Caveolin-1 peptide exerts cardioprotective effects in myocardial ischemia-reperfusion via nitric oxide mechanism

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Young, Lindon H., Yasuhiro Ikeda, and Allan M. Lefer. Caveolin-1 peptide exerts cardioprotective effects in myocardial ischemia-reperfusion via nitric oxide mechanism. Am J Physiol Heart Circ Physiol 280: H2489–H2495, 2001.—Caveolin-1 is a protein constituent of cell membranes. The caveolin-1 scaffolding region (residues 82–101) is a known inhibitor of protein kinase C. Inhibition of protein kinase C results in maintained nitric oxide (NO) release from the endothelium, which attenuates cardiac dysfunction after ischemia-reperfusion (I/R). Therefore, we hypothesized that the caveolin-1 scaffolding region of the molecule, termed caveolin-1 peptide, might attenuate postsischemic polymorphonuclear neutrophil (PMN)-induced cardiac dysfunction. We examined the effects of caveolin-1 peptide in isolated ischemic (20 min) and reperfused (45 min) rat hearts reperfused with PMNs. Caveolin-1 peptide (165 or 330 μg) given intravenously 1 h before I/R significantly attenuated postsischemic PMN-induced cardiac dysfunction, as exemplified by left ventricular developed pressure (LVDP) (P < 0.01) and the maximal rate of developed pressure (+dP/dt max) (P < 0.01), compared with I/R hearts obtained from rats given 0.9% NaCl. In addition, caveolin-1 peptide significantly reduced cardiac PMN infiltration from 195 ± 5 PMNs/mm² in untreated hearts to 103 ± 5 and 60 ± 5 PMNs/mm² in hearts from 165 and 330 μg caveolin-1 peptide-treated rats, respectively (P < 0.01). PMN adherence to the rat coronary vasculature was also significantly reduced in rats given either 165 or 330 μg caveolin-1 peptide compared with rats given 0.9% NaCl (P < 0.01). Moreover, caveolin-1 peptide-treated rat aortas exhibited a 2.2-fold greater basal release of NO than vehicle-treated aortas (P < 0.01), and this was inhibited by Nω-nitro-L-arginine methyl ester. These results provide evidence that caveolin-1 peptide significantly attenuated PMN-induced post-I/R cardiac contractile dysfunction in the isolated perfused rat heart, probably via enhanced release of endothelium-derived NO.

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proteins (3, 13, 19, 26). Inhibition of PKC in endothelial cells results in inhibition of superoxide release (15, 33). In addition, PKC also inhibits eNOS, thereby attenuating NO release from endothelial cells (8). Therefore, substances that inhibit PKC, such as caveolin-1, tend to inhibit superoxide generation and augment NO release from endothelial cells. Enhanced NO release coupled with inhibition of superoxide release from the endothelium attenuate endothelial dysfunction and hence retard PMN infiltration into cardiac tissue, thereby preventing PMN-induced cardiac contractile dysfunction after I/R (10, 12, 20).

The major purpose of the present study was to examine the effect of systemic administration of caveolin-1 peptide on cardiac contractile function in the isolated perfused rat heart after PMN-induced I/R injury and to ascertain the mechanisms of any such effect. To this end, we assessed 1) the number of PMNs adhering to the coronary vasculature and infiltrating into the cardiac tissue and 2) the NO release from the rat aortic endothelium. These studies thus enabled us to determine that caveolin-1 peptide is able to modulate endothelial NO release and PMN adherence to the endothelium in a manner that could significantly account for its cardioprotective effect.

METHODS

Isolated Rat Heart Preparation

Male Sprague-Dawley rats (275–325 g) were anesthetized with 60 mg/kg pentobarbital sodium injected intraperitoneally. Sodium heparin (1,000 units) was also administered intraperitoneally. Hearts were rapidly excised, the ascending aortas were cannulated, and retrograde perfusion of the heart was initiated with a modified Krebs-Henseleit (K-H) buffer maintained at 37°C at a constant pressure of 80 mmHg. The K-H buffer had the following composition (in mmol/l): 17 dextrose, 120 NaCl, 25 NaHCO3, 2.5 CaCl2, 0.5 EDTA, 5.9 KCl, and 1.2 MgCl2. 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 flowmeter (model T106, Transonic Systems). Left ventricular developed pressure (LVDP) and the maximal rate of developed pressure (dP/dt max) were monitored using a MacLab data acquisition system (AD Instruments) in conjunction with a Power Macintosh 7600 computer (Apple Computers). LVDP, dP/dt max, and coronary flow were measured every 5 min for 15 min to equilibrate the hearts and obtain a baseline measurement. LVDP was defined as left ventricular end-systolic pressure minus left ventricular end-diastolic pressure. After 15 min, the flow of K-H buffer was reduced to zero to induce global ischemia for 20 min. After ischemia, hearts were perfused for 5 min with 200 x 10^6 PMNs resuspended in 5 ml of K-H buffer plus 5 ml of plasma at a rate of 1 ml/min.

Groups of Isolated Perfused Hearts

The following groups of isolated perfused rat hearts were used.

Group 1: sham I/R. Hearts not subjected to ischemia and not perfused with PMNs (n = 6).

Group 2: sham I/R + caveolin-1 peptide. Hearts treated with caveolin-1 peptide (330 μg) not subjected to ischemia and not perfused with PMNs (n = 6).

Group 3: I/R. Hearts subjected to ischemia but reperfused without PMNs (n = 6).

Group 4: I/R + caveolin-1 peptide. Hearts treated with caveolin-1 peptide (330 μg) and subjected to ischemia but reperfused without PMNs (n = 6).

Group 5: I/R + PMNs. Hearts subjected to ischemia and reperfused with PMNs (n = 7).

Group 6: I/R + PMNs + caveolin-1 peptide (165 μg). Hearts treated with caveolin-1 peptide (165 μg) and subjected to ischemia and reperfused with PMNs (n = 7).

Group 7: I/R + PMNs + caveolin-1 peptide (330 μg). Hearts treated with caveolin-1 peptide (330 μg) and subjected to ischemia and reperfused with PMNs (n = 6).

Group 8: I/R + PMNs + caveolin-1 peptide (330 μg) + L-NAME. Hearts treated with caveolin-1 peptide (330 μg) and subjected to ischemia and reperfused with PMNs and Nω-nitro-L-arginine methyl ester (L-NAME; 50 μM) (n = 5).

Previous studies have shown that sham I/R hearts given PMNs exhibited no changes from initial control values (11). Data were recorded every 5 min for the first 30 min after reperfusion and at the 45-min time point. Rats were anesthetized with ethyl ether and then given either caveolin-1 peptide (165 μg, approximating 2.5 μM in the blood, or 330 μg, approximating 5 μM in the blood) or 0.9% NaCl intravenously 1 h before the experiments. Caveolin-1 peptide (molecular weight = 2,518; amino acid residues 82–101, Genemed Synthesis) was prepared in 0.9% NaCl, pipetted into 0.5-ml aliquots, and stored at −20°C. Aliquots were thawed once just before intravenous injection. In an additional series, 50 μM L-NAME (Sigma), a NO synthase inhibitor, was added to the perfusate at the start of reperfusion of five I/R hearts perfused with PMNs given caveolin-1 peptide (330 μg). After each experiment (i.e., at 45 min of reperfusion or the equivalent time in control hearts), hearts were placed in 4% paraformaldehyde and stored at 4°C for later histological analysis.

Isolation of Plasma

Blood was collected from the aorta in citrate phosphate buffer (Sigma) over a period of 1 min just before isolation of the rat heart. The blood was centrifuged at 10,000 g for 10 min. Thereafter, the plasma was decanted and used for infusion to I/R hearts. Five milliliters of plasma collected from a single rat were used for each perfused heart.

Isolation of PMNs

Sprague-Dawley rats (350–400 g) were anesthetized with ethyl ether and given glycogen intraperitoneally (14 ml of 0.5% glycogen dissolved in PBS). Sixteen to eighteen hours later, the rats were anesthetized with ethyl ether and then given either caveolin-1 peptide (165 μg, approximating 2.5 μM in the blood, or 330 μg, approximating 5 μM in the blood) or 0.9% NaCl intravenously 1 h before the experiments. Caveolin-1 peptide (molecular weight = 2,518; amino acid residues 82–101, Genemed Synthesis) was prepared in 0.9% NaCl, pipetted into 0.5-ml aliquots, and stored at −20°C. Aliquots were thawed once just before intravenous injection. In an additional series, 50 μM L-NAME (Sigma), a NO synthase inhibitor, was added to the perfusate at the start of reperfusion of five I/R hearts perfused with PMNs given caveolin-1 peptide (330 μg). After each experiment (i.e., at 45 min of reperfusion or the equivalent time in control hearts), hearts were placed in 4% paraformaldehyde and stored at 4°C for later histological analysis.
Determination of PMN Infiltration of Cardiac Tissue

Three rat hearts from each of the eight experimental groups were used for histological analysis. Ten areas of each rat heart were counted for PMN infiltration. Hearts were dehydrated in graded ice-cold acetone washes (50–100%). The heart tissue was then embedded in plastic and sectioned into 4-μm serial sections and placed onto glass slides. Sections were then placed in 100% ethanol for 5 min to remove the plastic and rehydrated in tap water for 1 min. Thereafter, hematoxylin was applied to the sections for 7 min, and the sections were rinsed in running tap water for 30 s. Eosin stain was then applied to the sections for 2 min, followed by a second running tap water rinse for 30 s. The number of infiltrated PMNs was counted by light microscopy. We also counted intravascular PMNs that adhered to the vascular endothelium in cardiac tissue to determine the effect of caveolin-1 on PMN adherence to the coronary vascular endothelium. These results are expressed as intravascular and infiltrated PMNs per millimeter square area of cardiac tissue.

Measurement of NO Release from Rat Aortic Segments

Rats were given either 330 μg of caveolin-1 peptide intravenously in the systemic circulation or an equal volume of 0.9% NaCl, as described above. One hour later, the aortas were isolated after pentobarbital sodium anesthesia, and the excised aortas were immersed in warm oxygenated K-H...
solution. Aortas were cleaned of adherent fat and connective tissue, and rings 6–7 mm in length (i.e., ~5–7 mg of tissue) were carefully prepared. Aortic rings were cut, opened, and fixed by small pins, with the endothelial surface facing up, in 24-well culture dishes containing 1 ml of K-H solution. After equilibration at 37°C, NO released into the K-H solution was measured. NO was measured according to the method of Guo et al. (7) using a calibrated NO meter (Iso-NO; World Precision Instruments; Sarasota, FL) connected to a polarographic internally shielded NO electrode. NO released into the medium was reported as picomoles per milligram of aortic tissue. After the basal NO release was determined, 200 µM L-NAME was added to the K-H bathing solution, and basal NO release was reassessed 30 min later.

Statistical Analysis

All data are presented as means ± SE. The data for LVDP and +dP/dt max were analyzed by ANOVA using post hoc analysis with the Scheffé's test. Probability values of <0.05 were considered to be statistically significant.

RESULTS

To determine whether systemic administration of caveolin-1 peptide exerted direct effects on cardiac contractile function in acute myocardial I/R, we perfused nonischemic control rat hearts isolated from rats that received 330 µg caveolin-1 peptide 1 h before constant

Fig. 3. A: histological assessment of total intravascular and infiltrated PMNs in isolated perfused rat heart samples taken from 3 rats per group and 10 areas per heart. B: histological assessment of intravascular PMNs that adhered to the coronary vasculature in isolated perfused rat heart samples taken from 3 rats per group and 10 areas per heart. All values are mean numbers of PMNs per millimeter squared of heart area ± SE. Both the number of PMNs that adhered to the coronary vasculature and infiltrated into postreperfusion cardiac tissue were significantly attenuated by caveolin-1 peptide, but these effects were overridden by L-NAME.
pressure perfusion for 80 min at 80 mmHg. Perfusion of caveolin-1 peptide-treated sham I/R hearts without PMNs did not result in any significant change in LVDP (Fig. 1) or +dP/dt_max (Fig. 2) over the entire 80-min observation period, indicating that caveolin-1 peptide did not exert any direct effect on cardiac contractile function. Moreover, perfusion of untreated I/R hearts without PMNs did not result in any significant sustained attenuation in any of the cardiac function variables measured at the end of the observation period, indicating that global ischemia did not provoke a sustained cardiac dysfunction in this model of I/R. LVDP did decrease to 50% of initial control at 15 min after the onset of reperfusion but by 45 min after the onset of reperfusion had recovered to 88 ± 6% of initial control in I/R hearts not given PMNs at reperfusion.

However, I/R rat hearts perfused with PMNs experienced a marked and sustained reduction in cardiac contractile function compared with the first six groups. A decrease in LVDP and +dP/dt_max of >50% was observed at 45 min after the onset of reperfusion. In contrast, I/R rat hearts that received caveolin-1 peptide and were reperfused with PMNs exhibited a significant attenuation of cardiac contractile dysfunction (i.e., markedly higher LVDP and +dP/dt_max, Figs. 1 and 2). The 330-μg caveolin-1 peptide-treated group exhibited a significant improvement in final LVDP and +dP/dt_max compared with the I/R + PMN group (P < 0.01; Figs. 1 and 2). The 165-μg caveolin-1 peptide-treated group also exhibited a significant but less dramatic improvement in final LVDP compared with the I/R + PMN group (Figs. 1 and 2). However, the cardioprotective effects of caveolin-1 peptide (330 μg) were significantly blocked by L-NAME after the onset of reperfusion (Figs. 1 and 2).

The significant deficit in cardiac contractile performance in I/R hearts we observed is closely correlated to the presence of infiltrated PMNs after ischemia. We observed a 47 ± 5 and 69 ± 8% attenuation of PMN infiltration into postreperfused cardiac tissue in the 165- and 330-μg caveolin-1 peptide-treated groups compared with 0.9% NaCl-treated I/R + PMN rats (P < 0.01) (Fig. 3A). A significant reduction in PMN adherence (46 ± 7 and 70 ± 10%) was also observed in the coronary vascular endothelium from 165- and 330-μg caveolin-1 peptide-treated rat hearts, respectively, compared with those isolated from untreated rats (P < 0.01) (Fig. 3B). The significant attenuation of PMN infiltration into cardiac tissue and adherence to the coronary vascular endothelium in caveolin-1 peptide-treated hearts was significantly blocked by L-NAME (Fig. 3, A and B).

Basal release of NO from caveolin-1 peptide-treated rat aortic segments was significantly increased compared with control rat aortic segments (P < 0.01) (Fig. 4). Basal NO release was enhanced by more than twofold in caveolin-1-treated aortic segments compared with control aortic segments (26 ± 1 vs. 12 ± 1 pmol NO/mg aortic tissue) (Fig. 4). The addition of L-NAME totally inhibited NO release in these rat aortic segments. These findings point toward a significant

NO-enhancing effect of caveolin-1 peptide on rat endothelium.

**DISCUSSION**

The present study demonstrates that the caveolin-1 peptide exerts significant cardioprotective effects against PMN-mediated reperfusion injury in the isolated perfused rat heart. These cardioprotective effects of the caveolin-1 peptide were characterized by a significant restoration of LVDP and +dP/dt_max compared with PMN perfused I/R hearts obtained from rats receiving only 0.9% NaCl as a vehicle. The cardioprotective effects of the caveolin-1 peptide are most likely due to significantly reduced adherence of PMNs to the vascular endothelium, thereby resulting in a significant reduction in PMN infiltration into postischemic cardiac tissue (10, 12). Regarding the accessibility of the caveolin-1 peptide we employed, we utilized a 20 amino acid portion of the caveolin-1 protein (molecular weight = 2,518; residues 82–101). This small peptide can be better understood to inhibit PKC in intact cells, because it inhibits PKC activity in cell membrane caveolae (19). A significant fraction of PKC-α is known to colocalize with caveolin-1 within the caveolae of plasma membranes (26). Therefore, it is not necessary for the caveolin-1 peptide to gain access to the cytoplasm to inhibit PKC. Moreover, the caveolin-1 peptide may exert its antineutrophil and cardioprotective effects by enhancing physiological amounts of NO production from the vascular endothelium, because the cardioprotective effects of caveolin-1 peptide-treated hearts were significantly blocked by L-NAME (7, 12).

Physiological concentrations of NO are known to attenuate PMN adherence to the vascular endothelium.
(2, 10, 12), thus reducing PMN infiltration into cardiac tissue. In this regard, NO has been shown to be a physiological inhibitor of leukocyte-endothelium interaction by suppressing upregulation of endothelial cell adhesion molecules (e.g., P-selectin) (2, 10–12). In contrast, ischemia followed by reperfusion results in a release of reactive oxygen species, particularly superoxide (28), via activation of NADPH oxidase activity from endothelial cells (15, 33). In this setting, superoxide released from the endothelium and from PMNs adhered to the endothelium can quench endogenous NO release and potentiate endothelial dysfunction, leading to further increased PMN adherence and infiltration into the coronary vasculature and surrounding tissue resulting in aggravated cardiac contractile dysfunction (14, 22).

Recently, Oka et al. (19) has shown that the caveolin-1 peptide was able to dose dependently inhibit the classic and atypical PKC isozymes at concentrations of 1–10 μM. PKC inhibition results in enhanced NO release from the endothelium by at least two mechanisms. First, PKC downregulates eNOS activity, leading to reduced endothelium-derived NO production from endothelial cells (8, 23). Inhibition of PKC will therefore counteract this downregulation of eNOS and enhance NO release (8). Second, PKC is required for stimulating NADPH oxidase activity and thus enhances superoxide production in endothelial cells (15, 33). Inhibition of PKC therefore suppresses superoxide production from endothelial cells and minimizes the quenching of NO by superoxide radicals in these cells. The combination of superoxide inhibition and enhanced NO release in the coronary vasculature could account for the cardioprotective effect of caveolin-1 peptide in myocardial I/R in the presence of PMNs. Inhibition of PKC also inhibits P-selectin expression (18). P-selectin is rapidly upregulated (i.e., 10 min after the onset of reperfusion) (30) and promotes PMN adhesion to the vascular endothelium (12). It is highly unlikely that the caveolin-1 peptide exerts its cardioprotective effects by directly increasing cardiac contractility, because we observed no increases in LVDP or +dP/dt_{max} in nonischemic perfused rat hearts given caveolin-1 peptide.

In contrast to our results, other investigators (9, 16) have shown that endogenous caveolin-1 inhibits eNOS activity. The nature of the inhibition by caveolin-1 peptide on eNOS activity is reversible and antagonized by Ca^{2+}-calmodulin (9, 16). When intracellular Ca^{2+} concentration rises via receptor stimulation, caveolin-1 binding to eNOS dissociates and can be replaced by calmodulin binding (16). Calmodulin binding to eNOS then stimulates NO release (16). It is possible that the caveolin-1 peptide did not have an appreciable degree of inhibition of eNOS when given intravascularly or that the effects of PKC inhibition were sufficient to compensate for the negative regulation of caveolin-1 on eNOS activity. Alternatively, Xia et al. (32) have shown that caveolin-1 peptide suppresses eNOS production of reactive oxygen species when l-arginine is not available as a substrate for NO synthesis, so that caveolin-1 peptide may shift the product profile of eNOS away from superoxide radicals toward NO production in I/R. Consistent with this possibility, acute infusion of l-arginine at reperfusion has been shown to attenuate reperfusion injury (31).

In summary, our results are the first to show a cardioprotective effect of caveolin-1 peptide in myocardial I/R injury. Caveolin-1 peptide was able to significantly attenuate cardiac contractile dysfunction in isolated perfused I/R rat hearts compared with similarly perfused hearts isolated from 0.9% NaCl-treated rats. 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. Caveolin-1 peptide also appears to stimulate endothelium-derived NO release, which significantly contributes to these effects via downregulation of leukocyte-endothelium interaction.

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