Myocardial Expression of a Dominant-Negative Form of Daxx Decreases Infarct Size and Attenuates Apoptosis in an In Vivo Mouse Model of Ischemia/Reperfusion Injury

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Background—Apoptosis has been described extensively in acute myocardial infarction and chronic heart failure. Because Daxx (death-associated protein) appears to be essential for stress-induced cell death and acts as an antisurvival molecule, we tested the hypothesis that Daxx is involved in myocardial ischemia/reperfusion–induced cell death in vivo.

Methods and Results—Transgenic mice overexpressing a dominant-negative form of Daxx (Daxx-DN) under the control of the β-actin promoter and control wild-type mice underwent an ischemia/reperfusion protocol: 40 minutes of left coronary artery occlusion and 60 minutes of reperfusion. Area at risk and infarct size were measured after dual staining by triphenyltetrazolium chloride and phthalocyanine blue dye. Apoptosis was measured in the ischemic versus the nonischemic part of the left ventricle by terminal deoxynucleotidyl transferase–mediated dUTP biotin nick end labeling staining, enzyme-linked immunosorbent assay, and Western blotting of caspase-3, caspase-8, and poly(ADP-ribose) polymerase. The mitogen-activated protein kinase status was investigated by Western blot analysis. Comparison between groups was assessed by ANOVA or Student t test (statistical significance: \(P<0.05\)). Left ventricle tissues from transgenic mice expressed Daxx-DN at the protein level. Area at risk/left ventricle values were comparable among groups. Infarct size/area at risk was 45% reduced in Daxx-DN versus wild-type mice (\(P<0.001\)). This cardioprotection was maintained for a 4-hour reperfusion. Ischemia/reperfusion-induced apoptosis was significantly decreased and ERK1/2 prosurvival pathway was activated in ischemic Daxx-DN hearts.

Conclusions—Our study clearly indicates that Daxx participates in myocardial ischemia/reperfusion proapoptotic signaling in vivo. (Circulation. 2007;116:2709-2717.)

Key Words: apoptosis ▪ Daxx ▪ infarction ▪ ischemia ▪ reperfusion

A poptosis has been described extensively in numerous diseases including acute myocardial infarction and chronic heart failure.1-5 The participation of a highly regulated, energy-dependent form of cell death during myocardial ischemia/reperfusion may lead to novel therapeutic interventions.6 This possibility is supported by studies in which inhibition of apoptosis by a variety of pharmacological and genetic approaches results in a smaller infarction size.7-9 Although these experiments demonstrate a causal role for apoptosis in myocardial infarction, the apoptosis signaling pathways functional during myocardial ischemia/reperfusion have not yet been delineated fully in vivo.

To date, the death receptor/extrinsic and mitochondrial/intrinsic pathways are the most heavily studied pathways of cell death in myocardial ischemia/reperfusion. Once activated, these 2 different apoptotic pathways initiate a “caspase cascade” leading to the final destruction of the cell. Apoptotic signaling via external stimuli involves the binding of specific ligands to death receptors. Fas (TNFRSF6/CD95/APO-1) is a prototypic death receptor belonging to the tumor necrosis factor (TNF) receptor (TNFR) superfamily.10 These death receptors are expressed on the cell surface as preassociated...
heterotrimeric and on engagement by their ligands undergo a conformational change that favors homotypic interactions with other death domain–containing proteins such as FADD (Fas-associated protein with death domain). FADD recruits pro-caspase-8 through an additional homotypic interaction via its “death effector domain.” This protein complex, therefore, formed by Fas, FADD, and pro-caspase-8, is called DISC (death-inducing signaling complex). Efficient DISC assembly induces autoproteolytic cleavage, which subsequently activates the cell death pathway. Alternatively, cell death can be triggered internally by initiation of the mitochondrial pathway. A dynamic balance of proapoptotic and antiapoptotic Bcl-2 proteins controls mitochondrial permeability. Permeabilization of the outer mitochondrial membrane leads to the release of cytochrome c and other proapoptotic factors. The release of cytochrome c triggers the formation of a complex containing Apaf-1, dATP, and pro-caspase-9, which is then autocleaved to process the downstream caspases, such as caspase-3. Cross talk between death receptor and mitochondrial pathways has been demonstrated. For example, the activated caspase-8 can induce the proteolytic cleavage of Bid, which initiates cytochrome c release from mitochondria.

Other less well-defined cell death signaling mechanisms, such as Daxx/ASK1/MAP kinases–mediated apoptosis, have recently been described in noncardiac cells. Daxx has been implicated in the modulation of apoptosis in response to various stimuli, including ultraviolet irradiation and oxidative stress. The cellular localization and function of Daxx is controlled by the relative concentration of the apoptosis signal-regulating kinase 1 (ASK1). In the nucleus, Daxx colocalizes with the promyelocytic leukemia protein (PML), whereby it plays a direct role in transcriptional regulation. On oxidative stress, Daxx relocates from the nucleus to the cytoplasm by binding to ASK1, and this correlates with the activation of the JNK pathway and cell death.

Because Daxx appears to be essential for stress-induced cell death and acts as an antisurvival molecule, we tested the hypothesis that Daxx is involved in myocardial ischemia/reperfusion–induced cell death in vivo. Because Daxx-deficient mice exhibit early embryonic lethality, they are unsuitable to directly test this hypothesis. To overcome this limitation, we used transgenic mice that overexpress a dominant-negative form of Daxx under the control of the β-actin promoter. WT and Daxx-DN mice underwent a 40-minute coronary occlusion followed by reperfusion. Two protocols were applied according to the reperfusion time: IR 1H if the reperfusion period lasted 1 hour of reperfusion and IR 4H if it lasted 4 hours. Shams were subjected to the same protocol except for the reversible coronary occlusion. The black box represents the period of ischemia. C, Hematoxylin-eosin histology of LV myocardium sections of WT (top) vs Daxx-DN (bottom) mice at different ages (a, d, 7 weeks; b, e, 11 weeks; c, f, 12 months) (magnification ×40).

**Methods**

**β-Actin-Daxx-DN Transgenic Mice**

To maintain heterozygosity, β-actin-Daxx-DN (+/−) transgenic mice expressing a dominant-negative form of Daxx under the control of the β-actin promoter were crossed with C57BL/6 mice. The transgene derives from the mouse Daxx-DN cDNA encoding amino acids 628 to 739, which correspond to the Fas binding domain of Daxx (Figure 1A). Transgenic lines had no overt phenotype and were compared with wild-type (WT) transgenic littermates. All the exper-
Immunoblotting

paraffin-embedded LV sections (4 m) were harvested at the end of surgery. Terminal transferase-mediated dUTP-biotin nick-end labeling (TUNEL) was performed with fluorescein-dUTP (In Situ Cell Death Detection Kit; Roche Diagnostics). After TUNEL, the sections were blocked with 2% bovine serum albumin in phosphate-buffered saline and incubated with anti-α-actinin antibody (EA-53; Sigma) and biotinylated isocyt B4 (IB4; Sigma) to label cardiomyocytes and endothelial cells, respectively.21 Labeling was revealed with the use of extravidin-Cy3 and anti-mouse IgG conjugated to cyanine 5. Cell nuclei were stained with DAPI (Sigma). Slides were visualized on a LEICA-DMR2A microscope and a Biorad MRC 1024 confocal laser scanning microscope. Adobe Photoshop was used to prepare final figures.

Immunoblotting

Transmural samples of nonischemic or ischemic areas of LV were rapidly frozen in liquid nitrogen. The LV tissues were homogenized with a grinder in cold phosphate-buffered saline supple-
dystrophy in Daxx-DN mice compared with WT littermates (Figure 1C).

Expression of Daxx-DN Protein in LV
Western blot analysis of lysates from LV tissues (ischemic or nonischemic) with an antibody specific for the mouse Daxx protein revealed the expression of the endogenous Daxx protein (120 kDa) and Daxx-DN transgene (17 kDa) exclusively in lysates from the transgenic animals. The relative expression level of the Daxx-DN transgenic protein was 38.1/88.6 in percentage of the endogenous Daxx protein estimated amount (n=4 for WT or Daxx-DN samples of LV) (Figure 2A).

Reduction of Infarct Size in Daxx-DN Mice
After incubation in TTC, slices from WT mice hearts showed a large homogeneous myocardial infarct in the anterior and lateral free wall of the LV. There was a subepicardial rim of surviving myocardium adjacent to the infarct. Daxx-DN mice had smaller infarcts than control littermates (Figure 2B).

Average area at risk/LV mass and infarct size/area at risk were determined in Daxx-DN and WT mice subjected to ischemia/reperfusion. In the group subjected to reperfusion for 1 hour (IR 1H group), average Daxx-DN infarct size/area at risk was 45% smaller than in WT animals (27.4±3.0%, n=10 versus 50.1±2.1%, n=14; P<0.001). In the IR 4H group, average Daxx-DN infarct size/area at risk was also markedly reduced (36%) compared with WT mice (24.5±5.7%, n=7 versus 42.7±3.7%, n=6; P<0.05). There was no statistical difference in the reduction of infarct size measured in Daxx-DN animals subjected to 1 hour versus 4 hours of reperfusion (27.4±3.0%, n=10 versus 24.5±5.7%, n=7; P=NS) (Figure 2C). Area at risk/LV mass was unchanged among groups (P=NS): for the IR 1H group, WT:
Apoptosis in Daxx-DN Mice

Inhibition of Ischemia/Reperfusion-Induced Apoptosis in Daxx-DN Mice

TUNEL analyses were performed on LV tissue sections from WT and Daxx-DN mice (IR 1H group). TUNEL-positive nuclei were determined by randomly counting 18 fields in the ischemic and nonischemic areas of both WT (n=3) and Daxx-DN (n=3) mice.

At high-power magnification (×40) of the infarcted zone, the percentage of TUNEL-positive nuclei reached 22.6±5.0% in WT versus 10.0±3.0% in Daxx-DN mice (P<0.01; n=18). The nonischemic regions exhibited low levels of TUNEL staining (3.5±1.0% in WT versus 3.8±1.3% in Daxx-DN; n=18) compared with the infarcted zone (ischemic versus nonischemic; P<0.001 for WT and P=NS for DN). TUNEL-positive nuclei identity was determined by colocalizing with sarcomeric (α-actinin) and vascular endothelium–specific (IB4) markers. Only 11.2±1% among TUNEL-positive nuclei were associated with IB4 staining, whereas the remaining nuclei (88.8±1%) were localized in association with the sarcomeric marker (for a total of 197 counted nuclei in n=3 fields) (Figure 3A and 3B).

In WT mice, DNA fragmentation was increased in the ischemic portion of LV tissues compared with the nonischemic portion (1.10±0.18 versus 0.31±0.05; n=8; P<0.001). As shown in Figure 3C, the amount of soluble nucleosome in the ischemic portion was markedly reduced in Daxx-DN animals compared with WT (1.10±0.18, n=8 versus 0.57±0.15, n=8; P<0.01).

To analyze the molecular mechanisms of the cell death pathways involved in the ischemia/reperfusion process, we compared the activity of caspase-3 and -8 in both the nonischemic and the ischemic tissue lysates from the WT and the Daxx-DN animals. All nonischemic LV samples (n=3) showed a strong signal for a band at 32 kDa corresponding to the inactive proform of caspase-3 (Figure 4A). By contrast, the inactive proform of caspase-3 was reduced in the ischemic LV tissues obtained from WT animals. Consistently, the active form of caspase-8 was detected mainly in the ischemic LV tissues obtained from WT animals. During apoptosis, active PARP (116 kDa), a known substrate of caspase-3, is inactivated by proteolytic cleavage, which generates an 85-kDa fragment. In WT mice, the PARP fragment was more abundant in the ischemic portion of LV tissues compared with the nonischemic, whereas PARP cleavage was reduced in Daxx-DN animals (Figure 4A).

Modulation of Ischemia/Reperfusion-Induced MAP Kinase Activation in Daxx-DN Mice

Phosphorylation patterns of ERK1/2, JNK1/2, and P38 MAP kinases were analyzed with antibodies specifically recognizing their phosphorylated forms in the presence of pharmacological inhibitors (Figure 4B). Ischemia/reperfusion (IR 1H) significantly induced phosphorylation of MAP kinases in the ischemic versus nonischemic area for ERK1/2 (P<0.05; n=9...
Daxx-DN hearts) and for JNK1/2 (P=0.01; n=10 WT and Daxx-DN hearts). A slight increase, although not significant (ischemic versus nonischemic; P>0.05), was observed for the phosphorylation of P38 in WT (n=13) and Daxx-DN (n=13) hearts and for the phosphorylation of ERK1/2 in WT hearts (n=13).

As illustrated in Figure 4C, phosphorylation of ERK1/2 was significantly increased in the ischemic part of the LV obtained from Daxx-DN compared with WT mice (P<0.01). By contrast, there was no difference in JNK1/2 and P38 MAP kinase phosphorylation in the ischemic area from WT and Daxx-DN mice (P=0.97 and P=0.80, respectively) (Figure 4D and 4E).

**Discussion**

In the present study, we tested the hypothesis that Daxx is involved in myocardial ischemia/reperfusion-induced cell death in vivo. Our results show a marked reduction in both infarct size and cardiac apoptosis in transgenic mice that overexpress a dominant-negative form of Daxx. Our study clearly indicates that Daxx participates in myocardial ischemia/reperfusion proapoptotic signaling in vivo.

**Myocardial Ischemia/Reperfusion-Induced Cell Death**

The participation of apoptosis in cell death during myocardial ischemia/reperfusion remains controversial. A plethora of studies show apoptotic cell death in different models of animal and human ischemic heart disease.1–5,24 This contrasts with the relatively few reports stating that apoptosis is not detected in the ischemic heart.25,26 These discrepancies could be due to the variability among infarct models (ischemia with or without reperfusion) and the methods used to detect apoptosis. Ischemic injury is thought to initiate apoptosis, but reperfusion seems to be necessary for substantial apoptotic cell death to occur.1,26,27 In our ischemia/reperfusion murine model, apoptosis in the ischemic part of the LV was identified by both TUNEL staining and enzyme-linked immunosorbent assay and confirmed by analysis of caspase-3–mediated PARP proteolytic cleavage.

Genetic manipulation of proteins regulating apoptosis in murine models has been crucial in identifying their involvement in myocardial ischemia/reperfusion-induced cell death. For example, overexpression of the antiapoptotic Bcl-2 protein reduces apoptosis and protects against myocardial ischemia/reperfusion injury in transgenic...
mice. Our data clearly demonstrate that cardiac expression of a dominant-negative form of a protein acting in the apoptotic cascade is associated with significant in vivo cardioprotection. These results are consistent with other studies that have found a beneficial effect of specific in vivo antiapoptotic interventions in myocardial ischemia/reperfusion.²⁷⁻²⁹

**Involvement of the Death Receptor Pathway in Ischemia/Reperfusion-Induced Cell Death**

The TNFR superfamily members are cell surface cytokine receptors controlling cell fate decisions, eg, proliferation, differentiation, and cell death.²⁸ A prototype of the death receptor TNFR subfamily is Fas (CD95; APO-1). Binding of the Fas ligand to its membrane receptor induces an intracellular cascade initiated by the formation of DISC. In this complex, the adapter protein FADD allows the recruitment of pro-caspase-8 molecules that are autoactivated and in turn provokes downstream caspase-3 processing. Involvement of the Fas receptor pathway remains controversial in myocardial ischemia/reperfusion-induced cell apoptosis. Under physiological conditions, cardiac myocytes are relatively insensitive to Fas ligand–induced cell death.²⁷,²⁹ Moreover, mice treated systemically with an activating Fas antibody die from massive hepatic apoptosis but exhibit no cardiac damage.³⁰ Jeremias et al³¹ suggested that CD95/Fas/APO-1 is directly involved in cell death after myocardial ischemia. In agreement, Lee et al³² demonstrated that Fas receptor–deficient (lpr) mice exhibit smaller infarcts compared with WT animals and that the levels of soluble Fas ligand expression during ischemia/reperfusion correlate with infarct size. In vitro studies also demonstrate that hypoxia sensitizes cardiac cells to the effects of Fas ligand.³³ However, Gomez et al³⁴ suggest that mitochondria are preferentially involved in mice cardiomyocyte death after ischemia/reperfusion injury and that the Fas pathway plays a relatively minor role.

**Daxx Participates in Myocardial Ischemia/Reperfusion Proapoptotic Signaling In Vivo**

Daxx was originally described by Yang et al¹⁶ as a novel protein that specifically binds to the death domain of the receptor Fas in the cytoplasm and potentiates Fas-induced apoptosis. However, a large proportion of Daxx molecules are nuclear and associate with the PML and other subnuclear domains.³⁵ Daxx is a promiscuous interactant and has been reported to associate with numerous proteins involved in cell death regulation.³⁶ The subcellular localization of Daxx is determined by the relative concentration of ASK1, which controls the dual function of Daxx as a transcriptional repressor in the nucleus and as a proapoptotic signal mediator in the cytoplasm.¹⁹

Kim et al¹⁷ demonstrate that Daxx expression is necessary for apoptotic signaling triggered by oxidative stress in HeLa and HEK 293 cells. Their report shows that H₂O₂ treatment rapidly induces the expression of endogenous Daxx at the transcription level. Inhibition of Daxx upregulation with antisense oligonucleotides prevents H₂O₂-induced cell apoptosis. In agreement, Yaniv et al³⁷ demonstrate that the Daxx-ASK1-JNK pathway is the main apoptotic signaling cascade that operates when Fas is activated after H₂O₂ sensitization. In a recent study, Jung et al³⁸ examined the role of Daxx on chemical hypoxia and, through the use of localization-specific Daxx mutants, demonstrated that the subcellular localization of Daxx determines its role in ischemic cell death. In response to simulated ischemia, Daxx is exported to the cytoplasm, and cell death is mediated via JNK activation. Overall, there is no evidence for the role of Daxx in myocardial ischemia/reperfusion-induced cell death in vivo.

With the use of transgenic mice that overexpress a dominant-negative form of Daxx, our results show that expression of Daxx-DN in LV tissues subjected to ischemia/reperfusion contributes to reduce both infarct size and cardiac apoptosis in mice hearts in vivo. To our knowledge, this is the first indication that Daxx participates in myocardial ischemia/reperfusion proapoptotic signaling in vivo. Because the mouse Daxx-DN transgene contains the Fas binding domain of Daxx, it is reasonable to suggest that the Fas-Daxx pathway is required for cell death in the ischemic and reperfused heart. Surprisingly, we also show that the active form of caspase-8 is more abundant in the ischemic portion of LV tissues obtained from WT compared with Daxx-DN animals. These results indicate that the Fas-FADD-caspase-8 axis is downregulated in our transgenic animal hearts on ischemia/reperfusion and suggest a cross talk between Fas-Daxx and Fas-FADD-caspase-8 pathways.

To study the level at which Daxx regulates cell death in vivo, we examined the expression of several stress-activated protein kinases, including JNK1/2, P38, and ERK1/2. These kinases control both cell survival and death. Accumulating evidence suggests that Daxx can activate the JNK pathway in isolated cells. Indeed, the expression of a dominant-negative mutant of Daxx inhibits JNK activation on ultraviolet radiation.³⁹ Paradoxically, we did not observe differences in the phosphorylation levels of both JNK1/2 and P38 between WT and Daxx-DN hearts. These findings are not totally surprising because the strength and the timing of stress-activated protein kinase activation might differ depending on cell types and stimuli. For example, Daxx silencing significantly enhances the early phase of JNK activation but inhibits its late phase.⁴⁰ Remarkably, we show that ERK1/2 phosphorylation is significantly increased to a greater extent in the ischemic LV tissues obtained from Daxx-DN compared with WT mice. This observation is consistent with numerous studies in which ERK1/2 activation is associated with protection against apoptosis in cardiac myocytes.⁴¹ ⁴³ Thus, MEK1-ERK1/2 signaling has been shown previously to protect the mouse heart from ischemia/reperfusion injury in vivo.⁴² ⁴⁴ The control mechanisms that balance cell survival against cell death are not well understood. However, most data in the literature support the concept that JNK1/2 and P38 can both facilitate or inhibit apoptosis, whereas ERK1/2 always protects against cell death.

Altogether, our results show that transgenic Daxx-DN expression protects against ischemic/reperfusion-induced apoptosis and that this cardioprotection is likely mediated by an increase in ERK1/2 phosphorylation.
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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Lethal reperfusion injury is caused by restoration of coronary blood flow after an ischemic episode. This phenomenon culminates in apoptotic death of cardiac cells that were viable immediately before myocardial reperfusion. The participation of a highly regulated form of cell death during myocardial ischemia/reperfusion may lead to novel therapeutic interventions in the reperfusion phase. However, the apoptosis signaling pathways functional during myocardial ischemia/reperfusion have not yet been delineated fully in vivo. In this study, we show that Daxx, a novel protein that specifically binds to the death domain of the receptor Fas, participates in myocardial ischemia/reperfusion proapoptotic signaling in vivo. With the use of transgenic mice overexpressing a dominant-negative form of Daxx, our study shows a marked reduction in both infarct size and cardiac apoptosis in transgenic animals. This cardioprotection was associated with a significant increase in ERK1/2 phosphorylation. The Fas–Daxx pathway is emerging as a new target for cardioprotection. These findings could lead to new strategies to improve clinical outcomes in acute myocardial infarction.
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