Ischemic preconditioning blocks BAD translocation, Bcl-x_L cleavage, and large channel activity in mitochondria of postischemic hippocampal neurons

Takahiro Miyawaki*†, Toshihiro Mashiko†, Dimitry Ofengeim*, Richard J. Flannery‡, Kyung-Min Noh*, Sho Fujisawa*, Laura Bonanni§, Michael V. L. Bennett*¶, R. Suzanne Zukin*¶, and Elizabeth A. Jonas‡

*Dominick P. Purpura Department of Neuroscience, Rose F. Kennedy Center, Albert Einstein College of Medicine, Bronx, NY 10461; ‡Departments of Internal Medicine and Cell Biology, Yale University, New Haven, CT 06520; †Department of Neurosurgery, Jichi Medical University, Tochigi 329-0498, Japan; and §Neurofisiopatologia, Università degli Studi Gabriele d'Annunzio di Chieti-Pescara, 65124 Pescara, Italy

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Transient forebrain or global ischemia induces delayed neuronal death in vulnerable CA1 pyramidal cells with many features of apoptosis. A brief period of ischemia, i.e., ischemic preconditioning, affords robust protection of CA1 neurons against a subsequent more prolonged ischemic challenge. Here we show that preconditioning acts via PI3K/Akt signaling to block the ischemia-induced cascade involving mitochondrial translocation of Bad, assembly of Bad with Bcl-x_L, cleavage of Bcl-x_L to form its prodeath fragment, ΔN-Bcl-x_L, activation of large-conductance channels in the mitochondrial outer membrane, mitochondrial release of cytochrome *c* and Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI), caspase activation, and neuronal death. These findings show how preconditioning acts to prevent the release of cytochrome *c* and Smac/DIABLO from mitochondria and to preserve the integrity of the mitochondrial membrane. The specific PI3K inhibitor LY294002 administered *in vivo* 1 h before or immediately after ischemia or up to 120 h later significantly reverses preconditioning-induced protection, indicating a requirement for sustained PI3K signaling in ischemic tolerance. These findings implicate PI3K/Akt signaling in maintenance of the integrity of the mitochondrial outer membrane.

Akt | ischemic tolerance | PI3K | postischemic neurodegeneration

Transient global or forebrain ischemia arising in humans as a consequence of cardiac arrest or cardiac surgery, or induced experimentally in animals, leads to selective and delayed neuronal death of hippocampal CA1 neurons and cognitive deficits (1–4). Injurious stimuli such as ischemia disrupt the integrity of the mitochondrial membrane, leading to the release of cytochrome *c* and activation of cysteine proteases including caspase-9, a critical "initiator" caspase, and caspase-3, a "terminator" caspase implicated in the execution step of apoptosis (5–7). Caspase-3 activation is critical to delayed neurodegeneration after ischemia (8). Global ischemia promotes expression of the prosurvival inhibitor-of-apoptosis (IAP) family member cIAP but causes release of Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI) from mitochondria, a factor that neutralizes the protective actions of IAPs and promotes neuronal apoptosis (1–4).

The Bcl-2 family member Bcl-x_L is a potent inhibitor of programmed cell death and acts by heterodimerization with other Bcl-2 family members to inhibit caspase activation, presumably by blocking release of cytochrome *c* (9, 10). Bcl-x_L is abundantly expressed in adult neurons (11, 12) and localizes to the outer mitochondrial membrane (13). N-terminal cleavage of Bcl-x_L to form its proapoptotic fragment, ΔN-Bcl-x_L, leads to appearance of large-conductance channels in the mitochondrial outer membrane (14–16). In hippocampal neurons these channels appear within 1 h of insult (16). Activation of large-conductance channels correlates with appearance of ΔN-Bcl-x_L in the mitochondria, is mimicked by introduction of recombinant ΔN-Bcl-x_L, and is blocked by introduction of a Bcl-x_L antibody via the patch pipette (16). These findings implicate ΔN-Bcl-x_L in the large-conductance channels observed in the mitochondrial outer membrane of insulted hippocampal CA1 neurons.

Ischemic tolerance is a well established phenomenon in which a brief ischemic insult (or preconditioning) protects CA1 neurons against a subsequent more prolonged ischemic challenge (17, 18). This protective action involves inhibition of activated caspase-3 (19). Neuroprotective strategies such as ischemic preconditioning promote phosphorylation of Akt at Ser-473 and phosphorylation/inactivation of Akt downstream targets; PI3K/Akt signaling is required for preconditioning-induced neuroprotection (20–22). Preconditioning intervenes downstream of proteolytic processing of caspase-3 to its active form, but caspase-3 activity is inhibited by endogenous inhibitors, and one of its targets, caspase-activated DNase (CAD, a DNase that catalyzes DNA fragmentation) is unaffected (19). Preconditioning blocks the mitochondrial release of Smac/DIABLO but not the ischemia-induced up-regulation of IAPs (19). In the absence of Smac/DIABLO, cIAP halts the caspase death cascade and arrests neuronal death.

The present study was undertaken to examine the molecular mechanisms by which ischemic preconditioning prevents the ischemia-induced increase in cytosolic cytochrome *c* and Smac/DIABLO. We show that ischemic preconditioning acts via PI3K signaling to prevent mitochondrial translocation of Bad, binding of Bad to Bcl-x_L, cleavage of Bcl-x_L to generate ΔN-Bcl-x_L, activation of large-conductance channels in the mitochondrial outer membrane, and activation of the caspase cascade leading to neuronal death. These findings give new insight into the molecular mechanisms of neuroprotection by ischemic preconditioning.

Results

Preconditioning Promotes Assembly of Akt with Bad. Akt promotes cell survival via phosphorylation and reduction in cytoplasm to mitochondrial translocation of downstream targets such as the proapoptotic Bcl-2 family member Bad (23). To examine whether PI3K is involved in ischemic tolerance, rats were subjected to sham operation, preconditioning [4-min four-vessel occlusion (4-VO); PC], ischemia (10-min 4-VO; Isch), preconditioning followed by

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¶To whom correspondence may be addressed at: Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Kennedy Center, Room 602, 1300 Morris Park Avenue, Bronx, NY 10461. E-mail: mbennett@aecom.yu.edu or zukin@aecom.yu.edu.

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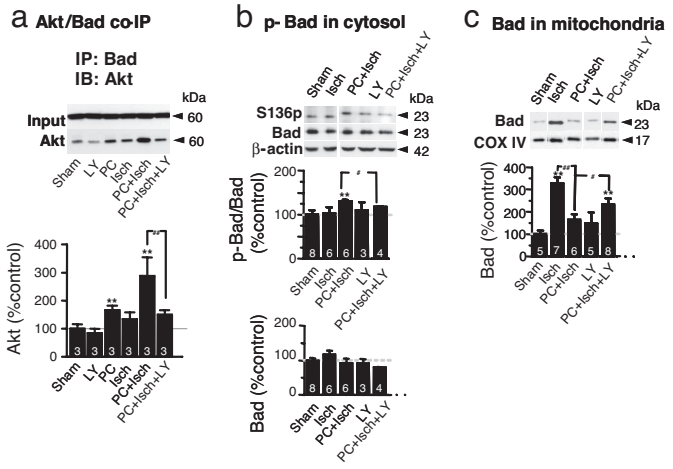

Fig. 1. Preconditioning promotes assembly of Akt with Bad, Akt-mediated Bad phosphorylation, and cytoplasmic retention of Bad. (*a*) Representative coimmunoprecipitation of Bad–Akt (*Upper*) and summary data (*Lower*). Preconditioning (PC) and preconditioning followed by ischemia (PC + Isch) promote assembly of Akt with Bad. The PI3K inhibitor LY294002 reverses the effect of PC. (*b*) Representative Western blot (*Top*) and summary data for p-Ser136-Bad (*Middle*) and total Bad (*Bottom*) in the cytosol. Preconditioning promotes Bad phosphorylation. $n = 3$–8 per group. (*c*) Western of total Bad in the mitochondria. $n = 5$–8 per treatment group. Cytochrome *c* oxidase IV (COX IV) served as loading control. Error bars indicate SEM. In all figures, significance between experimental and sham animals is indicated by *, and significance between PC + Isch and Isch groups is indicated by #. * and #, $P < 0.05$; ** and ##, $P < 0.01$.

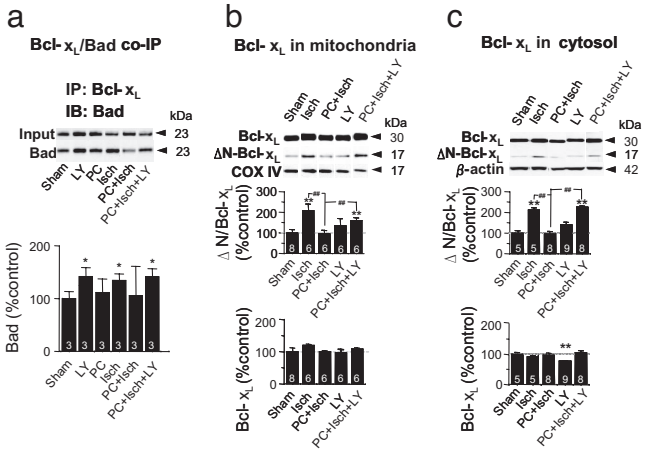

Fig. 2. Preconditioning acts via PI3K signaling to promote Bad/Bcl-x_L assembly and Bcl-x_L cleavage to generate ΔN-Bcl-x_L. (*a*) Coimmunoprecipitation of Bad/Bcl-x_L was increased over sham by LY, Isch, and PC + Isch + LY. (*b*) Western blot of mitochondrial Bcl-xL and ΔN-Bcl-x_L (*Top*) and summary data ΔN-Bcl-x_L/Bcl-x_L (*Middle*) and full-length Bcl-x_L (*Bottom*). $n = 6$ per group. (*c*) Western blot of cytosolic Bcl-x_L and ΔN-Bcl-x_L (*Top*) and summary data (*Middle* and *Bottom*). Animals were killed 1 h after the last surgery or 49 h after PC. Values of *n* are indicated on the histogram bars. Isch promoted (and PC prevented) Bcl-x_L cleavage. LY294002 (LY) attenuated the effects of preconditioning.

ischemia (4-min 4-VO, followed 48 h later by 10-min 4-VO; PC + Isch), or LY294002 injection (i.c.v.) just after sham operation (LY), 48 h after PC (PC + LY), + LY, or just after Isch in PC + Isch animals (PC + Isch + LY) (see details in supporting information (SI)). Preconditioning altered assembly/association of Akt with Bad, as demonstrated by coimmunoprecipitation with an antibody directed against Bad and probed for Akt. Ischemia did not detectably alter association of Akt with Bad, assayed at 1 h after reperfusion (Fig. 1*a*). Preconditioning itself induced a modest increase in assembly of Bad and Akt (to ≈170%, measured at 49 h after reperfusion) and markedly enhanced Bad/Akt interaction in animals subjected to ischemia 48 h after preconditioning (PC + Isch) (to ≈290% measured at 1 h after Isch; Fig. 1*a*). LY294002 did not itself alter assembly of Bad with Akt after 1 h but reduced the impact of preconditioning on Bad–Akt assembly (1 h after PC + Isch + LY, to ≈150%; Fig. 1*a*). These findings indicate that preconditioning promotes assembly of Akt with Bad and suggest a role for PI3K signaling in Bad phosphorylation.

Preconditioning Acts via PI3K Signaling to Prevent Mitochondrial Translocation of Bad. Bcl-2 family members such as Bad shuttle between the cytosol and mitchondria and, when associated with mitochondria, can promote apoptosis (5, 6, 9). PI3K/Akt-mediated phosphorylation of Bad promotes its binding to the cytoplasmic retention factor 14-3-3 and prevents translocation of Bad to mitochondria (23). To examine the impact of preconditioning on phosphorylation of Bad at Ser-136, we probed Western blots of cytosolic and mitochondrial fractions with antibodies against total and p-Bad (1 h after ischemia and reperfusion; Fig. 1 *b* and *c*). Ischemia alone did not detectably alter the phosphorylation status of Bad (Fig. 1*b*). Preconditioning also did not alter the phosphorylation status of Bad (measured 49 h after preconditioning; data not illustrated) but modestly enhanced Bad phosphorylation induced by subsequent ischemia (to 130 ± 4% of control; $P < 0.01$; Fig. 1*b Top*). LY294002 (1 h after i.c.v. injection) did not itself alter the phosphorylation status of Bad, but it significantly reduced the increase in phosphorylation induced in PC + Isch animals [1 h after LY294002 injection immediately after reperfusion; $P < 0.05$ for (PC + Isch) vs. (PC + Isch + LY); Fig. 1*b*]. Bad abundance in the cytosol was unaltered by all treatments (Fig. 1*b Bottom*).

To examine the impact of preconditioning on Bad translocation, we examined Bad abundance in the mitochondrial fraction 1 h after Isch. Isch promoted Bad translocation (to 328 ± 26% of control; Fig. 1*c*); preconditioning did not itself affect Bad distribution (measured at 49 h; data not illustrated) but markedly attenuated ischemia-induced translocation of Bad (to 163 ± 27% of control; $P < 0.01$ vs. Isch). LY294002 itself did not significantly affect the amount of Bad in the mitochondria but reduced the effect of preconditioning on Bad translocation after ischemia [mitochondrial accumulation of Bad to 232 ± 29% of control; $P < 0.01$ for (PC + Isch + LY) vs. (PC + Isch) and $P < 0.01$ vs. sham; Fig. 1*c*]. These findings indicate that preconditioning acts via PI3K signaling to promote assembly of Akt with Bad, phosphorylation of Bad, and its cytosolic retention, presumably as p-Bad, in the face of ischemia, consistent with a role in neuroprotection by preconditioning. Because PC + Isch shows only a small increase in the p-Bad/Bad ratio and total cytosolic Bad shows little change, it is unlikely that cytosolic Bad is reduced enough to account for the large reduction in mitochondrial Bad in PC + Isch animals compared with Isch animals. We hypothesize that only a small fraction of the Bad we measure by Western blotting is available for translocation to the mitochondria and that this fraction is greatly reduced by preconditioning and Bad phosphorylation. p-Bad was not detectable in the mitochondrial fraction from any treatment group (data not illustrated), in confirmation of others (24).

Ischemia and PI3K Blockade Promote Assembly of Bad with Bcl-x_L. Upon translocation to the mitochondria, Bad forms homodimers and heterodimers with other Bcl-2 family members and promotes apoptosis (25). To examine the impact of ischemia on assembly of Bad with Bcl-x_L, we performed coimmunoprecipitation with an anti-Bcl-x_L antibody and probed for Bad. Ischemia promoted assembly of Bad and Bcl-x_L in naïve animals assessed at 1 h after the last surgery (Fig. 2*a*). Moreover, Bad-Bcl-x_L association was

higher in LY and in PC + Isch + LY animals (Fig. 2*a*). These findings are consistent with the possibility that preconditioning acts via PI3K signaling to promote Bad phosphorylation and thereby prevent mitochondrial translocation of Bad and assembly of Bad with Bcl-x_L. However, values for PC and for PC + Isch animals were too variable for reliable interpretation.

Preconditioning Acts via PI3K Signaling to Maintain Full-Length Bcl-x_L. Ischemia promotes cleavage of Bcl-x_L to generate its prodeath fragment, ΔN-Bcl-x_L, which in turn activates large-conductance channel activity in the mitochondrial outer membrane (16). To examine the impact of preconditioning on production of ΔN-Bcl-x_L, we subjected animals to treatments as above, killed them at 1 h after sham operation or reperfusion, and analyzed samples of microdissected CA1 by Western blotting. Protein samples were sequentially probed for full-length Bcl-x_L followed by ΔN61-Bcl-x_L. Ischemia promoted cleavage of Bcl-x_L to generate its proapoptotic counterpart, ΔN61-Bcl-x_L, evident in mitochondria (211 ± 11% of control, $P < 0.01$; Fig. 2*b Middle*; see SI Fig. 6) and cytosolic fractions (207 ± 33% of control, $P < 0.01$; Fig. 2*c Middle*). Preconditioning did not itself alter the cleavage of Bcl-x_L (data not shown) but prevented ischemia-induced generation of ΔN61-Bcl-x_L in the mitochondria (95 ± 19% of control, $P > 0.05$; Fig. 2*b Middle*) and cytosol (97 ± 11% of control, $P > 0.05$; Fig. 2*c Middle*). LY294002 itself did not significantly alter the status of Bcl-x_L, but it reduced the action of preconditioning to prevent the ischemia-induced rise in ΔN61-Bcl-x_L in mitochondria (to 158 ± 15% of control, $P < 0.01$ vs. PC + Isch; Fig. 2*b Top*) and cytosol (to 235 ± 37% of control, $P < 0.01$ vs. PC + Isch; Fig. 2*c Middle*). The abundance of full-length Bcl-x_L was unaltered by all treatments, except for LY294002, which moderately reduced full-length Bcl-x_L abundance in the cytosol (to 76 ± 3% of control, $P < 0.01$; Fig. 2*c Bottom*). Abundance of full-length Bcl-x_L in the total cell lysate was unchanged by all treatments (see SI Fig. 7). Thus, preconditioning acts via PI3K signaling to promote Bad phosphorylation and prevent mitochondrial translocation of Bad and cleavage of Bcl-x_L to generate its prodeath fragment, ΔN61-Bcl-x_L.

Preconditioning Acts via PI3K Signaling to Prevent Ischemia-Induced Large-Conductance Channel Activity. Global ischemia promotes appearance of large-conductance (>750 pS) channel activity in the outer membranes of mitochondria isolated from postischemic hippocampus 1 h after reperfusion (16). Similar activity is induced in control mitochondria by introduction of recombinant ΔN61-Bcl-x_L via the patch pipette (data not shown), and the activity in postischemic mitochondria is blocked by an antibody to Bcl-x_L, consistent with a role for ΔN61-Bcl-x_L in channel formation (16). To examine the impact of preconditioning on large-conductance channel activity, we recorded by patch clamping mitochondria isolated from the whole hippocampus of control and experimental animals (at times corresponding to those above). Mitochondria from the hippocampus of sham-treated animals exhibited primarily small channel openings (up to 180 pS; Fig. 3*a*) at low frequency and a few intermediate conductance openings and were predominantly in the closed state (Fig. 3 *a* and *b*). In contrast, mitochondria from the hippocampus of PC or LY animals exhibited more frequent openings of intermediate conductance (180–750 pS; Fig. 3 *a* and *b*) and few large-conductance openings (> 750 pS; Fig. 3*c*). Mitochondria from postischemic hippocampus showed markedly increased large-conductance activity in confirmation of earlier work (16) (Fig. 3 *a* and *c*). However, intermediate- and large-channel activity after PC + Isch was near that after PC alone (Fig. 3 *a–c*). In mitochondria from PC + Isch + LY animals, large-channel activity was increased relative to PC + Isch; i.e., the protective effect of PC was reduced, although not significantly (Fig. 3 *a–c*).

The current–voltage relations of the large-conductance channels were linear with a reversal potential near zero (Fig. 3*d*). The current–voltage relations of the large-conductance activity of the mitochondria from PC + Isch + LY animals were also linear with a reversal potential near zero (Fig. 3*e*). These findings indicate that preconditioning acts via PI3K signaling to prevent the appearance of large-conductance channel activity in the mitochondrial outer membrane.

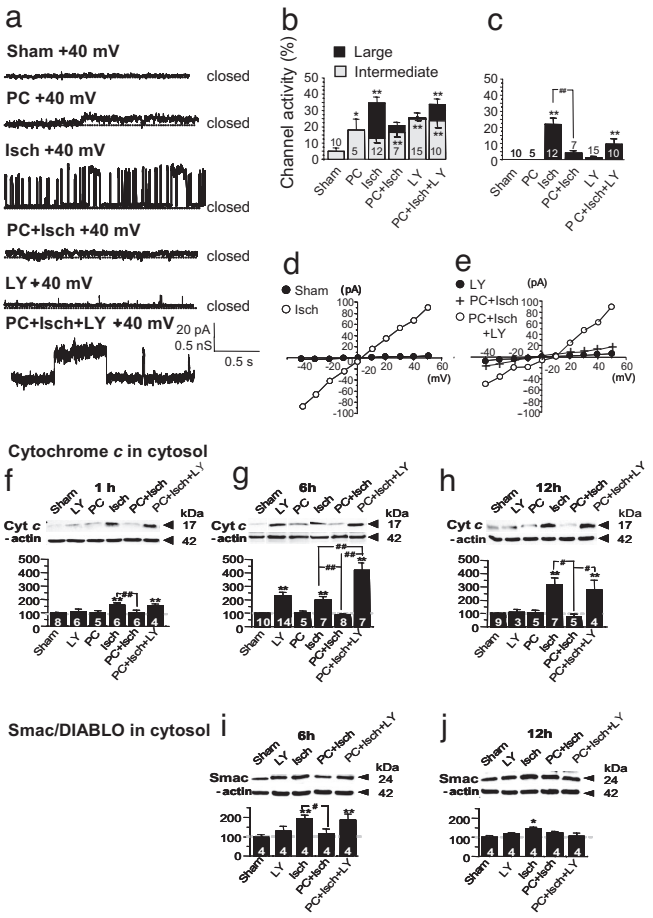

Fig. 3. Preconditioning acts via PI3K signaling to prevent ischemia-induced formation of large-conductance channels in mitochondrial outer membrane and mitochondrial release of cytochrome *c* and Smac/DIABLO. (*a*) Sample patch recordings from the outer membrane of mitochondria isolated from the hippocampus of indicated groups. The lowest level for the PC + Isch + LY trace is 45 pA. (*b* and *c*) Histograms of percent recording time in which a patch showed open intermediate-conductance (180–750 pS, stippled lines) or large-conductance (>750 pS, black) channels for recordings like those in *a*; *n* values are indicated. (*d*) Current–voltage relations for a representative large-conductance channel and an inactive patch recorded from mitochondria from postischemic and control animals, respectively. (*e*) Current–voltage relations for a large-conductance channel recorded from a PC + Isch + LY and low-conductance channels from LY and PC + Isch animals. (*f–h*) Western blot of cytosolic cytochrome *c* at 1, 6, and 12 h (plus 48 h for PC). (*i* and *j*) Western blot of cytosolic Smac/DIABLO at 6 and 12 h. *n* values are indicated on bars.

Preconditioning Acts via PI3K Signaling to Prevent Mitochondrial Release of Proapoptotic Proteins. Injurious stimuli that are sufficiently potent promote the mitochondrial release of cytochrome *c* and Smac/DIABLO (5–7). To examine a possible role for PI3K signaling in protection of the mitochondrial outer membrane by preconditioning, we subjected animals to the procedures described above and examined the subcellular distribution of cytochrome *c* and Smac/DIABLO by Western blot analysis of mitochondrial and cytosolic fractions at 1, 6, and 12 h after reperfusion (or corresponding times in sham-operated, PC, and LY animals). Ischemia increased the abundance of cytochrome *c* in the cytosol of CA1,

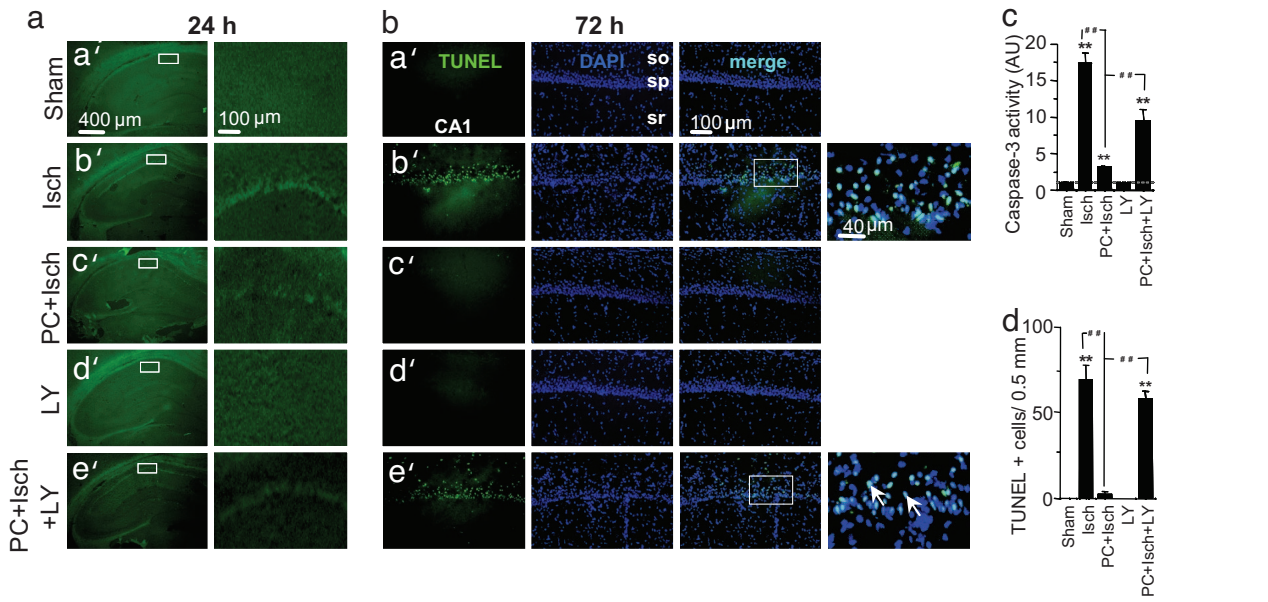

Fig. 4. Preconditioning acts via PI3K signaling to reduce caspase-3 activity and neuronal death in postischemic hippocampus. (*a*) Caspase-3 activity at 24 h. (*b*) TUNEL positivity (green) and DAPI staining (blue) of coronal brain sections at the level of the dorsal hippocampus at 72 h. Treatment rows and magnifications are as indicated. (*c*) Summary data for caspase activity, fluorescence in arbitrary units (AU). (*d*) Quantification of TUNEL-positive cells.

evident at 1 h (to 160 ± 18% of control; Fig. 3*f*), 6 h (to 196 ± 27% of control; Fig. 3*g*), and 12 h (to 314 ± 55% of control; Fig. 3*h*) after reperfusion, as assessed by Western blot analysis. Ischemia also increased the abundance of Smac/DIABLO in the cytosol at 6 h (to 191 ± 22% of control, $P < 0.01$) and to a lesser extent at 12 h (to 144 ± 9% of control, $P < 0.05$; Fig. 3 *i* and *j*). These data are in confirmation of previous studies (refs. 19 and 26 but see ref. 27). Preconditioning alone had little effect on cytosolic cytochrome *c*, but it blocked the ischemia-induced increases in cytosolic cytochrome *c* at all times of measurement (Fig. 3 *f–j*: ∗ and ∗∗, $P < 0.05$ and 0.01 vs. sham; # and ##, $P < 0.05$ and 0.01 vs. ischemia alone) and Smac/DIABLO at 6 h (Fig. 3*i*; $P < 0.05$). Neither Isch nor PC caused a significant change in the mitochondrial fraction (at 6 h and 12 h; data not illustrated, but see ref. 19). Together, these findings indicate that preconditioning prevents the mitochondrial release of cytochrome *c* and Smac/DIABLO in the face of ischemic insults (19). LY294002 alone did not significantly alter cytosolic levels of cytochrome *c* at 1 and 12 h (Fig. 3 *f* and *h*) or Smac/DIABLO at 6 and 12 h (Fig. 3 *i* and *j*) but transiently increased cytochrome *c* release at 6 h (to 231 ± 23% of control; Fig. 3*g*, $P < 0.01$). Moreover, in PC + Isch + LY animals the protective effects of preconditioning were reduced; ischemia-induced cytochrome *c* release was greater than in PC + Isch animals [at 1 h to 151 ± 17% of control (Fig. 3*f*), at 6 h to 418 ± 56% of control (Fig. 3*g*), and at 12 h to 277 ± 75% of control (Fig. 3*h*); ∗∗, $P < 0.01$ for all times). Collectively, these data indicate that PI3K activity is required for the protective effect of preconditioning on the mitochondrial outer membrane.

Preconditioning Acts via PI3K Signaling to Block Caspase-3 Activity. Cytochrome *c* release triggers formation of the apoptosome, which proteolytically processes procaspase-9 to generate the active caspase-9 (28, 29), which in turn cleaves procaspase-3 to form the active caspase-3, a terminator protease implicated in the execution step of apoptosis (30, 31). To directly measure caspase-3 activation, we used FAM-DEVD-FMK, a fluorescein-tagged analog of zDEVD-FMK. FAM-DEVD-FMK is a potent and cell-permanent inhibitor of caspase-3, which enters cells and binds irreversibly to the active site of caspase-3, and thus provides a fluorescent indicator of the abundance of activated caspase-3 (while inactivating it). In sections of control brain, abundance of activated caspase-3 was low (Fig. 4 *a* and *c*). Global ischemia induced a dramatic increase in activated caspase-3 in CA1, determined here at 24 h (see also ref. 19) ($P < 0.01$; Fig. 4 *a* and *c*). The increase in activated caspase-3 was region-specific in that it was not observed in the resistant CA3 or dentate gyrus. Preconditioning largely prevented the ischemic-induced increase in activated caspase-3 in CA1 pyramidal neurons ($P < 0.01$; Fig. 4 *a* and *c*). LY294002 alone did not significantly induce caspase-3 activity, but it reduced the protective effect of preconditioning on ischemia-induced formation of activated caspase-3 ($P < 0.01$; Fig. 4 *a* and *c*). These results confirm that preconditioning prevents the ischemia-induced increase in activated caspase-3 and indicate that PI3K signaling is required for this effect.

Preconditioning Acts via PI3K Signaling to Prevent Neuronal Apoptosis. Caspase-3 cleaves the inhibitory subunit of caspase-activated DNase (ICAD) of the CAD-ICAD heterodimer to generate activated CAD (5–7). CAD or DNA fragmentation factor (DFF) catalyzes internucleosomal cleavage of genomic DNA (19). If preconditioning indeed acts upstream of CAD activation, it would be expected to block DNA fragmentation in CA1 neurons destined to die. TUNEL is a labeling method in which a terminal transferase "tags" nicked ends present in fragmented DNA. To examine DNA fragmentation in neurons undergoing apoptosis, we labeled brain sections from control and experimental animals at 72 h after ischemia with TUNEL to assess DNA fragmentation and DAPI to assess cell numbers and possible alterations in nuclear morphology. In sections from control brain, TUNEL labeling was undetectable in CA1 (Fig. 4 *b*, *a′*, and *d*) and other subfields of the hippocampus (data not illustrated). Global ischemia induced a marked increase in the incidence of TUNEL-positive CA1 neurons, evident at 72 h after ischemia [$P < 0.01$; Fig. 4 *b*, *b′* (higher magnification in *Right*), and *d*]. Preconditioning completely blocked ischemia-induced DNA fragmentation indicated by TUNEL ($P < 0.01$; Fig. 4 *b*, *c′*, and *d*). LY294002 alone did not elicit TUNEL labeling, but it greatly reduced the protective effect of preconditioning on ischemia-induced TUNEL positivity ($P < 0.01$; Fig. 4 *b*, *e′*, and *d*). These results indicate that preconditioning acts via PI3K signaling to prevent ischemia-induced neuronal apoptosis and that, whereas

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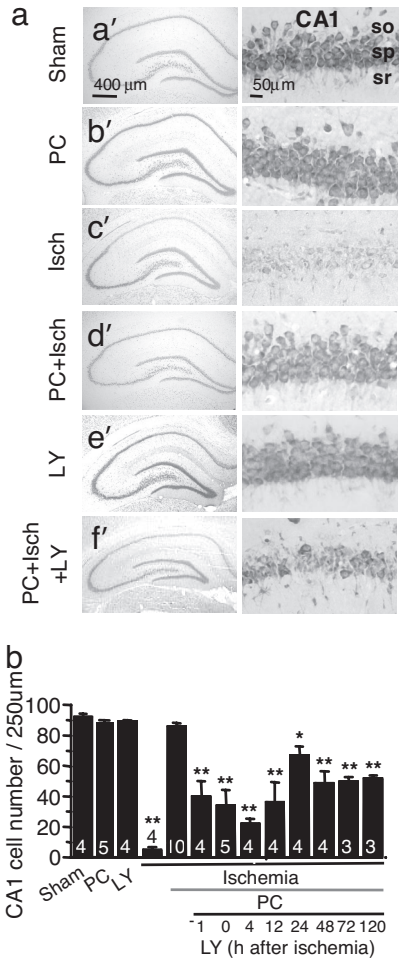

Fig. 5. The PI3K inhibitor LY294002 reduces ischemic tolerance as late as 120 h after ischemia. (*a*) Toluidine blue-stained coronal brain sections at the level of the dorsal hippocampus. Treatments are as indicated, with higher magnification in *Right*. Animals were terminated 9 days after preconditioning, 7 days after LY294002 alone, and 7 days after ischemia in PC + Isch and PC + Isch +LY experiments. (*f′*) LY294002 (single injection) was administered at 4 h after PC + Isch. (*b*) Neuronal counts. PC and LY alone were no different from sham. LY (single injection at the indicated times relative to Isch) decreased the PC-induced protection against neurodegeneration in animals terminated 7 days after Isch. *n* values are shown for each bar.

blockade of PI3K elicits transient cytochrome *c* release, it does not elicit caspase-3 activation, DNA fragmentation, or neuronal death.

Preconditioning Acts via PI3K Signaling to Protect Insulted CA1 Neurons. To examine whether PI3K is involved in the neuroprotection provided by preconditioning, animals were subjected to sham operation, PC, Isch, PC + Isch, LY, or PC + Isch + LY. Neuronal death in CA1 was assessed histologically in animals terminated 7 days after sham operation, LY294002 injection alone, ischemia alone, or ischemia in preconditioned animals with LY294002 injection at times from 1 h before to 120 h after reperfusion. Ischemia caused extensive and selective death of pyramidal cells in the CA1 layer ($P < 0.01$ vs. sham; Fig. 5*a*, compare *c′* with *a′*), in confirmation of other work (1, 4). These findings were quantified by neuronal counts (Fig. 5*b*). Preconditioning did not itself induce neuronal damage (Fig. 5 *a*, *b′*), but it afforded robust protection of CA1 neurons against the subsequent ischemic insult (Fig. 5 *a*, *d′*). LY294002 by itself did not induce neuronal death (Fig. 5 *a*, *e′*), but it largely reversed preconditioning-induced neuroprotection when given at 4 h after ischemia (Fig. 5*a*, *f′*). LY294002 reduced preconditioning-induced ischemic tolerance when given 1 h before ischemia or even 120 h after ischemia ($P < 0.01$; Fig. 5*b*). These results suggest that continued PI3K activity is necessary for preconditioning-induced neuroprotection and that ischemia causes a change lasting at least 5 days that can induce cell death if PI3K is inhibited. Maintained PI3K inhibition with multiple injections of LY294002 might lead to greater block of ischemic tolerance.

Discussion

Ischemic preconditioning affords robust protection of CA1 neurons against a subsequent severe ischemic challenge. Although the molecular mechanisms underlying ischemic tolerance are only partially understood, a crucial role for PI3K/Akt signaling is clear. Here we show that preconditioning acts via PI3K/Akt signaling to block ischemia-induced mitochondrial translocation of Bad, cleavage of Bcl-x_L to form its prodeath fragment, ΔN-Bcl-x_L, activation of large-conductance channels in the mitochondrial outer membrane, mitochondrial release of cytochrome *c* and Smac/DIABLO, caspase activation, DNA fragmentation, and neuronal death. The specific PI3K inhibitor LY294002 administered *in vivo* 1 h before or up to 120 h after ischemia significantly reverses preconditioning-induced protection, indicating a requirement for sustained PI3K signaling in this protection. A caveat concerning the mitochondrial channel recordings is that the mitochondria were not specifically from CA1. Subsequent experiments show that ischemia-induced large-conductance channels are more frequent in CA1 than in CA3 mitochondria, but effects of preconditioning and LY294002 have not yet been evaluated.

Our finding that PI3K activity is required for preconditioning-induced neuroprotection is consistent with findings of others that wortmannin administered before preconditioning reduces ischemic tolerance and Akt phosphorylation in global (20) and focal (32) ischemia. Others showed that Akt phosphorylation is increased by preconditioning (21) and that LY294002 increases death in CA1 after a moderate ischemic insult (33). Moreover, in a very different model, neonatal rat brain slices, PI3K blockade during and immediately after hypoxia reduces Akt phosphorylation and promotes mitochondrial release of cytochrome *c* and the onset of neuronal apoptosis (34).

We observed that LY294002 alone, in the absence of a neuronal insult, activates intermediate-conductance (but few large-conductance) channels in the mitochondrial outer membrane, whereas ischemia activates both intermediate- and large-conductance channels. These findings implicate large-conductance channels in neuronal death. LY294002 alone also promotes transient release of cytochrome *c*, which does not lead to caspase-3 activity or neuronal death. That neurons can survive cytochrome *c* release was unexpected, because a rise in cytochrome *c* was thought to lead inexorably to postischemic cell death (5–7). A more recent study shows that microinjection of cytochrome *c* into the cytosol of neurons is not sufficient to cause cell death (35). Whereas ischemia promotes mitochondrial release of cytochrome *c* and Smac/DIABLO, PI3K blockade promotes transient release of cytochrome *c* only. The enhanced cytosolic levels of cytochrome *c* are evident at 6 h but not at 1 h or 12 h. Moreover, LY294002 does not promote Bcl-x_L cleavage, caspase-3 activity, DNA fragmentation, or cell loss. These findings are consistent with a quantitative model in which cell death requires that sufficient cytochrome *c* must be present for sufficiently long; neurons may survive if the integrity of the mitochondrial outer membrane is not fully breached, if affected mitochondria seal up, or if they are removed by autophagy.

We previously showed that preconditioning causes substantial caspase-3 activation from 12 to 48 h afterward (earlier than the measurements here), but without cell death (19). Caspase-3 is still activated by ischemia in preconditioned animals, but the activation is transient, and the caspase-3 activity is inhibited. We did not determine whether preconditioning elevates cytochrome *c* in the

cytosol before caspase-3 activation. This study also showed that preconditioning protects the integrity of the mitochondrial outer membrane against a later severe ischemic insult in that cytochrome *c* and Smac/DIABLO are not released (19). The present study confirms and extends these findings by indicating mediation by the PI3K pathway.

It was surprising that LY294002 reduces protection even 120 h after ischemia. We presume that removal of PI3K activity reveals ischemia-induced changes that persist in the presence of PI3K activity and that can still cause cell death when PI3K is inhibited.

Our study has further dissected the mitochondrial cell death signaling pathway in CA1 after ischemia. Although the neuroprotective mechanisms after preconditioning have not been fully elucidated, an inference from the present study is that preconditioning leads to preservation of the integrity of the mitochondrial membrane in the face of ischemic insults and thereby enables CA1 neurons to survive. Diverse protective strategies may intervene at different steps in the caspase cascade. An understanding of the molecular mechanisms underlying ischemic tolerance should help in the design of novel neuroprotective strategies for intervention in the neuronal death associated with stroke, cardiac arrest, and various neurodegenerative disorders.

Materials and Methods

Preconditioning, Global Ischemia, and Drug Injection. Male Sprague–Dawley rats (100–150 g) were subjected to preconditioning, global ischemia, or preconditioning followed by ischemia, by four-vessel occlusion (preconditioning, 4 min; ischemia, 10 min), followed by reperfusion, as described (36, 37). LY294002 (12.5 mM in 5 μl of 25% DMSO) was administered by a single injection into the right lateral ventricle at a flow rate of 1 μl/min immediately after the last surgery. PC, Isch, and PC + Isch animals in the same series were injected with vehicle only.

Mitochondrial Preparation. Mitochondria were isolated from control and experimental rat hippocampus and purified by centrifugation over a discontinuous Ficoll gradient (38). The mitochondrial fraction was frozen on dry ice and stored at − 80°C until use.

Caspase-3 Activity, TUNEL, and Histology. Caspase-3 activity assays were performed on sections of fresh-frozen rat brain using the FAM-DEVD-FMK caspase detection kit according to the manufacturer's instructions (APO LOGIX; Cell Technology). To detect DNA fragmentation, animals were killed at 72 h after the last surgery and coronal sections (18 μm) of brain were processed for TUNEL and DAPI-labeled with an *in situ* cell death detection kit as per the manufacturer's instructions (Roche Molecular Biochemicals) as described (19).

Western Blot Analyses and Coimmunoprecipitation. For Western blots, protein samples of CA1 were prepared and run on SDS/PAGE as described (39). For coimmunoprecipitation experiments, samples were precipitated with the indicated antibody (IP), precipitates were resuspended and run on SDS/PAGE, and membranes were blotted with a second antibody (IB).

Additional Details. For more details see *SI Materials and Methods*.

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