Laboratory Investigation

Pathways Involved in Interleukin-1β–Mediated Murine Cardiomyocyte Apoptosis

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Accumulating evidence suggests that interleukin-1 (IL-1) signaling plays an essential role in the pathogenesis of heart failure by inducing cardiomyocyte apoptosis, but the mechanisms of this process are poorly defined. We further explored these molecular pathways.

We isolated cardiomyocytes from neonatal mice and then cultured and stimulated them with murine IL-1β in vitro. Cell apoptotic ratios were measured by means of flow cytometry. Expression of effector molecules was analyzed by means of enzyme-linked immunosorbent assay, Western blotting, and real-time quantitative polymerase chain reaction. The results showed that IL-1β induced murine cardiomyocyte apoptosis through a release of cytochrome c into cytoplasm and through caspase 3 activation. Simultaneously, IL-1β signaling promoted expression of endonuclease G and high-temperature requirement protein A2 messenger RNA. Survivin and X-linked inhibitors of apoptosis protein (IAP), members of the IAP family, were inhibited on the messenger RNA level during IL-1β–mediated cardiomyocyte apoptosis.

We found that IL-1β signaling during cardiomyocyte apoptosis in vitro induced the activation of caspase-dependent and caspase-independent pathways, and inhibited IAPs. Understanding the molecular mechanisms involved in IL-1β–mediated cardiomyocyte apoptosis might assist in the design of therapeutic approaches to protect cardiomyocyte function and prevent heart failure. ***(Tex Heart Inst J 2015;42(2):109-16)***

Interleukin-1 (IL-1), a multifunctional proinflammatory cytokine, was originally described as the first endogenous pyrogen. It can exert complex biological effects by modulating gene expression in a wide variety of cell types and inflammatory conditions.[1-3] Interleukin-1 consists of 2 distinct ligands (IL-1α and IL-1β) with high-sequence homology; however, only IL-1β can be released from cytosol to exocellular space after activation.[4,5] In addition, IL-1 is consistently induced and activated after tissue injury, and it appears to mediate reparative responses.[6,7] In the pathogenesis of cardiovascular diseases, IL-1 signaling promotes the formation of atheromatous lesions, increases vascular inflammation, and triggers plaque destabilization.[8,9] After myocardial infarction, IL-1 signaling critically regulates the inflammatory response and is involved in the development of adverse remodeling by increasing the expression of matrix metalloproteinases.[7,10] Emerging evidence suggests that IL-1 signaling has an essential role in heart failure by suppressing cardiac function, promoting myocardial hypertrophy, and even inducing cardiomyocyte apoptosis.[7,11]

Interleukin-1β has induced rat cardiomyocyte apoptosis that was associated with activation of Bcl-2 family members.[11] Although the activation of pro-apoptotic pathways mediated by IL-1 stimulation during cardiomyocyte injury has been confirmed, the mechanism involved in this process is poorly defined. Coincidentally, a marked upregulation of IL-1 has been found in ischemic hearts.[12-14] Therefore, understanding the mechanism of IL-1β–mediated cardiomyocyte apoptosis is important in the designing of optimal therapeutic approaches to counter heart failure. In the current study, we used cardiomyocytes isolated from neonatal mice and explored the molecular pathways involved in IL-1β–mediated cardiomyocyte apoptosis in vitro, including caspase-dependent pathways, caspase-independent pathways, and inhibitor of apoptosis proteins (IAPs).

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Materials and Methods

We used the neonatal (1–3 d after birth) C57BL/6J mouse in accordance with our institution's guidelines for the care and use of animals. We referred to standard methods for the primary culture of neonatal rat cardiomyocytes and modified them for the neonatal mouse.[11,15] First, each mouse heart was gently removed into a Petri dish that contained cold Gibco® phosphate-buffered saline solution (Life Technologies, part of Thermo Fisher Scientific Inc.; Grand Island, NY). The atria were discarded. The ventricles were dissected into small pieces with use of fine scissors and were placed into a new 50-mL tube (Corning Life Sciences; Tewksbury, Mass) that contained 0.25% Gibco trypsin-EDTA solution (Life Technologies) and 0.2% type II collagenase solution (Sigma-Aldrich Co. LLC; St. Louis, Mo). The tube was incubated at a temperature of 37 °C in a water bath for approximately 30 min, with gentle stirring.

The neonatal mouse cardiomyocytes were isolated by means of digestion with collagenase and trypsin. During the first 3 days of culture, we added fibroblast growth inhibitor to the medium, to purify the cardiac myocytes from the isolated population. The digestion was stopped by the addition of a Gibco minimal essential medium that contained 10% Gibco fetal bovine serum (Life Technologies).

After being washed twice with the medium in a centrifuge for 5 min at 250 ×g at a temperature of 4 °C, the isolated cells were placed into 6-well plates (Corning Costar Corporation; Corning, NY) at a density of 2×10^5 cells/cm^2 in a humidified 5% Co_2 incubator at a temperature of 37 °C. The next day, the unattached cells were removed and fresh medium was added, supplemented with fibroblast growth inhibitor (0.1 mmol/L of bromodeoxyuridine). The culture medium was changed daily until the cells were used; fibroblast growth inhibitor was added only on the first 3 days. By means of our method, the purity of the cardiomyocytes exceeded 90%, as determined from the results of cardiac troponin I immunostaining (Abcam; Cambridge, Mass).

After routine culture in minimal essential medium and 10% fetal bovine serum for another 3 to 5 days, the cardiomyocytes were transferred into a new dish (concentration, 1×10^6 cells/mL) and were incubated with the addition of 10 ng/mL of recombinant murine IL-1β (PeproTech; Rocky Hill, NJ) for 72 hr. The cardiomyocytes were then dispersed into single cells with use of trypsin and were analyzed by means of flow cytometry under annexin V staining.

Flow Cytometric Analysis

The cultured cardiomyocytes were dispersed into single cells with use of 0.25% trypsin-EDTA solution. Phycoerythrin-conjugated BD Pharmingen™ annexin V (BD Biosciences; San Jose, Calif) was added into each sample, with incubation in the dark for 15 min at a temperature of 25 °C. Flow cytometry was performed on a BD FACSCalibur™ flow cytometer (BD Biosciences) within 1 hr after staining, and the data were analyzed with use of FlowJo software (FlowJo LLC; Ashland, Ore).

Enzyme-Linked Immunosorbent Assay Analysis

The cultured cardiomyocytes were harvested from the plates with use of trypsin, and the cytosolic fraction of every sample (10^6 cells/sample) was generated with use of a cell fractionation kit (Abcam). The level of cytochrome c in the cytoplasm was determined with use of a cytochrome c quantitative enzyme-linked immunosorbent analysis (ELISA) kit (Abcam).

Western Blot Analysis

We separated the whole-cell protein extracts from the harvested cardiomyocytes by means of sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred them to Millipore® Durapore® PVDF Membrane Filters (EMD Millipore; Billerica, Mass). After the membranes were incubated with use of an anti-active (cleaved) caspase-3 antibody (EMD Millipore), we added a horseradish peroxidase-conjugated secondary antibody. Finally, the bands were detected with use of an enhanced chemiluminescence assay, and the grayscales were analyzed.

Real-Time Quantitative Polymerase Chain Reaction

The total RNA of the cardiomyocytes was extracted with use of Invitrogen™ TRIzol® RNA Isolation Reagent (Life Technologies), and the complementary DNA was synthesized with use of Invitrogen™ SuperScript® II Reverse Transcriptase (Life Technologies). Table I shows the primers used to test for the following: apoptosis-inducing factor (AIF), cellular inhibitor of apoptosis protein-1 and -2 (c-IAP1 and c-IAP2), endonuclease G, glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*), high-temperature-requirement protein A2 (HtrA2/Omi), second mitochondria-derived activator of caspase (Smac/Diablo), survivin, and X-linked inhibitor of apoptosis protein (XIAP). Messenger RNA (mRNA) was quantified with use of a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.; Hercules, Calif). The data were analyzed by means of the $2^{-\Delta\Delta Ct}$ method, and the fold change in the expression of the target gene relative to the internal-control gene (*GAPDH*) was determined.

Statistical Analysis

Results are presented as mean ± SD. Significance between groups was evaluated by means of the Student *t* test with use of SPSS 20.0 for Windows (IBM Corporation; Armonk, NY). A *P* value less than 0.05 was considered statistically significant.

TABLE I. Primers Used to Synthesize Complementary DNA of Cardiomyocytes by Means of Real-Time Quantitative Polymerase Chain Reaction

Effector Molecule	Primer
AIF	TTGGTCTGGTGGATAGTAGTTTGC/AGAAGATGACACCTTTGCCGTAG
c-IAP1	TGTGGCCTGATGTTGGATAAC/GGTGACGAATGTGCAAATCTACT
c-IAP2	TTTGAATCCAGCCAACAGTCTG/TGATTTGCTCGGAAGTTCACAG
Smac/Diablo	GTTGGAGACCACTTGGATGACAG/AGCCAACTTGGTTTCTGCCTT
Endonuclease G	GCGAACTTACCAAAATGTCTATGTC/GCTCCAGAGGGATGGTCTCAT
HtrA2/Omi	GGACTCCCTCAAAACAACGTG/GAAGGGATGGCAAAGGAGATT
Survivin	GAGGCTGGCTTCATCCACTG/ATGCTCCTCTATCGGGTTGTC
XIAP	ACTTCCCAAGTAGTAGTCCTGTT/TATTGCCGCATGACAACTGAA
GAPDH	GCCTTCCGTGTTCCTACC/AGAGTGGGAGTTGCTGTTG

AIF = apoptosis-inducing factor; c-IAP = cellular inhibitor of apoptosis protein; *GAPDH* = glyceraldehyde-3 phosphate dehydrogenase; HtrA2/Omi = high-temperature-requirement protein A2; Smac/Diablo = second mitochondria-derived activator of caspase; XIAP = X-linked inhibitor of apoptosis protein

Results

In comparison with the control samples (which did not undergo IL-1β incubation), the IL-1β–incubated groups had a significantly higher cell apoptotic ratio (36.7% ± 8.4% vs 5.5% ± 2.3%, $P=0.0034$) (Fig. 1). Interleukin-1β therefore induced apoptosis of murine cardiomyocytes in vitro.

Endonuclease G and HtrA2/Omi. Endonuclease G and HtrA2/Omi were involved in caspase-independent pathways during IL-1β–mediated cardiomyocyte apoptosis. To explore the molecular mechanisms of this apoptosis, we checked principal effectors involved in the caspase-dependent and caspase-independent pathways. The IL-1β–incubated cardiomyocytes had significantly higher levels of cytosolic cytochrome c (31 ± 4.4 vs 6 ± 0.4 ng/mL per 10^6 cells; $P=0.0014$) than did the unincubated control samples, as determined by means of ELISA analysis. The released cytochrome c in cytoplasm triggered the formation of the apoptosome complex that leads to cell apoptosis via a caspase-dependent pathway. The results of Western blot analysis revealed that the cardiomyocytes had significantly higher active (cleaved) caspase-3 expression after IL-1β incubation than did the control samples (Fig. 2). As a central executioner caspase, caspase 3 was activated in the cardiomyocytes under IL-1β treatment. These results indicate that IL-1β signaling triggers the caspase-dependent apoptotic pathway in cardiomyocytes.

In regard to the caspase-independent pathways, the results of real-time quantitative polymerase chain reaction (PCR) analysis revealed that the IL-1β–incubated cardiomyocytes had higher endonuclease G and HtrA2/Omi mRNA expression than did the control samples (endonuclease G, $P=0.0084$; and HtrA2/Omi, $P=0.0483$). However, we found no significant difference in Smac/Diablo or AIF expressions of mRNA between the groups, with or without IL-1β incubation (Fig. 3). Interleukin-1β signaling also triggered the caspase-independent apoptotic pathways, which were mediated by endonuclease G and HtrA2/Omi.

Interleukin-1β Signaling and Inhibition of Survivin and XIAP during Apoptosis. Further, we wanted to know whether anti-apoptotic molecules such as IAPs were involved in IL-1β–mediated cardiomyocyte apoptosis. The results of real-time quantitative PCR analysis indicated that IL-1β–incubated cardiomyocytes had lower survivin and XIAP mRNA expression than did the control samples (survivin, $P=0.0001$; and XIAP, $P=0.0069$). We unexpectedly found that c-IAP1 and c-IAP2, two other IAPs, had higher mRNA levels after IL-1β incubation (c-IAP1, $P=0.0227$; and c-IAP2, $P=0.0003$) (Fig. 4). Therefore, IL-1β signaling simultaneously mediated the inhibition of IAP family members, including survivin and XIAP during cardiomyocyte apoptosis.

Discussion

The results of abundant studies of cardiac biology suggest that the actions of IL-1β are highly complex, depending on cell type.[7,16,17] To better define the mechanisms involved in cytokine-induced cardiomyocyte apoptosis, Ing and colleagues[11] examined the effects of IL-1β with and without the combination of other inflammatory cytokines, such as interferon-γ and tumor necrosis factor-α. The investigators found that IL-1β alone was sufficient to induce apoptosis of neonatal

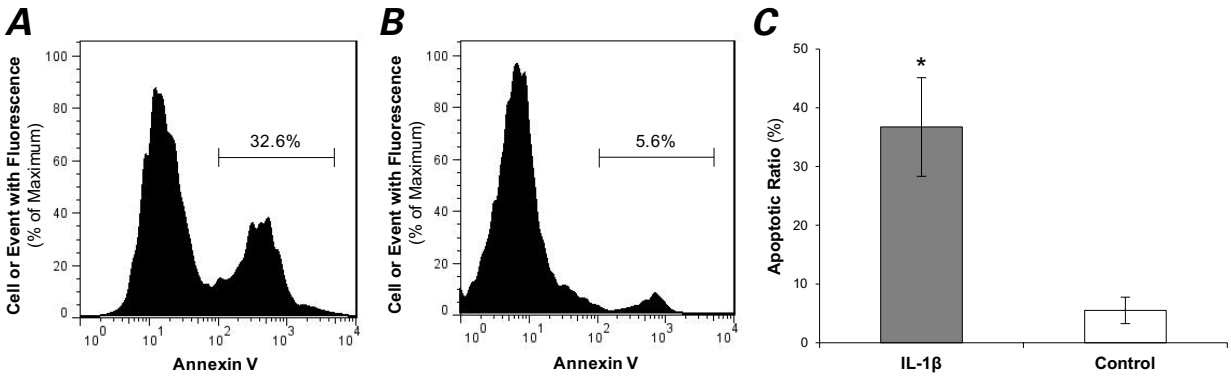

Fig. 1 *Apoptosis of murine cardiomyocytes with interleukin-1β (IL-1β) incubation in vitro. After 72 hours, flow cytometry showed that* ***A****) interleukin-1β–incubated cardiomyocytes had more annexin V-positive cells than did* ***B****) unincubated control samples. Data are representative of 3 different experiments.* ***C****) Graph shows the apoptotic ratio of cardiomyocytes with and without IL-1β incubation (mean ± SD of 3 experiments, *P <0.01). P <0.05 was considered statistically significant.*

rat cardiomyocytes, which was associated with expression of nitric oxide and Bcl-2 family members. In that study, pro- and anti-apoptotic genes (*Bak* and *Bcl-xL*) of cardiomyocytes were upregulated after IL-1β stimulation.[11] Coincidentally, we found apoptosis in response to IL-1β treatment on murine cardiomyocytes in our experiments. A statistically significant increase in the apoptosis of cardiomyocytes was achieved after 72-hr incubation with IL-1β, whereas there was no significant difference between these samples and the unincubated control samples at 24 or 48 hr (data not shown). This suggests that IL-1β triggers other intracellular signaling associated with cardiomyocyte apoptosis, instead of priming cell-death programs directly. The results of other studies to determine the molecular mechanisms of this process have revealed that cardiomyocytes express a higher level of active caspase 3, a principal effector in caspase-dependent pathways, after IL-1β incubation in vitro. The apoptotic signals from the death receptor and mitochondrion usually converge to caspase 3. As a central-executioner caspase, caspase 3 can be activated by the upstream cascades, which results in the orderly dismantling and removal of cells.[18] Accordingly, the apoptotic process of cardiomyocytes cannot be reversed after caspase pathways are activated by IL-1β stimulation.

Apoptosis is dependent on caspase activation, which leads to substrate cleavage and ultimately cell death.[19] However, in some processes, cells undergo apoptosis without caspase activation, and this is called caspase-independent cell death. Caspase-independent cell death occurs in response to most intrinsic apoptotic cues and shares some characteristics with the caspase-dependent process, such as mitochondrial outer-membrane permeabilization.[20,21] This last process leads to the release of various proteins from the mitochondrial intermembrane space, including cytochrome c, AIF, endonuclease G, HtrA2/Omi, and Smac/Diablo. [21-26] The released

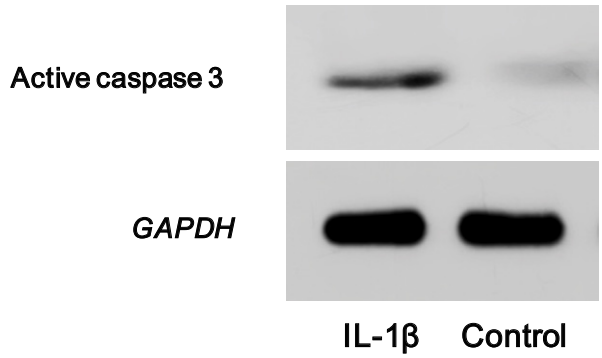

Fig. 2 *Western blot results show activation of caspase 3 in cardiomyocytes after 72-hr incubation in 10 ng/mL of interleukin-1β (IL-1β) versus unincubated control samples (control gene, glyceraldehyde-3 phosphate dehydrogenase [*GAPDH*]). Data are representative of 3 different experiments.*

cytochrome c interacts with other molecules (such as Apaf-1 and caspase 9) and forms the apoptosome complex in cytoplasm, which leads to activation of the apoptotic program via a caspase-dependent pathway.[27] After the IL-1β treatment, we found that the cardiomyocytes released cytochrome c into cytoplasm. This finding became further evidence of the activation of caspase-dependent apoptotic pathways in cardiomyocytes mediated by IL-1β signaling (Fig. 5).

In addition, cytochrome c, AIF, endonuclease G, HtrA2/Omi, and Smac/Diablo have been shown to induce caspase-independent cell death after mitochondrial release. Our results of quantitative PCR analysis showed that IL-1β signaling in cardiomyocytes triggered the caspase-independent apoptotic pathways that were mediated by endonuclease G and HtrA2/Omi (Fig. 5). Endonuclease G is a nuclear-encoded mitochondrial nuclease that reportedly functions in apoptosis.[28,29] During cell apoptosis, endonuclease G can

be released from the mitochondria, translocate to the nucleus, and cooperate with other nucleases to trigger DNA fragmentation.[23,30,31] In addition, endonuclease G expression can be promoted under certain initial stimuli during cell apoptosis.[32-34] Our results illuminated the fact that IL-1β signaling initiated the death program in cardiomyocytes by increasing the expression of endonuclease G.

The serine protease HtrA2/Omi is found in the mitochondria and can be released during cell apoptosis.[24,35] Activated (cleaved) HtrA2/Omi exposes an amino terminal motif resembling that of the IAP inhibitor Smac/Diablo. The interaction between HtrA2/Omi and IAP family members antagonizes their inhibition of caspase activity and protection from apoptosis.[36,37] In our study, IL-1β signaling significantly increased HtrA2/Omi expression, which might amplify the antagonistic action on IAP family members and promote cardiomyocyte apoptosis.

In addition to the pro-apoptotic molecules discussed above, we explored anti-apoptotic factors. The IAPs are structurally related anti-apoptotic proteins that were initially identified in baculoviruses. Mammalian IAPs, such as c-IAP1, c-IAP2, survivin, and XIAP, are able to block apoptosis either by binding and inhibiting caspases or through caspase-independent mechanisms.[38-40] This family of proteins has become increasingly prominent in the apoptotic process. The results of our quantitative PCR analysis indicated that IL-1β signaling mediated the inhibition of survivin and XIAP in cardiomyocyte apoptosis. The modulation of survivin and XIAP would attenuate their inhibitory function on

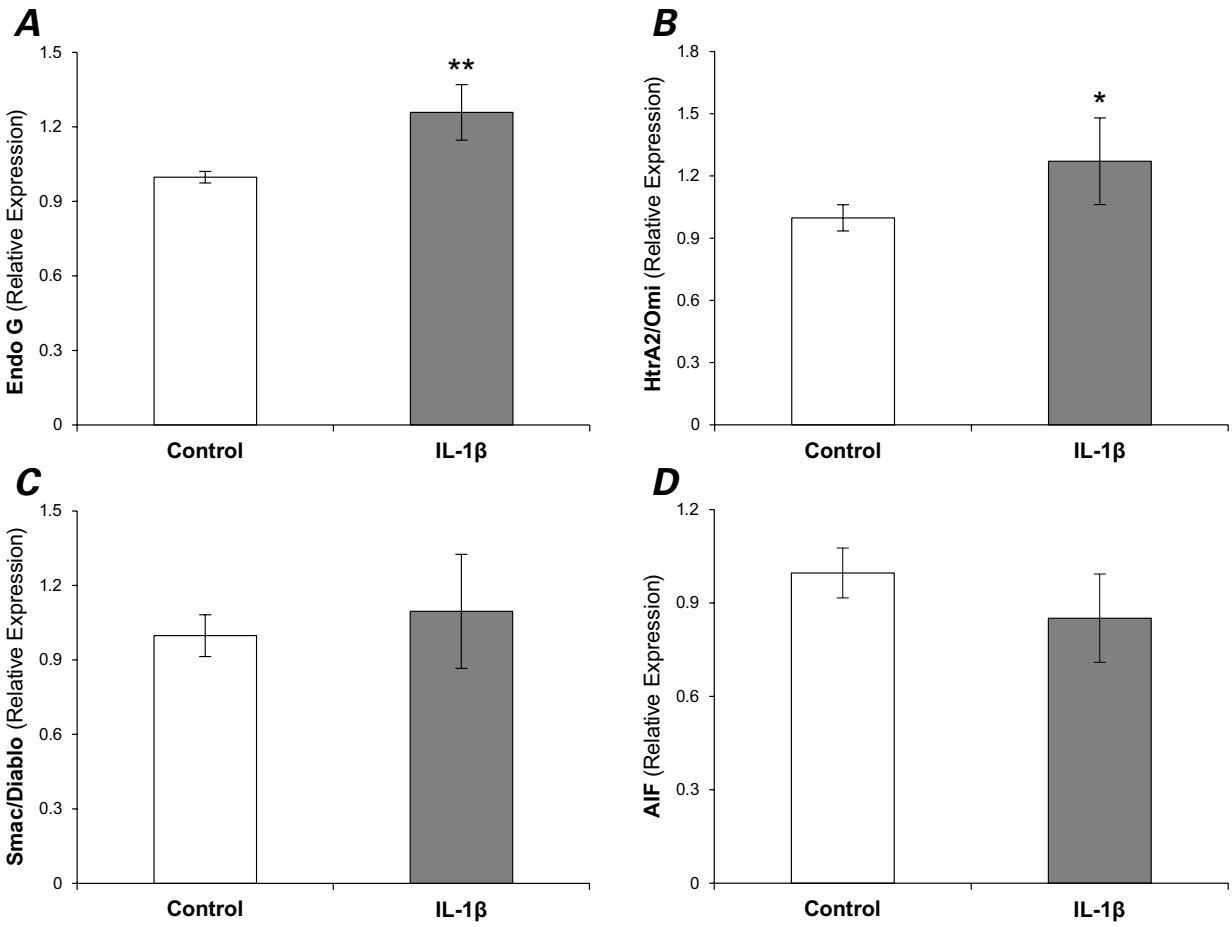

Fig. 3 *Graphs show caspase-independent pathways during interleukin-1β (IL-1β)–mediated cardiomyocyte apoptosis. After 72-hr incubation with 10 ng/mL of IL-1β (control samples underwent no IL-1β incubation), the cardiomyocytes were harvested and used for RNA isolation. Real-time quantitative polymerase chain reaction analysis was performed for gene expression of effectors in the caspase-independent pathways: IL-1β–incubated cardiomyocytes had significantly higher* ***A****) endonuclease G (Endo G) and* ***B****) high-temperature-requirement protein A2 (HtrA2/Omi) messenger RNA expression than did control samples. There were no differences in* ***C****) second mitochondria-derived activator of caspase (Smac/Diablo), or* ***D****) apoptosis-inducing factor (AIF) messenger RNA expression.*

*P <0.05, **P <0.01

Results are shown as mean ± SD of 3 experiments with appropriate primers. P <0.05 was considered statistically significant.

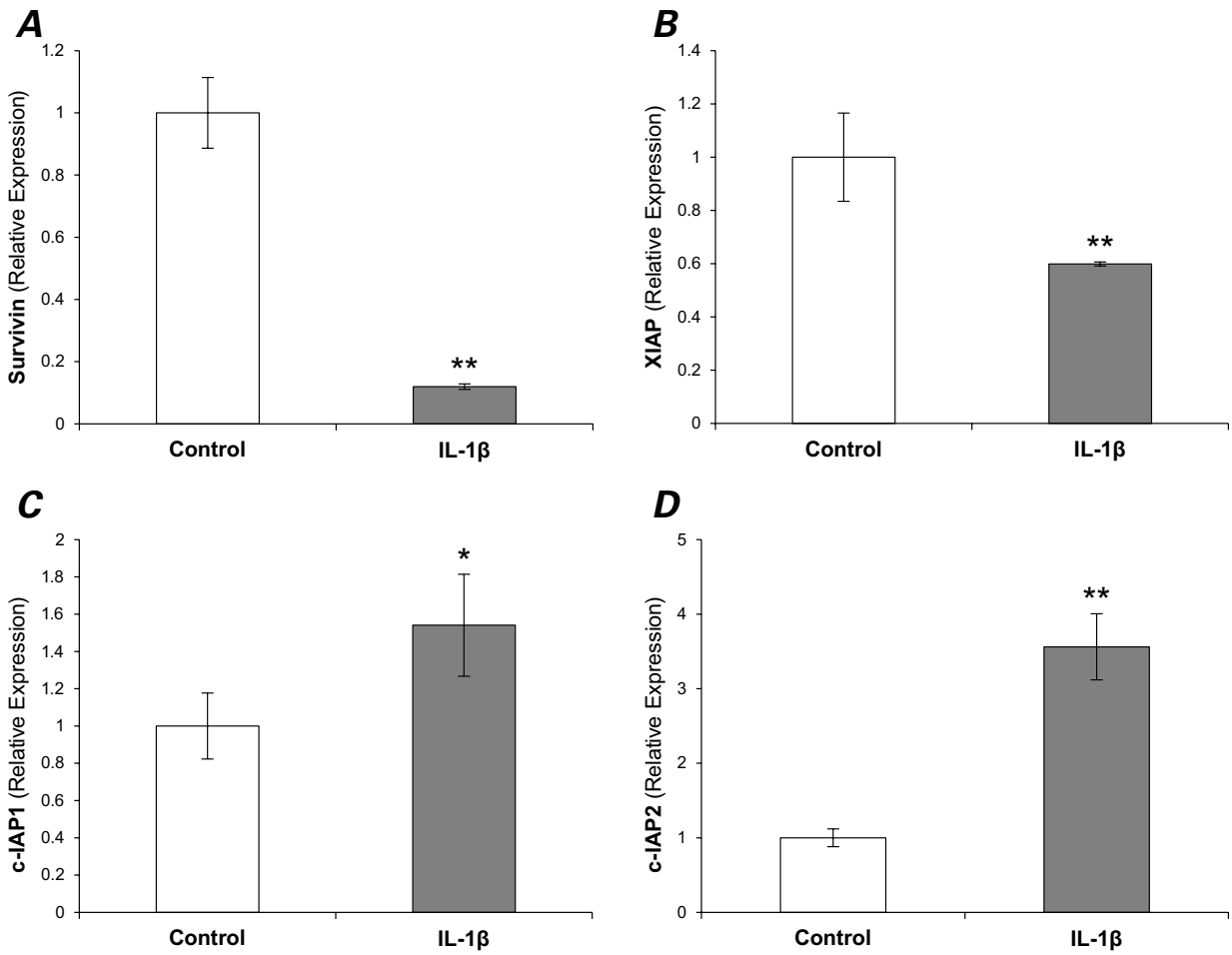

Fig. 4 *Graphs show expression of inhibitor of apoptosis proteins (IAPs) during interleukin-1β (IL-1β)–mediated cardiomyocyte apoptosis. After 72-hr incubation with 10 ng/mL of IL-1β (control samples underwent no IL-1β incubation), the cardiomyocytes were harvested and used for RNA isolation. In comparison with control samples, significant differences were seen in the expression of IAPs in the IL-1β–incubated cardiomyocytes, including* ***A****) survivin,* ***B****) X-linked inhibitor of apoptosis protein (XIAP),* ***C****) cellular inhibitor of apoptosis 1 (c-IAP1), and* ***D****) cellular inhibitor of apoptosis 2 (c-IAP2), upon real-time quantitative polymerase chain reaction analysis.*

*P <0.05, **P <0.01

Results are shown as mean ± SD of 3 experiments with appropriate primers. P <0.05 was considered statistically significant.

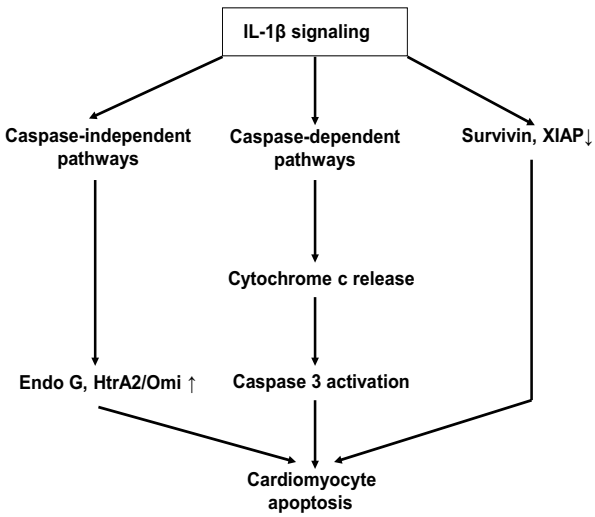

Fig. 5 *Schematic diagram shows pathways involved in interleukin-1β (IL-1β)–mediated cardiomyocyte apoptosis in vitro. Interleukin-1β signaling induced the release of cytochrome c from the mitochondria into cytosol and induced the activation of caspase 3 to trigger the caspase-dependent apoptotic pathway in the cardiomyocytes. Interleukin-1β signaling also induced the activation of caspase-independent apoptotic pathways through the upregulation of endonuclease G (Endo G) and high-temperature-requirement protein A2 (HtrA2/Omi) expression. Simultaneously, IL-1β signaling mediated the inhibition of survivin and X-linked inhibitor of apoptosis protein (XIAP), members of the inhibitor of apoptosis proteins family, to attenuate anti-apoptotic effects during cardiomyocyte apoptosis.*

apoptosis, which would consequently promote the pro-apoptotic progression of cardiomyocytes. Nevertheless, we found higher levels of both c-IAP1 and c-IAP2 on the mRNA level after IL-1β stimulation. This suggests that IL-1β signaling precisely modulates the apoptotic process of cardiomyocytes by inducing or inhibiting specific gene expression.

A limitation of this study is that we observed the activation of various molecules without providing information on the interactions of various pathways that result in a net increase in cardiomyocyte apoptosis. In future studies, we expect to identify the dominant pathways that result in cardiomyocyte apoptosis, and we plan to explore the interactions of the molecules involved in the various pathways. Another limitation is that this study was performed with the use of cultured cardiomyocytes. To confirm the findings from in vitro experiments, an animal model of heart failure should include IL-1 neutralization and IL-1 receptor antagonism.

Extensive experimental evidence has already suggested an important role for IL-1 signaling in cardiovascular diseases.[7,8,13,41,42] Interleukin-1 signaling seems to be a promising therapeutic target in a variety of cardiovascular conditions, including heart failure. The inhibition of IL-1 signaling might reduce the progression of cardiomyocyte apoptosis and preserve heart function. The current study has revealed that IL-1β signaling induces the activation of caspase-dependent and -independent pathways and the inhibition of IAPs during cardiomyocyte apoptosis in vitro. Understanding the molecular mechanisms involved in IL-1β–mediated cardiomyocyte apoptosis might assist in the design of optimal therapeutic approaches for cardiomyocyte survival.

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