ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 9 & 10, 2006

Forum Review

Redox Stress and the Contributions of BH3-Only Proteins to Infarction

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ABSTRACT

Ischemia followed by reperfusion is the primary cause of tissue injury and infarction during heart attack and stroke. The initiating stimulus is believed to involve reactive oxygen species that are produced during reperfusion when electron transport resumes in the mitochondria after suppression by ischemia. Programmed death has been shown to be a significant component of infarction, and evidence indicates that multiple pathways are initiated during both ischemia and reperfusion phases. Major infarction is preceded by severe ischemia that includes hypoxia, intracellular acidosis, glucose depletion, loss of ATP, and elevation of cytoplasmic calcium. The superimposition of a reactive oxygen surge on the latter condition provides the impetus for maximal damage. Compelling evidence implicates mitochondria not only as the source of initiating ROS but also as the focal sensors that translate the redox stress signal into a cellular-death response. Pivotal to this response are the BH3-only proteins that are activated by death signals and regulate mitochondrial communication with executioner proteins in the cytoplasm. The BH3-only proteins do this by controlling the activity of pores and channels in the outer mitochondrial membrane. To date at least six BH3-only proteins have been shown to contribute to ischemia–reperfusion death pathways in heart and/or brain; these include Bnip3, PUMA, Bid, Bad, HGTD-P, and Noxa. Here we review the evidence for these cell-death pathways and discuss their relevance to ischemic disease and infarction. *Antioxid. Redox Signal.* 8, 1667–1676.

INTRODUCTION

Although components of both the intrinsic and extrinsic pathways have been reported to contribute to programmed death in ischemia–reperfused hearts and brains, the intrinsic pathway is most frequently implicated. In the extrinsic pathway, receptor-specific ligands bind to death domain–containing receptors, which undergo trimerization and recruit the adapter proteins FADD and TRADD. Binding of the adapter proteins to the death domains initiates the proteolytic cleavage of procaspases 8, 10, and 2, leading to the activation of caspases 7, 6, and 3 (7, 12, 77, 92). Tumor necrosis factor-α (TNF-α) and Fas ligand as well as other cytokine receptors have been implicated in animal models of myocardial ischemia as well as in the progression of heart failure in patients. However, blocking the intrinsic pathway with antagonists of the TNF-αR provided no clinical benefit during myocardial ischemia, and it was recently demonstrated that genetic deletion of the Fas receptor or Fas ligand did not affect infarct size in a mouse model of MI (87). These observations suggest that infarction due to ischemia–reperfusion can occur independent of the activities of TNF-α and Fas-L signaling. The intrinsic apoptotic pathway is controlled by Bcl-2 family members that regulate the release of proapoptotic proteins and accessory molecules from the intermembrane space of the mitochondria. Both phases of ischemia and reperfusion provide signals that combine to induce and activate proapoptotic Bcl-2 proteins. Hypoxia, an obligatory component of ischemia, primes the intracellular environment for apoptosis by influencing multiple metabolic and signaling pathways, including electron transport and mitochondrial ion channels, calcium flux, intracellular pH, glucose, and ATP. Hypoxia can

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induce the endoplasmic reticulum (ER) stress response that activates the proapoptotic BH3-only protein PUMA, and hypoxia mediates the direct induction of several other proapoptotic genes, including Bnip3, Noxa, and HGTD-P through the hypoxia-inducible transcription factor 1-α (HIF-1α) (3, 43, 50). Antioxidant levels may be reduced during ischemia, contributing to reactive oxygen species (ROS) accumulation at reperfusion (39, 48). A primary role for oxidative stress in myocardial injury is supported by studies on transgenic mice over- or underexpressing antioxidant pathway genes (9, 39). The contribution of the Bcl-2 family is similarly supported by transgenic mouse studies with gain or loss of function of Bcl-2 family genes (37, 86).

OXIDATIVE STRESS

Pivotal roles for ROS in myocardial infarction (MI), ischemia–reperfusion injury, and heart failure are well documented (12, 93). ROS are generated primarily from mitochondria within the ischemic tissues and secondarily from NAD(P)H oxidases in vascular cells and myocytes, as well as from neutrophils infiltrating the sites of injury. ROS are also generated as a by-product of ATP catabolism; xanthine oxidase and hypoxanthine levels increase during ischemia, and superoxide is produced when hypoxanthine is oxidized to xanthine by xanthine oxidase and again when xanthine is further oxidized to urate. In the vasculature, ROS interact with NO, generating the highly reactive peroxinitrite ($ONOO^{\bullet}$) radical, with the dual effect of decreasing the bioavailability of NO, causing vasoconstriction, and aggravating oxidative damage. ROS initiate primary damage by direct oxidation of cellular components including proteins, lipids, and DNA, and secondarily, they modulate signaling pathways that influence cell fate. Protein kinases including protein kinase C mitogen-activated kinases and phosphoinositol-3 kinases and associated phosphatases are particularly relevant to the regulation of Bcl-2 proteins. In reperfused ischemic hearts, the high level of oxidative stress is caused by a combination of increased ROS production and decreased antioxidant defense (31, 40). Genetic overexpression of catalase (51), glutathione peroxidase (GPX) (104), or Cu/ZnSOD (9, 13, 94) confers cardioprotection, whereas deficiency of GPX (102) or Cu/ZnSOD (103) exacerbates the extent of ischemic injury to the myocardium. Similar trends have been reported in stroke models. Despite this, results from experiments testing the cardioprotective function of exogenous enzymatic and nonenzymatic antioxidants have been variable, and patient studies, mostly negative (57, 61, 65, 72). As a consequence, neither antioxidant nor anti-inflammatory strategies are used in the clinical treatment of MI (23, 39, 48). The inability to target antioxidants to the mitochondria probably accounts for the lack or therapeutic benefit, a possibility that is supported by the greater protection afforded by MnSOD (mitochondrial) compared with Mg/ZnSOD. Similarly, whereas caspase inhibitors limit cardiac myocyte apoptosis after ischemia–reperfusion in animal models, they have not been shown to be clinically therapeutic (2, 8, 74, 85, 97).

Bcl-2 FAMILY OF APOPTOSIS REGULATORS

The Bcl-2 proteins comprise a protein family of at least 22 members that regulate programmed cell death pathways in response to a wide variety of stimuli (4, 35, 46, 79). In addition to Bcl-2, the genes in this family include *Bcl-Xl, Mcl-1, A1, Bcl-W,* and *CED-9* that are anti-apoptotic, and *Bak, Bax, Bcl-XS, Diva,* and *Mtd/Bok* that are proapoptotic (*Bcl-XS* arises from the same gene as *Bcl-XL* by alternative splicing and a shift in the reading frame). These proteins are usually associated with the cell membranes, particularly the mitochondria, ER, and nuclear envelope, where they are anchored by a COOH-terminal domain. Bcl-2 proteins contain regions of amino-acid sequence similarity known as Bcl-2 homology (BH) domains. These domains are involved in protein–protein interactions, including homo- and heterooligomerization with other Bcl-2 family members. Bcl-2 has four domains (BH1–BH4), although only the BH4 domain is required for the anti-apoptotic function (51). Individual Bcl-2 family members may remain in the cytosol or be loosely membrane bound and translocate into the membrane after a death signal is received (1, 70, 91). 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 antiapoptotic members is an important feature of the regulation. Bcl-2 has been attributed antioxidant, prooxidant and proton-translocating properties (36, 80, 84), and a major function of the Bcl-2 family as a whole is to determine the on/off state of channels and pores in the outer mitochondrial membrane, including the mitochondrial apoptosis-inducing channel (MAC) and the mitochondrial permeability transition pore (MPTP) [reviewed in (15, 19, 27, 28)].

Bax and Bak are the prototypic proapoptotic Bcl-2 family members and contain three BH domains (BH1–BH3). Cells lacking these two proteins do not undergo outer mitochondrial membrane permeabilization in response to apoptotic stimuli and are resistant to multiple apoptotic stimuli (16). Bax is either loosely attached to the outer mitochondrial membrane or sequestered in the cytosol through interactions with different protein factors including humanin, Ku70, 14-3-3 isoforms, HSP-70, and ARC (29, 30, 59, 62, 75, 83). The active part of Bax is the C-terminal tail, which, in the latent state, is sequestered by proline 68 into a hydrophobic pocket formed by its BH1, BH2, and BH3 domains (60, 76). Activation involves a conformational change that releases Bax from its sequestered state, exposes the C-terminus, and allows translocation to the mitochondrial membrane, where oligomerization with Bak creates a transmembrane pore through which apoptotic factors are released from the mitochondrial intermembrane space. The activation of Bax and Bak is regulated by the third class of Bcl-2 proteins that contain a single BH3 domain, known as the BH3-only proteins.

In some animal ischemia models of coronary occlusion, myocardial levels of Bcl-2 proteins have been shown to decline, while proapoptotic Bax increases (35). Overexpression of Bcl-2 reduces apoptosis in some models of myocardial and neuronal ischemia (41, 49). Increasing the amount of anti-

apoptotic Bcl-2 through overexpression in cardiac myocytes has also been shown to inhibit apoptosis caused by ischemia and reperfusion both *in vitro* and *in vivo* (37, 56, 86).

BH3-ONLY PROTEINS

Bnip3, Bid, Bad, PUMA, and Noxa are members of the so-called BH3-only subfamily of Bcl-2 family proteins that antagonize the activity of prosurvival proteins, stimulate prodeath proteins, and promote apoptosis (45, 69, 91). All of these proteins are implicated in death pathways associated with ischemic damage in the heart and brain. These proteins do not possess the same (BH1, BH2, BH4) protein binding domains as the other Bcl-2 family members but are related through the common BH3 domain that with the exception of Bnip3 is required for hetero- and homodimerization. Related members of this group include Bik, Blk, Hrk, BimL, and Nix. Nix has been linked with apoptosis mediated by G-αq-mediated hypertrophy and dilated cardiomyopathy (106).

Bnip3 was originally identified as a Bcl-XL or E1B 19K-binding protein (108). Bnip3 is expressed at low levels in most organs under normal (nonischemic) conditions but is induced by hypoxia, ischemia, and reperfusion (6, 25, 47, 91, 98). Overexpression of Bnip3 by transfection of the cDNA into HEK-293 cells results in membrane translocation and initiation of the death pathway (91). In cotransfection experiments, Bnip3 was shown to interact with both Bcl-2 and Bcl-XL (69). These interactions require the transmembrane domain but not the BH3 domain, suggesting that the interaction is dissimilar to other BH3-only proteins and perhaps not specific (69). Deletion of the transmembrane domain eliminates the death function of Bnip3 and converts the remaining N-terminal portion into a dominant negative with protective functions (69). Replacement of the transmembrane domain with an ER-targeted domain from the cytochrome *b*5 protein prevented mitochondrial localization of transfected Bnip3 and reduced the death function by 50% (69, 91). Interestingly, Bnip3 with cytochrome *b*5 transmembrane domain retained the Bcl2/Bcl-XL binding property either in purified protein mixes or when cotransfected into 293 cells (69). These results suggest that Bnip3 can kill effectively even when targeted to nonmitochondrial sites. In cardiac myocytes, Bnip3 transfection alone is not sufficient to induce death but requires coincident hypoxia and/or acidosis (47). Therefore, Bnip3 death functions may be cell-type specific; indeed, adult skeletal and cardiac muscle as well as solid tumors contain high levels of Bnip3, but the cells remain fully viable (24, 91). Our results indicate that the death function of Bnip3 is activated in cardiac myocytes by acidic pH or by reoxygenation and probably involves one or more posttranslational modifications. Acidosis and reoxygenation both dramatically increased the stability and abundance of Bnip3 protein in myocytes supporting levels fivefold to 10-fold higher than hypoxia alone [(47) and Graham RM and Webster KA, unpublished)]. In the case of acidosis, enhanced stability is associated with enhanced membrane interaction and may involve a pH-mediated change in protein conformation that promotes membrane insertion (Frazier et al., *Antioxid Redox Signal* this issue pp. 1625–1634).

The Bnip3-mediated death pathway has characteristics of both apoptosis and necrosis including DNA fragmentation and MPTP opening but no activation of caspases. Van de Velde *et al.* (91) reported that transfection of 293 cells with Bnip3 causes death independently of Apaf-1, caspase activation, and cytochrome *c* release despite MPTP opening. Cell death involved increased reactive oxygen generation by mitochondria, cytoplasmic vacuolation, and mitochondrial autophagy. We also found that opening of the MPTP was necessary for Bnip3-mediated death of cardiac myocytes during exposure to hypoxia and acidosis but death occurred without significant caspase activation (47). These studies present a puzzling paradox and set Bnip3 apart from the other BH3-only proteins PUMA, Bid, Bad, and Noxa that initiate a classic intrinsic apoptotic pathway (99). Although cotransfected Bnip3 binds to Bcl-2 and Bcl-XL, these interactions have not been demonstrated for the endogenous protein. If these interactions were an integral part of the death pathway they would promote activation of the death functions of Bax and Bak and initiate the caspase cascade. Because this is not the case, it is possible that the interactions of Bnip3 with prosurvival Bcl-2 proteins is not part of the pathway. Another paradox involves opening of the MPTP that is also associated with release of intermembrane space proteins and caspase activation. In our studies, the death functions of Bnip3 were blocked by MPTP inhibitors cyclosporine A and DUB, but caspases were not activated (47). A possible explanation for both of these apparent anomalies involves the recently described mitochondrial apoptosis-induced channel (MAC). MAC is a voltage-independent outer mitochondrial membrane channel that was discovered by patch-clamping mitochondria isolated from FL5.12 cells in which apoptosis was induced by withdrawal of interleukin-3 (IL-3) [see (18, 53) for reviews]. MAC is structurally and pharmacologically distinct from the MPTP; it is activated early in the intrinsic apoptosis pathway, possibly by association with activated Bax and/or Bak and can transport cytochrome *c*. MAC may be the channel that initiates the intrinsic mitochondrial apoptotic pathway, whereas MPTP promotes a pathway more akin to necrosis (53). The paradoxes discussed here could be explained if Bnip3 activates MPTP but not MAC. Recent results from our group support this possibility; we found that calpains and caspase-independent DNases, but not caspases, are activated downstream of Bnip3 and MPTP during death by hypoxia–acidosis (26, 89). The lack of involvement of MAC may explain why cytochrome *c* is not released, but the pathway is sensitive to MPTP-selective inhibitors.

Bid is one of the most abundant and widespread mammalian BH3-only proteins. It is strongly expressed during development and remains high in many adult tissues, including the heart (21). In healthy cells, Bid is cytosolic or loosely membrane associated and functionally inert. It can be cleaved by caspases, granzyme B, and possibly calpains that remove the N-terminal fragment and create an active form known as tBid that relocates to the mitochondria, where it facilitates the opening of both MAC and MPTP, probably by interacting with Bax, and the release of proapoptotic proteins, cytochrome *c,* and Apaf-1 (53). Like Bnip3, the membrane targeting of tBid is not fully understood but may involve increased hydrophobicity of the cleaved fragment and/or

spontaneous oligomerization within the membrane, analogous to the self-aggregating properties of membrane-disrupting toxins such as aerolysin (18, 53). In one pathway, Bid is cleaved by caspase 8 after stimulation of cell death through the Fas/Fas-L or TNF-receptor pathway. Cleaved Bid translocates to the mitochondria and activates the intrinsic pathway. More caspases are activated, and more Bid is cleaved, providing a positive-feedback loop for maximal Bid cleavage and amplification of the intrinsic pathway. Truncated Bid can be further posttranslationally modified by the addition of a myristyl (long-chain unsaturated fatty acyl) group, which may promote direct interaction with Bax or Bak (107). In artificial membranes, tBid associates with Bax to produce a voltage-independent channel that can transport cytochrome *c* and has similar characteristics to MAC. Dejean *et al.* reported that MAC-like activities and cytochrome *c* release are observed when recombinant t-Bid is added to isolated mitochondria containing endogenous Bax and Bak, suggesting that t-Bid may activate MAC in vivo (17, 44).

A role for Bid has been clearly demonstrated in the apoptosis pathways associated with neuronal and myocardial ischemia *in vitro* and *in vivo* (10, 11, 67). In both cases, blocking Bid either pharmacologically or genetically reduced infarct volumes by about 50%. In the heart, Bid was activated by calpain cleavage; in the brain, Bid was cleaved by caspase 8.

Bad is another BH3-only protein that resides in the cytoplasm in healthy cells and translocates to mitochondria in response to death signals. The BH3 domain of Bad mediates its death-promoting activities by heterodimerization and neutralization of Bcl-XL and related prosurvival proteins. Growth and survival factors inhibit the death-promoting activity of Bad by stimulating phosphorylation at multiple sites, in particular Ser-112, Ser-136, and Ser-155. Phosphorylation at these sites by the survival kinases Akt (protein kinase B), protein kinase A, and MAPKAP kinase-1 (RSK1) promotes binding of Bad to 14-3-3 proteins, sequestering it away from the mitochondria. The 14-3-3 proteins are conserved dimeric regulators that have been shown to bind more than 100 cellular proteins [reviewed in (54)]. Phosphorylation at Ser/Thr sites targets proteins to 14-3-3 complexes, and Bax and tBid as well as Bad have been found associated with 14-3-3 (63, 81).

Phosphorylation of Bad by the Akt kinase at Ser-136 is an important point of cross-talk between survival kinases and death pathways. Activation of Akt by insulin or IGF-1 protects the heart against ischemic injury; Akt has also been linked with the powerful protection that is afforded by ischemic preconditioning (101). Other targets for Akt include FOXO transcription factors, caspases, glucose-transport proteins, and the mTOR complex (96). Inagaki *et al*. (38) and Murriel *et al.* (58) reported recently that myocardial ischemia–reperfusion promotes the translocation of PKC-delta to the mitochondria and this correlates with significant increases in the levels of Bad, and decreases in Bad and Akt phosphorylation. Inhibition of PKC translocation at the onset of reperfusion reduced cytochrome *c* leakage from mitochondria and apoptosis. This protection correlated with enhanced Akt activity, attenuated Bad levels, increased Bad phosphorylation, and was associated with the almost complete elimination of infarction in their model.

PUMA (p53-upregulated modulator of apoptosis) is another BH3-only protein that has been implicated in numerous apoptotic pathways involving developmental signals and cellular stress. PUMA was originally identified as a target for the tumor-suppressor transcription factor p53 and shown to play the leading role in p53-mediated apoptosis [reviewed in (105)]. PUMA can bind and sequester all of the prosurvival Bcl-2 proteins and initiates the intrinsic death pathway by activating proapoptotic Bax and Bak (99). PUMA is activated at the transcriptional level by p53 and is induced by all stimuli that activate p53, including DNA damage and oxidative stress (20, 105). In addition, PUMA mediates multiple p53-independent death pathways initiated by diverse cytotoxic stimuli, including cytokine deprivation and exposure to glucocorticoids, the kinase inhibitor staurosporine, phorbol esters, and conditions that induce ER stress and the unfolded-protein response (20, 100). Recently PUMA was implicated in the apoptotic death of neurons during brain ischemia and cardiac myocytes during myocardial ischemia–reperfusion (71, 90). In the heart, infarct sizes in the PUMA-null mice were reduced by 50% compared with wild-type or heterozygous mice, apoptotic indices were dramatically reduced, and postischemic recovery was significantly improved. Therefore, PUMA, Bnip3, Bid, and Bad are all implicated in the death pathways that are activated by ischemia.

PUMA differs from Bnip3, Bid, Bad, and Noxa insofar as its pathway of induction and activation by ischemia–reperfusion is not known. In the ischemic heart, PUMA induction does not require p53 (90, 95). Considering the apparently strong impact of PUMA on reperfusion injury, identification of its upstream regulation is extremely important. Unlike Bnip3 and Noxa, which are induced by hypoxia through promoter elements that bind the transcription factor HIF-1α, PUMA has not been reported to possess such a function and probably does not respond directly to hypoxia. So how is PUMA induced by ischemia? One possibility is that it is regulated indirectly through components of the ER-stress pathways that have been described in ischemic heart and brain (71, 100). ER stress during ischemia is predicted to occur under conditions of ATP depletion, acidosis, and abnormal ER/SR-calcium handling. In isolated hippocampal neurons, PUMA is induced by treatment of cells with the ER-stress mediators tunicamycin, a protein glycosylation inhibitor, and thapsigargin, an ER calcium ATPase inhibitor (71). Transcriptional activation of PUMA coincides with induction of the early growth response factor-1 (EGR-1) and the ER-stress–specific transcription factor CHOP, although the involvement of these factors has not been confirmed.

Two other factors that have been implicated in the transcriptional activation of PUMA are p73, a homologue of p53, and E2F1, a transcription factor that binds the retinoblastoma (Rb) protein and controls cell cycle–regulatory genes (33, 55). The relations are complex; E2F1 and p73 both transactivate PUMA directly by binding and activating the promoter. E2F1 also transactivates p73, and this may amplify the induction of PUMA (see Fig. 1). Similarly, p73 has multiple and complex roles in apoptosis. In addition to inducing PUMA, p73 also stimulates the transcription of Bax (proapoptotic) and Scotin, a protein required for initiating the ER-stress pathway (22, 88). Brains of E2F-null mice are resistant to

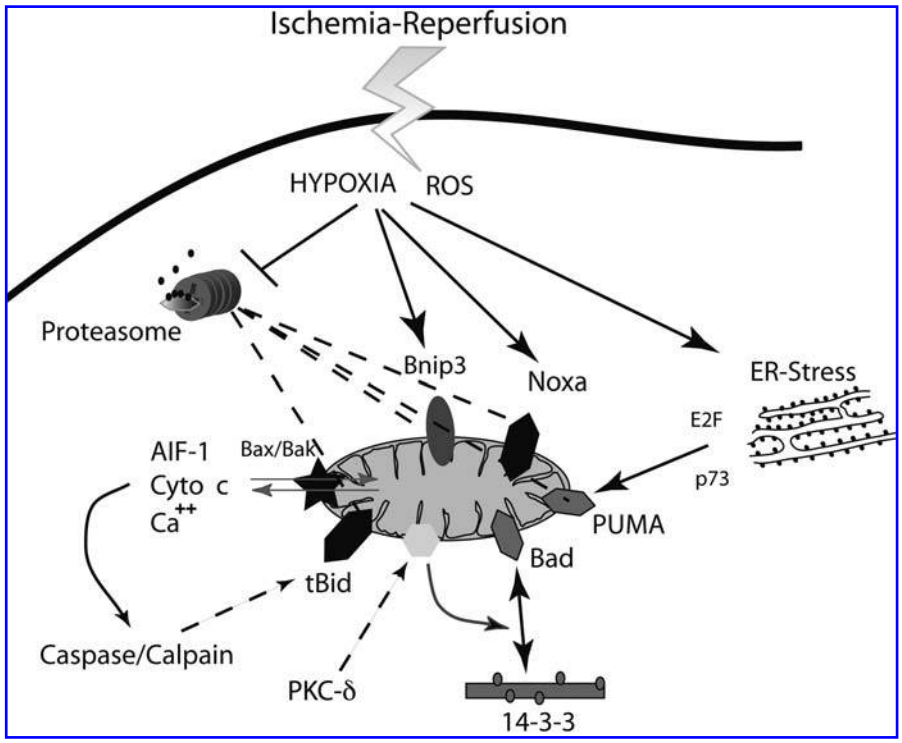

FIG. 1. Multiple BH3-only proteins mediate cell death by ischemia in the heart. Hypoxia and oxidative stress are primary consequences of ischemia in cardiac myocytes. The BH3-only proteins Bnip3 and Noxa are transcriptionally induced by hypoxia through HIF-1α binding sites in their promoters. Bnip3 is further activated by acidosis and reoxygenation. PUMA is activated indirectly by ischemia–reperfusion through the ER stress response and by transcription factors E2F and p73. Activation of p73 may be through the inactivation of the ubiquitin ligase Itch, which is sensitive to oxidative stress. Reduced Itch activity during ischemia would stabilize p73 and enhance production of PUMA. During ischemia–reperfusion, calpains are activated, and these can cleave Bid, which is normally sequestered in an inactive form in the cytoplasm. Cleavage of Bid promotes the translocation of the cleavage product, tBid, to the mitochondria. During ischemia–reperfusion, PKC-δ translocates to the mitochondria, and this modulates the phosphorylation state of Bad, perhaps by activating a phosphatase. When Bad is dephosphorylated, it is released from 14–3-3 proteins, where it is inactive, and translocates to the mitochondria. In addition to p73, Bnip3, tBid, and Noxa are all regulated by ubiquitination and proteasomal degradation. The latter activity may be suppressed during ischemia–reperfusion. Bnip3, PUMA, Bad, and tBid probably act together to neutralize Bax and Bak and permeabilize the mitochondria, promoting cell death by apoptotic and necrotic pathways.

focal ischemia, and at least one study reported that apoptosis of cardiac myocytes exposed to simulated ischemia also requires active E2F (32, 52). If E2F and/or p73 contribute to the induction of PUMA in the heart by hypoxia and/or ischemia, these conditions must activate these factor(s). One way that this could occur would be by changes in the posttranslational regulatory pathways. Some evidence suggests this. Both p53 and p73 are subject to ubiquitination and proteasomal degradation. p53 degradation is determined by its binding to the murine double-minute 2 (MDM2) ubiquitin ligase, and p73, by binding to the Hect ubiquitin ligase, Itch (73). Binding of p73 to Itch promotes ubiquitination and rapid proteasome-dependent degradation. This process normally keeps p73 at very low levels in most cells. Under conditions of stress such as DNA damage or oxidative stress, Itch is downregulated, and p73 protein levels increase. Because this p73 regulatory pathway was discovered very recently, it is not yet known whether Itch or p73 levels change during ischemia in the brain or heart. If Itch levels do not change, another pathway that could mediate an increase of p73 and PUMA in the ischemic heart is by reduced activity of the proteasome. Proteasomal activity in the heart is significantly inhibited by ischemia–reperfusion (68).

NOXA was initially identified as a PMA-responsive gene in leukemia cells (34) and subsequently shown to be a BH3-only family member regulated by both p53 and HIF-1α (43, 64) via their respective promoter-binding elements. Regulation by hypoxia and p53 is a common feature of Noxa and PUMA. Kim *et al.* (69) showed that Noxa promotes a classic intrinsic pathway of apoptosis that involves cytochrome *c* release and caspase activation. Using Soas-2 osteogenic sarcoma cells, they also demonstrated that the Noxa-mediated death pathway involved ROS generation, a property that may be shared with the Bnip3 death pathway (69, 91). Noxa expression is increased during transient brain ischemia and antisense reduced infarct size (43). Noxa expression is also increased in the heart subjected to ischemia–reperfusion (Spiga M-G and Webster KA, unpublished data).

HGTD-P and RTP801 are HIF-1α–regulated genes that may contribute to regulation of programmed death during exposure to hypoxia and ischemia (50). HGTD-P has the properties of a classic inducer of the intrinsic pathway, demonstrating integration into the mitochondrial membrane, release of cytochrome *c,* and caspase activation. Inhibition of HGTD-P with siRNA protected neuronal cells from hypoxia-mediated death (50). Roles for HGTD-P in stroke or myocardial ischemia *in vivo* have not been described. RTP801 was initially isolated by subtraction microarray screens of C6 glioma cells comparing aerobic and hypoxic culture conditions (42, 82). RTP801 levels increased after transient ischemia in the brain, and overexpression increased the sensitivity of neurons to oxidative stress and ischemic injury. Subsequent studies showed that RTP801 may have a protective rather than a death-promoting role during exposure to hypoxia. RTP801 activates the

tumor-suppressor TSC1/TSC2 complex and downregulates mTOR activity, thereby slowing growth and conserving energy. This property may provide a selective advantage during chronic hypoxia under conditions in which energy is depleted (5, 14, 66, 78). Cells with disrupted TSC1/TSC2 or RTP801 accumulate abnormally high levels of HIF-1α, maintain normal mTOR activity, and continue to divide under hypoxia. The latter properties may contribute to the proliferation of these mutant cells in hypoxic tumors. The mechanism of proapoptosis by RTP801 has not been reported but may be restricted to terminally differentiated, nondividing cells.

SIGNIFICANCE OF MULTIPLE BH3-ONLY PATHWAYS IN ISCHEMIC DEATH

The BH3-only proteins that have been causally linked with ischemic injury include PUMA, Noxa, Bid, Bad, and Bnip3. Pathways that activate PUMA, including ER-stress, EF2, and p73, also activate Noxa (88). Although Noxa has not yet been implicated in myocardial ischemia, it has been shown to contribute to neuronal ischemic damage (43). Noxa is also directly induced by hypoxia through Hif-1α and is probably activated in ischemic hearts [(71), and Spiga M-G and Webster KA, unpublished data). PUMA, Bid, Bad, and Noxa pathways all converge on the intrinsic mitochondrial pathway through direct or indirect interactions with Bax and/or Bak (99). PUMA and Bid bind and neutralize all Bcl-2 prosurvival proteins, whereas Bad and Noxa recognize only a subset and as a consequence are weaker killers that may require complementary family members for efficient killing. Enhanced mitochondrial ROS production may contribute to the death-promoting functions of Bnip3, Noxa, and RTP801. To account for the contributions of multiple BH3-only (and hypoxia-inducible non-BH3) proteins to death and infarction, it is probable that they act in concert in wild-type hearts and brains, neutralizing prosurvival Bcl-2 proteins, maximizing the activation of Bax and Bak, and opening the MAC and MPTP. By damaging mitochondria and contributing to permeability changes, Bnip3 may act in concert or independent of the other BH3-only proteins. Further work is required to determine the relations between the different component BH3-only proteins and to identify the optimal interventions for protecting the heart against these combined death pathways.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants HL44578, HL69812 (to K.A.W.), NIH HL07294 (to N.H.B.), American Heart Association fellowships (M-G. S., R.M.G., J.W.T., and A.W.), and by a Walter G. Ross endowed chair in Vascular Biology (K.A.W.).

ABBREVIATIONS

BH(1–4), Bcl-2 homology domains 1–4; MAC, mitochondrial apoptosis-inducing channel; MAPK, mitogen-activated protein kinase; MI, myocardial ischemia; MPTP, mitochondrial permeability transition pore; NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; TNF-αR, tumor necrosis factor-α receptor.

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First submission to ARS Central, April 27, 2006; date of acceptance, May 2, 2006.

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