Attenuation of neutrophil-mediated myocardial ischemia-reperfusion injury by a calpain inhibitor

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Calpains are Ca\(^{2+}\)-dependent neutral cysteine proteases that are ubiquitously expressed in all tissues and have a variety of regulatory functions within mammalian cells (1, 30, 32). There are two major isoforms of calpain, calpain I (or \(\mu\)-calpain) and calpain II (or \(m\)-calpain), which require micromolar and millimolar concentrations, respectively, of intracellular calcium for activation (1). Inhibition of calpain activity has been shown to reduce organ injury associated with ischemia-reperfusion (I/R) of the brain (21, 37), liver (12, 13) and heart (11, 22, 33, 34, 39, 40). Furthermore, recent in vivo and in vitro studies have shown that calpain inhibitors attenuate the surface expression of P-selectin on endothelial cells (2) and prevent the nuclear translocation of the transcription factor nuclear factor-\(\kappa\)B (44), which is important for expression of a variety of cell adhesion molecules on the endothelium. However, the role of calpain on leukocyte-endothelium interaction or neutrophil-mediated organ injury is not fully understood.

It has been established that polymorphonuclear leukocytes (PMNs) play a pivotal role in the setting of myocardial I/R injury, contributing to microvascular plugging, cardiac contractile dysfunction, and enhanced cardiomyocyte necrosis (5, 8, 18). On reperfusion, PMNs accumulate in the coronary microvasculature and infiltrate into cardiac tissue (6, 8). These transmigrated PMNs can provoke tissue injury by the release of cytotoxic substances, including reactive oxygen species, chemotactic factors, and proteolytic enzymes (7, 8). PMNs can also contribute to endothelial dysfunction (19). Therefore, it is likely that compounds that inhibit PMN adherence to the vascular endothelium exert cardioprotective effects against PMN-induced I/R injury.

In the present study, we have tested a selective calpain inhibitor Z-Leu-Leu-CHO to determine whether it has beneficial effects on neutrophil-mediated I/R injury using isolated perfused rat hearts. Z-Leu-Leu-CHO is over 100-fold more selective for calpain than for proteosomal enzymes (IC\(_{50}\) = 1.2 \(\mu\)M) (36). In this experimental model, postschismic cardiac contractile dysfunction is primarily induced by perfusion of PMNs. We assessed the effects of this calpain inhibitor on cardiac contractile function and PMN adhesion and infiltration in the postreperfused hearts. We also related these effects of Z-Leu-Leu-CHO to endothelial P-selectin expression in coronary microvessels.

METHODS

Isolation of PMNs and plasma. Sprague-Dawley rats (350–400 g) were used as neutrophil donors, anesthetized with ethyl ether, and given a 14-ml intraperitoneal injection of 0.5% oyster glycogen (Sigma) dissolved in phosphate-buffered saline (PBS). Sixteen to eighteen hours later, the rats were anesthetized with ethyl ether, and the neutrophils were harvested by peritoneal lavage in 30 ml of 0.9% NaCl as previously described (43). The peritoneal lavage fluid was
centrifuged at 250 g for 20 min at 4°C. The PMNs were then washed in 15 ml of PBS and centrifuged at 250 g for 10 min at 4°C. Thereafter, the PMNs were resuspended in 2.5 ml of PBS and 10–12 rat samples were pooled before use. The neutrophil preparations were >90% pure and >95% viable according to microscopic analysis and exclusion of 0.3% trypan blue, respectively.

Plasma was isolated from a single rat in each cardiac perfusion experiment to infuse along with the PMNs to more closely simulate the conditions present in vivo. Blood was collected from the aorta in citrate phosphate buffer just before the heart was isolated (see Isolated rat heart preparation). The blood was centrifuged immediately in a refrigerated centrifuge at 10,000 g for 10 min. The plasma was decanted and later infused at reperfusion in the I/R hearts.

Isolated rat heart preparation. Rats were anesthetized with 60 mg/kg ip pentobarbital sodium. Heparin sodium (1,000 units) was also administered intraperitoneally. After plasma isolation, the hearts were rapidly excised. The ascending aorta was cannulated, and retrograde perfusion of the heart was initiated with a modified Krebs bicarbonate buffer maintained at 37°C and at a constant pressure of 80 mmHg. The Krebs bicarbonate buffer had the following composition (in mM/l): 120 NaCl, 25 NaHCO3, 2.5 CaCl2, 0.5 EDTA, 5.9 KCl, 1.2 MgCl2, and 17 glucose. The perfusate was aerated with 95% O2-5% CO2 and equilibrated at a pH of 7.3–7.4. Two side arms in the perfusion line proximal to the heart inflow cannula allowed for infusion of PMNs and plasma directly into the coronary inflow line. Coronary flow was monitored by a transit time flowmeter (model TI06, Transonic Systems). Left ventricular (LV) developed pressure (LVDP) and the maximal rate of development of LVDP (+dP/dt max) were monitored using a pressure transducer (model SPR-524, 2.5 Fr, Millar Instruments) that was positioned in the LV cavity. LVDP was defined as LV end-systolic pressure minus LV end-diastolic pressure. Coronary flow, LV pressure, and +dP/dt max were recorded using a MacLab data-acquisition system (ADI Diagnostics) in conjunction with a Power Macintosh 7600 computer (Apple Computers).

Perfused heart experimental protocol. LVDP, +dP/dt max, and coronary flow were measured every 5 min for 15 min to obtain a baseline measurement. After 15 min of equilibration, the flow of Krebs bicarbonate buffer was reduced to zero to induce global ischemia. This total global ischemia was maintained for 20 min. Coronary flow was then reestablished by returning coronary perfusion pressure to 80 mmHg. At reperfusion, hearts were infused for 5 min with 200 × 106 PMNs resuspended in 5 ml of Krebs bicarbonate buffer along with 5 ml plasma at a rate of 1 ml/min. In some experiments, the selective calpain inhibitor Z-Leu-Leu-CHO (BioMol Labs, MW = 362.5) was added to plasma at a final concentration of 10 and 20 μM. Sham I/R hearts were not perfused with PMNs and plasma. Previous studies (16) showed that sham I/R hearts given PMNs exhibited no changes from initial control values. The hearts were allowed to reperfuse for a total of 45 min, during which time cardiodynamic data were recorded every 5 min for the first 30 min and at the 45-min time point. After each experiment, hearts were rinsed in Krebs bicarbonate buffer, placed in 4% paraformaldehyde, and then stored at 4°C for later histologic analysis as previously described (43). The number of infiltrated PMNs was determined by light microscopy. Moreover, we also counted the intravascular PMNs that adhered to the vascular endothelium in cardiac tissue to determine the effect of Z-Leu-Leu-CHO on PMN adherence to coronary vascular endothelium. These results are expressed as intravascular and infiltrated PMNs per millimeter squared area of cardiac tissue.

Immunohistochemistry. Immunohistochemical localization of P-selectin was investigated by using the avidin-biotin immunoperoxidase technique (Vectastain ABC reagent, Vector Laboratories) according to a previously described method (38). Tissue sections, which were prepared as mentioned above, were treated with 0.25% trypsin (Sigma) to improve reagent penetration. Blocking serum (horse) was applied to the tissue for 30 min to reduce nonspecific binding, and the tissue sections were then incubated for 24 h with specific primary antibodies. In particular, P-selectin was detected with the monoclonal antibody PB1.3 at a dilution of 1:100. PB1.3 is a monoclonal antibody that recognizes only P-selectin, which is expressed on the endothelial cell surface and does not bind to intracellular P-selectin (38). The tissue was then incubated with the biotinylated secondary antibody, and peroxidase staining was carried out using 3,3′-diaminobenzidine. Expression of adhesion molecules was determined by microscopic observation of the brown peroxidase reaction product on the coronary microvascular endothelium of the tissue sections. Positive staining was defined as a vessel displaying brown reaction product on >50% of the circumference of its endothelium. The percent of microvessels staining positively was then calculated.

Measurement of superoxide release from rat PMNs. We examined the effect of Z-Leu-Leu-CHO (10 and 20 μM) on phorbol 12-myristrate 13-acetate (PMA; 15 nM)-stimulated rat PMNs (5 × 106 cells). The superoxide anion release by PMNs was measured spectrophotometrically by the reduction of ferricytochrome C (100 μM, Sigma) as previously described (42).

Statistical analysis. All data in the text and figures are presented as means ± SE. All data were subjected to ANOVA with the use of post hoc analysis with the Bonferroni-Dunn test. Probability values <0.05 were considered to be statistically significant.

RESULTS

To determine whether the calpain inhibitor exerts direct effects on cardiac contractile function, we perfused nonischemic control rat hearts (i.e., sham I/R hearts) without PMNs for 80 min under normal flow conditions. Figure 1 illustrates the time course of LVDP changes in the five key groups of this study. Perfusion of sham I/R hearts in Krebs bicarbonate buffer with Z-Leu-Leu-CHO did not result in any sustained change in LVDP over the entire 80-min observation period, indicating that Z-Leu-Leu-CHO did not exert any direct effect on cardiac contractile function. Moreover, perfusion of untreated I/R hearts in the absence of PMNs did not result in any sustained alteration in any of the final cardiac function variables measured (Figs. 2 and 3), indicating that global ischemia in the absence of PMNs does not provoke severe prolonged cardiac dysfunction in this model of I/R. Ischemia for 20 min, followed by reperfusion, produced a transient cardiac dysfunction, such that LVDP was depressed by 31 ± 6% 15 min after reperfusion (Fig. 1). This transient cardiac dysfunction recovered to 93 ± 4% of initial control value by 45 min postreperfusion (Fig. 1 and 2).

I/R rat hearts perfused with PMNs experienced a more severe and sustained reduction in cardiac con-
CHO (20 and 3). In contrast, I/R hearts treated with Z-Leu-Leu- 
creased only 16 45 min postreperfusion; Figs. 2 and 3). LVDP de-
calpain inhibitor (10 I/R PMN). All values are expressed as means 
cant contractile dysfunction, which was attenuated by the calpain inhibitor. All values are expressed as means ± SE. **P < 0.01 from final +dP/dt max of I/R+PMNs.

The marked deficit in postreperfusion cardiac performance can be largely attributed to the presence of PMNs soon after reperfusion because the same I/R protocol in the absence of PMNs produced only small alterations in cardiodynamics at 45-min postreperfusion. PMN adhesion and infiltration in I/R hearts occurred to a significantly greater degree in hearts perfused with PMNs than in any other group (Fig. 4, A and B). However, the total numbers of adhered and infiltrated PMNs in postreperfused Z-Leu-Leu-CHO-treated hearts (10, 20 μM) were 63–70% less than that observed in nontreated I/R hearts (P < 0.01, Fig. 4A). Furthermore, 61–68% fewer PMNs adhered to the vascular endothelium in Z-Leu-Leu-CHO-treated hearts (10 and 20 μM) than those observed in nontreated I/R hearts (P < 0.01, Fig. 4B). These antiadherence effects in hearts perfused with the calpain inhibitor are a major factor contributing to the attenuated infiltration of PMNs into the reperfused myocardium.

ttractile function, because LVDP was depressed to 52 ± 6% and +dP/dt max by 42 ± 7% of the initial control value at 45 min postreperfusion (P < 0.01, see Figs. 2 and 3). In contrast, I/R hearts treated with Z-Leu-Leu-CHO (20 μM) exhibited a significant attenuation of cardiac contractile dysfunction in the presence of PMNs (i.e., more normalized LVDP and +dP/dt max at 45 min postreperfusion; Figs. 2 and 3). LVDP decreased only 16 ± 5% and +dP/dt max decreased only 17 ± 6% in Z-Leu-Leu-CHO-treated hearts at 45 min postreperfusion (Figs. 2 and 3).

Fig. 2. Initial and final LVDP (in mmHg) in isolated perfused rat hearts before ischemia and after reperfusion. Hearts were perfused in the presence or absence of PMNs. PMNs induced a significantly greater degree in hearts perfused with PMNs than in any other group (Fig. 4, A and B). However, the total numbers of adhered and infiltrated PMNs in postreperfused Z-Leu-Leu-CHO-treated hearts (10, 20 μM) were 63–70% less than that observed in nontreated I/R hearts (P < 0.01, Fig. 4A). Furthermore, 61–68% fewer PMNs adhered to the vascular endothelium in Z-Leu-Leu-CHO-treated hearts (10 and 20 μM) than those observed in nontreated I/R hearts (P < 0.01, Fig. 4B). These antiadherence effects in hearts perfused with the calpain inhibitor are a major factor contributing to the attenuated infiltration of PMNs into the reperfused myocardium.

To determine whether the endothelium of I/R hearts perfused with Z-Leu-Leu-CHO exhibited any changes in adhesion molecule expression that could account for the low degree of PMN involvement, we performed immunohistochemical analysis of P-selectin expression on the rat coronary microvascular endothelium. Figure 5 summarizes these results. I/R hearts that were reperfused with PMNs and not treated with the calpain inhibitor exhibited a relatively high basal P-selectin expression on the coronary microvascular endothelium. However, Z-Leu-Leu-CHO-treated hearts (10 and 20 μM) exhibited a 41–60% decrease in P-selectin expression. The significant extent of this effect is such that it likely acts as a major mechanism for the cardioprotection exerted by inhibition of calpain.

Z-Leu-Leu-CHO-treated rat PMNs (10 and 20 μM, n = 3) modestly inhibited superoxide release in PMNs stimulated with PMA by 16 ± 9% (10 μM) and 20 ± 9%
compared with control PMNs treated with PMNs but that were perfused without the calpain inhibitor. These results indicate a significant attenuation of cardiac contractile dysfunction by the calpain inhibitor. It is unlikely that this calpain inhibitor directly stimulated cardiodynamics (i.e., exerted a coronary vasodilation or a positive inotropic effect) because there were no significant changes in LVDP or +dP/dt max in nonischemic hearts perfused with Z-Leu-Leu-CHO. The cardioprotective effects of the calpain inhibitor are most likely due to a significantly reduced adherence of PMNs to the coronary endothelium, thereby resulting in fewer PMNs infiltrating into post-reperfused cardiac tissue and therefore less release of cytotoxic mediators by these transmigrated neutrophils.

DISCUSSION

The major purpose of this study was to determine whether a selective calpain inhibitor exerts a cardioprotective effect against PMN-mediated I/R injury in the isolated perfused rat heart. It is well accepted that myocardial ischemia followed by reperfusion results in PMN-mediated cardiac contractile dysfunction (16, 43). In the present study, the cardioprotective effect of the calpain inhibitor was characterized by a significant preservation of postreperfusion LVDP and +dP/dt max compared with those of hearts subjected to I/R and PMNs but that were perfused without the calpain inhibitor. The modest inhibition of superoxide release of Z-Leu-Leu-CHO observed at these concentrations was not significantly different from control PMNs and is most likely not a major mechanism to explain the cardioprotective effects of Z-Leu-Leu-CHO.

Nitric oxide (NO) is known to attenuate PMN adherence to the vascular endothelium by suppressing up-regulation of endothelial cell adhesion molecules (3, 4) resulting in a reduction in PMN infiltration into the tissue (17). However, basal release of NO from the coronary endothelium is diminished very early after myocardial I/R (20). It has been shown that the reduction of endothelium-derived NO further facilitates PMN adherence to the vascular endothelium (20) via upregulation of P-selectin expression (3). P-selectin is an important cell adhesion molecule for PMN adherence to the endothelium and is expressed on the surface of the endothelium (24). There is abundant evidence to show the role of the selectins, particularly P-selectin, in myocardial I/R injury (9, 15, 25). P-selectin is necessary for leukocyte rolling a prerequisite for PMN adhesion to vascular endothelium under physiological conditions. It has been shown that P-selectin can be rapidly upregulated (i.e., maximally at 10–20 min) and translocated to the endothelial surface after ischemia.

(20 μM) compared with control PMNs not treated with Z-Leu-Leu-CHO, whereas superoxide dismutase (4 μg/ml), used as a positive control, inhibited superoxide release from PMA-stimulated rat PMNs by 93 ± 7%. The modest inhibition of superoxide release of Z-Leu-Leu-CHO observed at these concentrations was not significantly different from control PMNs and is most likely not a major mechanism to explain the cardioprotective effects of Z-Leu-Leu-CHO.

Fig. 4. Histologic assessment of total intravascular and infiltrated PMNs (A) and intravascular adhered PMNs (B) to the coronary vascular endothelium in isolated perfused rat hearts taken from 3 rats per group. Ten areas were analyzed per heart. All values are the means of PMNs/mm² ± SE. The calpain inhibitor significantly attenuated the number of PMNs (A) infiltrated into postreperfusion cardiac tissue (B) as well as PMNs adhered intravascularly.

Fig. 5. Immunohistochemical analysis of P-selectin expression in rat hearts subjected to I/R. Ischemic hearts were reperfused in the presence or absence of PMNs. The presence of P-selectin-positive staining was then analyzed. All values are expressed as mean percentage values ± SE. Numbers inside bars represent number of fields counted in each of 3 hearts per group.
the onset of reperfusion (38). Thus it is likely that P-selectin played a pivotal role in the I/R injury observed in the present study. Our immunohistochemistry results clearly showed that P-selectin expression on the vascular endothelial surface is markedly attenuated in hearts perfused with the calpain inhibitor compared with the endothelium of hearts perfused in the absence of the inhibitor. Therefore, the cardioprotective effect of Z-Leu-Leu-CHO appears to be related to inhibition of P-selectin expression on the vascular endothelial surface, resulting in fewer PMNs infiltrating the postreperfused cardiac tissue. Although the calpain inhibitor modestly attenuated superoxide formation, this effect could not explain the marked cardioprotective effects observed in this study. However, others (27, 28) have reported that calpain is known to be involved in neutrophil activation. Thus it is possible that the calpain inhibitor could have inhibited neutrophil activation and thus attenuated the neutrophil-mediated cardiac dysfunction observed in the present study. This effect may also be independent of P-selectin inhibition.

Because calpain activity is calcium dependent, it has been demonstrated that calpains play a harmful role in several pathological states associated with a Ca\(^{2+}\) overload, such as myocardial I/R (22). Increased calpain activity has been reported in the setting of myocardial ischemia (hypoxia) and/or reperfusion (10, 14, 23, 31, 33, 40, 41) and seems to have a critical role in breakdown of myocardial proteins resulting in tissue damage (26, 35). Furthermore, the cardioprotective effects of calpain inhibitors have been reported after ischemia (11, 22, 33, 34, 39, 40). However, the cardioprotective effects of a calpain inhibitor shown in the present study are significantly different from those of other studies because the postischemic cardiac dysfunction is induced by perfusion of PMNs. In this study, we employed a model of isolated perfused rat hearts subjected to I/R in the presence of PMNs. We clearly demonstrated that cardiac contractile dysfunction was not caused by ischemia alone, but rather PMNs exert major deleterious effects in the setting of I/R. Using this experimental model, we have previously shown the cardioprotective effects of other substances that inhibit expression of P-selectin on the vascular endothelium (16) also inhibit leukocyte-endothelium interaction (29). These reports strongly suggest that the cardiac contractile dysfunction observed in this model of I/R is mediated by leukocyte-endothelium interaction. Moreover, these findings indicate that this model is suitable for analyzing potentially beneficial compounds that may inhibit expression of P-selectin, such as Z-Leu-Leu-CHO in the present investigation.

In summary, we have demonstrated the cardioprotective effects of a selective calpain inhibitor. This inhibitor significantly attenuated neutrophil-induced cardiac contractile dysfunction in isolated perfused I/R rat hearts compared with similarly perfused hearts in the absence of the inhibitor. These cardioprotective effects appear to be closely related to inhibition of PMN adherence to the vascular endothelium, resulting in fewer PMNs infiltrating the cardiac tissue. The calpain inhibitor also appears to inhibit expression of P-selectin on the vascular endothelium, which significantly contributes to these effects via downregulation of leukocyte-endothelium interaction.

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REFERENCES

CALPAIN INHIBITOR IN ISCHEMIA-REPERFUSION


