Different Signaling Pathways Induce Apoptosis in Endothelial Cells and Cardiac Myocytes During Ischemia/Reperfusion Injury

Tiziano M. Scarabelli, Anastasis Stephanou, Evasio Pasini, Laura Comini, Riccardo Raddino, Richard A. Knight, David S. Latchman

Abstract—Apoptosis contributes, with necrosis, to the cardiac cell loss after ischemia/reperfusion injury. The apoptotic cascade is initiated either by mitochondrial damage and activation of caspase-9 or by death receptor ligation and activation of caspase-8. In the present study, performed in the isolated rat heart exposed either to ischemia alone or ischemia followed by reperfusion, cleavage of caspase-9 was observed primarily in endothelial cells. Conversely, caspase-8 cleavage was only found in cardiomyocytes, where it progressively increased throughout reperfusion. Addition of a specific caspase-9 inhibitor to the perfusate before ischemia prevented mitochondrial apoptosis, whereas preischemic infusion of a specific caspase-8 inhibitor affected only myocyte apoptosis. Additionally, caspase-8–mediated BID processing was observed only during reperfusion. Production of tBID then sustains mitochondrial injury and perpetuates caspase-9 activation. (Circ Res. 2002;90:745-748.)

Key Words: apoptosis ■ endothelium ■ myocytes ■ ischemia ■ reperfusion

Apoptosis is an active form of cell suicide affecting both endothelial cells and cardiac myocytes during ischemia/reperfusion injury. Mitochondrial damage leads to activation of the initiator protease, caspase-9, which then propagates the cascade of downstream caspases. In contrast, ligation of death receptors, such as Fas, activates caspase-8, which also then processes downstream effector enzymes. Enzymatically active caspase-8 cleaves BID, and the truncated protein, tBID, relocates to the mitochondria where it induces caspase-9 processing.

In this study, we have addressed three questions in the isolated rat heart: the level of enzymatic activity of the two initiator caspases during ischemia versus reperfusion; the selective contribution of these caspases to apoptosis of endothelial cells and myocytes; and the role played by BID in linking the two pathways.

Materials and Methods

Treatment Protocols
Isolated Langendorff-perfused rat hearts were randomized to 11 groups, each of at least 6 animals. The care and use of the animals in this study were in accordance with the Guidance of the Animal (Scientific Procedures) Act 1986, UK. The animals were purchased from Charles River Italia S.p.a. (a division of Charles River Laboratories, Wilmington, Mass). The control group was buffer-perfused for 60 minutes; 4 ischemia/reperfusion control groups and as many treated groups were exposed to 35 minutes of global ischemia either alone or followed by 5, 60, and 120 minutes of reperfusion, respectively. Before induction of ischemia, treated hearts were perfused for 20 minutes either with a specific and irreversible inhibitor of caspase-8 (Z-IEDT.fmk; C8i) or caspase-9 (Z-LEHD.fmk; CASPASE-9i), at a dose of 0.07 μmol/L. Finally, 20 minutes before ischemia, two other treated groups subjected to 35 minutes of global ischemia and 120 minutes of reperfusion received either a pan-caspase inhibitor (Z-VAD.fmk; 0.1 μmol/L) or a specific and irreversible inhibitor of caspase-3 (Ac-DEVD.cmk; 0.07 μmol/L; C3i). All caspase inhibitors were from Calbiochem.

Caspase-8 and Caspase-9 Enzymatic Activity Measurement
Cardiac activation of caspase-8 and caspase-9 was evaluated in tissue extracts using commercial kits (BioVision; see online data supplement available at http://www.circresaha.org).

Immunocytochemical Staining
Myocardial sections were stained with antibodies recognizing the cleaved active form of caspase-8 and caspase-9 (BioVision; see online data supplement). Other sections were stained with TUNEL, labeled with anti-desmin (Research Diagnostics Inc) or anti–von Willebrand antibodies (Boehringer Mannheim Biochemica), counterstained with propidium iodide, and finally analyzed by confocal fluorescent microscopy.

Western Blotting
Antibodies anti–cytochrome c (Cytc) and anti-BID (Santa Cruz Biotechnology) were used to process frozen samples from each heart by Western blotting (see online data supplement).

Statistical Analysis
Significance was evaluated using the ANOVA test. A value of P<0.05 was considered significant.
Results

In agreement with our earlier results in cultured myocytes, enzymatic assay shows that caspase-9 activation starts during ischemia and increases during reperfusion. In contrast, caspase-8 is functionally active only in hearts exposed to ischemia/reperfusion (Figure 1A).

By immunocytochemistry, in hearts receiving only ischemia, expression of cleaved caspase-9 is significantly increased in cardiomyocytes and even more in endothelial cells (4.7 ± 0.65% and 6.8 ± 0.54%, respectively; P < 0.05 versus controls) (Figure 1B). In the same hearts exposed to ischemia alone, consistent with the level of caspase enzymatic activity, cleavage of caspase-8 is not observed in any cell type. The proportion of endothelial cells positive for activated caspase-9 rises dramatically in ischemic/reperfused hearts, peaking after 1 hour of reperfusion (35.9 ± 3.1%; P < 0.001 versus control). In contrast, cleavage of caspase-9 in cardiomyocytes remains stable after 5 and 60 minutes of reperfusion and halves at 120 minutes of reperfusion. Finally, in

Figure 1. Caspase activation and TUNEL positivity in endothelial cells (EC) and cardiomyocytes (CM) after ischemia/reperfusion (I/R). Caspase-8 (C8) and caspase-9 (C9) activation was measured by cleavage of specific substrates (A) and by the percentage of EC and CM stained with antibodies against activated C8 and C9 (B). Relative AFC fluorescence is fold increase activity compared with buffer-perfused hearts. C. Percentage of TUNEL-positive EC and CM in control hearts exposed to I/R and treated hearts after infusion of caspase-8 and caspase-9 inhibitors (C8i and C9i). Data are expressed as mean ± SEM. #P < 0.05, *P < 0.05, **P < 0.01, and ***P < 0.001 vs equivalent I/R controls.

Figure 2. Cleavage of BID after ischemia/reperfusion (I/R). A, Fold increase expression of tBID, and the effects of Z-VAD and caspase-8 and caspase-9 inhibitors (C8i and C9i) on the appearance of tBID after 35 minutes of ischemia (I) and 60 minutes of reperfusion (R) (B). Kinetics during reperfusion of cytosolic translocation of CytC and tBID appearance in the presence of C8i (C) or C9i (D).
hearts exposed to ischemia/reperfusion, cleavage of caspase-8 is not observed in endothelial cells. However, the proportion of cardiomyocytes positive for cleaved caspase-8 progressively increases throughout reperfusion, reaching its maximum value after 120 minutes (8.9±1.6%; P<0.01 versus control).

The percentage of TUNEL-positive endothelial cells and cardiomyocytes in the hearts pretreated with caspase-8 and caspase-9 inhibitors (i) is reported in Figure 1C. Preischemic infusion of caspase-9i dramatically reduces endothelial apoptosis at all 3 time points of reperfusion. The decrease of cardiomyocyte apoptosis, in contrast, is less pronounced and becomes statistically significant only after 60 minutes of reperfusion. The administration of caspase-8i before ischemia consistently prevents TUNEL positivity in cardiomyocytes throughout reperfusion, without significantly affecting endothelial cell death.

Cleavage of BID assessed by Western blotting is seen in ischemic/reperfused hearts, but not in hearts exposed to ischemia alone (Figure 2A). Additionally, BID processing is greatly reduced by either Z-VAD or caspase-8i given before ischemia but is not affected by preischemic administration of either caspase-9i or caspase-3i (Figure 2B). To test whether tBID mediates communication between activated C8 and the
mitochondrial death machinery in the intact heart, we evaluated the time kinetics of both BID processing and mitochondrial release of Cytc throughout ischemia/reperfusion in hearts pretreated with caspase-8i and caspase-9i (Figures 2C and 2D). After inhibition of caspase-8, processing of BID is not observed. However, leakage of Cytc, reflecting the extent of direct mitochondrial injury in the absence of tBID, is seen after 5 and 60 minutes of reperfusion but disappears by 120 minutes (Figure 2C). In contrast, when caspase-9 is inhibited and only caspase-8 is active, BID processing proceeds throughout reperfusion and Cytc relocates not only at 5 and 60 minutes of reperfusion but also after 120 minutes (Figure 2D; for ischemia/reperfusion control, see online data supplement). At this time, tBID production parallels the peak of cytosolic relocation of Cytc, which is not observed when BID processing is prevented by inhibition of caspase-8.

Similar results are found evaluating the proportion of cleaved caspase-8 and caspase-9 in hearts subjected to ischemia/reperfusion after their selective inhibition (Figure 3A). Pretreatment with caspase-8i consistently reduces not only cleavage of caspase-8 in cardiomyocytes but also that of caspase-9 in both endothelial cells and cardiomyocytes. In contrast, the caspase-9i diminishes activation of caspase-9 in endothelial cells and cardiomyocytes but does not affect the proportion of cells positive for cleaved caspase-8. Hearts pretreated with caspase-8i, although not caspase-9i, similarly show reduction of caspase-8 and caspase-9 enzymatic activities (Figures 3B and 3E). Additionally, whereas both endothelial cells and cardiomyocytes are positive for active caspase-9 after ischemia alone and after ischemia/reperfusion, cleaved caspase-8 staining is only seen in cardiomyocytes after ischemia/reperfusion (Figures 3C and 3D). Figure 3D confirms this identification of the cell types by staining the same sections with anti–von Willebrand and anti-desmin antibodies.

Discussion

This study shows that caspase-9 is activated during ischemia and remains activated throughout reperfusion in the intact rat heart exposed to ischemia/reperfusion injury. In contrast, processing of caspase-8 is only triggered during reperfusion. Apoptosis of endothelial cells seems to be mediated completely by caspase-9 activation. Cardiomyocyte apoptosis relies on active caspase-9 during ischemia and early in reperfusion but shows an increasing dependence on caspase-8 activation later in reperfusion. Therefore, different initiator caspsases are processed during different phases of ischemia/reperfusion injury and differentially trigger apoptosis in endothelial cells and cardiomyocytes.

The apparent resistance of endothelial cells to apoptosis after death receptor ligation and caspase-8 activation may reflect their high levels of expression of FLICE (FLIP inhibitory protein), an endogenous inhibitor of caspase-8 activation. Synthesis of death receptor ligands such as FasL and tumor necrosis factor-α has been demonstrated in isolated rat hearts early in reperfusion, and hearts from mice with a dysfunctional Fas receptor have fewer apoptotic cells after reperfusion. This suggests that cardiomyocyte apoptotic death during reperfusion may be mediated by Fas ligation and is consistent with our observation of caspase-8 activation only during reperfusion. However, others have shown a critical role for free radicals and mitochondrial damage in the cardiomyocyte apoptosis induced by ventricular pacing, and the relative contribution of these two mechanisms to myocyte death remains to be fully clarified.

Our results on differential initiator caspase activation depend partly on the use of selective caspase inhibitors. Although the absolute specificity of these may be questioned, their use at submicromolar concentrations together with their in vivo effects (see Figures 3A and 3B) suggests that specific inhibition is being obtained under the experimental conditions used. Similarly, the previously documented specificity of the antibodies for the cleaved active forms of the respective caspases is supported by the general agreement between the immunostaining data and the enzymatic activity assays.

Our data further suggest that sustained activation of caspase-9 seen during reperfusion may depend on caspase-8-mediated cleavage of BID. Other caspsases, such as caspase-3, as well as non–caspase proteases such as calpain, have also been shown to cleave BID, resulting in its mitochondrial translocation. Although the caspase-8 inhibitor reduces BID cleavage, the caspase-3 inhibitor is ineffective. Our data do not, however, exclude a contribution by calpain to BID cleavage.

These data, showing differences in initiator caspase activation over time, and between endothelial cells and cardiomyocytes, suggest that apoptosis after ischemia/reperfusion injury is not a homogenous process. Further understanding of the differential contribution of caspase-8 and caspase-9 may reveal new selective targets for minimizing cell loss after infarction.

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Correction to: Different Signaling Pathways Induce Apoptosis in Endothelial Cells and Cardiac Myocytes During Ischaemia-Reperfusion Injury

As part of an investigation by the University College of London (UCL), concerns were raised regarding certain figures in three American Heart Association journals.1–3 To address these concerns, the authors of these articles have prepared the following corrections:

For the article by Scarabelli et al in Circulation (Apoptosis of endothelial cells precedes myocyte cell apoptosis in ischaemia/reperfusion injury. Circulation 2001;104:253–256.), concerns were raised regarding Figure 2d, which was used inadvertently in a subsequent publication. To avoid any misunderstanding, the authors have corrected the panel with a replicate Figure performed at much the same time as the original but in a different laboratory.

For the article by Lawrence et al in Circulation (KATP Channel gene expression is induced by urocortin and mediates its cardioprotective effect. Circulation, 2002; 106: 1556–1562.), to address the concerns raised about Figure 3a, the first author, Dr Lawrence, repeated the experiment, reproduced the induction of the KATP channel with an appropriate actin control, and corrected the panel.

For the article by Scarabelli et al in Circulation Research (Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischaemia-reperfusion injury. Circ Res. 2002;90:745–748.), to address the concerns raised about Figure 2d, the authors completed two new distinct sets of experiments. As the same loading control was used twice for both Figure 2c and Figure 2d, the authors repeated both experiments, using a caspase 8 inhibitor (Figure 2c) and a caspase 9 inhibitor (Figure 2d), respectively and corrected these panels.

The authors apologize for these errors, which have been corrected in the online version of each article.

References
ONLINE SUPPLEMENTARY INFORMATION

METHODS

*Western Blot analysis for whole tissue extract and cytosol fractions.*

Cytosolic fractions were generated using the digitonin-based subcellular fractionation method, essentially described previously (Ekert et al. J. Cell. Biol. 2001;152,483-490). Tissues were digitonin-permeabilized by gently homogenizing with a Dounce homogenizer in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 1mM PMFS, 10µg/ml aprotinin, 20µg/ml leupeptin, containing 250 mg/ml digitonin). Lysates were centrifuged at 1,000 x g for 5 min at 4 °C. The supernatants (30 ml cytosolic fractions) or whole tissue lysate (30 ml) were supplemented with 5 x SDS-PAGE loading buffer, subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with anti-active caspase 8, anti-active caspase 9, anti-Bid and anti-cytochrome c, followed by horseradish peroxidase-conjugated secondary antibody and autoradiography with enhanced chemiluminescence.
Caspase-8 and -9 enzymatic activity measurement in whole tissue extract and cytosol fractions

Cardiac activation of Caspase-8 and caspase-9 was evaluated in tissue extracts using commercial kits purchased from BioVision (Caspase-8/Flice and Caspase-9/Mch6 Fluorometric Assay Kit, Biovision; Mountain View, CA, USA). The following changes were made to the recommended protocol. Cardiac ventricular tissue from each group was placed in ice-cold lysis buffer and subsequently homogenized. The homogenates were centrifuged at 750 x g for 5 mins at 4° C. Supernatants were then centrifuged at 10000 x g for 15 mins at 4° C. Enzyme reactions were performed with 300 µg of cytosolic proteins per assay and a final concentration of 50 µM of AFC (7-amino-4-trifluoromethyl coumarin)-conjugated IEDT and LEHD, substrates specific for caspase-8 and -9 respectively. Samples were then read in a fluorimeter equipped with a 400-nm excitation and a 505-nm emission filter. Fold-increase in C3 activity was determined by comparing fluorescence of AFC in control and treated hearts with buffer perfused control.

Characterisation of anti-active caspase-8 and -9 antibodies

Fig. A: Anti-active caspase-8

The antibody does not detect a signal corresponding to the catalytically inactive procaspase 8 (c. p60) in either control or ischemia/reperfused samples. However, it does detect a band corresponding to one of the catalytically active caspase subunits (p10) only
in the ischemia/reperfused sample. This demonstrates that the antibody only recognises a cleaved enzymatically active caspase-8 fragment. The Figure also demonstrates that procaspase-8 cleavage does not occur in control, but only in ischemia/reperfused tissues.

**Fig. B: Anti-active caspase-9**

The antibody does not detect a band corresponding to the inactive procaspase-9 (c. p46). It does detect a band (p37) corresponding to the cleaved enzymatically active enzyme in the ischemia/reperfused sample. This demonstrates that procaspase-9 cleavage does not occur in control tissues, but only following ischemia/reperfusion.

**RESULTS**

**Fig. C: BID cleavage and Cytochrome c (Cytc) translocation following ischemia (I) and ischemia followed by reperfusion (R) in the absence of caspase inhibitors.**

Total and cytosolic extracts from Control (C) hearts and hearts exposed to ischemia alone or to ischemia followed by the times of reperfusion shown were subjected to Western blotting as described above and the blots probed with the antibodies indicated. The first panel shows total cellular cytc, the second the appearance of cytc translocated from mitochondria to the cytosol during reperfusion. The third panel demonstrates the production of the cleaved form of BID (tBID). The final panel shows actin staining as a protein loading control.
Fig. A - active caspase-8

Control 35'I+60'R

p60

p10

Actin
Fig. B - active caspase-9

Control 35'I+60'R

p46
p37
Actin