of 20 000 specific gene sequences from rat and mouse libraries (these experiments were carried out in collaboration with Incyte, Inc., Freemont, CA, USA). Results from one such set of screens, subjected to analysis using an Excel 98 program, are shown in Fig. 5A. A gene transcript identified as the Bcl-2 family member *BNIP3* appeared as the second most strongly induced gene. Hypoxia-inducible marker genes, including metallothionein-1 (MT-1), heme oxygenase (HO), pyruvate dehydrogenase kinase (PDK), glucose transporter (GLUT), tyrosine amino transferase (TAT) and triosephosphate isomerase (TPI), are also indicated. The microarray data were confirmed by northern and western blot analyses of BNIP3 (Fig. 5B,C; for details, see Kubasiak et al., 2002). BNIP3 mRNA levels increased progressively during hypoxia and peaked after 8 h at similar levels in both neutralized and acidic conditions. BNIP3 mRNA was degraded after 48 h of hypoxia in acidic but not in neutral pH media in parallel with cell death. BNIP3 protein accumulated more rapidly under acid pH and peaked at a significantly higher level than the pH-neutral samples. There were no corresponding changes in Bax, Bak or β-actin proteins. These results demonstrate that hypoxia activates *BNIP3* transcription, and the protein may be stabilized by acidosis. Fig. 5D shows that *BNIP3* transcripts also increased in a Langendorf-perfused intact rat heart preparation subjected to 1 h of no-flow ischemia or 2 h of perfusion with hypoxic medium. Procedures for these experiments are described in detail in Webster et al. (1999) and Kubasiak et al. (2002).

Activation of BNIP3 by acidosis

These results demonstrate a correlation between BNIP3 accumulation and cardiac myocyte death during hypoxia–acidosis. Further experiments were able to confirm a functional relationship using antisense suppression of the *BNIP3* gene transcripts. As expected, treatment of myocytes with BNIP3 antisense oligonucleotides almost completely prevented hypoxia–acidosis cell death (not shown; see Kubasiak et al.,

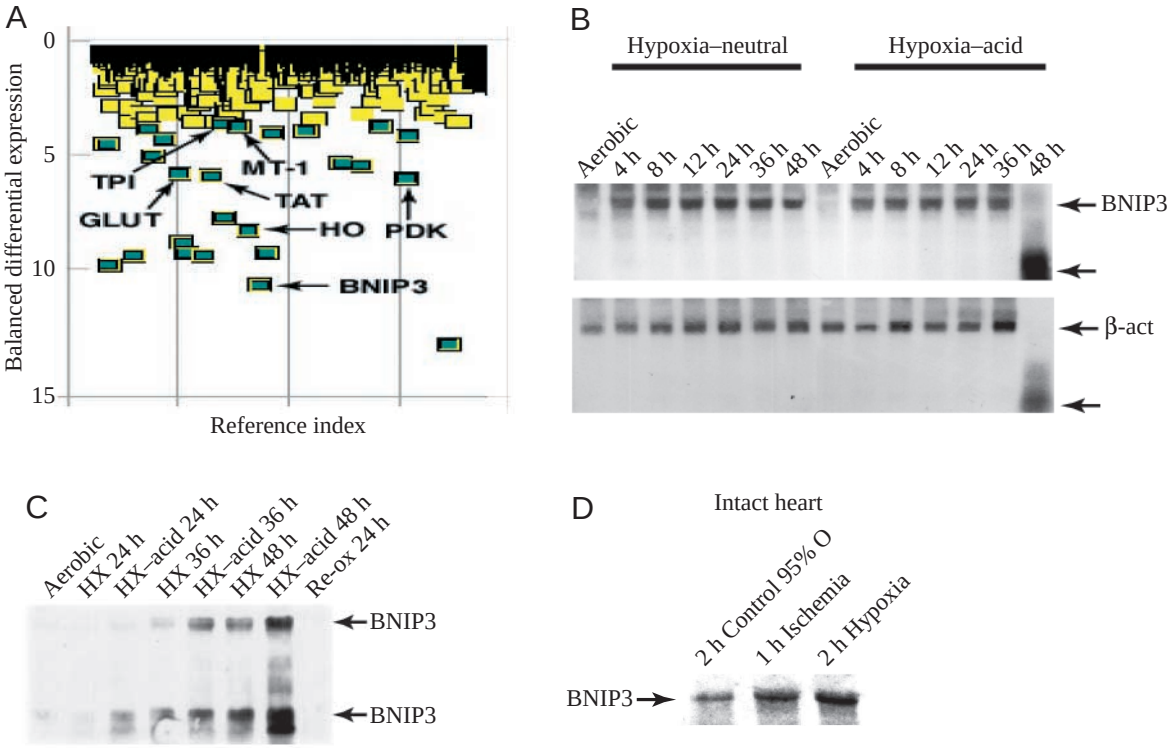

Fig. 5. (A) Analysis of mRNA and protein from aerobic and hypoxic (24 h) cardiac myocytes. Parallel plates of cardiac myocytes were cultured under aerobic or hypoxic conditions for 24 h and then harvested for RNA extraction. RNA was purified and reverse transcribed by standard procedures. Differentially labeled cDNAs were hybridized to arrays of 20 000 specific gene sequences (known and unknown cDNAs and expressed sequence tags) from combined rat and mouse libraries (Incyte Inc., Freemont, CA, USA). Data were analyzed using Excel 98 software. Hypoxia-regulated marker genes including heme oxygenase (HO), glucose transporter (GLUT), pyruvate dehydrogenase kinase (PDK), triosephosphate isomerase (TPI), tyrosine amino transferase (TAT) and metallothionein (MT-1) are shown. (B) Northern blot of cardiac myocyte RNA extracted from hypoxic cultures. The top gel shows BNIP3; the bottom gel shows β-actin. (C) Western blot analysis of proteins from hypoxic cardiac myocytes as in A. Anti-BNIP3 recognizes two bands at ~60 kDa and 30 kDa, corresponding to SDS-resistant homodimers and monomers, respectively. Lower panels show the same blot probed with anti-Bax, Bak and β-actin. (D) Rat hearts were removed and perfused by the Langendorf method, as described previously (Webster et al., 1999). Hearts were subjected to no flow for 1 h or to perfusate equilibrated with 100% N_2 for 2 h. RNA was analyzed by northern blot, as above.

2002). However, these results do not explain why cell death requires acidosis because BNIP3 accumulated during hypoxia at both neutral and acidic pH. Therefore, acidosis may be required to activate BNIP3. Because Bcl-2 proteins can regulate the MPTP by integrating into the outer mitochondrial membrane, we hypothesized that activation may involve a pH-dependent translocation from the cytosolic compartment to membrane compartments, particularly mitochondrial membranes. To test this possibility, cells were separated into subcellular fractions after exposure to hypoxia and hypoxia–acidosis. Membranes were prepared and treated with strong alkali to dislodge loosely membrane-associated protein (Kubasiak et al., 2002). Results from western blots of untreated and alkali-solubilized fractions are shown in Fig. 6. BNIP3 levels were initially detected in the alkalinized cytoplasmic fraction at 12 h of hypoxia and increased progressively at 24 h and 48 h. BNIP3 was present exclusively in the mitochondrial fraction of hypoxic samples without alkali treatment and was primarily mitochondrial in hypoxia–acidosis samples. Alkaline treatment caused a significant shift of BNIP3 into the cytoplasmic fraction from hypoxic–neutral but not hypoxic–acidic treatments. In 48 h hypoxia–neutral samples, 73% of BNIP3 was in the cytoplasm compared with <10% of the hypoxia–acidosis sample. These results show that acidosis promotes a stronger alkali-resistant association of BNIP3 with mitochondrial membranes and support our hypothesis that BNIP3 activation involves the pH-mediated membrane integration.

Hypoxia–acidosis: an atypical programmed death pathway

Overexpression of BNIP3 by transient transfection of cell lines has been reported to activate a necrosis-like pathway that

includes early loss of plasma membrane integrity (Ray et al., 2000; Vande Velde et al., 2000). We did not find this to be the case for hypoxia–acidosis (Kubasiak et al., 2002). Progressive hypoxia–acidosis with >60% cell death caused <20% loss of membrane integrity even at the late time points as determined by Trypan blue exclusion or lactate dehydrogenase (LDH) release (Fig. 7A). These results favor a more apoptosis-like pathway. However, as shown in Fig. 7B, the death pathway was unaffected by the broad-range caspase inhibitor Boc-D (or ZVAD; not shown) at a concentration that completely blocked apoptosis by staurosporine, implying atypical apoptosis. Carrying these arguments a step further, BNIP3 associates with mitochondrial (and ER) membranes and has been shown to hererodimerize with the protective proteins Bcl-2 and Bcl-XL (Ray et al., 2000). Our experiments confirm that the opening of the MPTP is also a central feature of death by hypoxia–acidosis. Treatment of cultures with the MPTP inhibitors bongrekic acid (BA) and decylubiquinone (DUB) significantly blocked DNA ladders (Fig. 8A), and Fig. 8B shows directly that the MPTP opens during hypoxia–acidosis. Aerobic cultures retained the mitochondrial dye MitoTracker Red whereas 48 h hypoxia–acidotic mitochondria did not. These studies support an atypical pathway of cell death that retains plasma membrane integrity, requires MPTP opening but involves no caspase activation. The principal steps in the pathway are illustrated in Fig. 9.

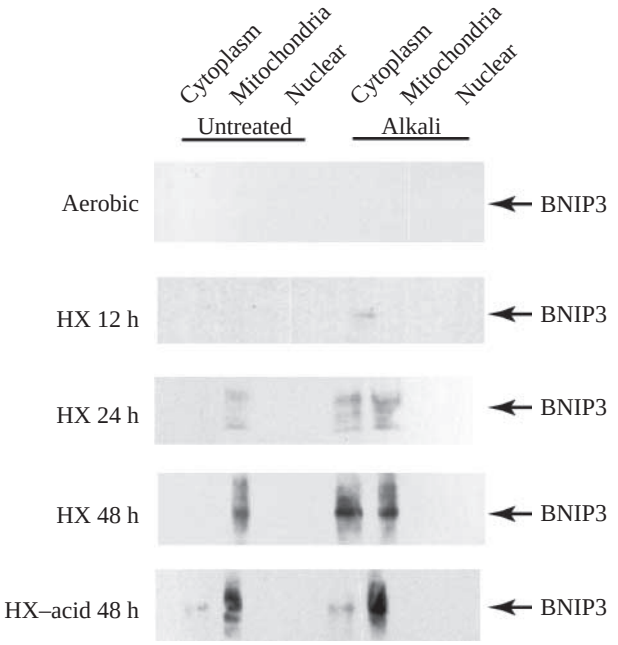

Fig. 6. Association of BNIP3 with subcellular fractions. Cardiac myocytes were subjected to hypoxia as described in Fig. 1. At the indicated times, cells were harvested, rinsed and lysed (Kubasiak et al., 2002). Lysed cells were untreated (left panels) or subjected to alkaline solubilization (right) (Kubasiak et al., 2002). The pH of each sample was verified by sampling small aliquots. After treatments, samples were separated into subcellular fractions and analyzed by western blots. Blots were stripped and re-probed with anti-succinate dehydrogenase (Upstate Biotechnology, Lake Placid, NY, USA) probes to define the purity of fractions. Results are representative of two separate experiments.

Integration and significance of the BNIP3 pathway

All of the results from this laboratory support the argument that chronic severe hypoxia is not necessarily a lethal stress for cardiac myocytes. Lethal stress is imposed by secondary effects of hypoxia including energy depletion, metabolite accumulation (acidosis) or reoxygenation. The combination of hypoxia with acidosis signals a greater stress than hypoxia alone because it indicates that the microenvironment is limited in its life-sustaining capacity. Numerous signals from the

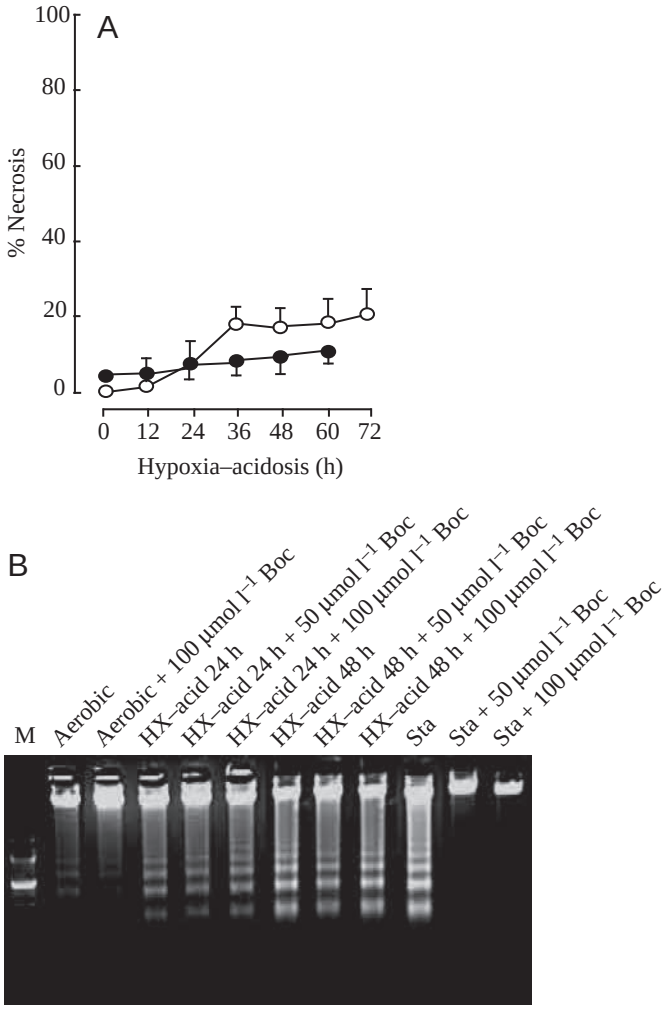

Fig. 7. Characteristics of programmed death by BNIP3. (A) Cardiac myocytes were subjected to hypoxia–acidosis as described in Fig. 2. At the indicated times, samples of media were taken for analysis of lactate dehydrogenase (LDH) activity (open circles), or plates were stained with Trypan blue (filled circles) (Kubasiak et al., 2002). Data are expressed as % of cells stained with Trypan blue or % LDH released relative to total LDH in homogenates. (B) Cardiac myocytes were subjected to hypoxia–acidosis in the absence or presence of the broad-range caspase inhibitor Boc-D as indicated. Staurosporine (Sta; 0.1 μmol l^{-1} for 8 h) is shown as a positive control.

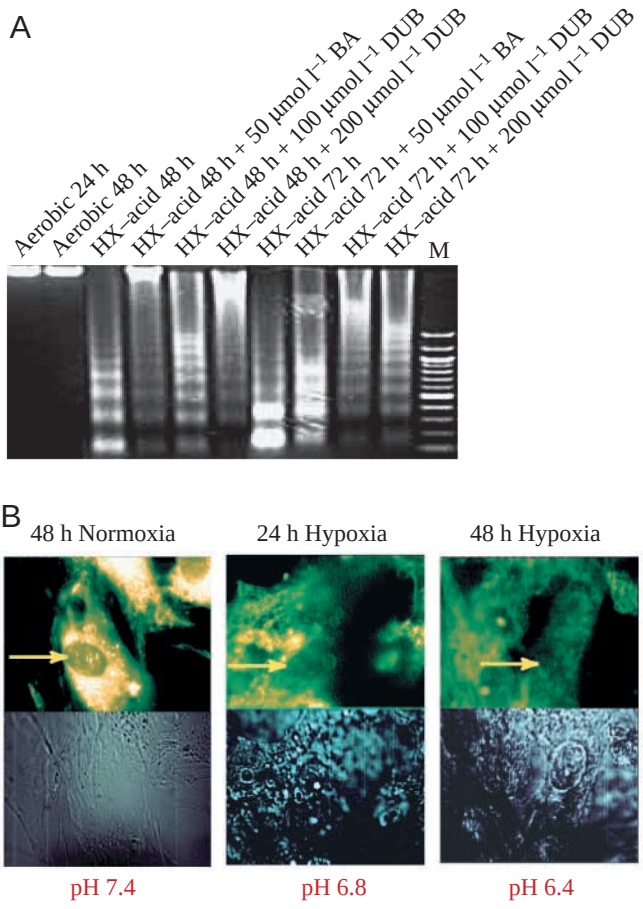

Fig. 8. (A) Cardiac myocytes were subjected to hypoxia–acidosis in the absence or presence of the mitochondrial permeability pore (MPTP) inhibitors bongrekic acid (BA) or decylubiquinone (DUB), as indicated. (B) Cardiac myocytes were exposed to normoxic or hypoxia–acidosis conditions. At the times indicated, cells were loaded with MitoTracker Red dye and analyzed by confocal microscopy as described (Kubasiak et al., 2002). Arrows indicate intense staining around nuclei in aerobic myocytes and reduced staining under hypoxia. Results are representative of three experiments.

extracellular space communicate to intracellular targets through ion channels. In the case of pH, excess protons are expelled from the intracellular compartment of cardiac myocytes through the actions of three main transporters, the Na^+/H^+ exchanger, the Na^+/HCO_3^- cotransporter and the vacuolar proton-ATPase (Karwatowska-Prokopczuk et al., 1998; Lagadic-Gossmann et al., 1992; Lazdunski et al., 1985). Under conditions of hypoxia or ischemia, each of these exchangers contributes to the maintenance of neutral intracellular pH by extruding protons. The activity of each transporter is regulated at least in part by the myoplasmic membrane pH gradient (Lazdunski et al., 1985). A decrease of [pH]o below intracellular pH ([pH]i) requires that protons are transported against the proton gradient, and as this gradient increases, transporter activities decrease, and the [pH]i falls correspondingly. If the extracellular pH falls low enough, proton extrusion stops; this has been reported to occur at a [pH]o of ~6.0 for Na^+/H^+ exchange in chick cardiac myocytes (Lazdunski et al., 1985). Therefore, when extracellular protons are not cleared, decreased [pH]o will be paralleled by a corresponding or even greater drop of [pH]i. Massive cell death in our model of hypoxia–acidosis occurs between pH 6.5 and 6.0 and this probably coincides with the failure of the Na^+/H^+ exchanger. However, apoptosis is initiated well before there is significant loss of ATP, and the pathway is unlikely to involve significant contributions from the ATP-sensitive

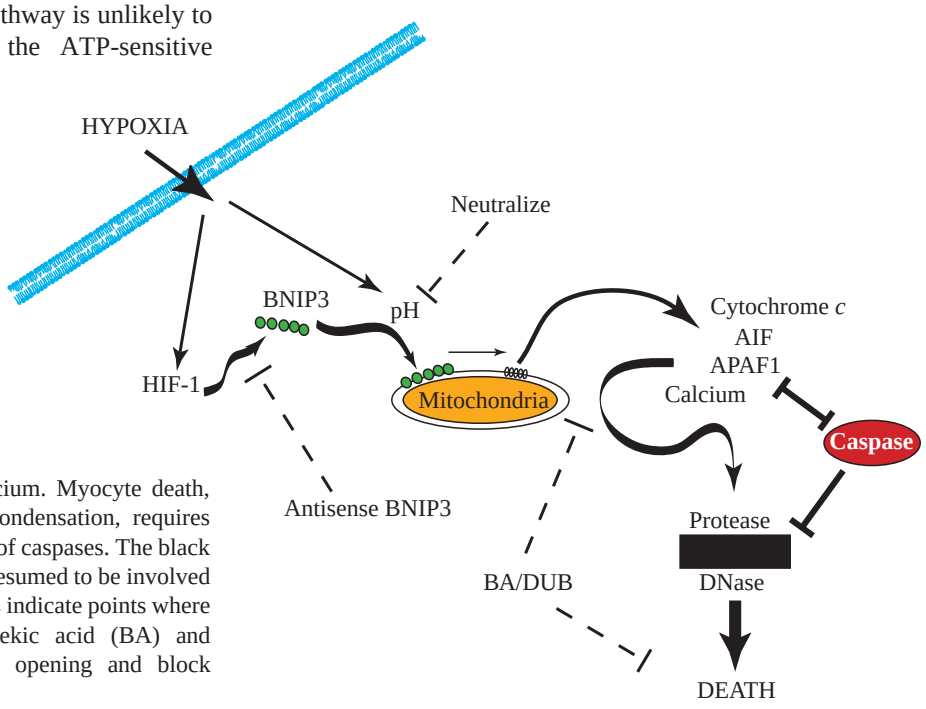

Fig. 9. Pathway of cardiac myocyte death by exposure to hypoxia–acidosis. Hypoxia mediates accumulation of hypoxia-inducible factor-1α (HIF-1α), and activated HIF-1 induces transcription of BNIP3. BNIP3 is loosely membrane bound at neutral pH but translocates into membranes, including mitochondria, when the pH decreases. Acidosis is caused by anaerobic metabolism. BNIP3 stimulates opening of the mitochondrial permeability transition pore (MPTP), releasing pro-apoptotic factors including apoptosis inducing factor (AIF), cytochrome *c* and calcium. Myocyte death, involving DNA fragmentation and nuclear condensation, requires MPTP opening but does not involve activation of caspases. The black box indicates that the proteases and DNases, presumed to be involved in cell death, are not yet identified. Broken lines indicate points where the death pathway can be blocked. Bongrekic acid (BA) and decylubiquinone (DUB) both inhibit MPTP opening and block myocyte death.

channels, either the vacuolar ATPase or the ATP-sensitive K^+ channel (Forgac, 1989; Gottlieb et al., 1996; Karwatowska-Prokopczuk et al., 1998).

The extreme effect of acidosis on survival of myocytes under hypoxia may explain why other groups have reported that hypoxia alone promotes apoptosis (Tanaka et al., 1994; Long et al., 1997; Matsui et al., 1999; Kang et al., 2000; Regula et al., 2002). BNIP3 is induced under all conditions of hypoxia and may undergo low-level activation even at neutral [pH]o. Although our results show that acid build-up drives newly synthesized BNIP3 into membranes, it is likely that prolonged hypoxia will promote a continuous low-level BNIP3 activation because of the increased effective proton activity. Hypoxic cells produce >10-fold more acid than aerobic cells with a corresponding increase of proton flux. This condition must lead to the establishment of a new ionic equilibrium involving a net increase of intra- and extracellular H^+ (Webster and Bishopric, 1992; Webster et al., 1994). This change may be sufficient to stimulate low-level BNIP3 translocation in some cells and a consequent low-level activation of the death program. Consistent with this model, proton pumps have been shown to play a role in apoptosis signaling (Anversa and Kajstura, 1998; Karwatowska-Prokopczuk et al., 1998), and acidosis has been shown to correlate with apoptosis in a number of other systems (Gottlieb et al., 1995; Perez-Sala et al., 1998).

Loss of cardiac myocytes is a central feature of heart disease of both ischemic and non-ischemic origin (reviewed in Anversa and Kajstura, 1998; Kajstura et al., 1998; Narula et al., 1996). It has been described in multiple regions of the myocardium during infarction, hibernation (Heusch and Schulz, 1996) and during both ischemia and subsequent reperfusion (Chen et al., 1997; Fliss and Gattinger, 1996). Death pathways involving necrosis, apoptosis and oncosis have been described (reviewed in Kajstura et al., 1996; Ohno et al., 1998). The probability that hypoxia and acidosis coexist in diseased and/or infarcted myocardial tissue is high because of the disrupted vasculature and elevated lactic acid production in ischemic tissue (Chen et al., 1997; Krayenbuehl and Hess, 1992). Direct measurements of myocardial tissue pH indicate that it drops by 1–2 units within 10 min of ischemia (Marzouk et al., 2002). Therefore, BNIP3 may be expected to play a significant role in cell loss during ischemic heart disease. Combined hypoxia and acidosis reflects a greater hemodynamic disruption than acidosis or hypoxia alone, and the dual signal may provide a selective advantage by activating the death pathway only as a last resort. Acidosis may also activate BNIP3 in skeletal muscle to allow myocyte drop-out under conditions of severe ischemia or hypoxia where the probability of irreversible damage is higher.

Further work is required to characterize this pathway of cardiac myocyte death. We need to determine the contribution of BNIP3 to tissue loss in the intact heart during ischemic heart disease and during infarction. To what extent does this pathway contribute to acute and chronic cell loss during coronary artery disease and congestive heart failure? If this is substantial then it will be important to develop methods to selectively block the pathway. Previous work from this and another laboratory (Kubasiak et al., 2002; Regula et al., 2002) indicates that N-terminal deletions of BNIP3 that lack the transmembrane domain are protective. It may be possible to develop cell-permeable peptides or other small organic mimics that can block BNIP activation very specifically. Finally, the requirement of this death pathway for MPTP opening but absence of caspase activation remains a paradox.

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References

Adams, J. M. and Cory, S. (2001). Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem. Sci.* **26**, 61-66.

Allen, D. G., Lee, J. A. and Smith, G. L. (1989). The consequences of simulated ischaemia on intracellular Ca^{2+} and tension in isolated ferret ventricular muscle. *J. Physiol.* **410**, 297-323.

Allen, D. G. and Orchard, C. H. (1987). Myocardial contractile function during ischemia and hypoxia. *Circ. Res.* **60**, 153-168.

Anversa, P. and Kajstura, J. (1998). Myocyte cell death in the diseased heart. *Circ. Res.* **82**, 1231-1233.

Bishopric, N. H., Andreka, P., Slepak, T. and Webster, K. A. (2001). Molecular mechanisms of apoptosis in the cardiac myocyte. *Curr. Opin. Pharmacol.* **1**, 141-150.

Boise, L. H., Gottschalk, A. R., Quintans, J. and Thompson, C. B. (1995). Bcl-2 and Bcl-2-related proteins in apoptosis regulation. *Curr. Top. Microbiol. Immunol.* **200**, 107-121.

Bond, J. M., Chacon, E., Herman, B. and Lemasters, J. J. (1993). Intracellular pH and Ca^{2+} homeostasis in the pH paradox of reperfusion injury to rat neonatal cardiac myocytes. *Am. J. Physiol.* **265**, C129-C137.

Bruick, R. K. (2000). Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc. Natl. Acad. Sci. USA* **97**, 9082-9087.

Budinger, G. R., Duranteau, J., Chandel, N. S. and Schumacker, P. T. (1998). Hibernation during hypoxia in cardiomyocytes. Role of mitochondria as the O_2 sensor. *J. Biol. Chem.* **273**, 3320-3326.

Buja, L. M., Eigenbrodt, M. L. and Eigenbrodt, E. H. (1993). Apoptosis and necrosis: basic types and mechanisms of cell death. *Arch. Pathol. Lab. Med.* **117**, 1208-1214.

Buja, L. M. and Entman, M. L. (1998). Modes of myocardial cell injury and cell death in ischemic heart disease. *Circulation* **98**, 1355-1357.

Chen, C., Ma, L., Linfert, D. R., Lai, T., Fallon, J. T., Gillam, L. D., Waters, D. D. and Tsongalis, G. J. (1997). Myocardial cell death and apoptosis in hibernating myocardium. *J. Am. Coll. Card.* **30**, 1407-1412.

Crompton, M. (2000). Bax, Bid and the permeabilization of the mitochondrial outer membrane in apoptosis. *Curr. Opin. Cell Biol.* **12**, 414-419.

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.

Dougherty, C. J., Kubasiak, L., Prentice, H., Andreka, P., Bishopric, N. H. and Webster, K. A. (2002). Activation of c-Jun *N*-terminal kinase promotes survival of cardiac myocytes after oxidative stress. *Biochem. J.* **362**, 561-571.

Dougherty, C. J., Kubasiak, L. A., Frazier, D. P., Li, H., Xiong, W.-C., Bishopric, N. H. and Webster, K. A. (2004). Mitochondrial signals initiate the activation of c-Jun *N*-terminal kinase (JNK) by hypoxia-reoxygenation. *FASEB J.* **18**, 1060-1070.

Earshaw, W., Martins, L. and Kaufmann, S. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* **68**, 383-424.

Fliss, H. and Gattinger, D. (1996). Apoptosis in ischemic and reperfused rat myocardium. *Circ. Res.* **79**, 949-956.

Forgac, M. (1989). Structure and function of vacuolar class of ATP-driven proton pumps. *Physiol. Rev*. **69**, 765-796.

Gottlieb, R. A., Burleson, K. O., Kloner, R. A., Babior, B. M. and Engler, R. L. (1994). Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J. Clin. Invest*. **94**, 1612-1628.

Gottlieb, R. A., Giesing, H. A., Zhu, J. Y., Engler, R. L. and Babior, B. M. (1995). Cell acidification in apoptosis: granulocyte colony-stimulating factor delays programmed cell death in neutrophils by up-regulating the vacuolar H+ ATPase. *Proc. Natl. Acad. Sci. USA* **92**, 5965-5968.

Gottlieb, R. A., Gruol, D. L., Zhu, J. Y. and Engler, R. L. (1996). Preconditioning in rabbit cardiomyocytes. *J. Clin. Invest*. **97**, 2391-2398.

Gottlieb, R. A. (2003). Cytochrome P450: major player in reperfusion injury. *Arch. Biochem. Biophys.* **420**, 262-267.

Green, D. and Reed, J. (1998). Mitochondria and apoptosis. *Science* **281**, 1309-1314.

Heusch, G. and Schulz, R. (1996). Hibernating myocardium: a review. *J. Mol. Cell Cardiol*. **28**, 2359-2372.

Hochachka, P. W., Clark, C. M., Holden, J. E., Stanley, C., Ugurbil, K. and Menon, R. S. (1996). Unifying theory of hypoxia tolerance: Molecular/metabolic defence and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* **93**, 9493-9498.

Hockenbery, D. M. (1995). Bcl-2, a novel regulator of cell death. *BioEssays* **17**, 631-638.

Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M, Milliman, C. L. and Korsmeyer, S. J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241-251.

Horwitz, L. D., Fennessey, P. D., Shikes, R. H. and Kong, Y. (1994). Marked reduction in myocardial infarct size due to prolonged infusion of an antioxidant during reperfusion. *Circulation* **89**, 1792-1801.

Jennings, R. B., Steebergen, C. and Reimer, K. A. (1995). Myocardial ischemia and reperfusion. *Monogr. Pathol*. **37**, 47-80.

Kajstura, J., Cheng, W., Reiss, K. and Clark, W. A. (1996). Apoptotic and necrotic myocyte cell death are independent contributing variables of infarct size. *Lab. Invest.* **74**, 86-107.

Kajstura, J., Leri, A., Finato, N., Loreto, C., Beltrami, C. A. and Anversa, P. (1998). Myocyte proliferation in end-stage cardiac failure in humans. *Proc. Natl. Acad. Sci. USA* **95**, 8801-8805.

Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T. and Bredesen, D. E. (1993). Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* **262**, 1274-1276.

Kang, P. M., Haunsetter, A., Aoki, H., Usheva, A. and Izumo, S. (2000). Morphological and molecular characterization of adult cardiomyocyte apoptosis during hypoxia and reoxygenation. *Circ. Res*. **87**, 118-125.

Karwatowska-Prokopczuk, E., Nordberg, J. A., Li, H. L., Engler, R. L. and Gottlieb, R. A. (1998). Effect of vacuolar proton ATPase on pHi, Ca^{2+}, and apoptosis in neonatal cardiac myocytes during metabolic inhibition/recovery. *Circ. Res.* **82**, 1139-1144.

Korsmeyer, S. J. (1995). Regulators of cell death. *Trends Genet*. **11**, 101-105.

Krayenbuehl, H. P. and Hess, O. M. (1992). Postischemic myocardial dysfunction: the hibernating myocardium. *J. Myocard. Ischemia* **4**, 49-58.

Kubasiak, L. A., Hernandez, O. M., Bishopric, N. H. and Webster, K. A. (2002). Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. *Proc. Natl. Acad. Sci. USA* **99**, 12825-12830.

Kumar, D. and Jugdutt, B. I. (2003). Apoptosis and oxidants in the heart. *J. Lab. Clin. Med*. **142**, 288-297.

Lagadic-Gossmann, D., Buckler, K. J. and Vaughan-Jones, R. D. (1992). Role of bicarbonate in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. *J. Physiol*. **458**, 361-384.

Lawrence, M. S., Ho, D. Y., Sun, G. H., Steinberg, G. K. and Sapolsky, R. M. (1996). Overexpression of Bcl-2 with herpes simplex virus vectors protects CNS neurons against neurological insults in vitro and in vivo. *J. Neurosci*. **16**, 486-496.

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.

Li, J. and Eastman, A. (1995). Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. *J. Biol. Chem*. **270**, 3203-3211.

Long, X., Boluyt, M. O., Hipolito, M. L., Lundberg, M. S., Zheng, J. S., O'Neil, L., Cirielli, C., Lakatta, E. G. and Crow, M. T. (1997). p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes. *J. Clin. Invest*. **99**, 2635-2643.

Long, X., Crow, M. T., Sollott, S. J., O'Neill, L., Menees, D. S., Hipolito, M., Boluyt, M. O., Asai, T. and Lakatta, E. G. (1998). Enhanced expression of p53 and apoptosis induced by blockade of the vacuolar proton ATPase in cardiomyocytes. *J. Clin. Invest*. **101**, 1453-1461.

Majno, G. and Joris, I. (1995). Apoptosis, oncosis, and necrosis: an overview of cell death. *Am. J. Pathol*. **146**, 3-15.

Mani, K. and Kitsis, R. N. (2003). Myocyte apoptosis: programming ventricular remodeling. *J. Am. Coll. Cardiol*. **41**, 761-764.

Marzouk, S. A., Buck, R. P., Dunlap, L. A., Johnson, T. A. and Cascio, W. E. (2002). Measurement of extracellular pH, K(+), and lactate in ischemic heart. *Anal. Biochem*. **308**, 52-60.

Matsui, T., Ling, L., del Monte, F., Fukui, Y., Franke, T. F., Hajjar, R. J. and Rosenzweig, A. (1999). Adenoviral gene transfer of activated phosphatidylinositol 3′-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. *Circulation* **100**, 2373-2379.

Narula, J., Haider, N., Virmani, R., DiSalvo, T. G., Kolodgie, F. D., Hajjar, R. J., Schmidt, Y., Semigran, M. J., Dec, G. W. and Khaw, B.-A. (1996). Apoptosis in myocytes in end-stage heart failure. *New Engl. J. Med.* **1182**, 1182-1189.

Narula, J., Hajjar, R. J. and Dec, G. W. (1998). Apoptosis in the failing heart. *Cardiol. Clinics* **16**, 691-710.

Neely, J. R. and Grotyohann, L. W. (1984). Role of glycolytic products in damage to ischemic myocardium. *Circ. Res*. **55**, 816-824.

Ohno, M., Takemura, G., Ohno, A., Misao, J., Hayakawa, Y., Minatoguchi, S., Fujiwara, T. and Fujiwara, H. (1998). "Apoptotic" myocytes in infarct area in rabbit hearts may be oncotic myocytes with DNA fragmentation: analysis by immunogold electron microscopy combined with in situ nick end-labeling. *Circulation* **98**, 1422-1430.

Perez-Sala, D., Collado-Escobar, D. and Mollinedo, F. (1998). Intracellular alkalinization suppresses lovaststin-induced apoptosis in HL-60 cells through the inactivation of a pH-dependent endonuclease. *J. Biol. Chem*. **270**, 6235-6242.

Pierce, G. N. and Czubryt, M. P. (1995). The contribution of ionic imbalance to ischemia/reperfusion-induced injury. *J. Mol. Cell Cardiol*. **27**, 53-63.

Ray, R., Chen, G., Vande Velde, C., Cizeau, J., Park, J. H., Reed, J. C., Gietz, R. D. and Greenberg, A. H. (2000). BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. *J. Biol. Chem.* **275**, 1439-1448.

Regula, K. M., Ens, K. and Kirshenbaum, L. A. (2002). Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circ. Res.* **91**, 226-231.

Reimer, K. A. and Ideker, R. E. (1987). Myocardial ischemia and infarction: anatomic and biochemical substrates for ischemic cell death and ventricular arrhythmias. *Hum. Pathol.* **18**, 462-475.

Shimada, Y., Hearse, D. J. and Avkiran, M. (1996). Impact of extracellular buffer composition on cardioprotective efficacy of Na^+/H^+ exchange inhibitors. *Am. J. Physiol*. **270**, H692-H700.

Shimizu, S., Eguchi, Y., Kamiike, W., Funahashi, Y., Mignon, A., Lacronique, V., Matsuda, H. and Tsujimotot, Y. (1998). Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. *Proc. Natl. Acad. Sci. USA* **95**, 1455-1459.

Tanaka, M., Ito, H., Adachi, S., Akimoto, H., Nishikawa, T., Marumo, F. and Hiroe, M. (1994). Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ. Res*. **75**, 426-433.

Todor, A., Sharov, V. G., Tanhehco, E. J., Silverman, N., Bernabei, A. and Sabbah, H. N. (2002). Hypoxia-induced cleavage of caspase-3 and DFF45/ICAD in human failed cardiomyocytes. *Am. J. Physiol. Heart Circ. Physiol*. **283**, H990-H995.

Valen, G. (2003). Cellular signalling mechanisms in adaptation to ischemia-induced myocardial damage. *Ann. Med*. **35**, 300-307.

Vande Velde, C., Cizeau, J., Dubik, D., Alimonti, J., Brown, T., Israels, S., Hakem, R. and Greenberg, A. H. (2000). BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol. Cell. Biol*. **20**, 5454-5468.

Vanoverschelde, J.-L. J., Wijns, W., Borgers, M., Heyndrickx, G., Depre, C., Flambeng, W. and Melin, J. A. (1997). Chronic myocardial hibernation in humans. *Circulation* **95**, 1961-1971.

Webster, K. A. and Bishopric, N. H. (1992). Molecular regulation of cardiac myocyte adaptations to chronic hypoxia. *J. Mol. Cell. Cardiol*. **24**, 741-751.

Webster, K. A., Discher, D. and Bishopric, N. H. (1993). Induction and nuclear accumulation of fos and jun proto-oncogenes in hypoxia cardiac myocytes. *J. Biol. Chem*. **268**, 16852-16859.

Webster, K. A., Discher, D. J. and Bishopric, N. H. (1994). Regulation of fos and jun immediate-early genes by redox or metabolic stress in cardiac myocytes. *Circ. Res*. **75**, 361-371.

Webster, K. A., Discher, D. J., Kaiser, S., Hernandez, O. M., Sato, B. and Bishopric, N. H. (1999). Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53. *J. Clin. Invest*. **104**, 239-252.

Webster, K. A., Discher, D. J., Hernandez, O. M., Yamashita, K. and Bishopric, N. H. (2000). A glycolytic pathway to apoptosis of hypoxic cardiac myocytes. Molecular pathways of increased acid production. *Adv. Exp. Med. Biol*. **475**, 161-175.

Williams, F. M., Kus, M., Tanda, K. and Wlliams, T. J. (1994). Effect of duration of ischaemia on reduction of myocardial infarct size by inhibition of neutrophil accumulation using an anti-CD18 monoclonal antibody. *Br. J. Pharmacol*. **111**, 1123-1128.

Yermolaieva, O., Xu, R., Schinstock, C., Brot, N., Weissbach, H., Heinemann, S. H. and Hoshi, T. (2004). Methionine sulfoxide reductase A protects neuronal cells against brief hypoxia/reoxygenation. *Proc. Natl. Acad. Sci. USA* **101**, 1159-1164.

Yoshida, T., Watanabe, M., Engelman, D. T., Engleman, R. M., Schley, J. A., Maulik, N., Ho, Y. S., Oberley, T. D. and Das, D. K. (1996). Transgenic mice overexpressing glutathione peroxidase are resistant to myocardial ischemia reperfusion injury. *J. Mol. Cell. Cardiol.* **28**, 1759-1767.

Zhang, H. M., Cheung, P., Yanagawa, B., McManus, B. M. and Yang, D. C. (2003). BNips: a group of pro-apoptotic proteins in the Bcl-2 family. *Apoptosis* **8**, 229-236.

Zuurbier, C. J., van Iterson, M. and Ince, C. (1999). Functional heterogeneity of oxygen supply-consumption ratio in the heart. *Cardiovasc. Res.* **44**, 488-497.