Caspase 3 activation is essential for neuroprotection in preconditioning

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Sublethal insults can induce tolerance to subsequent stressors in neurons. As cell death activators such as ROS generation and decreased ATP can initiate tolerance, we tested whether other cellular elements normally associated with neuronal injury could add to this process. In an *in vivo* model of ischemic tolerance, we were surprised to observe widespread caspase 3 cleavage, without cell death, in preconditioned tissue. To dissect the preconditioning pathways activating caspases, and the mechanisms by which these proteases are held in check, we developed an *in vitro* model of excitotoxic tolerance. In this model, antioxidants and caspase inhibitors blocked ischemia-induced protection against *N*-methyl-D-aspartate toxicity. Moreover, agents that blocked preconditioning also attenuated induction of HSP 70; transient overexpression of a constitutive form of this protein prevented HSP 70 up-regulation and blocked tolerance. We outline a neuroprotective pathway where events normally associated with apoptotic cell death are critical for cell survival.

Prior exposure to sublethal challenges can render neuronal tissue less vulnerable to severe insults (1). Preconditioning models share several key features, including limited window of efficacy, requirement for protein synthesis, involvement of ATP sensitive K^+ (K_{ATP}) channels, and heat-shock protein (HSP) induction (2, 3). However, the underlying mechanisms mediating neuroprotection remain undefined. The up-regulation of pro-survival elements within preconditioned cells seems to depend upon activation of pathways typically associated with degeneration. For example, generation of reactive oxygen species (ROS) is critical for induction of tolerance in cardomyocytes (4, 5) and neurons (6–9). Metabolic dysfunction also contributes to preconditioning, as decline in ATP/ADP ratios leads to mitochondrial K_{ATP} channel opening (8) and ROS production (10). In fact, neuronal preconditioning is attenuated with K_{ATP} antagonists (2), and K_{ATP} activators are neuroprotective (11).

Although ROS and energetic dysfunction contribute to preconditioning, little is known about how far these pathways progress before being halted, or the mechanism by which they are blocked. Here, we investigated the extent of activation of cell death pathways during ischemic preconditioning (IP) *in vivo* as well as the mechanism by which activation of these pathways results in tolerance *in vitro*. We propose a new model of IP in which neuroprotection depends upon activation of factors typically associated with neurodegeneration.

Materials and Methods

Focal IP, Immunohistochemistry, and Immunoblotting. Transient MCAO was performed on spontaneously hypertensive rats (12). Immunohistochemistry for activated caspase 3 was performed as described (12). At various times after preconditioning, tissue was harvested, and proteins were run by SDS/PAGE (13). For details, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

Tissue Culture and *in Vitro* Preconditioning. Cortical cultures (25–29 DIV) from E16 rats (14) were exposed to 3 mM KCN in a glucose-free balanced salt solution (150 mM NaCl/2.8 mM KCl/1 mM $CaCl_2$/10 mM Hepes, pH 7.2) for 90 min at 37°C, 5% CO_2. Twenty-four hours later, cells were exposed for 60 min to 100 μM *N*-methyl-D-aspartate (NMDA) and 10 μM glycine. Neuronal viability was determined 18–20 h later with a lactate dehydrogenase (LDH) release assay (14). Pooled data were expressed as the ratio of the LDH released in NMDA-treated cultures to that released in glycine-only-treated sister cultures. To compare across treatments with various compounds, some data were expressed as "NMDA toxicity." Given that most of the agents used to block preconditioning are relatively potent neuroprotective agents, all control cultures for these experiments received these same agents to ensure they did not block NMDA toxicity. For these experiments, LDH values for NMDA exposure in cells that had not been treated with KCN, but were given drugs, were considered 100% cell death. Statistical significance was assessed by parametric comparison between means.

Overexpression of HSC 70. To increase HSC 70 expression during preconditioning, cultures were incubated with 75 μg/ml HSC 70 for 48 h before exposure to KCN (15, 16). Negative control immunohistochemistry experiments were done in the absence of a permeabilizing agent to ensure cells had taken up the protein. There was marked HSC 70 degradation during the 72 h before NMDA exposure in our cell lysates as well as in the supernatant of cells continuously exposed to the protein (data not shown). Media from cells incubated in HSC 70 was harvested, and cells were washed extensively before NMDA exposure.

Results

Optimal Timing of IP *in Vivo*. A brief (10 min) middle cerebral artery occlusion (MCAO) protects against a permanent MCAO (PMCAO) performed 24 h later (12). When animals are preconditioned 1–7 days before the PMCAO, there is at least a 50% reduction in infarct volume. This neuroprotective effect was absent when PMCAO was performed <24 h after preconditioning and was appreciably decreased by 14 days (see Table 1, which is published as supporting information on the PNAS web site). Importantly, preconditioning treatment alone did not result in any observable damage, as assessed 24 h later. The time window of preconditioning reported here correlates well with the HSP 70 induction we previously observed (12).

IP Results in Caspase 3 Cleavage. Animals were treated with a preconditioning MCAO, in the absence of a subsequent PMCAO, and killed at various times. Immunocytochemical

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Abbreviations: K_{ATP}, ATP-sensitive K^+ channels; HSP, heat-shock protein; ROS, reactive oxygen species; IP, ischemic preconditioning; IT, ischemic tolerance; NMDA, *N*-methyl-D-aspartate; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion; PMCAO, permanent MCAO; PBN, *N*-tert-butyl-α-phenylnitrone; BAF, boc-aspartate fluoromethylketone.

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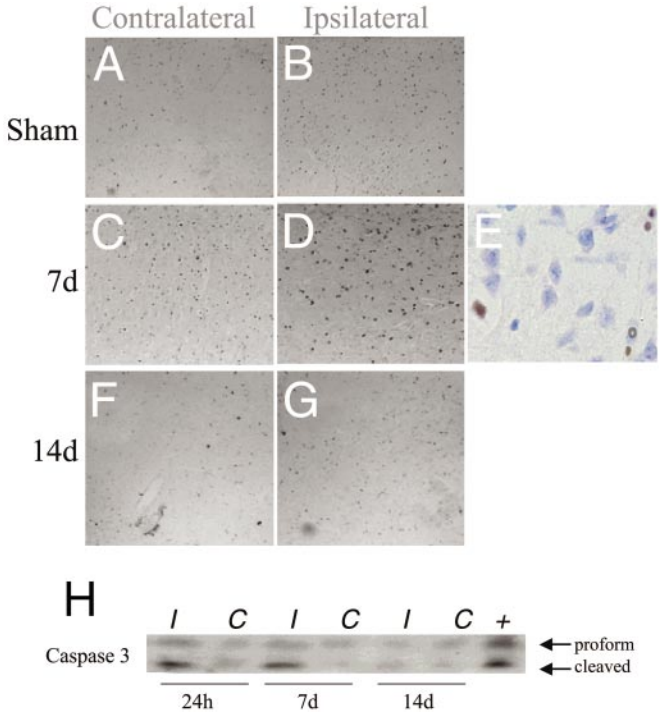

Fig. 1. Caspase 3 activation during *in vivo* IP. Animals were preconditioned (10-min MCAO) and killed at 24 h, 7 days, or 14 days ($n = 4$ per time point). This procedure has no associated neurotoxicity or behavioral deficits (12). Sections were taken from rostral-caudal 4.4 mm from the vertical-zone plane within the parietal cortex, and activated caspase 3 was detected immunohistochemically. Animals killed 7 days after sham surgery had no labeling in the cortex ipsilateral or contralateral to the MCAO (*A* vs. *B*). Appreciable caspase 3 cleavage was present in the ipsilateral cortex of animals killed 7 days after preconditioning (*C* vs. *D*). (*E*) Caspase activity in animals killed 7 days after MCAO was present only in a subset of Nissl-positive cells. (*F* and *G*) Caspase 3 cleavage was not evident at 14 days. (*H*) Western blot analysis revealed caspase 3 proteolysis 24 h and 7 days, but not 14 days, after MCAO preconditioning. *I*, ipsilateral cortex; *C*, contralateral. Positive controls (+), 1 μM staurosporine-treated cultures.

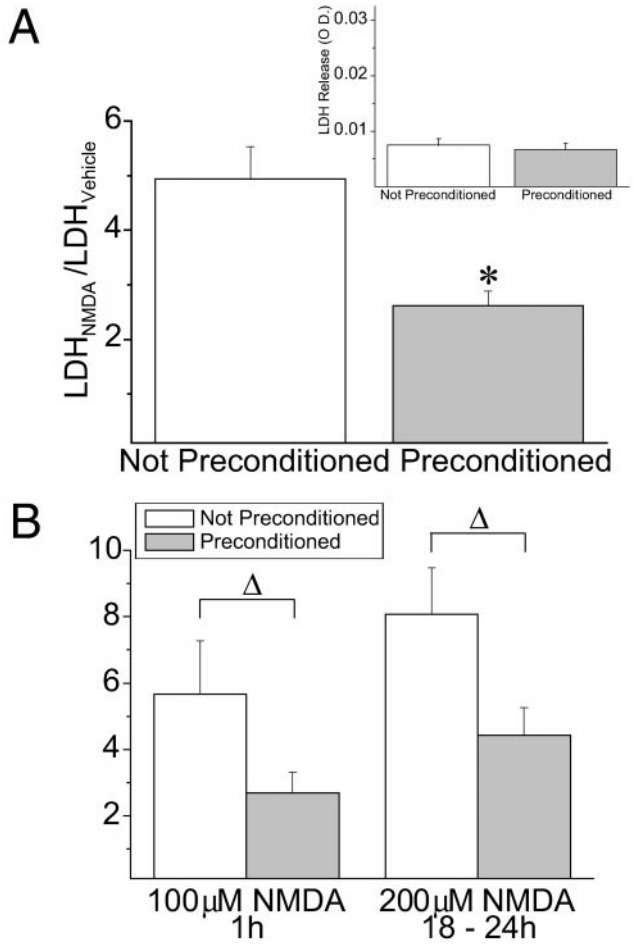

Fig. 2. *In vitro* model of IP. Prior exposure to KCN substantially decreases excitotoxicity. (*A*) Cells were preconditioned and then exposed for 1 h to 100 μM NMDA 24 h later. Data are pooled from >25 experiments and represent LDH values 18–20 h after NMDA exposure. (*Inset*) Pooled data from three experiments demonstrating that preconditioning is not toxic. Cells were exposed to control conditions or KCN without subsequent excitotoxin exposure. The *y* axis is the absolute amount of LDH released; optical density was assessed 24 h after the onset of chemical preconditioning. (*B*) Cortical cultures preconditioned with KCN, but then exposed to either 100 μM NMDA for 1 h or 200 μM NMDA for 18–20 h ($n = 5$), demonstrate that KCN pretreatment protects cells from substantial excitotoxic challenges. Asterisks are used when only two groups are compared; statistical analysis between groups is shown by brackets and Δ. Both asterisks and Δ represent $P < 0.05$.

detection of cleaved caspase 3 revealed a time-dependent activation of caspase 3 that coincided with the neuroprotective period (Fig. 1) but was absent at time points when there was no protection (6 h and 14 days; data not shown and Fig. 1*G*). These observations were confirmed by using immunoblots of parietal cortex derived from animals killed 24 h, 7 days, or 14 days after preconditioning (Fig. 1*H*).

IP *in Vitro*. Next, we developed an *in vitro* model of preconditioning by using KCN, an inhibitor of oxidative phosphorylation. We observed that a 90-min exposure to KCN (3 mM) in glucose-free media did not induce any cell death. This result was confirmed 24 h after KCN exposure by using LDH release (Fig. 2*A Inset*) and cell counts (data not shown). These conditions were used to precondition cells against a subsequent excitotoxic dose of NMDA (100 μM for 60 min) delivered 24 h later. There was near total neuronal loss in NMDA-treated cultures that had not been preconditioned, whereas KCN preconditioning resulted in substantial neuroprotection (Fig. 2).

IP *in Vitro* Is Time and Protein Synthesis Dependent. IP affords a limited duration of efficacy and is protein synthesis dependent *in vivo* (12, 17). We assessed this critical period *in vitro* by exposing cultures to 100 μM NMDA 24, 48, or 72 h after KCN preconditioning. Significant neuroprotection was evident only at the 24-h time point, where >50% of neurons were spared ($P < 0.05$; $n = 5$). Protection could also be blocked by the addition of the protein synthesis inhibitor cycloheximide (1 μg/ml; $P < 0.05, n = 4$). These data suggest that our *in vitro* model of preconditioning has several characteristics comparable to those described in our *in vivo* system (12, 17).

Caspase 3 Activation Is Present After IP *in Vitro*. We observed appreciable caspase 3 cleavage 6 h after KCN preconditioning in the absence of subsequent cell death *in vitro* (Fig. 3 *B* and *E*). Immunocytochemical analysis revealed that a subpopulation of neurons that had with no evidence of pyknosis or vaculation exhibited activated caspase 3 after KCN exposure. These observations are notable not only because there is no toxicity after caspase activation, but also because the caspase 3 immunoreactive cells comprise only a fraction of the total neurons within the cultures. Given that only 50% of the neurons within the cultures are spared by prior preconditioning, it is appealing to speculate

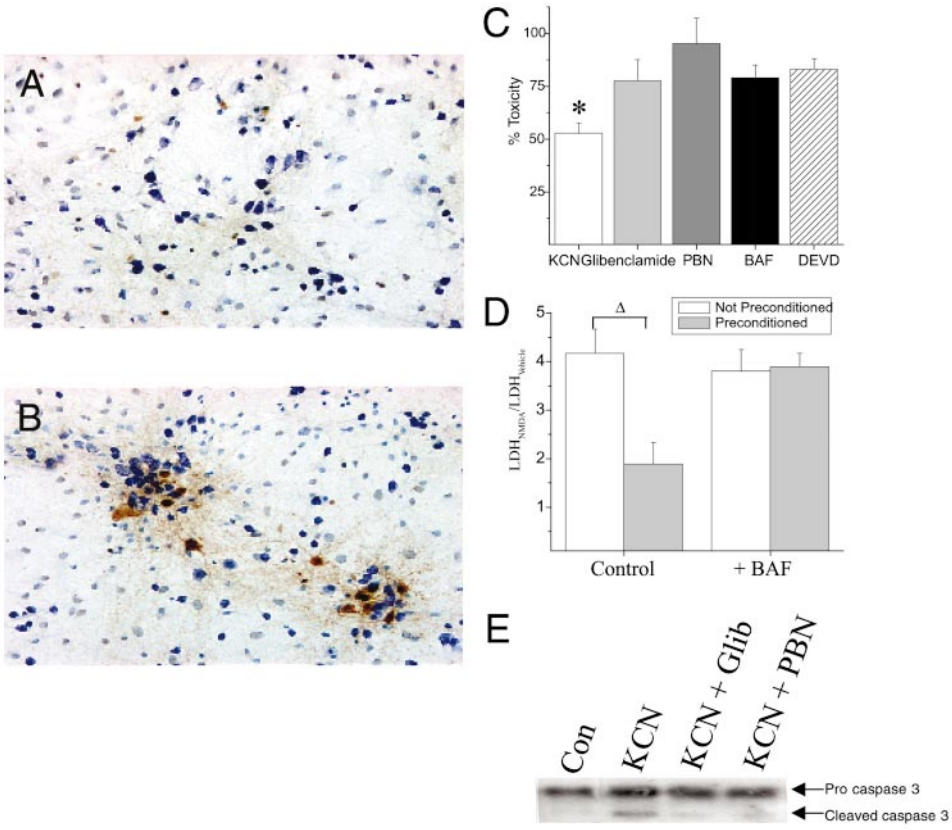

Fig. 3. Activation of K_{ATP} channels, generation of ROS, and caspase 3 activation are required for preconditioning. Cultures were exposed to control conditions (*A*) or KCN preconditioning (*B*) 6 h before being fixed with paraformaldehyde. Immunocytochemistry revealed activated caspase 3-positive cells throughout the preconditioned cultures (brown cells). Neurons in these experiments are stained deep purple with thionin. (*C*) Pooled data showing the effects of PBN, glibenclamide, or caspase inhibitors (BAF, DEVD) on KCN-induced preconditioning. Neuronal cultures were exposed to preconditioning in the presence of glibenclamide (1 μM), PBN (500 μM), BAF (10 μM), or DEVD (10 μM). Twenty-four hours later, cells were exposed to 100 μM NMDA for 60 min. Data were obtained from three to six independent experiments performed at least in duplicate (*, $P < 0.05$; all compounds tested significantly decreased KCN neuroprotection). (*D*) An individual experiment performed in triplicate demonstrates that there is no effect of the cysteine protease inhibitor on NMDA toxicity. Brackets and the Δ symbol are used to denote statistical analysis between groups where $P < 0.05$. Mixed cultures were exposed to KCN for 90 min in the presence of glibenclamide or PBN. Lysates were prepared 6 h later, and blots were probed with a caspase 3 antibody (*E*). The proform (36 kDa) of caspase 3 is shown with the upper arrow, whereas the lower arrow indicates the proteolytically active p20 subunit of caspase 3. Appreciable caspase 3 cleavage is observed in preconditioned cells at this and later times (data not shown), in spite of the fact that there is no subsequent cell death. Caspase cleavage is attenuated by both Glib and PBN, suggesting that K_{ATP} channel opening and ROS production occur upstream of caspase 3 cleavage.

that caspase 3 activation is critical for determination of cell fate after preconditioning.

K_{ATP} Blockers, ROS Scavengers, and Caspase Inhibitors Block Preconditioning. To evaluate the role of K_{ATP} opening, ROS generation, and limited caspase 3 proteolysis in the expression of ischemic tolerance (IT), we used a K_{ATP} blocker (glibenclamide), a free radical spin trap [*N*-tert-butyl-α-phenylnitrone (PBN)], a pan-caspase inhibitor [boc-aspartate fluoromethylketone (BAF)], or a more specific caspase 3,6,7,8,10 inhibitor, Asp-Glu-Val-Asp-Ala (DEVD). Exposure to any of these compounds during preconditioning resulted in an appreciable enhancement of subsequent NMDA toxicity (Fig. 3*C*). Pretreatment of cultures with these agents had no effect on NMDA toxicity itself in control cultures, which is attributable to the extensive washing before NMDA exposure (see Fig. 3*D*). As zVDVAD fmk (50 μM), YVAD fmk (20 μM) and the fmk control peptide (zFA fmk, 20 μM) did not affect preconditioning, caspases 1, 2, or 4 activation seem to play no role in this process (data not shown). Importantly, both glibenclamide and PBN treatment abolished the caspase 3 cleavage observed with preconditioning, suggesting that K_{ATP} channels and ROS contribute to caspase activation (Fig. 3*E*).

Bcl_{xl} and HSP 70 Are Induced at Times When Preconditioning Is Observed *in Vivo*. Multiple cell signaling pathways may provide neuroprotection against cell death after caspase cleavage. These factors can be divided into four groups: calbindins, the Bcl-2 family, the inhibitors of apoptosis (IAPs), and the HSPs (18, 19). Both Bcl_{xl} and HSP 70 were increased in the ipsilateral parietal cortex at times when protection is present (Fig. 4).

HSP 70 Induction Is Attenuated by Agents That Block Preconditioning *in Vitro*. Next, we assessed the temporal profile of HSP 70 and Bcl_{xl} expression after preconditioning *in vitro* and determined if agents that blocked preconditioning also blocked induction of these proteins. We observed a substantial increase in HSP 70 at the 24-h time point, but not 6 or 72 h after preconditioning (Fig. 5*A*). Importantly, BAF, glibenclamide, and PBN all blocked the increase in HSP 70 expression. These observations are consistent with a critical neuroprotective role for cell stress caused by ROS production, energetic stress, and caspase activation in leading to induction of HSP 70. Bcl_{xl} was increased at both 24 and 72 h after KCN exposure (Fig. 5*D*). Given that neuroprotection was only observed when cells were treated with NMDA 24 h, but not 72 h, after KCN exposure, the Bcl_{xl} expression pattern does not match well the temporal profile of a protein that would be a critical

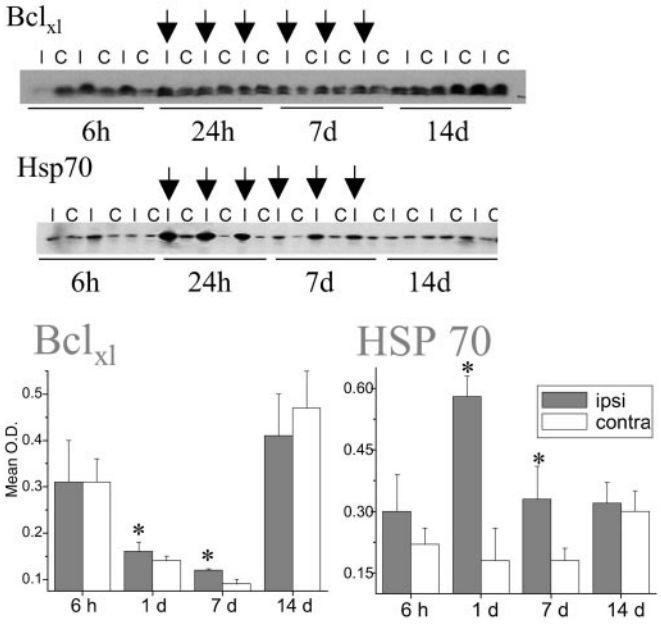

Fig. 4. HSP 70 and Bcl$_{xl}$ expression are increased *in vivo* at times when IP protection is observed. Proteins from animals killed at various time points after 10-min MCAO were isolated. Extracts were probed for the expression of members of the IAP, Bcl-2, and HSP family, as well as calbindin. Three different animals were used for each time point. Both Bcl$_{xl}$ and HSP 70 expression were increased in the ipsilateral cortex at times when preconditioning was observed (arrows indicate the lanes where increased protein expression was observed in these two groups). Comparison of optical densities revealed a small but significant increase in Bcl$_{xl}$ expression 1 and 7 days after preconditioning in the ipsilateral cortex (*, $P < 0.05$; $n = 3$). Larger increases in HSP 70 were observed in the ipsilateral cortex at these times ($P < 0.05$; $n = 3$).

mediator of IT in our *in vitro* system. Moreover, whereas both glibenclamide and BAF blocked Bcl$_{xl}$ induction, PBN did not (Fig. 5*E*). Taken together, these points suggest that the increase in this anti-apoptotic protein likely does not solely mediate the observed neuroprotection.

HSP 70 Is Induced by Caspase 3 Activation: A Model of Excitotoxic Tolerance. Based on the observation that caspase activation was required for HSP 70 induction as well as recent literature reports about HSC 70-binding partners (20, 21), we hypothesized that caspase activation during preconditioning is held in check by binding to the constitutively expressed HSP 70 homologue HSC 70. We speculated that depletion of caspase-binding proteins, including HSC 70, results in the activation of a positive-feedback cycle, leading to increased production of HSP 70. This up-regulation, then, is able to block normally lethal exposure to subsequent excitotoxic insults.

This model would make several predictions: (*i*) that the subpopulation of cells expressing activated caspase 3 would have increased HSP 70 expression; (*ii*) that enhanced expression of HSC 70 during preconditioning would block the subsequent increase in HSP 70 expression; and (*iii*) that increased levels of HSC 70 protein during preconditioning would block the induction of excitotoxic tolerance. To test the first prediction, cultures were exposed to KCN preconditioning and then fixed 7 h later. Although maximum induction of HSP 70 did not occur until 24 h, we determined in preliminary experiments that HSP 70 was beginning to be increased by 7 h (data not shown). We observed that a subpopulation of cells expressed increased HSP 70 (Fig. 6*A*) after KCN treatment, and that the majority of these cells also had appreciable levels of activated caspase 3 (Fig. 6*A*).

To test the second prediction of our model, that overexpression of HSC 70 would block the increase in HSP 70, we performed immunoblots and immunohistochemistry on cells that had been exposed to biotinylated HSC 70. Immunoblots of cell lysates exposed for 48 h to purified HSC 70 demonstrate a

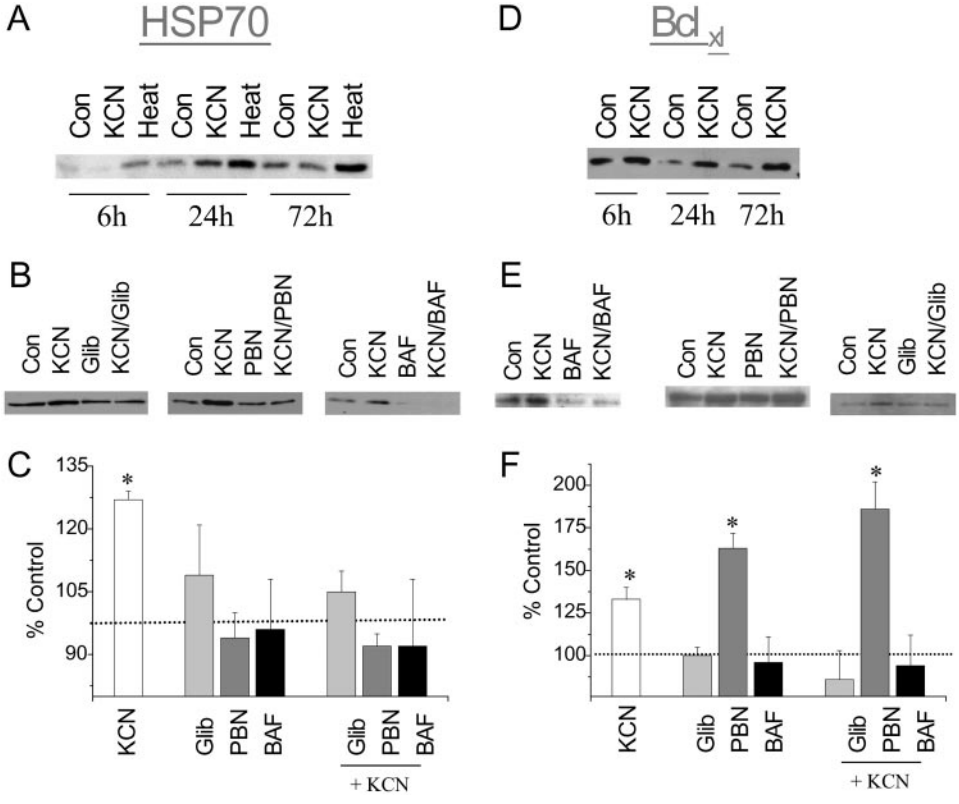

Fig. 5. Increased HSP 70, but not Bcl$_{xl,}$ is blocked by agents that block preconditioning *in vitro*. (*A*) Immunoblots of cells exposed to control conditions or KCN and harvested 6, 24, or 72 h later demonstrate a substantial increase of HSP 70 expression 24 h after KCN treatment. By using the 24-h time point, we evaluated the effects BAF, PBN, and glibenclamide (Glib) on the induction of HSP 70. (*B*) HSP 70 expression was blocked to a large extent by conditions that block preconditioning protection, suggesting a positive correlation of induction of this protein with the expression of IT. (*C*) Quantification of this effect confirmed that the significant elevation in HSP 70 expression was blocked by BAF, PBN, and glibenclamide ($P < 0.05$; $n =$ 3–4). Similar experiments were performed to evaluate the contribution of Bcl$_{xl}$ induction to IP. Bcl$_{xl}$ was increased at both 24 and 72 h (*D*). Both BAF and Glib blocked the increase in Bcl$_{xl}$ expression at the 24-h time point; however, PBN had no effect (*E*), an observation that was confirmed by densitometric quantification of blots ($P < 0.05$; $n =$ 3–4). This result suggests that Bcl$_{xl}$ expression does not correlate fully with the expression or causation of IT.

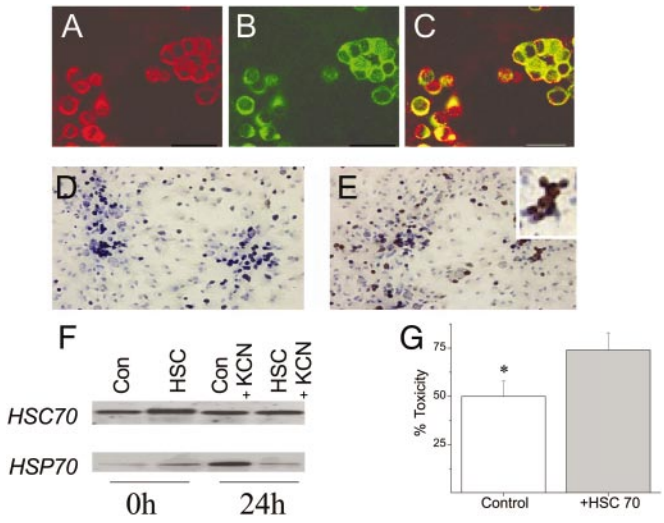

Fig. 6. HSC 70 depletion contributes to IP. HSP 70 expression was shown be induced in cells that have activated caspase 3. Cultures were exposed to KCN preconditioning and then fixed 7 h later. By using confocal microscopy, double immunostaining for HSP 70 (*A*) and activated caspase 3 (*B*) revealed extensive overlap in the cells expressing both proteins. Merged images are shown in *C*, and yellow cells have staining for both proteins. (Bar = 50 μm.) HSC 70 protein expression was increased immediately before preconditioning to determine whether it could block tolerance. Immunohistochemical staining of permeabilized cells exposed to control washes (*D*) or to 75 μg/ml biotinylated purified HSC 70 (*E*) reveals increased intracellular expression of the molecular chaperone immediately before cells being subjected to KCN. (*E Inset*) High-power magnification of intense brown HSC 70 expression in cells overlying the light purple glial bed. Immunoblots for intracellular HSC 70 reveal an increase in HSC expression after 48 h of exposure to purified protein (0 h) that was appreciably diminished 24 h after KCN treatment (*F*). Moreover, the increase in HSP 70 expression that is normally induced by KCN was blocked in cells that had been pretreated with HSC 70. A causal role of HSC 70 depletion in protection is suggested by toxicity experiments (*G*). Cultures previously exposed to HSC 70-purified protein and then preconditioned had increased cell death on subsequent exposure to NMDA, compared with cultures not pretreated with HSC 70 ($n = 3$). Asterisks denote a significant difference vs. matched cells that were pretreated with HSC but not preconditioned (*, $P < 0.05$).

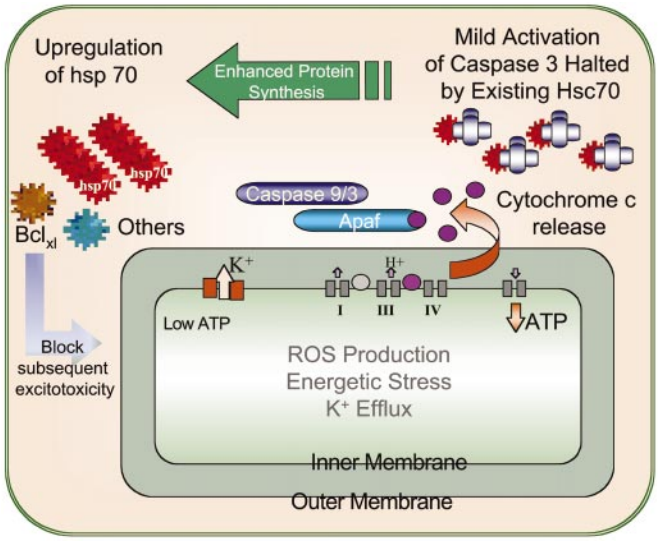

Fig. 7. Model of IP. Based on our observations that preconditioning elicits caspase cleavage and ROS generation, which are required for protection, and that this protection requires new protein synthesis, we propose the following pathway. We hypothesize that the initial energetic stress put on cells generates ROS and activation of mitochondrial K_{ATP} channels. These events likely contribute to limited cytochrome *c* redistribution and caspase 3 activation. Cleaved caspases are likely held in check by preexisting proteins such as HSC 70. When these proteins are depleted, we speculate that this depletion results in the activation of a positive-feedback cycle that leads to increased production of HSPs. This up-regulation is then able to block normally lethal exposure to excitotoxic insults.

marked enhancement of intracellular biotinylated HSC 70 expression (Fig. 6*F*, time 0 h) with minimal alteration in HSP 70 expression. Immunohistochemical detection of the biotin-labeled HSC 70 also revealed that many of the more intensely positive cells were neuronal in both their morphological features and that they were more intensely stained with Nissl substance than the underlying glial bed (Fig. 6*E*). There was no appreciable staining in cells that had been exposed to HSC 70 but were not permeabilized, suggesting that either the extensive washing that occurred as part of the immunohistochemistry removed the protein or that the majority of the purified protein was, in fact, taken up by the cells. Importantly, the increase in HSP 70 expression induced by KCN was blocked in cells which had been pretreated with HSC 70 (Fig. 6*F*). This result suggests a causal role of HSC 70 depletion in HSP 70 induction after preconditioning.

A final series of experiments were designed to test the prediction that HSC overexpression during preconditioning should diminish the expression of tolerance. Indeed, toxicity experiments confirmed that limited overexpression of HSC 70 during preconditioning was sufficient to block excitotoxic tolerance (Fig. 6*G*). Taken together, these data suggest that it is not so much the activation of caspases as it is the depletion of free pools of constitutively expressed HSPs by activated caspases that induces IT.

Discussion

We tested the hypothesis that cellular elements normally associated with neuronal cell death are involved in neuroprotection in IP, because appreciable caspase 3 cleavage was present during the tolerant period *in vivo*. To this end, we developed an *in vitro* model of preconditioning that expressed the hallmark features of IT, including requisite protein synthesis, involvement of mitochondrial K_{ATP} channels, and production of ROS (Fig. 7). A critical role for the opening of K_{ATP} channels has been established in a variety of models of preconditioning (8), and energetic dysfunction induced by KCN is sufficient to open K_{ATP} channels at the cell membrane (22). Opening of the K_{ATP} channel within the inner mitochondrial membrane decreases the mitochondrial membrane potential, thereby accelerating electron transfer and a net oxidation of the mitochondria (23). Oxidizing agents and ROS can also open K_{ATP} channels and contribute to preconditioning (10, 24, 25), potentially establishing a positive feedback loop for the expression of tolerance.

The role of K_{ATP} channels in the activation of mitochondria-initiated signals associated with apoptosis remains unclear. Recent work suggests that loss of the mitochondrial potassium and proton gradients results in cytochrome *c* release (26). It has also been shown that neuroprotective doses of the K_{ATP} openers may increase the release of cytochrome *c* from the mitochondria in neurons (ref. 27, but also see ref. 28).

Activation of caspases has historically been viewed as occurring downstream of the commitment to die decision. However, emerging evidence now suggests that multiple factors may provide protection against both apoptosome assembly and cleaved caspases. These factors include calbindin, the IAPs, the Bcl-2 family, and HSPs (18). Our preliminary experiments did not suggest a significant up-regulation of IAPs or calbindin during preconditioning. It has previously been suggested that tolerance is conferred on cells by the up-regulation of Bcl-2 and Bcl_{xl} (29). Although the up-regulation of Bcl_{xl} did, to a limited extent, parallel the time course of neuroprotection observed in both our models, several points suggest that other factors are likely to play a greater role in neuroprotection. For one, the induction of Bcl_{xl} was not very dramatic, particularly *in vivo*.

Moreover, the free radical spin trap, PBN, which blocks preconditioning, did not block the induction of Bcl_{xl}.

HSPs are highly conserved, abundantly expressed proteins with diverse functions (30). HSP 27 has been shown to protect against apoptotic cell death triggered by a variety of stimuli (31) and has been implicated in preconditioning, decreasing ROS production, and the blockade of cell death after cytochrome *c* release (8, 31, 32), yet in preliminary experiments, we observed no up-regulation of HSP 25 at the times when tolerance was present. Emerging evidence suggests that, in addition to important functions in protein refolding and transport, the HSP 70 family is also capable of binding and sequestering activated caspases, APAF and AIF, making them particularly appealing targets for a role in IT (6, 20, 21, 33). HSC 70 is the most abundant HSP found in cells; it is expressed constitutively and is only mildly inducible (30). HSP 70 is the major inducible form of HSPs (34). Not surprisingly, ischemic injury, ROS generation, and injuries that induce protein denaturation increase HSP 70 expression (34). Further, HSP 70 expression is regulated by transcription factors whose activity is increased by extracellular signal-regulated kinase phosphorylation (35), a process which has been implicated in preconditioning (36). Although the role of HSP 70 in preconditioning has been challenged (37), there have been extensive links of HSP 70 overexpression and tolerance in ischemic brain injury (3, 38). HSP 70 protects the brain against MCAO (34, 39), and we have previously reported an increase in HSP 70 expression in our *in vivo* model of preconditioning (12). We now present evidence that activation of K_{ATP} channels, ROS, and caspases leads to up-regulation of this neuroprotective protein.

Our observations suggest that caspases are activated during preconditioning, but are prevented from eliciting cell death. Moreover, if activation of this process is blocked, neuroprotection is lost. As new protein synthesis is required for tolerance, we hypothesize that sublethal caspase activation can, under some circumstances, up-regulate neuroprotective pathways. We propose that the initial energetic stress during preconditioning leads to limited activation of caspase 3. Once activated, caspases are held in check by sequestration with proteins such as HSC 70, thereby depleting the free pool of HSC 70, leading to increased synthesis of HSP 70.

Our proposed model predicts that cells that are protected against a secondary insult may also have appreciable levels of activated caspases in the absence of measurable toxicity. Consistent with this, neurons preconditioned to withstand a neurotoxic dose of staurosporine have been shown to have levels of cleaved caspase 3 which are as high as unprotected/naive cells, despite the fact that there was a marked reduction in death (40). It is important to note that the second insult need not be apoptotic in nature to be blocked by the increased expression of HSP 70. Given the diverse mechanisms by which HSP 70 can sequester, refold, and reprocess proteins, caspase activation itself may not need be critical for these chaperones to block subsequent injury (41).

As the signaling pathways responsible for IT become more fully understood, novel targets for the posttreatment protection from focal stroke brain injury may be identified. Further, pharmacological preconditioning agents that can protect the brain in high-risk individuals or before invasive cerebral surgical procedures may be developed. Finally, the strategies for intervention that are being developed to treat patients subject to invasive neurological procedures may need to be more critically evaluated, based on our observation that blockade of traditional cell death pathways may, in some instances, actually prove deleterious.

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