Inhibition of Caspase-3 Improves Contractile Recovery of Stunned Myocardium, Independent of Apoptosis-Inhibitory Effects

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OBJECTIVES
The aim of this study was to investigate whether the caspase-3 inhibitor Ac-DEVD-CHO functionally improves stunned myocardium.

BACKGROUND
Degradation of troponin I contributes to the pathogenesis of myocardial stunning, whereas the role of apoptosis is unknown. Caspase-3 is an essential apoptotic protease that is specifically inhibited by Ac-DEVD-CHO.

METHODS
Isolated working hearts of rats were exposed to 30 min of low-flow ischemia, followed by 30 min of reperfusion. Ac-DEVD-CHO (0.1 to 1 μmol/l) was added 15 min before ischemia/reperfusion or 5 min before reperfusion. Cardiac output, external heart power, left ventricular (LV) developing pressure and contractility (dp/dt max) were measured. Apoptosis was assessed by TUNEL staining and internucleosomal deoxyribonucleic acid fragmentation. Caspase-3 processing and troponin I cleavage were determined by immunoblotting. Caspase-3 activity was measured using a fluorogenic substrate.

RESULTS
The addition of Ac-DEVD-CHO before ischemia/reperfusion or before reperfusion dose-dependently and significantly (p < 0.05) improved post-ischemic recovery of cardiac output, external heart power, LV developing pressure and dp/dt max, compared with the vehicle (0.01% dimethyl sulfoxide). Ac-DEVD-CHO was similarly effective when given before reperfusion. Ac-DEVD-CHO blocked ischemia/reperfusion-induced caspase-3 activation, but cardiomyocyte apoptosis was unaffected. Troponin I cleavage was not inhibited by Ac-DEVD-CHO.

CONCLUSIONS
Caspase-3 is activated in stunned myocardium. Inhibition of caspase-3 by Ac-DEVD-CHO significantly improves post-ischemic contractile recovery of stunned myocardium, even when given after the onset of ischemia. The mechanism(s) of protection by Ac-DEVD-CHO appear to be independent of apoptosis. Inhibition of caspase-3 is a novel therapeutic strategy to improve functional recovery of stunned myocardium. (J Am Coll Cardiol 2001;38:2063–70) © 2001 by the American College of Cardiology

A significant reduction of coronary blood flow causes myocardial ischemia and contractile dysfunction. The cardiac dysfunction often persists for days, even after coronary artery perfusion has been re-established. This scenario of reversible post-ischemic contractile dysfunction is known as myocardial stunning (1). Depressed contractility, with reduced maximal force generation, is a hallmark of stunning (2–5).

Apoptosis is an evolutionary conserved process of programmed cell death in response to diverse stimuli, such as cardiac development or hypoxia (6). Increased cardiomyocyte apoptosis has been reported in patients with heart failure and experimental myocardial infarction (MI) (7–10). The caspase family of cellular proteases initiates and executes apoptotic cell death (11). Caspase-3, a pivotal effector caspase, is an essential protease of the apoptotic machinery. Caspase-3 proteolytically cleaves a number of death substrates and activates endonucleases, leading to internucleosomal deoxyribonucleic acid (DNA) fragmentation, a hallmark of apoptosis (12). Individual caspases differ in their substrate recognition sequences, which has allowed generation of inhibitors like Ac-DEVD-CHO that specifically inhibit caspase-3 (3). Ac-DEVD-CHO is a tetrapeptide (Asp-Glu-Val-Asp) based on the caspase-3 substrate recognition motif, with an acetylate group coupled to its N-terminal (for enhanced chemical stability) and an aldehyde group conjugated to its C-terminal (for irreversible inactivation of the caspase-3 catalytic cysteine residue) (13,14).

In a rabbit and rat model of MI, administration of a broad-spectrum caspase inhibitor before infarction significantly reduced the infarct size and rate of apoptotic cells in the area at risk (9,10). However, it is unknown whether caspase inhibitors functionally improve stunned myocardium and whether they are effective when given after the onset of myocardial ischemia. The latter would be more relevant to the clinical situation.

Therefore, we investigated the effects of the caspase-3 inhibitor Ac-DEVD-CHO in an isolated working-heart rat model of myocardial stunning. Our data suggest that inhibition of caspase-3 reduces myocardial stunning when...
initiated after the onset of ischemia (I), but before reperfusion (R). Thus, caspase-3 inhibition represents a novel therapeutic strategy to improve contractile functional recovery of stunned myocardium.

**METHODS**

**Isolated working-heart rat model.** Male, 4- to 6-week-old Sprague Dawly rats were anesthetized with pentobarbital (60 mg/kg intraperitoneally [IP]), heparinized (500 IU/100 g body weight IP). The hearts were removed, and the aorta was mounted onto a 1.4-mm cannula and attached to a perfusion apparatus (Hugo Sachs Electronic). The hearts were perfused with oxygenated (95% oxygen, 5% carbon dioxide), noncirculating Tyrode’s solution (in mmol/l): 124.6 NaCl; 4.0 KCl; 2.2 CaCl₂; 1.1 MgCl₂; 24.9 NaHCO₃; 0.3 NaH₂PO₄; and 11.1 glucose (pH 7.4) at a perfusion pressure of 51 mm Hg. After equilibration for 15 min, the perfusion was switched to the antegrade working-heart mode, with a preload of 11 mm Hg and an afterload of 51 mm Hg. Aortic pressure was measured through a pressure transducer (Hugo Sachs Electronic). Left ventricular pressure (LVP) was measured by using a microtipped catheter (SPR 407, 2F, Millar Instruments). The maximal rise in LVP was obtained with an electronic microtip catheter (SPR 407, 2F, Millar Instruments).

**Experiments groups and protocols.** In 8 to 10 animals in each experimental group, global low-flow ischemia was induced by reducing the coronary flow to 10%, resulting in a reduction of aortic pressure from 51 to 11 mm Hg. Low-flow ischemia was maintained for 30 min, followed by 30 min of reperfusion. External heart power (EHP) per gram of left ventricular (LV) wet weight was calculated: EHP₅₇ (mJ/g per min) = pressure × volume work/heart rate. Dimethyl sulfoxide (DMSO; 0.01%; Sigma) as vehicle or Ac-DEVD-CHO in 0.01% DMSO final (0.1 to 1 μmol/l; Alexis) was started 15 min before ischemia or 5 min before reperfusion and given throughout the reperfusion period.

**Immunoblotting.** Tissue was homogenized in lysis buffer (10 mmol/l tris-HCl, pH 8.0; 1% Triton X-100; 0.32 mol/l sucrose; 5 mmol/l EDTA; and 1 mmol/l phenylmethylsulfonyl fluoride), and proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), transferred to PVDF membranes and immunoblotted, as described (15), with the following primary antibodies: anti-troponin I (clone C5), anti-mouse actin (both Chemicon) or anti-caspase-3 (p17 subunit, Santa Cruz Biotecology). Bound antibodies were studied by chemiluminescence (Amersham).

**Caspase-3 and calpain enzymatic assays.** Myocardial tissue was homogenized as described previously, and 200 μg of protein was used in 700 μl of caspase-assay buffer (100 mmol/liter HEPES, pH 7.5; 0.32 mol/l sucrose; 100 mmol/l NaCl; 0.1% CHAPS; 2 mmol/l dithiothreitol; 10 μg/ml aprotinin; 10 μg/ml leupeptin; and 10 μg/ml pepstatin A), with 0.24 mmol/l of DEVD-AFC as the fluorogenic caspase-3 substrate, as described (16). For measuring calpain-like activity, 200 μg of protein was used in 600 μl of calpain-assay buffer (60 mmol/l imidazol, pH 7.5; 5 mmol/l l-cysteine; 0.2% Triton X-100; 5 mmol/l CaCl₂; 5 mmol/l dithiothreitol; 10 μg/ml aprotinin; 10 μg/ml leupeptin; and 10 μg/ml pepstatin A), with 0.24 mmol/l of fluorogenic calpain substrate I (Calbiochem). Purified calpain I (5 U; Calbiochem) was used as indicated, and after 60 to 100 s, calpain inhibitor I (Roche) or Ac-DEVD-CHO was added to the reaction mixture, while the increase in fluorescence was still linear.

**Detection of apoptosis.** The DNA strand breaks were analyzed in situ using 5-μm sections with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL), as described (15). Internucleosomal DNA fragmentation (“DNA laddering”) was detected by incubation of phenol-chloroform extracted DNA with 5 U of Klenow polymerase and 0.5 μCi of (α-³²P)-dCTP, followed by gel electrophoresis, as described (16).

**Statistical analysis.** All data are presented as the mean ± SEM. Comparisons were performed by analysis of variance for comparison of multiple measurements or by the paired or unpaired Student t test. A Bonferroni correction for multiple comparisons was used to determine the level of significance. A p value <0.05 was considered statistically significant.

**RESULTS**

Ac-DEVD-CHO improves post-ischemic contractile recovery in a model of myocardial stunning of isolated working hearts of rats. As an experimental model of myocardial stunning, we used isolated working hearts of rats (17). Figure 1A illustrates the experimental protocol: after initial stabilization for 15 min, the hearts were treated with Ac-DEVD-CHO (0.1 to 1 μmol/l) or vehicle (0.01% DMSO) for 15 min before ischemia (8 to 10 hearts per experimental group). After 30 min of low-flow ischemia, reperfusion was established for 30 min. For the first 15 min of reperfusion, the hearts were perfused in the Langendorff mode to allow for recovery. Then, perfusion was switched back to the working-heart mode. Under these conditions,
the residual 10% coronary flow during ischemia largely prevented MI (17).

In the control group, cardiac output, external heart power, (LV) developing pressure, myocardial contractility and aortic and coronary flow (data not shown) severely decreased during the ischemic period and remained depressed during reperfusion (Fig. 1B). Treatment of isolated hearts with 0.1 to 1 μmol/l of Ac-DEVD-CHO (starting 15 min before ischemia) did not affect cardiac performance at baseline or during ischemia. However, Ac-DEVD-CHO dose-dependently and significantly (p < 0.05 for 1 μmol/l) improved the contractile recovery of post-ischemic myocardium. Just 1 μmol/l of Ac-DEVD-CHO significantly enhanced cardiac output, external heart power, LV developing pressure, myocardial contractility (Fig. 1B) and aortic and coronary flow (data not shown).

Figure 2 shows that only 20% to 45% (calculated values relative to pre-ischemic baseline value) of post-ischemic recovery of the various variables was observed in the vehicle-treated group. This indicates severe cardiac dysfunction, even after restoration of perfusion. Treatment with 1 μmol/l of Ac-DEVD-CHO increased the relative recovery of all variables to 60% to 85% of the pre-ischemic values (Fig. 2). Ac-DEVD-CHO is equally effective when administered before reperfusion. To investigate whether Ac-DEVD-CHO protects the myocardium from stunning when administered after the onset of ischemia, Ac-DEVD-CHO (1 μmol/l) was started 5 min before reperfusion. This scenario more closely resembles the common clinical situation of patients presenting with acute coronary syndromes.

As shown in Figure 2, the group treated with 1 μmol/liter of Ac-DEVD-CHO, started 5 min before reperfusion, led
Ac-DEVD-CHO blocks ischemia/reperfusion-induced activation of caspase-3. Next, we investigated the effects of ischemia/reperfusion and Ac-DEVD-CHO on the activation of caspase-3. Caspase-3 exists as an inactive zymogen of 32 kD and is activated by proteolytic processing into p17 subunit in ischemic hearts. The effect of Ac-DEVD-CHO started 5 min before ischemia (top) or 5 min before reperfusion (bottom). Note the similar beneficial effect of Ac-DEVD-CHO on the relative recovery. *p < 0.05 versus vehicle-treated group. Open bars = 0.01% DMSO; solid bars = 1 μmol/l of Ac-DEVD-CHO.

Effects of Ac-DEVD-CHO on apoptosis. Increased apoptosis has been implicated in MI (9,10). Because Ac-DEVD-CHO can block apoptosis in various cell types (12), we determined the rate of apoptotic cells by TUNEL staining and internucleosomal DNA fragmentation.

As shown in Figure 3B, we detected single TUNEL-positive cells in the nonischemic myocardium, as well as in the vehicle-treated and Ac-DEVD-CHO–treated myocardium subjected to ischemia/reperfusion. The TUNEL-positive cells appeared to be mostly cardiomyocytes. Quantitative analysis (5 sectors of ~500 cells/sector; n = 4 hearts in each group) revealed a significant (p < 0.05) increase in the number of TUNEL-positive cells in both the vehicle-treated (0.38 ± 0.18%) and Ac-DEVD-CHO–treated (0.42 ± 0.31%) groups, compared with nonischemic control group (0.05 ± 0.03%).

This increase in the rate of myocardial apoptosis during ischemia/reperfusion was confirmed by the internucleosomal DNA fragmentation assay. Compared with the nonischemic control group, ischemia/reperfusion caused an increase in DNA strand breaks, with the typical “ladder” pattern (Fig. 3B, right). The ischemia/reperfusion-induced increase in apoptosis was similar in the vehicle-treated and Ac-DEVD-CHO–treated groups.

Effects of Ac-DEVD-CHO on troponin I degradation and calpain activity. Because the beneficial effect of Ac-DEVD-CHO on contractile recovery was largely independent of apoptosis, we investigated whether Ac-DEVD-CHO may inhibit other cysteine proteases, such as calpain. Because calpain-mediated cleavage of the thin-filament regulatory protein troponin I has been suggested as a molecular mechanism of stunning (1), we investigated the effects of Ac-DEVD-CHO on troponin I degradation and calpain activity.

Immunoblotting with an anti–troponin I antibody showed increased appearance of the characteristic troponin I fragment in vehicle-treated and Ac-DEVD-CHO–treated hearts subjected to ischemia/reperfusion (Fig. 4A). However, Ac-DEVD-CHO started 5 min before reperfusion did not quantitatively alter the troponin I degradation.

Using a fluorogenic calpain substrate, we were unable to blocked activation of caspase-3 induced by ischemia/reperfusion.

To determine catalytic activity of caspase-3 in rat heart extracts, we used a fluorogenic caspase-3 substrate (Fig. 3A, right). When compared with the control group, caspase-3–like activity was more than fivefold increased by ischemia/reperfusion in the vehicle-treated group (p < 0.05). Treatment with Ac-DEVD-CHO from 5 min before reperfusion abolished activation of caspase-3, with proteolytic activity remaining close to baseline levels (p < 0.05 vs. vehicle-treated group).
reproducibly detect measurable calpain-like proteolytic activity in any of the rat hearts (data not shown). To address a potential nonspecific effect of Ac-DEVD-CHO on calpain, we determined the effects of Ac-DEVD-CHO on purified calpain I in vitro. The addition of calpain to its substrate caused a linear increase in fluorescence (Fig. 4B). While the increase was still linear, calpain inhibitor I or Ac-DEVD-CHO was added. Although 100 nmol/l of calpain inhibitor I almost completely inhibited calpain I, the same concentration of Ac-DEVD-CHO had little inhibitory effect. Quantitative evaluation of the remaining catalytic activity after addition of the inhibitor (Fig. 4C) showed that the cysteine protease calpain could be principally inhibited by the aldehyde Ac-DEVD-CHO at higher concentrations (>1 μmol/l). However, 10-fold higher concentrations of Ac-DEVD-CHO were needed to achieve an equal inhibition of calpain, compared with calpain inhibitor I. Taken together, these results indicate that, in vivo, Ac-DEVD-CHO does not act primarily through calpain inhibition.
DISCUSSION

The caspase-3 inhibitor Ac-DEVD-CHO significantly improves post-ischemic contractile recovery of isolated working hearts of rats. Interestingly, Ac-DEVD-CHO was effective even when given after the onset of low-flow ischemia. The effects of caspase-3 inhibition appeared to be largely independent of cardiomyocyte apoptosis.

Myocardial stunning and caspase-3 activation. The role of apoptosis in the pathogenesis of myocardial stunning is unknown. Because caspase-3 is a key protease that executes apoptosis, we investigated whether caspase-3 inhibition reduces myocardial stunning in an isolated working-heart rat model. This model is well established and allows study of myocardial contractility in the intact heart, independent of compounding factors, such as sympathetic activity and activation of an immune response (17). Indeed, caspase-3 was activated in our model of myocardial stunning. Thus, our data confirm and extend two recently published studies demonstrating activation of caspase-3 in a rat and rabbit model of MI (9,10). As hypothesized, Ac-DEVD-CHO was able to block the
ischemia/reperfusion-induced activation of caspase-3 in our experimental model.

**Ac-DEVD-CHO functionally improves stunned myocardium.** In contrast to the vehicle, the caspase-3 inhibitor Ac-DEVD-CHO dose-dependently and significantly improved post-ischemic contractile recovery. Just 1 μmol/l led to almost a doubling of all contractile variables to 60% to 85% of the pre-ischemic values. These good recovery rates principally achievable with an appropriate pharmacologic intervention also demonstrate that low-flow ischemia/reperfusion in our model causes mainly myocardial stunning but little if any MI. Importantly, Ac-DEVD-CHO was equally potent in reducing myocardial stunning when given 5 min before reperfusion, instead of before the induction of ischemia. This scenario being given resembles the clinical situation.

**Myocardial stunning and apoptosis.** Although the beneficial effect of caspase inhibitors in experimental MI has been attributed to a reduced rate of apoptosis (7,10), our data do not support a role for the inhibition of apoptosis as the mechanism by which Ac-DEVD-CHO acts on the contractile recovery of stunned myocardium. Ac-DEVD-CHO neither reduced the number of TUNEL-positive cells, nor the amount of internucleosomal DNA fragmentation. Although ischemia/reperfusion-induced caspase-3-activation was associated with a minor increase in the number of apoptotic cells and fragmented DNA, there was no significant effect of DEVD-CHO on the degree of apoptosis. Thus, apoptosis of cardiac myocytes in this experimental setting seems to be mediated by a caspase-independent pathway. Indeed, a recent study suggests that other signalling pathways can induce apoptosis independent of the caspase cascade (18). Moreover, it remains to be clarified whether an increase of apoptosis of ~0.3% has any significant outcome with regard to the early functional integrity of the heart. Therefore, the profound improvement in post-ischemic contractile recovery by the caspase-3 inhibitor Ac-DEVD-CHO appears to be independent of apoptosis in the experimental setting used. These findings indicate that the integrity of the contractile apparatus may be adversely affected by caspase-3 activation in the myocardium during stunning.

**Degradation of contractile proteins during myocardial stunning.** Selective troponin I degradation has been reported in some (1–5,19), but not all (20), models of myocardial stunning. Transgenic overexpression of the major proteolytic troponin I product in the heart is sufficient to recapitulate many aspects of myocardial stunning (21). Subsequent studies suggested calpain-mediated cleavage of troponin I as an underlying mechanism (2–5,19), and calpain inhibitor I is able to reduce infarct size (22) and improve myocardial stunning (23). Because Ac-DEVD-CHO may inhibit other cysteine proteases, such as calpain, we investigated whether Ac-DEVD-CHO may inhibit troponin I degradation. Consistent with published reports (2–5,19), ischemia/reperfusion in isolated working hearts of rats resulted in partial troponin I degradation, which was not quantitatively affected by Ac-DEVD-CHO. In vitro, purified calpain could be inhibited by Ac-DEVD-CHO. However, 1 μmol/l of Ac-DEVD-CHO achieved only partial calpain inhibition, indicating that it acts in vivo, primarily through pathway(s) other than calpain inhibition. However, we cannot exclude that Ac-DEVD-CHO exerts some of its beneficial effects by partial calpain inhibition. Calpain or other proteinase(s) may play a pathogenic role in our model, because we achieved only incomplete recovery with Ac-DEVD-CHO.

Alternatively, Ac-DEVD-CHO may inhibit cleavage of other contractile proteins. Through a data bank search with caspase cleavage motifs, we identified a putative caspase cleavage site (DEVD) in cardiac troponin C (12). However, immunoblots of rat hearts with troponin C antibodies did not show any troponin C degradation during ischemia-reperfusion (data not shown). Further experiments will be required to identify the molecular target(s) of caspase activation during stunning.

**Conclusions.** Taken together, in an isolated working-heart rat model of myocardial stunning, inhibition of ischemia/reperfusion-induced caspase-3 activation by Ac-DEVD-CHO results in a substantial improvement of post-ischemic contractile recovery. The observed effects appear to be independent of suppression of apoptosis, but most likely involve both caspase and calpain inhibition. Regardless of the underlying mechanism(s), the use of a caspase-3 inhibitor represents a potentially clinically relevant, novel therapeutic strategy to reduce myocardial stunning.

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**REFERENCES**