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Original Research Communication

Pharmacology of Caspase Inhibitors in Rabbit Cardiomyocytes Subjected to Metabolic Inhibition and Recovery

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ABSTRACT

Protection of ischemic myocardium is an important unmet need in reperfusion therapy of acute myocardial infarction. Myocardial ischemia and reperfusion induce necrosis and apoptosis in cardiomyocytes. Caspase processing and activation are critical steps in most receptor and nonreceptor pathways of apoptosis. Caspase inhibitors have been shown to reduce ischemia reperfusion injury in cardiac muscle. Information about dose response and time of administration are needed to optimize the design of preclinical studies. We used isolated adult rabbit cardiomyocytes subjected to metabolic inhibition (MI) and recovery to examine the role of caspases and caspase inhibitors, the dose response, and the timing of administration. *In vitro* inhibitory concentrations (K_i) were determined for purified caspases. Cardiomyocytes subjected to MI were treated with peptidomimetic fluoromethyl ketone inhibitors of caspases before or during MI, or at recovery. Caspase inhibitors were most effective when added before MI and included throughout recovery, but were partially protective if added after MI. The optimal concentration of the inhibitors tested was ~10 μM. Protection was sustained when cells were allowed to recover for 4 or 24 h. These results suggest that caspase activation is an important component of myocyte injury mediated by MI and recovery. Low doses of caspase inhibitors were identified that reduce injury in this model system, and further investigations using *in vivo* models are warranted. Antioxid. Redox Signal. 3, 113–123.

INTRODUCTION

PROTECTION OF ISCHEMIC MYOCARDIUM is an important unmet need in the clinical therapy of myocardial infarction by reperfusion. The phenomenon of preconditioning suggests that myocyte death after ischemia and reperfusion is preventable. Preconditioning is the phenomenon wherein a brief nonlethal period of ischemia reduces tissue death during a subsequent prolonged episode of ischemia and reperfusion. It is the most effective way to reduce tissue death in experimental myocardial ischemia and has been observed in patients (3, 17, 27, 32). Intensive investigation into receptor-mediated signaling pathways (e.g., adenosine, bradykinin, and others) through protein kinases is ongoing in hopes of identi-

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fying new interventions for clinical application. However, the ultimate mechanisms by which preconditioning reduces myocyte death is not clear.

Ischemia and reperfusion result in myocyte cell death by both necrosis and apoptosis (8, 11, 16). Others and we have shown that prevention of apoptosis is an important mechanism of preconditioning in model systems (11, 33). Apoptosis is a stereotyped, energy-dependent process of cell death characterized by activation of proteases, membrane blebbing, mitochondrial alterations, and fragmentation of chromosomal DNA. Apoptosis can be activated by receptor pathways (e.g., Fas, tumor necrosis factor-α) and nonreceptor detection of cell injury (e.g., detection of DNA damage). Specific cellular processes of apoptosis are modulated, for example, by p53, c-myc (7), and members of the Bcl-2 family (13, 15, 26). Attempts to interfere with biochemical events in apoptosis may ameliorate cell injury associated with myocardial infarction. The so-called "death proteases" of apoptosis, caspases, appear to activate several of the final pathways responsible for the morphology of apoptosis (2, 19, 21, 24). These enzymes are highly conserved throughout evolution; the prototypic caspase Ced-3 identified in *C. elegans* is required for programmed cell death to occur. Caspases are present as proenzymes; they can become activated by upstream proteases (e.g., granzyme B or caspase-8) (4, 28), by mitochondrial release of cytochrome *c* and interaction with APAF-1, dATP, and caspase-9 (18, 20, 38), or by a positive feedback loop involving the MEKK/JNKK/JNK pathway (2). They also may be regulated by nitrosylation (22, 23). Because proteolytic cleavage is irreversible, caspase activation has been viewed as the "point of no return" in the induction of apoptosis (10). Activation of caspase-9 at the mitochondria is critical for the non–receptor-mediated pathway of apoptosis. Thus, inhibition of caspases has the potential for therapeutic application in myocardial ischemia and reperfusion. In rabbit cardiomyocytes, we found that the caspase inhibitor carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk) reduced cell death after metabolic inhibition (MI) (12). *In vivo* confirmation was obtained in a rat myocardial ischemia and reperfusion model where Z-VAD-fmk reduced infarct size, but pharmacokinetics and dose response were not studied (39). Critical questions for application of caspase inhibition in preclinical models include dose response and timing of treatment, e.g., will treatment at time of reperfusion be expected to salvage myocardium?

To study the effects of timing of caspase inhibition on myocyte cell death, and characterize dose response of several caspase inhibitors, we utilized isolated adult rabbit cardiomyocytes subjected to MI and recovery. Cardiomyocytes subjected to MI were treated at various times with IDN #1965, IDN #1529, or IDN #1501, peptidomimetic fluoromethyl ketone inhibitors of caspases, or an inactive peptide fluoromethyl ketone (IDN #1672). Inhibitory concentrations *in vitro* were determined for each compound. Our results show that caspase activation is an important aspect of myocyte cell death by MI and recovery, and that caspase inhibitors at micromolar concentrations have the potential to reduce ischemia reperfusion-induced cell death in myocardial infarction, even when administered after the injury.

MATERIALS AND METHODS

Compounds used in this study

The following compounds were synthesized and provided by IDUN Pharmaceuticals: IDN #1501: *N*-[(benzyloxycarbonyl)alaninyl]-3-amino-4-oxo-5-fluoropentanoic acid; IDN #1529: *N*-[(indole-2-carbonyl)alaninyl]-3-amino-4-oxo-5-fluoropentanoic acid; IDN #1965: *N*-[(1,3-dimethylindole-2-carbonyl)valinyl]-3-amino-4-oxo-5-fluoropentanoic acid; IDN #1672: *N*-[benzyloxycarbonyl)valinyl]-3-amino-1-fluoro-2-butanone. IDN #1501, IDN #1529, and IDN #1965 are peptidomimetic fluoromethyl ketone inhibitors of caspases; IDN #1672 was used as a control for the fluoromethyl ketone moiety. All these compounds were dissolved to 20 m*M* in dimethyl sulfoxide (DMSO) and stored at −20°C. The compounds will be referred to by number throughout the text.

In vitro assays of compounds

The effective inhibitory concentrations of these compounds were tested against recombinant caspases [prepared as described for caspase-3 (25)] using a fluorogenic substrate (Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) for caspase-3 and -8, Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin) for caspase-1) as previously described (1, 37). Their activity against Fas-mediated apoptosis in Jurkat cells was tested by adding the compounds 1 h before the addition of anti-Fas antibody and scoring apoptosis by conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) after 24 h of culture.

The caspase inhibitors were also tested for their ability to inhibit calpain II and cathepsin B (both from Calbiochem). Enzymes were diluted in ICE buffer (25 m*M* HEPES, 1 m*M* EDTA, 0.1% CHAPS, 10% sucrose; pH 7.5 for calpain II, pH 6.0 for cathepsin B) with a final concentration of 13.3 m*M* dithiothreitol. Also, for calpain II, $CaCl_2$ was added to a final concentration of 16.67 m*M*. The enzymes were allowed to incubate for 30 min at room temperature in the ICE buffer with dithiothreitol prior to the start of the assay. The inhibitors were diluted from 20 m*M* stock solutions in DMSO into ICE buffer at the optimal enzyme pH for the enzyme and added to a 96-well plate. Substrates (succinyl-Leu-Tyr-7-amino-4-methylcoumarin, 100 μM for calpain II; and benzyloxy-Arg-Arg-7-amino-4-methylcoumarin, 25 μM for cathepsin B) were diluted into ICE buffer at the same pH from a 20 m*M* DMSO stock and added to the wells. The enzymes were then added to the wells and the fluorescence measured over 90 min with readings at 1-min intervals using a Cytofluor (PerSeptive Biosystems) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Caspase activity assay of isolated myocytes

These investigations conform to the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, 1996). Cardiomyocytes were washed in phosphate-buffered saline, resuspended in lysis buffer, and sonicated as described (29). The samples were clarified by a 30-min microfuge spin. Latent caspase activity was activated by incubating 300 μg of cell lysate for 1 h at room temperature with 200 ng of recombinant caspase-8 (Pharmingen, La Jolla, CA, U.S.A.), 0.1 m*M* dATP, and 10 μg/ml cytochrome *c*. Caspase activity was measured using 200 μM Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA) as substrate. Results are reported as milli-optical density units per minute. Caspase-8 at this concentration has no activity toward DEVD-pNA (data not shown).

Cell isolation and culture

Ventricular myocytes were isolated from collagenase-perfused rabbit hearts as described previously (12). After centrifugation over Ficoll, 70–90% of the myocytes were viable (rod-shaped). In all subsequent analyses, a viable myocyte is defined as a rod-shaped, striated cell. Previous experiments indicated that all rod-shaped cells reduced the tetrazolium salt (MTT) and excluded trypan blue (12). The cells were allowed to rest in Dulbecco's modified Eagle medium with 5% fetal bovine serum, streptomycin, penicillin, and Fungizone (culture medium) for 30 min before use in experimental protocols.

MI protocol

MI was induced by placing cells in the following buffer containing the following (in m*M*): NaCl 106, KCl 4.4, $MgCl_2$ 1.0, $NaHCO_3$ 38, $CaCl_2$ 2.5, 2-deoxyglucose 20, NaCN 1.0, pH 6.6. In these experiments, the cells were exposed to MI buffer for 40 min in a humidified incubator containing 5% CO_2. This treatment consistently resulted in a reduction of viability by ~40% when scored 4 h or 24 h later.

Experimental protocols

The experimental protocols are illustrated in Fig. 1. In brief, before the experiment, the cells were equilibrated in culture medium for 30 min. Between each treatment period, cells were washed twice with culture medium. After either 4 or 24 h of recovery, cells were fixed in 4% formalin and scored. In *throughout* group, the compounds were present throughout the entire experiment. In the *pre-MI* groups, the compound was added either 45, 15, or 5 min

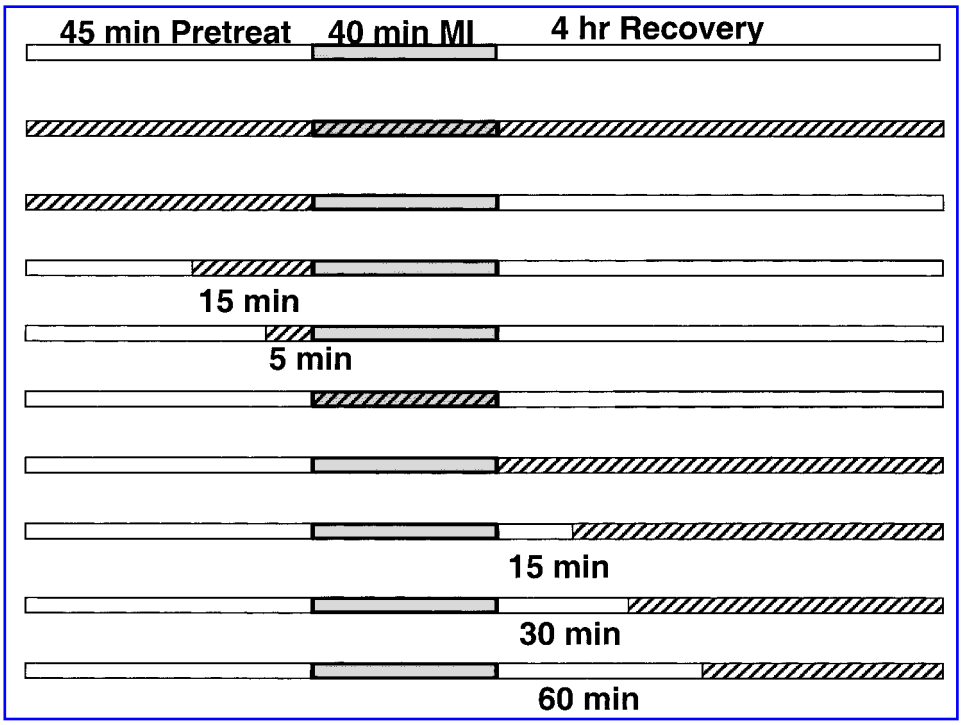

FIG. 1. Treatment conditions for MI and for addition of caspase inhibitors. MI (40 min) is denoted by lightly shaded box. The presence of caspase inhibitors is denoted by hatched lines. Medium changes were accomplished with two washes. Protocols were designed to test nine different time courses of caspase inhibitors in the medium in addition to control (top bar).

prior to MI, and then removed by washing before MI. In *with MI* group, the compounds were added with MI buffer only. In *at recovery* group, the compounds were present only during the recovery period. In 15-, 30-, and 60-min *post-recovery* groups, the compounds were added to the cells after 15, 30, and 60 min of recovery. Each experiment included one culture medium control, and one DMSO control. IDN #1672 (10 μM) was used as a control for the fluoromethyl ketone moiety. In each series, the number of experiments indicates the number of rabbit heart isolations used. During all experiments, cells were stored at 37°C in a 5% CO_2 atmosphere except during buffer changes.

Scoring of viable cells

After either 4-h or overnight recovery, cells were fixed in 4% formalin and placed on microscope slides. Approximately 300–400 cells were counted for determination of viability by the rod-shaped criteria by an observer blinded to conditions. In experiments in which the cells were evaluated by two blinded observers, the interobserver scores differed by <10%. Results were normalized as percent viable relative to control cells.

In situ nick translation assay

Cardiomyocytes were fixed and applied to sides, then stained as described (9) in terminal deoxynucleotidyl transferase reaction buffer containing 200 mM potassium cacodylate, 25 mM Tris, 0.25 mg/ml bovine serum albumin, 2.5 mM $CoCl_2$, 0.05 mM biotin-dUTP, and 0.5 U/μl terminal deoxynucleotidyl transferase, followed by detection with streptavidin-alkaline phosphatase and detection with substrate (SK5400; Vector Laboratories, Burlingame, CA, U.S.A.).

Statistical analysis

Experimental data were expressed as means ± SEM of the percent rod-shaped myocytes relative to DMSO-treated control cells. Differences between the experimental groups were evaluated by analysis of variance.

RESULTS

Activities of IDN compounds against recombinant caspases

Using recombinant caspases and fluorogenic substrates, we assessed relative inhibitory activities of the compounds. These results are shown in Table 1. It can be seen that IDN #1529 is the most potent inhibitor of all three caspases, whereas #1501 and #1965 are relatively more active against caspase-8 than against caspase-1 or caspase-3. The IDN compounds have much weaker *in vitro* activity against cathepsin B and calpain. The caspase inhibitors have roughly similar efficacy in protecting Jurkat cells against Fas-mediated apoptosis and are active at concentrations that are ~10-fold lower than that needed to protect cardiomyocytes against MI and recovery (see below).

Comparison of protective effects of caspase inhibitors

Previous work with the related caspase inhibitor Z-VAD-fmk had shown cytoprotection of cardiomyocytes subjected to MI and recovery at a concentration of 100 μM, with partial protection at 10 μM (12). To determine the range of activity of the caspase inhibitors in the myocyte system, we assessed IDN #1965, IDN #1529, and IDN #1501 with the compounds present for 45 min before MI. As shown in Fig. 2, the protective effect of these compounds was maximal at 10 μM. The concentration of 10 μM was used for subsequent experiments. IDN #1672 used at 10 μM had no protective effect. In contrast, these compounds are much more potent in Jurkat cells induced to undergo apoptosis with Fas ligation, with an IC_{50} of 0.6 μM for IDN #1501 and 0.3 μM for IDN #1965.

Specificity of inhibitors for caspases

Because it was possible that the caspase inhibitors used at these concentrations might inhibit other cysteine proteases, we examined whether calpain inhibitor II might confer protection. Consistent with previous reports (35, 36), we found that calpain inhibitors are protective in our model system (Fig. 3).

Effect of longer recovery time on protection by caspase inhibitors

Because it was possible that caspase inhibition might simply postpone inevitable cell death, we examined myocytes cultured for 4 and 24 h after MI. Caspase inhibitors were included 45 min before MI, during MI, and for the full duration of the recovery period. As shown in Fig. 4, there was no increase in cell death during the interval from 4 to 24 h, indicating that inhibition of caspases did not extend the time of cell death within the 24-h interval tested. Studies by ourselves and others have suggested that caspase activation is rapid following an apoptotic stimulus (1, 9, 31). These considerations raised the possibility that exposure to an irreversible caspase inhibitor for a

Table 1. Relative Activities of Various Protease Inhibitors Against Recombinant or Purified Enzymes *In Vitro* Using Fluorogenic Substrate

	1501	*1529*	*1965*	*1672*
Enzyme assay:				
Caspase-1	0.82	0.06	1.4	>50
Caspase-3	2.4	0.19	0.96	>50
Caspase-8	0.07	0.03	0.06	>50
Cathepsin B	ND	1,600	1860	ND
Calpain I	ND	81	24.3	ND
Calpain II	ND	1,200	11.6	ND
Cell culture:				
Jurkat/anti-Fas	0.64	0.33	0.35	>50

"Jurkat/anti-FAS" refers to the *in vivo* ability to block apoptosis in Jurkat cells treated with anti-Fas antibody. The relative K_i values are shown (in μM). ND, not tested.

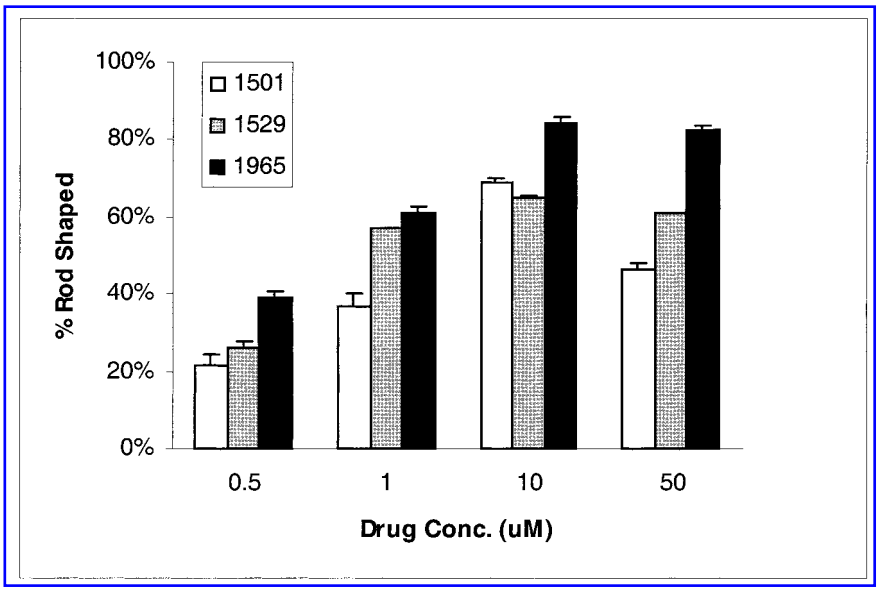

FIG. 2. Dose–response curves for caspase inhibitors. The ability of caspase inhibitors to prevent cell death (*y*-axis) mediated by MI and recovery was tested using the compounds at various concentrations (*x*-axis) during a 45-min preincubation. Error bars represent the standard error of three separate experiments.

limited time might be as effective as prolonged exposure. We tested this by comparing cell viability after MI and recovery with IDN #1965 present for only the first hour after recovery, with IDN #1965 included for the full 4 h of recovery, and with IDN #1965 included for 24 h of recovery. All conditions gave equivalent protection.

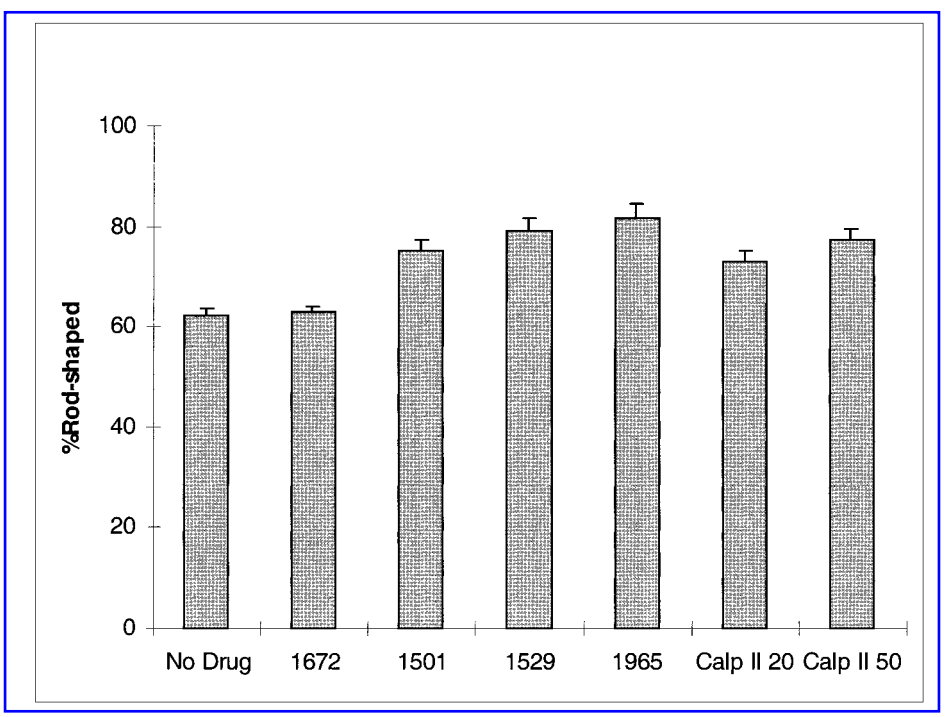

FIG. 3. Effect of a variety of protease inhibitors on loss of viability after MI and recovery. Cardiomyocytes were subjected to MI and 4 h of recovery, and then scored for rod-shaped morphology. Results are normalized to untreated control (100%). IDN compounds 1672, 1501, 1529, and 1965 were used at 10 μM, and calpain inhibitor II (Calp II) was used at 20 and 50 μM. Error bars represent the standard error of three separate experiments.

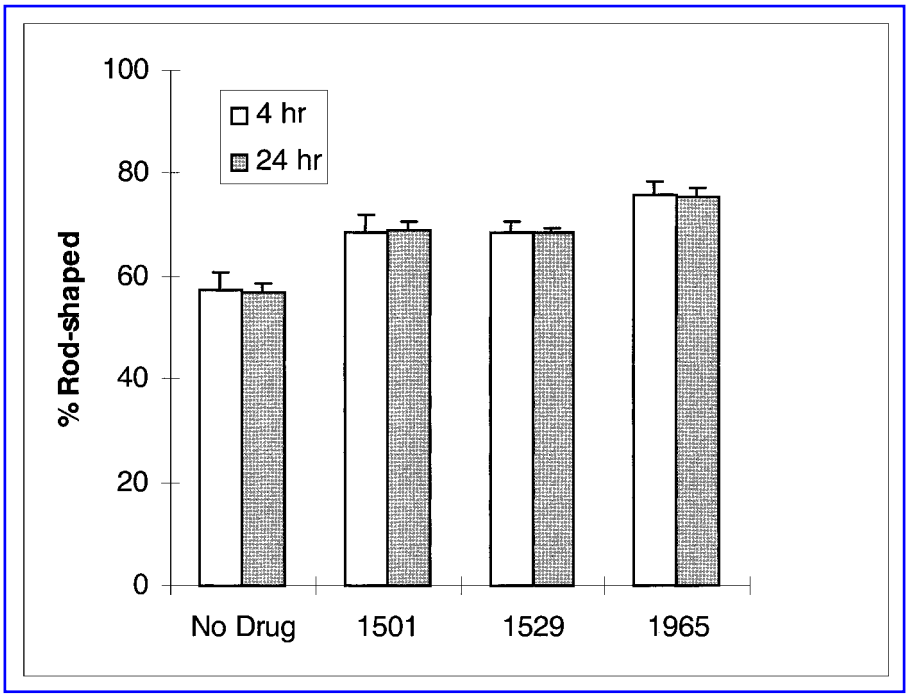

FIG. 4. Measurement of cell viability at 4 and 24 h. Cardiomyocytes were subjected to MI and allowed to recover for 4 or 24 h, and then viability was scored. Results are normalized to untreated control. Error bars represent the standard error of three separate experiments.

Time course

The foregoing results indicated that one or more caspases were activated as a consequence of MI and recovery, and that caspase inhibition could protect myocytes against the morphologic manifestations of this injury. We next evaluated the minimum time of drug exposure needed to confer protection (Fig. 5). We pretreated cells with IDN #1965 and IDN #1501 for 5, 15, or 45 min before MI, and then evaluated viability. We found that 45 min of pretreatment with IDN #1965 or IDN #1501 preserved rod-shaped morphology to a similar extent to that achieved by including the caspase inhibitor in the medium throughout the entire experiment. However, reducing the exposure time to 15 or 5 min before MI resulted in a decrease in protection. However, even the brief (5 min) pretreatment had a statistically significant protective effect compared with no drug ($p < 0.01$). These results suggest that in whole cells, a preincubation time of between 15 and 45 min is needed for maximal protection.

Effect of delayed addition of caspase inhibitors

We used the caspase inhibitors as a probe to learn whether caspases become activated during MI or upon recovery. We reasoned that if caspase activation and substrate cleavage occurred during MI, then adding caspase inhibitors at the time of recovery would not confer protection. If, however, caspase activity were not significant until recovery, then adding caspase inhibitors at the beginning of recovery would still be protective, although the protection might be diminished due to slow uptake of the compound into the cell. We found that adding the peptide inhibitors at the time of recovery was almost as protective as adding it during the period of MI (Fig. 5), indicating that if caspases were activated during MI, the execution phase is sometime later during recovery. To examine the activity during recovery, we tested the effect of adding IDN #1965 at various times after the beginning of recovery. We found that a delay of 15 min was less protective, and the effect was completely lost with a delay of 30 or 60 min.

Detection of caspase activity

To demonstrate that caspase activity can be recovered from control rabbit cardiomyocytes, we prepared cell lysates and assayed them for activity. No spontaneous activity could be de-

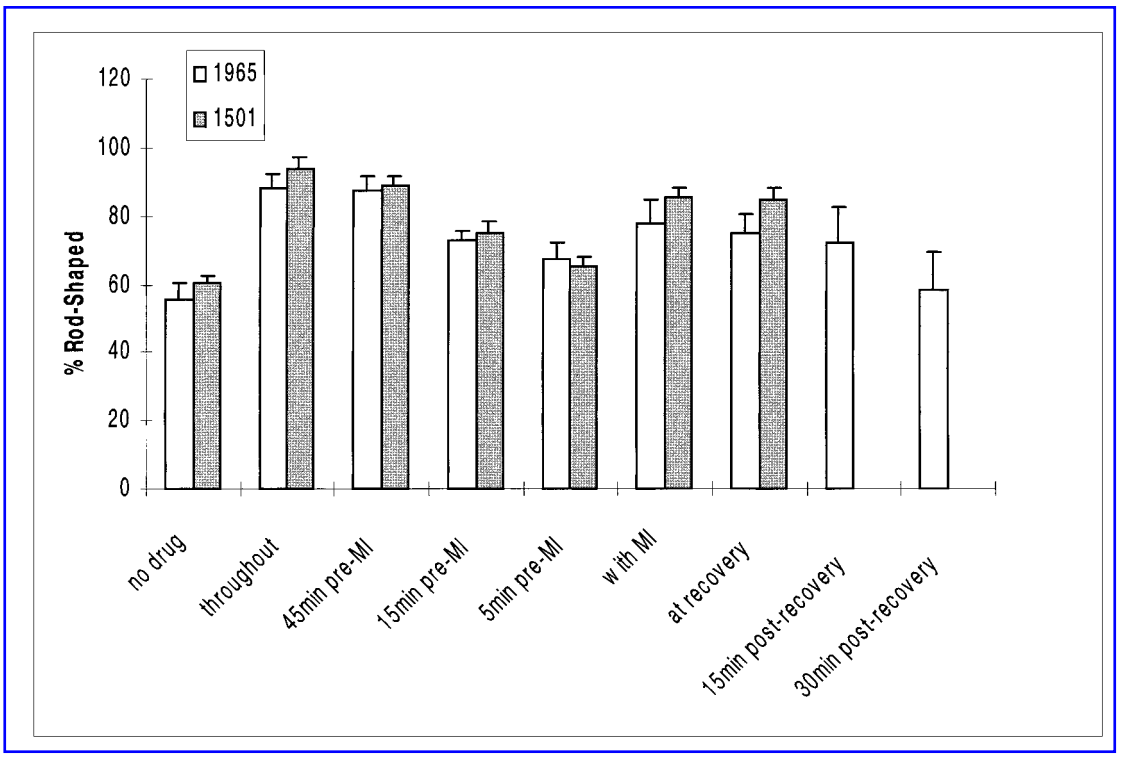

FIG. 5. Efficacy of caspase inhibitors as a function of time of addition. Caspase inhibitors (10 μM) were added at the times indicated in Fig. 1, and the ability of the compounds to prevent cell death was assessed at the end of a 4-h recovery period. Error bars represent the standard error of three independent experiments. For the experiments in which caspase inhibitors were added after recovery, only IDN #1965 was used.

tected in the cell lysates, but the addition of catalytic amounts of caspase-8 and cytochrome *c* in the presence of dATP was sufficient to generate DEVD-pNA cleaving activity. Cardiomyocyte cell extracts representing 300 μg of protein yielded cleaving activity of 0.172 ± 0.006 and 0.275 ± 0.011 milli-optical density units/min in two independent experiments. Lysates from metabolically inhibited cells were not examined.

Nick translation assay of cardiomyocytes subjected to MI and recovery

We used terminal transferase dUTP nick end labeling (TUNEL) to detect cardiomyocytes that had begun to degrade their chromatin after MI and recovery. Consistent with our previous study (12), we found that MI and recovery resulted in TUNEL-positive nuclei in the majority of rounded-up cells; rod-shaped cells were labeled less frequently. Caspase inhibition reduced the number of TUNEL-positive nuclei in rounded-up cells, as well as the total number of rounded cells (27.8% rounded cells with positive nuclei in MI and recovery versus 12.5% in MI and recovery with 10 μM IDN #1965).

DISCUSSION

Our studies indicate that inhibition of caspases protects cardiomyocytes against the morphologic manifestations of cell death that occur with MI and recovery. MI and recovery have been used to simulate ischemia/reperfusion injury. Several key features of ischemia/reperfusion are reproduced in the isolated myocyte/MI model that support its use: (i) ATP depletion; (ii) intracellular ionic alterations, including cytoplasmic acidification, sodium influx, and calcium overload; and (iii) preconditioning. The isolated myocyte system examines cell-autonomous responses to energy depletion, but provides no information as to cell–cell interactions, signaling from extracellular matrix and cytokines, wall tension, and electrical excitation, which would contribute to the over-

all response seen in the intact heart. These studies do not examine other parameters of cardiomyocyte function, such as metabolic activity or contractility. However, we have previously shown that preservation of rod-shaped structure correlates well with mitochondrial function measured by the MIT assay, and with preservation of an intact plasma membrane measured by trypan blue exclusion (12). In these studies, we found that TUNEL positivity correlated closely with loss of rod-shaped morphology. However, because DNA nicking is a late event in the process of cell death, and because the TUNEL assay is a relatively insensitive assay, we used the more informative criteria of preservation of rod-shaped morphology. Both death processes may be activated in the same cell; inhibition of apoptosis is not useful if the cell is going to die by necrosis. Therefore, rather than consider whether cell rounding represents apoptosis or necrosis, we consider that it is more important to assess whether the cell death is preventable.

We show that peptidomimetic fluoromethyl ketone inhibitors of caspases are effective, although 10-fold higher concentrations of drug are needed to protect cardiomyocytes against MI and recovery than to protect Jurkat cells against apoptosis mediated by Fas ligation. This may be due to differences in the relative specificity of these caspase inhibitors for their targets, the magnitude of caspase activation, a difference in drug penetration into the cell, differences in receptor-mediated (Fas) versus mitochondrial injury pathways, or other non-caspase death pathways active after MI and recovery. For example, IDN #1965 has a lower K_i for caspase-8 (Fas pathway) than caspase-3 (both pathways), and thus would be expected to have greater potency for Fas-mediated apoptosis.

It is possible that the caspase inhibitors at these concentrations may be active against other proteases. However, the profiles of activity of the different caspase inhibitors *in vitro* are not consistent with inhibition of calpains as the predominant mechanism by which they confer protection. It was important to test for inhibition of calpain by the caspase inhibitors because inhibition of calpain has previously been shown to protect against ischemic injury (35, 36). We confirmed those findings in this model. Although some investigators have used the operational definition that if caspase inhibition prevented cell death, it must have been apoptotic cell death, this may be an oversimplification. Recent studies have demonstrated "cross-talk" between caspases and calpain, thus blurring the distinction between death pathways mediated by calpain (necrotic) and caspases (apoptotic) (6, 30, 34).

The observation that caspase inhibition is partially effective even if added 15 min after the beginning of recovery suggests that irreversible cellular injury due to caspases does not occur (in the majority of cells) until sometime later. This is consistent with the finding that there are energy-dependent steps in apoptosis both before and after caspase activation (5). In the metabolically inhibited cell, the apoptotic process may be arrested until energy is restored during recovery. To date, animal studies have involved pretreatment with caspase inhibitors before coronary artery occlusion (14, 39). Our studies suggest that caspase inhibitors administered after coronary occlusion at the time of restoration of blood flow (in the setting of acute myocardial infarction) may salvage myocardium. Effective concentrations in the range of 1–10 μM in the myocardium will be required. Furthermore, testing administration of these compounds after coronary occlusion, as will be required in the clinic, is warranted. Caspase inhibition holds promise as a useful clinical intervention in acute myocardial infarction.

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ABBREVIATIONS

DEVD-pNA, Asp-Glu-Val-Asp-*p*-nitroanilide; DMSO, dimethyl sulfoxide; MI, metabolic inhibition; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal transferase dUTP nick end labeling; Z-VAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone.

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