# Caspase Inhibition Prevents Cardiac Dysfunction and Heart Apoptosis in a Rat Model of Sepsis

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**Despite intensive therapy, severe septic shock is commonly associated with myocardial dysfunction and death in humans. No new therapies have proven efficiency against cardiovascular alterations in sepsis. Here, we addressed the question of a beneficial effect of pharmacological inhibition of caspases on myocardial dysfunction following endotoxin treatment. Hearts from rats treated with endotoxin (10 mg/kg, intravenously) were isolated 4 h posttreatment for analysis. Assessment of myocardial contractility *ex vivo* and detection of apoptosis were performed. Hearts from endotoxin-treated rats displayed multiple caspase activities and also typical apoptosis pattern as detected by TUNEL, DNA fragmentation assays, and cytochrome *c* release as compared with control rats. z-VAD.fmk (3 mg/kg, intravenously), a broad spectrum caspase inhibitor (but not the irrelevant peptide z-FA.fmk), in coinjection with endotoxin, not only reduced caspase activities and nuclear apoptosis but also completely prevented endotoxin-induced myocardial dysfunction evaluated 4 h and even 14 h after endotoxin challenge. These data indicate that caspase activation plays an important role in myocardial cell dysfunction. Moreover, these results suggest that inhibitors of caspases may have important therapeutic applications in sepsis.**

Endotoxin, or bacterial lipopolysaccharide, depresses myocardial contractility in laboratory animals, and is also responsible for cardiac dysfunction associated with human sepsis (1). Scoring systems developed to predict mortality in patients with sepsis have shown a strong association between myocardial decompensation and mortality from sepsis (2). This association results from the unique vulnerability of the heart to the effects of sepsis. Although sepsis-induced myocardial depression is a well-defined entity in the clinical literature (3), the physiological basis of the myocardial contractility reduction is still poorly understood.

Numerous reports have demonstrated that sepsis exerts some of these effects through the induction of host responses (for review, *see* Rackow and Astiz [4]). Endotoxin stimulates monocytes and macrophages and thereby elicits a cascade of proinflammatory cytokines. Among them, tumor necrosis factor (TNF)-α, alone or in association with interleukin (IL)-1β, has been reported to be responsible for cardiac depression in both myocytes *in vitro* (5) and instrumented hearts *in vivo* (6). Thus, overproduction of TNF-α, which is also synthesized locally within the myocardium (7), represents one of the most critical factors for the development of myocardial depression in sepsis. Proposed mechanisms of TNF-α-induced myocardial depression include the modulation of secondary factors (8) such as nitric oxide (9), intermediates of the sphingomyelinase pathway (9), and calcium flow (10, 11). In addition, it has been postulated that chronic exposure to TNF-α can also provoke the inhibition of the mitochondrial function (12). Recently, it has been demonstrated that relevant levels of TNF-α induce apoptosis in rat cardiomyocyte *in vitro* (13).

Apoptosis is a well defined type of cell death (14). Dysregulation of apoptosis has been involved in the initiation of many human diseases including heart failure and ischemia reperfusion injury (for review *see* Krown and coworkers [8]). In most cases, the initiation and execution phases of the apoptotic process involve activation of a family of aspartate-specific cysteine proteases called caspases (15). Schematically, caspases can be divided into initiating and effector caspases. Caspases that are rapidly activated as a consequence of cell membrane signaling events including oligomerization of death receptors (CD95 or TNF-α receptors) and recruitment of adaptator proteins are classified as initiating or upstream caspases (e.g., caspases-2, -8, and -10) (14). For example, caspase-8 activation occurs at the initiation of the TNF-α-mediated apoptotic pathway (14). In contrast, numerous apoptotic stimuli lead to the activation of the effector or downstream caspases (e.g., caspase-3, -6, and -7) responsible for the cleavage of crucial substrates in the final degradation phase (14).

Two main apoptotic pathways have been described and converge on the activation of the downstream caspases (15). The first one integrates different proapoptotic signals at a mitochondrial level and triggers the release from the mitochondria of proapoptotic factors including cytochrome *c*. After its release into the cytosol, cytochrome *c* is then associated with other molecules (Apaf-1, dATP/ATP, procaspase-9) in an "apoptosome" complex, which is capable of activating caspase-9 (14, 15). Subsequently, active caspase-9 cleaves and activates caspase-3. The second pathway is independent of mitochondria and involves the recruitment and activation of upstream procaspases by the activated death receptors. Then, the active upstream caspases directly activate the downstream ones, such as caspase-3, in a proteolytic cascade. Furthermore, the existence of a crosstalk between these two apoptotic pathways has also been demonstrated. For example, Bid, a proapoptotic member of the Bcl-2 family, is cleaved by the upstream caspase-8 and truncated Bid acts on mitochondria to release cytochrome *c* (16).

Although the potential therapeutic interest of caspase inhibition has been documented in several animal models of acute diseases including cerebral and cardiac ischemia, no information is available on the role of caspases and the consequence of their inhibition in the heart failure observed in sepsis. In the present study, we explored the implication of caspases in the cardiac dysfunction generated in rats infused with *Escherichia coli* endotoxin, a classical model of sepsis. First, we used multiple criteria to document caspase activation and caspase-mediated apoptosis in heart following endotoxin treatment. Second, pharmacological experiments were performed with a broad spectrum caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp-

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fluoromethylketone (z-VAD.fmk) to prevent the effects mediated by caspases. Finally, we examined the influence of caspase inhibition on the myocardial contractility depression observed in instrumented hearts of endotoxin-treated animals. Our results provide the first evidence that caspase activation plays an important role in endotoxin-induced myocardial cell dysfunction.

## METHODS

### *In Vivo* Treatment

Adult male Sprague–Dawley rats (250–300 g) (Dépré, Saint Doulchard, France) were housed for 6 d in groups of six in standard cages and supplied *ad libitum* with laboratory chow and tap water. Treatments were administered intravenously, via the dorsal penine vein, after brief ether anesthesia. Overall, six groups of rats were studied: untreated control animals, rats treated with z-VAD.fmk, *N*-benzyloxycarbonyl-Phe-Asp-fluoromethylketone (z-FA.fmk), or endotoxin alone, and rats treated with both endotoxin and z-VAD.fmk or z-FA.fmk. z-VAD.fmk (Bachem, Basel, Switzerland) and z-FA.fmk (Enzyme Systems, Dublin, CA) were dissolved in dimethyl sulfoxide (DMSO) 20 mg/ml and a 3 mg/kg dose was injected in 500 μl saline. Sham-treated and endotoxin-treated rats were injected, respectively, with 500 μl saline and 10 mg/kg of endotoxin from *Escherichia coli* serotype 055:B5 (Sigma, St. Louis, MO) in 500 μl saline, using the same amount of DMSO as in peptide-treated rats.

Four hours (or 14 h for some experiments) after treatment, rat hearts were prepared for either cardiac function assessment or for *in vitro* assays. In parallel, sera from blood samples were collected via abdominal aorta puncture. All experiments were conducted in accordance with institution guidelines for the care and use of laboratory animals.

### Histological Studies

After euthanasia by pentobarbital overdose, the heart was excised and the left ventricle (LV) apex was cross-sectioned into specimens of 5 mm. Specimens were fixed in 10% formalin (Sigma) and embedded in paraffin. Paraffin sections were 4 μm thick and a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method was performed as described elsewhere (17). The "*in situ* cell death detection kit" (Boehringer, Mannheim, Germany) was used according to manufacturer's instructions with minor modifications. Briefly, after stripping of nuclear proteins through incubation with 20 μg/ml proteinase K (Sigma) for 15 min at room temperature, samples were washed three times in phosphate-buffered saline (PBS) and incubated with the TUNEL reaction mixture (TdT in the label solution 1:80 final concentration) for 1 h at 37° C. To avoid evaporation, samples were covered with a coverslip during incubation. At the end of the TUNEL reaction, cardiomyocytes were also labeled with a monoclonal antidesmin antibody (1:20 in PBS; Chemincon, Temecula, CA) for the identification of the phenotype of TUNEL-positive cells. Then, sections were incubated for 30 min with the Cy5-conjugated secondary antibody (1:800 in PBS; Chemicon). Counterstaining of nuclei was performed with propidium iodide 0.1 μg/ml. All sections were mounted in Vectashield H-100 medium (Vector Laboratories, Burlingame, CA) before analysis with a Leica TCS NT confocal microscope (Leica Microsystemes, Rueil Malmaison, France). TUNEL- positive controls were obtained by a DNase I treatment for 60 min at 37° C (40 U/ml; Sigma). For negative controls, TdT was replaced by PBS in the TUNEL reaction mixture.

### Determination of Cardiac Enzymes

Blood samples were used for measurement of serum cardiac enzyme creatine phosphokinase (CK). Quantification was made with a standard clinical automatic analyzer (model 911; Hitachi). CK activity of the rat heart was assessed as previously described (18) with minor modifications. A portion of the LV apex was separated, weighed, and homogenized in 2 ml of ice-cold buffer (0.25 M sucrose, 0.1 mM EDTA, and 10 mM 2-mercaptoethanol). The homogenates were then centrifuged at 30,000 × *g* at 4° C for 30 min and the supernatants were assayed for CK activity by use of a standard clinical automatic analyzer (Hitachi). CK activity was expressed as IU/ml $mg^{-1}$ of tissue.

### Determination of Caspase Activation

After euthanasia by pentobarbital overdose, the heart was excised and tissue was dissected, washed in ice-cold Krebs–Henseleit (KH) buffer solution containing NaCl 118 mM, KCl 4.75 mM, $KH_2PO_4$ 1.19 mM, $MgSO_4$ 1.19 mM, $CaCl_2$ 2.54 mM, $NaHCO_3$ 25 mM, EDTA 0.5 mM, and glucose 11 mM and immediately frozen in liquid nitrogen. Frozen tissue was subsequently ground into a powder using a mortar and pestle and resuspended with ice-cold lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA, 2 mM EGTA, Triton X-100, 0.1%) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml aprotinin and leupeptin. Homogenates were centrifuged at 14,000 × *g* for 10 min and the supernatants were used. Then 100 μg of proteins was diluted with assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 2 mM EDTA, 2 mM EGTA, Triton X-100, 0.1%, and incubated at 25° C with the colorimetric substrates (Biomol, Plymouth, PA): Ac-DEVD-*p*NA, 200 μM final concentration, Ac-YVAD-*p*NA, 200 μM final concentration, IETD-*p*NA, 200 μM final concentration, or LEHD-*p*NA, 200 μM final concentration in 96-well microtiter plates. Cleavage of the *p*-nitroaniline (*p*NA) dye from the peptide substrate was determined by the measure of absorbance of *p*NA at 405 nm in a microplate reader Digiscan (Asys Hitech, Cincinnati, OH). Results were calibrated with known concentrations of *p*-NA and expressed in picomole substrate cleaved/minute and per microgram proteins at 25° C.

### Immunoblotting

Frozen powdered tissue prepared as described above was resuspended in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 10 μg/ml aprotinin and leupeptin, and 5 mM PMSF. For caspase-3, lysates were centrifugated at 14,000 × *g* and 30 μg of supernatant was loaded on 12.5% polyacrylamide gels, electrophoresed, and transferred as previously described. Caspase-3 was detected using a polyclonal rabbit anti-caspase-3 antibody (BD Pharmingen, San Diego, CA), which recognizes both the 32-kD unprocessed procaspase-3 and the 17-kD subunit of the active caspase-3.

For cytochrome *c* release detection, cytosolic protein extracts were prepared by Dounce homogenization of LV apex tissue in a hypotonic buffer (buffer A 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin and aprotinin). Homogenates were centrifuged at 100,000 × *g* for 1 h at 4° C and the supernatant also called cytosolic S-100 fraction was used. Eight micrograms of proteins was separated by 12% SDS/PAGE and revealed with anti-cytochrome *c* monoclonal antibody (clone 7H8.2C12) (Pharmingen), which recognizes the denaturated form of cytochrome *c*.

### DNA Fragmentation Detection

For the detection of oligonucleosomes, a cell death detection enzyme-linked immunosorbent assay (ELISA) plus kit (Boehringer Mannheim, Switzerland) was used according to the manufacturer's instructions. Briefly, small pieces (40–50 mg) of heart were homogenized in the provided lysis buffer 45 min at room temperature, followed by centrifugation for 10 min at 2,000 rpm. Protein level was determined on the supernatants. Twenty microliters of the supernatant was subjected immediately to the ELISA test. For electrophoresis, DNA was extracted from cardiac tissue (LV apex) using a commercially available isolation kit (Genzyme TACS; R&D Systems, Minneapolis, MN). The DNA obtained was used in a ligation mediated polymerase chain reaction (LM-PCR) assay according to the manufacturer's instructions (Clontech Laboratories, Palo Alto, CA). This method allows us to reveal oligonucleosomal fragmentation that may be undetectable with other methods. DNA electrophoresis (10 ng/lane) was run through 1% agarose/ethidium bromide gel at 6 V/cm for 2.5 h.

### Cytokine Determination

Serum levels of IL-1β, IL-6, and TNF-α were determined in sera collected 4 h after endotoxin treatment by use of commercially available immunoassay kits (ELISA) specific for rat cytokines (Biosource International, CA). Reading was realized in a microplate reader Digiscan (Asys Hitech).

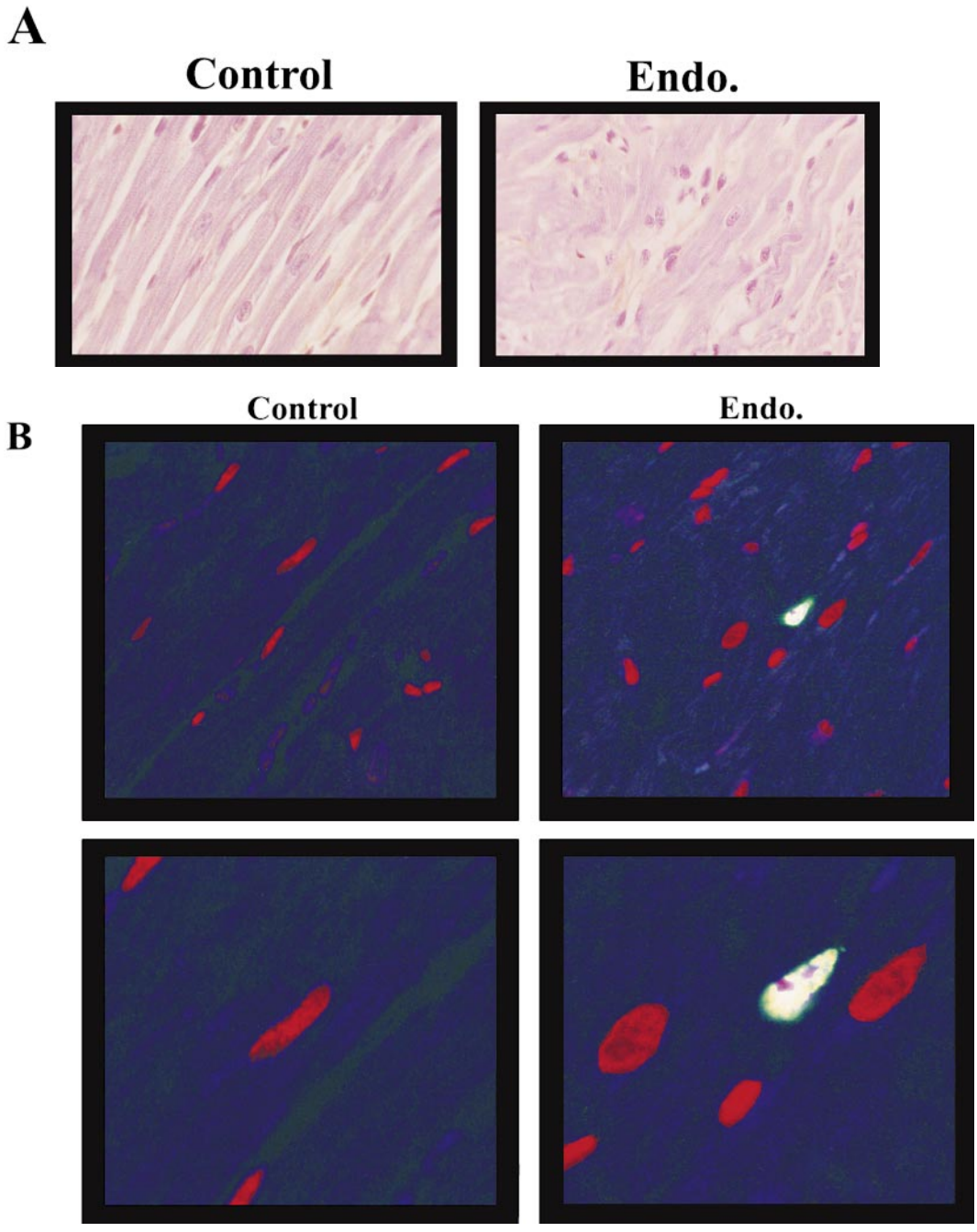


***Figure 1.*** Determination of myocardial cell apoptosis. (*A*) *In situ* analysis of myocardial sections stained with May–Grumwald–Giemsa. Four hours after treatment, 4-μm paraffin-embedded tissue sections were prepared from LV apex obtained from control (n = 4; *left panel*) or endotoxin-treated rats (n = 4; *right panel*). Original magnification: ×400. (*B*) TUNEL staining (*green/yellow*) of cardiac myocyte nuclei with counterstaining with PI (*red*) and anti-desmin antibody (*blue/purple*) of LV apex from control (n = 5; *left part*) and endotoxin-treated rats (n = 5; *right part*) 4 h after endotoxin treatment. Following endotoxin exposure, apoptotic cell death can occur in single cardiac myocytes (*upper panel*; *right part*). Original magnification: ×630. Zoom of nucleus shows a typical aspect of TUNEL-positive myocyte nucleus (*lower panel*, *right part*). (*C*) Quantification of apoptosis by DNA fragmentation assay (oligonucleosomes). Different portions of heart from control (n = 5) or endotoxin-treated rats (n = 5) were submitted to ELISA test (*see* METHODS) 4 h after endotoxin treatment. Results are expressed as OD/μg proteins. Results represent the means ± SEM. *$p < 0.01$ between controls and endotoxin-treated rats for each portion of heart.

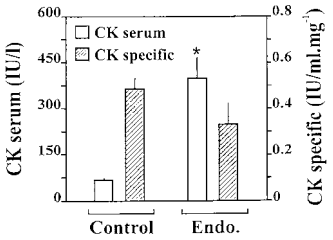


*Figure 2.* Assessment of myocardial injury 4 h after endotoxin challenge. CK levels were determined both in sera and in heart tissue (CK specific) as described in METHODS. For each group, seven rats were used for CK activity in sera and three for CK specific in tissue. Results are representative of the mean ± SEM. *$p < 0.01$ between two groups.

### Isolated and Perfused Heart Preparation

Myocardial contractile function was studied using a modified Langendorff isolated heart preparation as previously described (19). Briefly, following heparinization and ether anesthesia, the heart was rapidly excised and placed into ice-cold Krebs–Henseleit (KH) buffer solution. Then the heart was mounted onto a Langendorff heart perfusion apparatus and perfused in a retrograde fashion via the aorta at a constant flow rate of 10 ml/min with aerated (95% $O_2$–5% $CO_2$) KH buffer at 37° C. Cardiac contractile function was assessed using a water-filled latex balloon inserted into the left ventricular (LV) cavity and connected to a pressure transducer. This balloon was then adjusted to a left ventricular end-diastolic pressure (LVEDP) of 5 mm Hg. The heart was paced at 300 beats/min and allowed to equilibrate for 30 min. Left ventricular developed pressure (LVDP), its first derivatives ($dP/dt_{max}$ and $dP/dt_{min}$), and coronary perfusion pressure (CPP) were monitored and recorded on a chart recorder (Kontron, Basel, Switzerland). After baseline measurements, LVDP–LV preload relationships (LVEDP from −5 to 20 mm Hg) were obtained.

### Statistical Analysis

For *in vitro* and cardiac function studies, we tested for differences using an ANOVA procedure. When a significant difference was found, we identified specific differences between groups using a sequentially rejective Bonferroni procedure. After application of a Bonferroni correction, significance was achieved with $p < 0.01$ for comparisons with control. Data are presented as means ± SEM throughout. For cardiac function studies, we tested for differences in LVDP–LV volume preload relationship of the isolated hearts over LVEDP pressure using an analysis of variance for repeated measurements with Scheffé's post hoc test.

## RESULTS

### Characterization of Cardiac Cell Alterations Induced by Endotoxin

Histopathological evaluation (n = 4 rats in each group) of May–Grumwald–Giemsa-stained sections displayed alterations in tissue architecture including interstitial edema, infiltration of white blood cells, and slight modification of the myocytes shape in myocardium from endotoxin-treated rats (Figure 1A, *right panel*). No specific nuclear alteration was detected with this technique. However, tissue sections from endotoxin-treated hearts (n = 5 rats) stained by the TUNEL technique revealed the presence of TUNEL-positive nuclei compared with control hearts (n = 5 rats) (Figure 1B, *upper panel*). The three colors staining with TUNEL-FITC, propidium iodide, and Cy5-conjugated secondary antibody against monoclonal anti-desmin antibody revealed that nuclear myocytes were labeled by the TUNEL procedure in endotoxin-treated myocardium (Figure 1B, *lower panel*, *right part*). In contrast to control heart lysates, DNA oligonucleosomes were detectable in lysates of the LV apex, LV free (free wall, FW), interventricular wall (IVW), and the right ventricle of endotoxin-treated rat (n = 5 rats in each group) (Figure 1C). Four hours after endotoxin administration, a loss of myocardial CK specific activity and a significant increase of CK level in sera were observed (Figure 2). Overall, these data demonstrate that 4 h after endotoxin injection, myocardium contains cellular damages involving myocytes apoptosis in both the right and the left ventricle.

### The Broad Spectrum Caspase Inhibitor, z-VAD.fmk, Fully Inhibits Caspase Activation and Myocardial Apoptosis *In Vivo*

Four hours after endotoxin administration, caspase activities including caspase-1, -3, -8, and -9-like were assessed by measuring hydrolysis of the preferential substrates, Ac-YVAD-*p*NA, Ac-DEVD-*p*NA, Ac-IETD-*p*NA and Ac-LEHD-*p*NA,

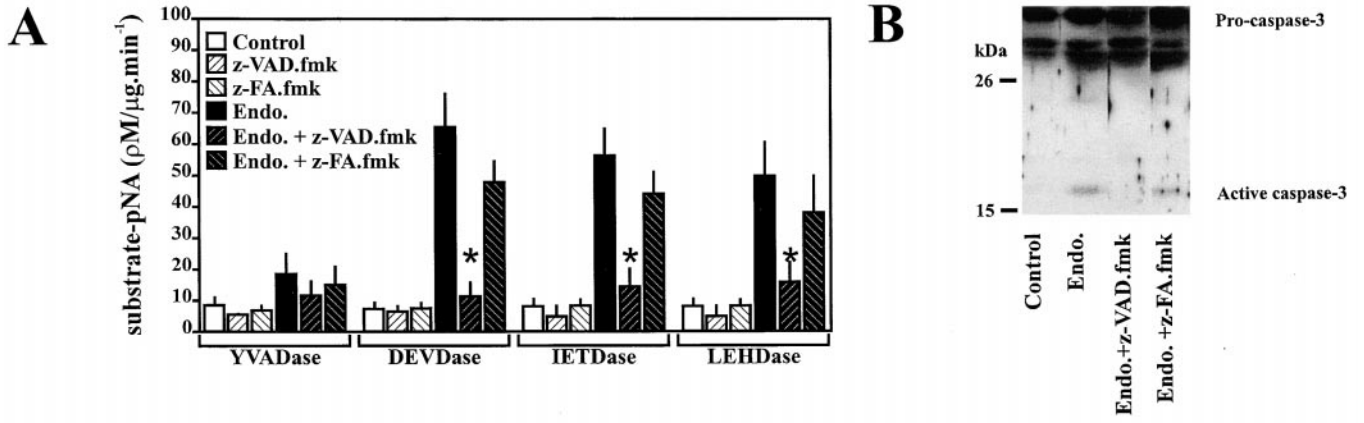


*Figure 3.* Effects of z-VAD.fmk on caspases activation induced by endotoxin. (*A*) Determination of multiple caspase activities. YVADase, DEVDase, IETDase, and LEHDase enzymatic activities were measured 4 h after *in vivo* treatments with specific *p*NA substrates as described in METHODS. Results are expressed as picomoles substrates *p*NA hydrolyzed/minute and per microgram of proteins. Data (mean ± SEM) of a representative experiment (n = 6 in each group) made in duplicate. *$p < 0.01$ versus endotoxin-treated group. (*B*) Effect of z-VAD. fmk on caspase-3 cleavage. Immunoblotting for caspase-3 was performed on heart lysates in control and endotoxin-treated rats for 4 h. z-VAD.fmk but not z-FA.fmk inhibits *in vivo* caspase-3 proteolytic cleavage in response to endotoxin. Pro-caspase-3 (32 kD) is cleaved into active subunits in myocardium from endotoxin-treated rats but not in myocardium from control. Z-VAD.fmk but not z-FA.fmk fully inhibited this process. Results are representative for three independent immunoblotting.

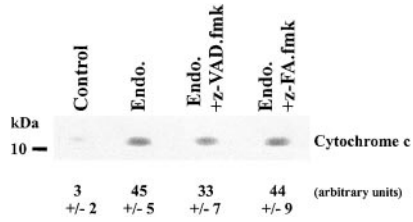


*Figure 4.* z-VAD.fmk does not prevent the induction of the cytochrome *c* release by endotoxin. Proteins from the cytosolic S-100 fractions were separated by 12% SDS/PAGE and were analyzed by immunoblotting for cytochrome *c* release. Four hours after injection, endotoxin induced cytochrome *c* release into the cytosol. z-VAD.fmk and z-FA.fmk failed to fully inhibit cytochrome *c* leakage. Four independent immunoblottings were scanned on a densitometer. The intensity (mean ± SEM) of the values obtained were expressed in arbitrary units.

respectively (n = 6 rats in each group). In our model, DEVDase (caspase-3-like), IETDase (caspase-8-like), and LEHDase (caspase-9-like) activities increased by 4- to 6-fold in myocardium prepared from endotoxin-treated rats (Figure 3A). No significant increase in YVADase (caspase-1-like) activity was observed under identical conditions (Figure 3A). Cotreatment with z-VAD.fmk (3 mg/kg) totally abrogated caspases-3, -8, and -9-like activities in myocardium of endotoxin-treated rats (Figure 3A). However, injection of z-FA.fmk (3 mg/kg), a structural analog of z-VAD.fmk in which the amino acid sequence VAD has been replaced, did not affect endotoxin-induced caspase activities in myocardium (Figure 3A). In contrast to control hearts, the proteolytic activation of caspase-3 was significant in endotoxin-treated myocardium as demonstrated by Western blot analysis (Figure 3B). Moreover, proteolytic caspase-3 activity was largely reduced by cotreatment with z-VAD.fmk, but not with z-FA.fmk endotoxin-treated myocardium (Figure 3B). Results of immunoblotting indicated that cytochrome *c*, in contrast to normal hearts, was substantially increased in cytosolic fraction of endotoxin-treated heart (Figure 4). The cytosolic accumulation of cytochrome *c* upon endotoxin treatment was not abrogated by the cotreatment with z-VAD.fmk or with z-FA.fmk (Figure 4). Finally, agarose gel electrophoresis confirmed that z-VAD.fmk (but not z-FA.fmk) protected myocardial cells from oligonucleosomal DNA fragmentation induced by endotoxin (Figure 5).

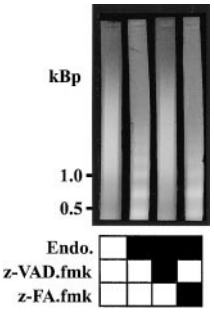


*Figure 5.* z-VAD.fmk but not z-FA.fmk inhibits nuclear apoptosis in heart from endotoxin-treated rats. Analysis by agarose gel electrophoresis. Endotoxin-induced nucleosomal ladders were visualized after LM-PCR assay (*see* METHODS), which were prevented by z-VAD.fmk and not by z-FA.fmk. Results are representative for three independent experiments.

### Injection of z-VAD.fmk Does Not Interfere with Endotoxin-induced Proinflammatory Cytokines Production *In Vivo*

Four hours after endotoxin administration, the level of IL-1β, TNF-α, and IL-6 cytokines in serum, measured by ELISA, was dramatically increased as compared with control (Figure 6). Endotoxin-induced cytokines production (n = 7 in each group, n = 3 in z-FA.fmk-treated groups) was not modified by the coinjection of z-VAD.fmk or z-FA.fmk (Figure 6). Consistent with this result, rats cotreated with z-VAD.fmk and endotoxin presented diarrhea, polypnea, and piloerection as well as those treated with endotoxin alone (data not shown).

### z-VAD.fmk (But Not z-FA.fmk) Fully Prevents Endotoxin-induced Myocardial Dysfunction Assayed *Ex Vivo*

As shown in Table 1, LVDP and its first derivatives (i.e., $dP/dt_{max}$ and $dP/dt_{min}$) were significantly decreased 4 h after administration of endotoxin as compared with control animals. Coronary perfusion pressure (CPP) levels remained unchanged (Table 1). Coinjection of z-VAD.fmk largely prevented LV systolic function alterations of endotoxin-treated hearts whereas the control peptide, z-FA.fmk, had no detectable effect (n = 8 in each group) (Table 1). Animals cotreated with endotoxin and z-VAD.fmk exhibited LVDP-preload relationships that were shifted upward compared with animals treated with endotoxin alone, suggesting improved systolic myocardial performance (Figure 7).

In another series of experiments, heart dysfunction and nuclear apoptosis were studied 14 h after endotoxin challenge. Coinjection of z.VAD.fmk at the same dose (3 mg/kg) largely prevented the depressive effect of endotoxin on LVDP (n = 6 in each group) (Table 2). Concomitantly, z-VAD.fmk was also active significantly reducing heart lysate oligonucleosome formation in endotoxin-treated rats (n = 3 in each group) (Table 2). Taken together, these observations strongly suggest that z-VAD.fmk does not delay but truly prevents myocardial dysfunction induced by endotoxin.

## DISCUSSION

In the present report, we demonstrated that apoptosis triggered myocardial cell death and that caspases are involved in cardiac depression induced by sepsis. Apoptosis has been extensively described as a determinant process in sepsis-associated cell death of different cell types including hepatocytes

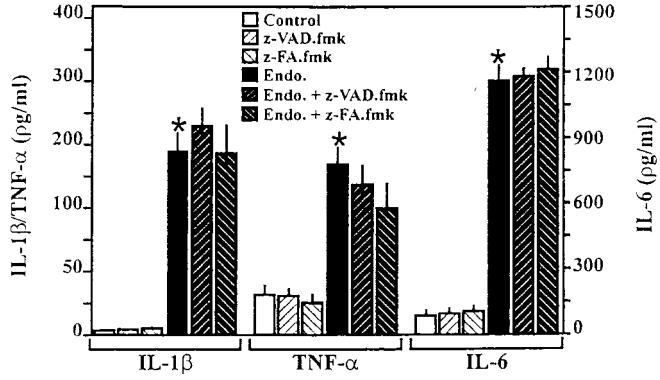


*Figure 6.* Effect of z-VAD.fmk on production of inflammatory cytokines induced by endotoxin. ELISA determination of inflammatory cytokines (IL-1β, TNF-α, IL-6) levels in sera from treated rats (n = 7 except for z-FA.fmk and endotoxin + z-FA.fmk, n = 3). *$p < 0.01$ between control and endotoxin groups.

**TABLE 1**

**ASSESSMENT OF CARDIAC FUNCTION 4 h AFTER TREATMENT***

| Group ($n = 8$) | LVDP (*mm Hg*) | CPP (*mm Hg*) | $dP/dt_{max}$ (*mm Hg/s*) | $dP/dt_{min}$ (*mm Hg/s*) |
|---|---|---|---|---|
| Control | 89 ± 5 | 39 ± 4 | 3,500 ± 200 | 1,950 ± 110 |
| Control + z-VAD.fmk | 89 ± 2 | 43 ± 7 | 3,740 ± 80 | 2,040 ± 50 |
| Control + z-FA.fmk | 90 ± 3 | 41 ± 2 | 3,420 ± 100 | 1,900 ± 150 |
| Endotoxin | 49 ± 7† | 44 ± 9 | 1,760 ± 250† | 930 ± 130† |
| Endotoxin + z-VAD.fmk | 79 ± 3‡ | 42 ± 5 | 3,160 ± 120‡ | 1,660 ± 60‡ |
| Endotoxin + z-FA.fmk | 50 ± 5† | 44 ± 4 | 1,950 ± 190† | 950 ± 90† |

*Definition of abbreviations*: CPP = coronary perfusion pressure; $dP/dt_{max}$ = maximum rate of LV pressure rise; $dP/dt_{min}$ = maximum rate of LV pressure fall; LVDP = left ventricular developed pressure; z-FA.fmk = *N*-benzyloxycarbonyl-Phe-Ala-fluoromethylketone; z-VAD.fmk = *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

* Results are expressed as mean ± SEM (n = 8 in each group).

*See* METHODS for treatment group design.

† $p < 0.01$ compared with control.

‡ $p < 0.01$ compared with endotoxin-treated rats.

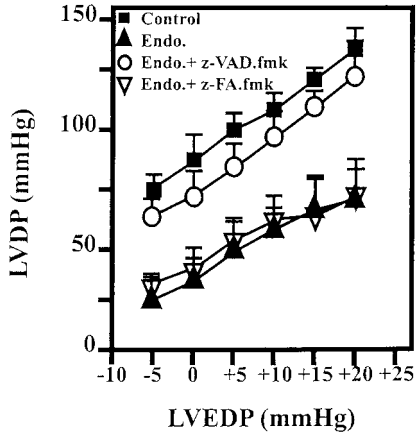


***Figure 7.*** Injection of z-VAD.fmk prevents cardiac dysfunction 4 h after exposure to endotoxin. Changes in preload on LV function in hearts from control, endotoxin-, endotoxin + z-VAD.fmk-, and endotoxin + z-FA.fmk-treated rats are displayed. (n = 8 in each group). Results are representative of the mean ± SEM. LV end-diastolic pressure was increased incrementally from −5 to 20 mm Hg in order to construct LV developed pressure–preload relationship curves. Compared with control, the LVDP-preload relationships were shifted downward (in the direction of myocardial contractility decrease) in endotoxin-treated rats. z-VAD.fmk + endotoxin–treated rat hearts exhibited a shift of LVDP–preload relationships upward compared with control and endotoxin-treated rat hearts (in the direction of myocardial contractility increase). $p < 0.01$ between endotoxin- and endotoxin + z-VAD.fmk-treated samples.

(20), enterocytes (21), or endothelial cells (21), but to our knowledge, no observation on endotoxin-mediated apoptosis in myocardial tissue has been reported. We observed cellular damages in the septic myocardium, including myocyte apoptosis and increased caspase activities. The broad spectrum caspase inhibitor, z-VAD.fmk, prevented the increase in endotoxin-induced caspase (-3, -8, -9)–like activity but did not influence cytokine levels. Finally, z-VAD.fmk admininistration prevented myocardial systolic dysfunction and apoptosis when studied 4 h and even 14 h after endotoxin administration.

It is only recently that apoptosis has been implicated as a major process in heart diseases including dilated cardiomyopathy, myocarditis, heart failure, and ischemic heart disease (for review see Davies [22]). This is due, at least in part, to technical difficulties in demonstrating apoptosis in heart tissues (22). For example, the TUNEL technique, the most extensively used method that reveals DNA strand breaks *in situ*, may in fact be both too sensitive and also able to produce equally false negatives (22, 23). Consequently, the use of several distinctive methods is required to demonstrate the occurrence of apoptosis (23). In this study, using different technical approaches, we provide strong evidence for the existence of apoptosis in heart from endotoxin-treated rats. First, *in situ* internucleosomal DNA fragmentation revealed the presence of TUNEL-positive cardiomyocyte nuclei in endotoxin-treated hearts. Second, the activity of several caspases, the proteolytic processing of the effector caspase-3, and the cytochrome *c* release into the cytosolic fraction bring additional evidence for apoptosis in endotoxin-treated rats. In parallel, we also observed some degree of myocardial cell injury as evidenced by histological alterations and release of myocardial CK. This finding is consistent with recent observations showing an increase in serum CK and cardiac troponin I in human septic shock (24).

Numerous apoptotic stimuli activate the caspase proteolytic cascade. Here, the increase in caspase-8 like activity observed in endotoxin-treated hearts probably resulted from the activation of the TNF-α-mediated death pathway, as the role of TNF-α in the pathogenesis of septic cardiomyopathy has already been documented (25). The importance of caspase-8 in myocardial tissue has been demonstrated by knockout studies showing abnormal heart development in caspase-8−/− mouse embryos (26). Activation of the effector caspase-3 is considered as a determinant of apoptosis in human heart failure (27). Here we observed proteolytic cleavage of procaspase-3 and caspase-3-like activity in heart from endotoxin-treated rats.

In our model, activation of caspases probably occurred through the mitochondrial apoptotic pathway as indicated by the release of cytochrome *c* from mitochondria and the presence of caspase-9-like activity in heart extracts. Here, cytochrome *c* release occurred, at least in part, independently of

**TABLE 2**

**ASSESSMENT OF CARDIAC FUNCTION AND DNA FRAGMENTATION 14 h AFTER TREATMENT***

| Group | LVDP (*mm Hg*) | CCP (*mm Hg*) | $dP/dt_{max}$ (*mm Hg/s*) | $dP/dt_{min}$ (*mm Hg/s*) | Nucleosome (*OD/μg*) |
|---|---|---|---|---|---|
| Control | 99 ± 4 | 40 ± 4 | 3,650 ± 200 | 2,050 ± 115 | 19 ± 4 |
| Control + z-VAD.fmk | 98 ± 3 | 43 ± 5 | 3,570 ± 120 | 2,140 ± 75 | 17 ± 2 |
| Endotoxin | 52 ± 6† | 41 ± 3 | 1,860 ± 150† | 850 ± 150† | 54 ± 4† |
| Endotoxin + z-VAD.fmk | 85 ± 8‡ | 42 ± 5 | 3,500 ± 100‡ | 1,950 ± 100‡ | 28 ± 8†,‡ |

*Definition of abbreviations*: CPP = coronary perfusion pressure; LVDP = left ventricular developed pressure; $dP/dt_{max}$ = maximum rate of LV pressure rise; $dP/dt_{min}$ = maximum rate of LV pressure fall; nucleosome = oligonucleosomal framgentation detected by ELISA (OD/μm protein); z-VAD.fmk = *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

* Results are expressed as mean ± SEM (n = 6 for cardiac function parameters; n = 3 for DNA fragmentation parameters).

*See* METHODS for treatment group design.

† $p < 0.01$ compared with control.

‡ $p < 0.01$ compared with endotoxin-treated rats.

upstream caspases as z-VAD.fmk administration, which inhibited caspase-8-like activity, was unable to totally prevent the cytochrome *c* release. Similar observations were recently made in cardiac myocytes in which the mitochondrial apoptotic pathway was activated by serum and glucose deprivation (28). These findings raise the possibility that experimental endotoxemia could initiate multiple apoptotic pathways in heart including both mitochondrial apoptotic pathways and death receptors pathways (e.g., TNF-α receptors ) activating upsteam caspases (e.g., caspase-8).

The pivotal role of caspases in our septic model was further demonstrated by the fact that injection of z-VAD.fmk (but not the irrelevant peptide z-FA.fmk) not only inhibited the activation/processing of caspases and nuclear apoptosis but also the cardiac dysfunction induced by endotoxin. It is to notice that the protective effect of z-VAD.fmk was a long-term effect observed even at 14 h after endotoxin challenge. This result is consistent with a recent report showing that z-VAD.fmk may prevent caspase-mediated apoptosis with significant reduction of infarct size and cardiac dysfunction (29). Similarly, single injection of z-VAD.fmk prevented late-onset (6 h and 12 h) hypoxia-induced cerebral injury, suggesting a long half-life of this peptidomimetic compound (30).

However, we cannot exclude that caspase activation induces cardiac dysfunction by a mechanism distinct from apoptotic cell death. Indeed, it has been demonstrated that caspase activation may occur in the absence of cell death (for review *see* Zeuner and coworkers [31]). In addition to their role in apoptosis, caspases influence the production of several cytokines (for review *see* Reed [32]). For example, the maturation of IL-1β is mediated predominantly by caspase-1 *in vivo*. Also, endotoxin-induced production of IL-6 and TNF-α is reduced in caspase-1 and –11 knockout mice (32). Nevertheless, our results suggest that injection of z-VAD.fmk does not interfere with endotoxin-induced proinflammatory cytokine production. Alternatively, recent data indicate the ability of several death receptor members to inhibit calcium signaling in lymphoid cells (33). In T lymphocytes, the CD95 stimulation via the sphingomyelinase pathway can inhibit TCR-induced calcium influx into the cytosol (33). Although the link between calcium signaling and caspase activation was not tested in this study, we can speculate that caspases, which are activated early after CD95 or TNF-receptor stimulation, could also be implicated in regulation of calcium signaling and, thus, myocyte contractile function.

In conclusion, these observations strongly suggest a central role of caspases in the onset of functional alterations in the septic heart. Finally, the efficacy of the broad spectrum caspase inhibitor, z-VAD.fmk, in preventing endotoxin-induced myocardial dysfunction suggests that inhibition of apoptosis could be a new promising adjunctive treatment of cardiodepression associated with sepsis.

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