Targeted deletion of Puma attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion

Ambrus Toth,1 John R. Jeffers,2 Philip Nickson,1 Jiang-Yong Min,3 James P. Morgan,3 Gerard P. Zambetti,2 and Peter Erhardt1

1Boston Biomedical Research Institute, Watertown, Massachusetts; 2Department of Biochemistry, St. Jude Children’s Research Hospital, Memphis, Tennessee; and 3Department of Medicine, Caritas St. Elizabeth’s Medical Center, Boston, Massachusetts

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Targeted deletion of Puma attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion. Am J Physiol Heart Circ Physiol 291: H52–H60, 2006. First published January 6, 2006; doi:10.1152/ajpheart.01046.2005—The p53-upregulated modulator of apoptosis (Puma), a BH3-only member of the Bcl-2 protein family, is required for p53-dependent and -independent forms of apoptosis and has been implicated in the pathomechanism of several diseases, including cancer, acquired immunodeficiency syndrome, and ischemic brain disease. The role of Puma in cardiomyocyte death, however, has not been analyzed. On the basis of the ability of Puma to integrate diverse cell death stimuli, we hypothesized that Puma might be critical for cardiomyocyte death upon ischemia-reperfusion (I/R) of the heart. Here we show that hypoxia-reoxygenation of isolated cardiomyocytes led to an increase in Puma mRNA and protein levels. Moreover, if Puma was delivered by an adenoviral construct, cardiomyocytes died by apoptosis. Under ATP-depleted conditions, however, Puma overexpression primarily induced necrosis, suggesting that Puma is involved in the development of both types of cell death. Consistent with these findings, targeted deletion of Puma in a mouse model attenuated both apoptosis and necrosis. When the Langendorff ex vivo I/R model was used, infarcts were ~50% smaller in Puma−/− than in wild-type mice. As a result, after I/R, cardiac function was significantly better preserved in Puma−/− mice than in their wild-type littermates. Our study thus establishes Puma as an essential mediator of cardiomyocyte death upon I/R injury and offers a novel therapeutic target to limit cell loss in ischemic heart disease.

apoptosis; necrosis

ISCHEMIC HEART DISEASE PREVAILS as the leading cause of morbidity and mortality in Western societies (24). During ischemia-reperfusion (I/R), cardiomyocytes undergo apoptosis and necrosis, which significantly compromise myocardial functional performance. Although these responses involve separate pathways, they share common mediators, allowing cross talk under certain conditions. In particular, mitochondrial membrane damage plays an important role in initiating apoptosis and necrosis (7, 9, 23, 34, 38, 53). Upon mitochondrial injury, the abundance of ATP favors the execution of apoptotic cardiomyocyte death; however, necrosis ensues under circumstances of impaired cellular energy status (7, 10, 20, 45).

Principal regulators of mitochondrial membrane integrity and function include the Bcl-2 protein family, which consists of three subgroups distinguished by structural homology. The antiapoptotic subgroup entails, among others, Bcl-2 and BclXL, which protect the mitochondrial membranes by interacting with and inhibiting the proapoptotic subgroup of effectors Bax and Bak (7–9, 48, 60). The BH3-only proteins (BH: Bcl-2 homology) represent the third subgroup (e.g., Nix, BNIP3, Bad, Bid, Noxa, and Puma) and share sequence homology with other Bcl-2-like proteins only in the BH3 region (40). They use this region to heterodimerize with Bcl-2/Bcl-xl, thereby releasing Bax/Bak, which then form homodimers and trigger cytochrome c release and caspase activation (7, 8, 40).

These characteristics of the Bcl-2 family members offer an intriguing rationale to protect the heart from I/R injury by upregulating antiapoptotic or repressing proapoptotic proteins. In agreement with this hypothesis, elevated expression of Bcl-2 or Bcl-xl, or ablation of Bax, promotes recovery from cardiac I/R injury (5, 18–20). Among the BH3-only proteins, Nix and BNIP3 have recently been implicated in cardiomyocyte death (14, 26, 58); however, little is known regarding the contribution of other BH3-only proteins to cardiac diseases. Puma (p53 upregulated modulator of apoptosis) is a recently identified BH3-only protein that binds Bcl-2 and Bcl-xl and induces apoptosis in a p53-dependent (DNA damage, hypoxia, or oncogenes) and -independent (serum withdrawal, glucocorticoids, kinase inhibitors, or phorbol esters) manner (15, 21, 37, 49, 56). Given the requirement for Puma to integrate and implement a number of diverse death signals, we hypothesized that Puma may contribute to cardiomyocyte death upon I/R injury.

In the present study, we have demonstrated that hypoxia-reoxygenation induces Puma expression in cultured cardiomyocytes and intact hearts. Consistent with this finding, targeted deletion of Puma significantly improved the tolerance of isolated mouse hearts to I/R by preventing apoptotic and necrotic cell death. Puma, therefore, appears to be a novel therapeutic target in ischemic heart disease.

MATERIALS AND METHODS

Isolation of primary cardiomyocytes and adenoviral infections. Neonatal cardiomyocytes were prepared using a Percoll gradient method as described elsewhere (50). Briefly, neonatal hearts from 1- to 2-day-old Sprague-Dawley rats were cut into three or four equal-sized pieces and digested in enzyme solutions [collagenase type II (Worthington Biochemicals) and pancreatin (Sigma)]. The enzymes were inactivated by addition of newborn calf serum, and the released cells were pooled and separated on a discontinuous Percoll gradient (top density = 1.059 g/ml, bottom density = 1.082 g/ml) by centrif-
ugation. The middle band at the interface of the two Percoll layers was collected. The cells were then plated onto gelatin-coated dishes or laminin-coated glass coverslips at a density of $1 \times 10^5$ cells/cm² and cultured in serum-containing medium (4:1 DMEM-medium 199, 10% horse serum, 5% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM glutamine) for 2 days. To prevent proliferation of nonmyocyte contamination, 0.2 mM bromodeoxuryridine was added to the culture medium.

Adenoviruses were purified by cesium chloride banding and titrated using the Adeno-X Rapid Titer kit (Clontech Laboratories). Adenoviral infection (multiplicity of infection = 10–50 plaque-forming units/cell) was performed in glucose-containing (ATP-depleted) DMEM containing Nutridoma and 5% horse serum (10, 45) for the indicated times. Wild-type (AdPuma) and BH3 domain-defective mutant Puma-α (AdΔBH3) adenoviral constructs were kindly provided by Dr. Jian Yu (55).

In vitro model of hypoxia-reoxygenation. At 2 days after isolation, neonatal rat cardiomyocytes were cultured in serum- and glucose-free DMEM under hypoxic conditions as described elsewhere (57), with some modifications. Hypoxia was achieved by incubation of cells in a humidified environment at 37°C in a three-gas incubator maintained with 1% O₂/9% CO₂/80% N₂ for 24 h. The medium was changed every 3 days. After 24 h of hypoxia, the cells were reoxygenated for 16 h in maintenance medium. Following hypoxia, cardiomyocytes were cultured in serum-containing medium (4:1 DMEM-medium 199, 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM glutamine) for 2 days. To prevent proliferation of nonmyocyte contamination, 0.2 mM bromodeoxuryridine was added to the culture medium.

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RESULTS

Hypoxia-reoxygenation leads to increased Puma mRNA and protein levels in isolated cardiomyocytes. Because Puma is required for hypoxia-induced apoptosis of colorectal cancer cells and is transcriptionally upregulated by I/R in hippocampal cells (42, 55), we hypothesized that Puma might contribute to the hypoxia-reoxygenation-induced apoptosis described earlier in cardiomyocytes (1, 4, 13, 34, 44, 59). First, using semiquantitative RT-PCR, we investigated the possibility that hypoxia-reoxygenation induces Puma mRNA expression in primary rat cardiomyocytes. Puma mRNA levels were increased after 2 h of hypoxia and remained higher than in the control sample for up to 8 h (Fig. 1A). We also observed enhanced Puma expression when a 2- or 4-h period of hypoxia was followed by reoxygenation, but Puma mRNA levels returned to baseline in the 8-h hypoxia-reoxygenation sample (Fig. 1A).

Next, we determined whether expression of Puma mRNA is translated into increased protein levels in cardiac cells. To carry out these studies, we used an anti-Puma antibody that was raised against an NH₂-terminal peptide sequence identical in mouse, rat, and human (Imgenex). The antibody detected a single adriamycin-inducible band in Puma-positive, but not Puma-negative, HCT116 colorectal cancer cells, demonstrating the specificity of this antibody (data not shown). After exposing cardiomyocytes to hypoxia or hypoxia-reoxygenation, we observed a significant increase in Puma protein levels compared with those of the control sample (Fig. 1B). Importantly, the time course of Puma protein induction was consistent with that of Puma mRNA expression.

In summary, Puma expression is triggered upon hypoxia-reoxygenation of cardiomyocytes; therefore, it might contribute to hypoxia-reoxygenation-induced cardiac cell death.
Isolated cardiomyocytes undergo apoptosis in response to ectopic Puma expression. To evaluate whether Puma induces apoptosis in cardiac cells, cardiomyocytes were infected with adenoviruses expressing a wild-type Puma-α protein, and apoptosis was scored by TUNEL assay. A low level of exogenous Puma expression and concomitant apoptosis were first apparent at 6 h after infection (Fig. 2). By 12 h, there was a marked increase in Puma protein, resulting in ~50% apoptosis (Fig. 2). By 24 h, a further increase in Puma expression was accompanied by >80% TUNEL positivity (Fig. 2). As a negative control, we infected cardiomyocytes with a virus encoding a BH3-deletion mutant Puma-α (ΔBH3). The mutant Puma ΔBH3 adenovirus resulted in higher Puma expression than the wild-type infection but did not lead to apoptosis, indicating that the adenovirus infection by itself was not sufficient to induce apoptosis (Fig. 2). Taken together, overexpression of Puma leads to cardiomyocyte apoptosis in a BH3-domain-dependent manner.

ATP depletion converts Puma-induced cardiomyocyte apoptosis to necrosis. In cultured cardiomyocytes, Puma predominantly induces apoptosis (Fig. 2A), but myocardial injury after I/R results in both apoptosis and necrosis (1, 3, 16). Therefore, if Puma plays a role in cardiac I/R, the apoptotic events triggered by its overexpression may be reverted to necrosis. To test this possibility, we analyzed the response of cardiomyocytes infected with Puma adenoviruses to ATP-depleted conditions.

Because of the time course of Puma expression and apoptosis (Fig. 2), we focused on 12-h virus infection, when extensive Puma-induced apoptosis was first observed, and determined the activity of caspase-3 as a hallmark of apoptosis. Infection with wild-type Puma adenovirus in the presence of glucose (and ATP) caused significant caspase-3 activation and apoptosis. This has been demonstrated by an increase in the proteolytically cleaved species of caspase-3, with a concomitant decrease of the noncleaved inactive form as shown by Western blot analysis (Fig. 3A). The same wild-type Puma infection in the absence of ATP (1 μM oligomycin in glucose-free medium over 3 h), however, did not lead to caspase-3 cleavage (Fig. 3A). The BH3-domain-defective Puma virus, which served as a negative control, did not trigger caspase-3 cleavage in any of the conditions (Fig. 3A). As expected, ATP depletion prevented activation of the apoptotic cascade by Puma.

We subsequently determined whether Puma induced necrosis, instead of apoptosis, in cardiomyocytes under ATP-depleting conditions. To analyze the extent of necrosis, we used EH-2 staining to assess the fraction of cells with membrane damage (Fig. 3B). A small but significant increase (~3-fold) in EH-2 staining was observed in wild-type Puma-expressing cells in the presence of ATP at 12 h after infection (Fig. 3, B and C). Because half of these cells are TUNEL positive (Fig. 2, B and C), a substantial percentage might represent late apoptotic cells with membrane damage. Similarly, expression of the ΔBH3 mutant in conjunction with ATP depletion slightly increased the number of EH-2-positive nuclei (Fig. 3, B and C). In sharp contrast, wild-type Puma expression combined with ATP depletion resulted in ~80% EH-2 positivity (Fig. 3, B and C). Because these cells did not undergo apoptosis (Fig. 3A), the decreased viability corresponds to caspase-independent necrotic cell death. These results demonstrate that Puma can trigger necrosis, rather than apoptosis, when ATP stores are limiting.

In summary, our findings indicate that Puma induces apoptosis in the presence of ATP, whereas it causes necrosis in ATP-depleted cells. Therefore, Puma might be a causal factor in cardiac cell death during I/R.

Puma deletion improves cardiac function and protects cardiomyocytes from cell death in isolated perfused hearts. To assess the role of Puma in cardiac I/R in intact hearts, we first examined whether Puma is induced upon I/R of isolated mouse hearts. Similar to our findings in response to hypoxia-reoxygenation (Fig. 1), Puma protein level was increased after 20 min of ischemia and remained elevated during posts ischemic reperfusion (Fig. 4A). Subsequently, we generated a Puma-knockout mouse line and compared the functional performance of Langendorff-perfused hearts of wild-type, Puma^+/−, and Puma^−/− mice.

Most importantly, we found a strikingly better posts ischemic functional recovery in hearts of Puma-knockout mice than in hearts of wild-type or heterozygous animals. No significant differences were observed in the baseline parameters of the animals, regardless of their genotype (Table 1). After 20 min of no-flow ischemia and 120 min of reperfusion, the LVDP, dP/dmax, and dP/dmin of wild-type hearts recovered only to 15 ± 3, 11 ± 3, and 11 ± 3% of their baseline values, respectively (Fig. 4, B–D). In sharp contrast, functional recovery of Puma^−/− hearts dramatically improved, with LVDP, dP/dmax, and dP/dmin reaching 73 ± 12, 77 ± 14, and 67 ± 14% of their baseline values, respectively, after 120 min of reperfusion (Fig. 4, B–D). The recovery did not differ between hearts heterozygous for Puma and their wild-type littermates (Fig. 4, B–D), indicating that reduced amounts of Puma protein are sufficient to exert prodeath activities. In accordance with the robust and significantly better recovery of LVDP, dP/dmax, and dP/dmin in Puma^−/− hearts, LVEDP was also consistently lower in Puma^−/− hearts during reperfusion than in their wild-type and heterozygous littermates: at the end of 120 min
of reperfusion, LVEDP was 9 ± 1.9 and 37 ± 2.8 mmHg in Puma−/− and wild-type hearts, respectively (Fig. 4E).

Because cell loss is a major contributor to I/R-induced cardiac injury, we analyzed the effect of Puma deletion upon I/R-induced necrosis and apoptosis in isolated perfused hearts. To measure necrosis, we determined the extent of the infarcted area by TTC as well as NBT staining in histological sections and measured LDH release in the coronary effluent. In the TTC and NBT assays, >50% of the myocardium became infarcted upon I/R in wild-type and heterozygous hearts. By contrast, deletion of Puma reduced the infarct size to half that of the wild-type control (27 and 22% by TTC and NBT assays, respectively; Fig. 5A). Consistent with the infarct size assays, release of LDH into the perfusion fluid was also significantly decreased by ~50% in Puma−/− compared with wild-type and heterozygous hearts (Fig. 5B). These results indicate that Puma is an important mediator of necrosis after cardiac I/R.

To measure apoptosis, we scored the TUNEL-positive cardiomyocytes in immunohistological sections. After 2 h of posts ischemic reperfusion, ~1% of the cells underwent apoptosis in wild-type as well as heterozygous hearts (Fig. 5C). This level of cell death is equivalent to a >10-fold increase com-
pared with the normoxic control and is in agreement with previous reports (22, 34, 54). Importantly, in Puma-knockout hearts, the degree of apoptosis was significantly reduced to ~0.3% (Fig. 5C).

Finally, we observed no change in the protein levels of several other Bcl-2 family members, including Bcl-2, Bcl-xL, and Bax, in Puma−/− hearts (Fig. 5D). Therefore, it seems very unlikely that the improved tolerance of Puma−/− hearts to I/R injury stemmed from altered expression of other Bcl-2 family proteins. Collectively, these findings establish Puma as a crucial mediator of I/R-induced myocardial dysfunction and cell death.

DISCUSSION

In the present report, we have identified the BH3-only Bcl-2 family protein Puma as an essential mediator of cardiomyocyte death in response to I/R injury. In recent studies, Puma has emerged as a critical regulator of cell death in cancer, acquired immunodeficiency syndrome, and ischemic brain disease and
has attracted considerable interest for several reasons (37, 39, 42, 56). 1) Puma is crucial for p53-dependent and -independent apoptosis (15, 21, 37, 49, 56). It is regarded as a major and specific apoptotic effector of p53, because Puma deficiency almost completely suppresses p53-dependent apoptosis but does not impair nonapoptotic functions (21, 49, 56). 2) Puma activation is regulated by multiple transcription factors, such as p53, p73, and E2F1, providing the molecular basis for a potential differential regulation of Puma expression (17, 35, 37, 55). 3) Puma activates its downstream apoptotic effectors Bax and Bak through binding to Bcl-2 or Bcl-xL. Thereby, Bax and Bak are released from inhibition and undergo homooligomerization, resulting in release of cytochrome c from mitochondria (28, 35). 4) Puma induction directly links the endoplasmic reticulum stress response to the mitochondrial apoptosis pathway in neurons during postischemic reperfusion of the forebrain (42).

Our most important observation is that targeted deletion of Puma rendered Langendorff-perfused mouse hearts resistant to I/R injury. During reperfusion of Puma−/− hearts, systolic and diastolic functions recovered close to their preischemic levels. Puma ablation improved postischemic functional recovery even better than Bax deletion and Bcl-2/Bcl-xL overexpression in other studies (5, 18–20). This might be the result of the upstream position of Puma in the regulatory network of Bcl-2 family proteins. For example, Puma exerts its effect through Bax and Bak (52, 60); therefore, in Bax−/− cells, Bak may still transmit the prodeath signals. Furthermore, Puma+/− and wild-type hearts responded similarly after I/R, suggesting that even reduced levels of Puma might be sufficient to induce cell death.

### Table 1. Baseline parameters

<table>
<thead>
<tr>
<th></th>
<th>Puma+/+ (n = 10)</th>
<th>Puma+/− (n = 7)</th>
<th>Puma−/− (n = 7)</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>23±1.2</td>
<td>24±1.5</td>
<td>24±1.1</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>212±8.5</td>
<td>216±10.8</td>
<td>216±12</td>
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<tr>
<td>Coronary flow, ml/min</td>
<td>1.78±0.1</td>
<td>1.78±0.17</td>
<td>1.71±0.3</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td>86±6</td>
<td>92±11</td>
<td>88±10</td>
</tr>
<tr>
<td>dP/dtmax, mmHg/s</td>
<td>2.012±71</td>
<td>2.087±98</td>
<td>2.056±103</td>
</tr>
<tr>
<td>dP/dtmin, mmHg/s</td>
<td>1.474±66</td>
<td>1.562±74</td>
<td>1.503±79</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>11.9±0.7</td>
<td>11.9±1.1</td>
<td>12±1.0</td>
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</tbody>
</table>

Values are means ± SE. Data were obtained after 20 min of baseline perfusion from hearts paced at 7 Hz. LVDP, left ventricular (LV) developed pressure; dP/dtmax and dP/dtmin, maximum rate of LV pressure rise and fall, respectively; LVEDP, LV end-diastolic pressure.

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![Fig. 4. Puma is essential for mediating postischemic injury of isolated perfused hearts. A: immunoblot analysis of Puma levels in wild-type (Puma+/+) mouse hearts after baseline perfusion (N), 20 min of ischemia (Isch), and ischemia + 120 min of reperfusion (IR). Actin is shown as loading control. Results are representative of ≥3 similar experiments. B–D: hearts from Puma+/+ (n = 10), Puma+/− (n = 7), and Puma−/− mice (n = 7) were exposed to 20 min of ischemia and 120 min of reperfusion according to the Langendorff method. Postischemic recovery of left ventricular developed pressure (LVDP, B), maximum rate of left ventricular increase (+dP/dtmax, C), and maximum rate of left ventricular pressure decrease (−dP/dtmin, D) is shown after 30, 60, 90, and 120 min of reperfusion compared with baseline values. E: left ventricular end-diastolic pressure (LVEDP) at beginning and end of ischemic period as well as after 30, 60, 90, and 120 min of reperfusion. Error bars, SE. *Significantly different from Puma+/+ (P < 0.01).](http://ajpheart.physiology.org/.../H57)
Thymocytes and neuronal cells from $Puma^{+/−}$ mice, however, displayed an intermediate apoptotic response upon γ-irradiation (21), implying that cardiomyocytes might be more sensitive to Puma than some other cell types. Alternatively, the different sensitivity of cells to Puma in the above experiments might also reflect stress stimulus-specific responses.

The improved functional recovery of Puma-knockout hearts most probably derived from decreased cardiomyocyte loss due to an alleviation of both apoptosis and necrosis. Consistent with this possibility, we found reduced levels of apoptosis by TUNEL assay and suppression of necrosis by measurement of infarct size and LDH release. In the Langendorff perfusion model, however, the contribution of necrosis to myocardial cell loss exceeds that of apoptosis (22, 34, 54). Therefore, it appears that, in the Langendorff model, deletion of Puma protects the heart against I/R predominantly by blocking necrosis. This is further supported by our in vitro observation suggesting that Puma induces necrosis under ATP-depleted conditions, which represent a consequence of ischemia. Our data imply that the switch between necrosis and apoptosis described earlier upon ATP depletion (45, 47) occurs downstream of Puma.

Although our studies indicate that Puma expression is induced in hypoxic cardiomyocytes and ischemic hearts and is required for cell death during I/R, the regulation of Puma activity is not completely understood. In all cell types investigated thus far, Puma is transcriptionally upregulated during apoptosis (15, 28, 35, 37, 42, 55), and p53 has been identified as a major upstream regulator (37, 55). However, the role of p53 in heart ischemia remains somewhat controversial. Earlier studies reported that p53 is dispensable for hypoxia-induced cardiomyocyte apoptosis (2, 51), whereas more recent studies demonstrated that p53 was induced by hypoxia-reoxygenation and I/R (27, 29, 30, 33, 36, 41). Moreover, treatment with the p53 inhibitor pifithrin-α attenuated the I/R-induced infarct size similarly to ischemic preconditioning (36). We also observed a slight improvement of functional recovery upon I/R in $p53^−/−$ mice (data not shown), suggesting that, to some extent, p53 might contribute to I/R injury in the heart. In light of the remarkable resistance of $Puma^−/−$ hearts to I/R (Figs. 4 and 5), it is most likely that transcription factors other than p53 play a role in Puma activation in cardiomyocytes. In recent studies, p73 and E2F1 have been described as activators of Puma transcription and as possible alternatives to p53 (17, 35). Additional studies are needed to determine the contribution of p73 and E2F1, as well as other factors, to the regulation of Puma expression in the heart during I/R.

Although hypoxia-reoxygenation has been demonstrated to induce primarily p53-independent apoptosis (2, 51), genotoxic stress-induced cardiomyocyte apoptosis is predominantly dependent on p53 (36, 43). Therefore, it is less likely that a recently reported dynamic interaction of Bcl-xL with p53 and Puma described upon genotoxic stress of cancer cells (6) plays a role in Puma expression during I/R.

**Fig. 5.** Puma deletion attenuates ischemia-reperfusion-induced necrosis and apoptosis. Hearts from $Puma^{+/+}$, $Puma^{+/−}$, and $Puma^{−/−}$ mice were exposed to 20 min of ischemia and 120 min of reperfusion according to the Langendorff method. A: infarct size expressed as percentage of total myocardium detected with triphenyltetrazolium chloride (TTC) and nitro blue tetrazolium chloride (NBT). Data were averaged from 5 hearts. Error bars, SE. *Significantly different from corresponding $Puma^{+/+}$ ($P < 0.01$). B: relative increase of lactate dehydrogenase (LDH) leakage during reperfusion (compared with normoxic baseline values) normalized to volume of coronary effluents. Samples of coronary effluents were collected during baseline perfusion and reperfusion. Data were averaged from $≥$7 hearts. Error bars, SE. *Significantly different from $Puma^{+/+}$ ($P < 0.01$). C: quantitative analysis of apoptosis in cryosections with TUNEL assay. Data were averaged from 3 experiments, and $≥$1,000 nuclei were counted per experiment. Error bars, SE. *Significantly different from $Puma^{+/+}$ ($P < 0.01$). D: immunoblot analysis of Bcl-xL, Bcl-2, Bax, and Actin levels in $Puma^{+/+}$ and $Puma^{−/−}$ hearts after baseline perfusion. Actin is shown as loading control. Results are representative of $≥$3 similar experiments.
a significant role in hypoxia-reoxygenation-induced cardiomyocyte apoptosis. Consistent with this, we did not detect p53 or Puma in Bcl-xL immunoprecipitates of cardiomyocytes exposed to hypoxia-reoxygenation (data not shown).

In recent reports, BNIP3, another BH3-only protein, has been implicated in the pathomechanism of I/R. It was shown that overexpression of BNIP3 by transcriptional activation upon hypoxia leads to cardiomyocyte death and, conversely, that downregulation of BNIP3 using antisense oligonucleotides protects isolated cardiomyocytes from hypoxia-induced death (14, 26). Although Puma and BNIP3 belong to the same subset (to P. Erhardt) and CA-63230 and CA-71907 (to G. P. Zambetti) and Cancer Science Foundation Grant Grant MCB-9982789 (to P. Erhardt), an American Heart Association fellowship (to A. Toth), and by American Lebanese Syrian Associated Charities.

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