LOX-1 inhibition in myocardial ischemia-reperfusion injury: modulation of MMP-1 and inflammation

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Li, Dayuan, Victor Williams, Ling Liu, Hongjiang Chen, Tatsuya Sawamura, Tamim Antaki, and Jawahar L. Mehta. LOX-1 inhibition in myocardial ischemia-reperfusion (I/R) injury. A recent study (8) found that ox-LDL decreases cardiac contractility in isolated perfused hearts, but the precise mechanism of action of ox-LDL remains unclear. Some studies (26, 33) show that LOX-1, a newly described lectin-like receptor for ox-LDL, facilitates the uptake of ox-LDL and mediates several of the biological effects of ox-LDL in endothelial cells. LOX-1 mediates ox-LDL-induced cell injury and leukocyte adhesion via the activation of oxidative stress-sensitive mitogen-activated protein kinase (MAPK) (20). Expression of LOX-1 gene is upregulated by ox-LDL, which facilitates leukocyte adhesion to the intima (25). A recent study (8) found that ox-LDL decreases cardiac contractility in isolated perfused hearts, but the precise mechanism of action of ox-LDL remains unclear. Some studies (26, 33) show that LOX-1, a newly described lectin-like receptor for ox-LDL, facilitates the uptake of ox-LDL and mediates several of the biological effects of ox-LDL in endothelial cells. LOX-1 mediates ox-LDL-induced cell injury and leukocyte adhesion via the activation of oxidative stress-sensitive mitogen-activated protein kinase (MAPK) (20). Expression of LOX-1 gene is upregulated by ox-LDL, thrombosis, angioplasty, and coronary bypass surgery. Injury to myocardium due to I/R causes release of cytokines such as interleukins and TNF-α. These cytokines activate leukocytes to generate free radicals that result in myocardial injury via lipid peroxidation, calcium overload, and apoptosis (27, 34, 35). In addition, cytokines induce adhesion molecule expression and promote leukocyte aggregation and adhesion to activated endothelium, resulting in the no-flow phenomenon (10). Large amounts of activated leukocytes migrate into the myocardium and release proteolytic enzymes, which further damage myocytes (10). Many investigators have shown that blockade of adhesion molecule reduces myocardial I/R injury (11). Other studies (4) indicate that matrix metalloproteinases (MMP; i.e., MMP-1) are also involved in acute I/R injury.

Oxidized low-density lipoprotein (ox-LDL) elicits endothelial dysfunction by reducing the expression of constitutive nitric oxide synthase (15) and enhancing expression of adhesion molecules on the endothelium, which facilitates leukocyte adhesion to the intima (25). A recent study (8) found that ox-LDL decreases cardiac contractility in isolated perfused hearts, but the precise mechanism of action of ox-LDL remains unclear. Some studies (26, 33) show that LOX-1, a newly described lectin-like receptor for ox-LDL, facilitates the uptake of ox-LDL and mediates several of the biological effects of ox-LDL in endothelial cells. LOX-1 mediates ox-LDL-induced cell injury and leukocyte adhesion via the activation of oxidative stress-sensitive mitogen-activated protein kinase (MAPK) (20). Expression of LOX-1 gene is upregulated by ox-LDL, thrombosis, angioplasty, and coronary bypass surgery. Injury to myocardium due to I/R causes release of cytokines such as interleukins and TNF-α. These cytokines activate leukocytes to generate free radicals that result in myocardial injury via lipid peroxidation, calcium overload, and apoptosis (27, 34, 35). In addition, cytokines induce adhesion molecule expression and promote leukocyte aggregation and adhesion to activated endothelium, resulting in the no-flow phenomenon (10). Large amounts of activated leukocytes migrate into the myocardium and release proteolytic enzymes, which further damage myocytes (10). Many investigators have shown that blockade of adhesion molecule reduces myocardial I/R injury (11). Other studies (4) indicate that matrix metalloproteinases (MMP; i.e., MMP-1) are also involved in acute I/R injury.

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angiotensin II, free radicals, and inflammatory cytokines such as TNF-α and shear stress (16, 22, 28, 30).

In the present study, we investigated whether the expression of LOX-1 is involved in the determination of I/R injury. We also examined the expression of MMP-1 and inflammatory cell recruitment in the I/R area.

MATERIALS AND METHODS

Animal model. Male Sprague-Dawley rats (250–300 g) were anesthetized with pentobarbital, intubated, and ventilated. A left thoracotomy was performed, and the left coronary artery (LCA) was ligated with 6-0 silk suture from its origin with a slipknot. Ischemia was confirmed by myocardial blanching and electrocardiogram evidence of injury. After total ischemia for 1 h, reperfusion was instituted for 1 h. In the sham control group (n = 10 rats), thoracotomy was performed without left anterior descending coronary artery ligation.

Materials. Anti-rat LOX-1 monoclonal antibodies were generated by immunizing BALB/c mice with rat LOX-1-Chinese hamster ovary (CHO) cells. Hybridoma from the splenocytes was prepared by standard procedures and screened by cell-surface immunobinding to bovine LOX-1-CHO cells. A functional blocking antibody (JTX-20) was selected for blocking Dil-labeled ox-LDL binding and uptake in bovine LOX-1-expressing cells, as described earlier (1, 14, 29). The immunostaining kit was purchased from Santa Cruz Biotechnology. The remaining reagents were purchased from Sigma, unless noted otherwise.

Expression of LOX-1 determined by semiquantitative RT-PCR. Total RNA (1 μg) extracted from I/R myocardium was reverse transcribed with Oligo dT (Promega) and Moloney murine leukemia virus RT (Promega) at 37°C for 1 h. The reverse-transcribed material (1.5 μl) was amplified with Taq DNA polymerase (Promega) using specific rat primers of LOX-1 (26). The products of PCR-amplicated samples were visualized on 1.5% agarose gels using ethidium bromide. Each specific mRNA band was normalized with a band of relative internal reference β-actin mRNA. Relative intensity of band of interest was analyzed with Scan-Gel-It software (Silk Scientific) and expressed as the ratio to a β-actin mRNA band.

Western blot assay. SDS-PAGE was performed on 10% separation gels with a 6% stacking gel. Proteins were transferred to nitrocellulose membrane (Bio-Rad). Blots were incubated with primary antibodies to anti-rat LOX-1 antibody with a 1:500 dilution at 4°C overnight. Blots were incubated with horseradish peroxidase-conjugated secondary antibody, and signal was detected with enhanced chemiluminescence (Amersham) (20, 21).

Immunostaining for MMP-1, adhesion molecules, and inflammatory cells. Immunostaining kit was performed according to the instruction of the manufacturer. In brief, 5-μm-thick sections from myocardial tissues were incubated with primary antibodies (2 h, 22°C), rinsed in phosphate-buffered saline, and incubated with anti-mouse IgG conjugated to tetramethylrhodamine (30 min, 22°C) (43). Immunostaining with type- and class-matched nonimmune IgGs (Scan Cruz) served as a negative control for each antibody used in the present study. Immunostaining was performed in multiple sections of the myocardium in all animals. For assessment of leukocyte accumulation, five different regions (I/R areas) were evaluated in eight hearts in each group.

Measurement of MAPK activity. Myocardial tissues from risk area were homogenized and lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. After being blocked, the membranes were incubated with 1:1,000 dilution phosphospecific p38 MAPK antibodies to the rat (Santa Cruz, CA). Thereafter, the membrane was stripped and reprobed with the p38 MAPK antibody.

Determination of infarct size. After 1 h of ischemia and 1 h of reperfusion, the heart was quickly removed and mounted on a Langendorff apparatus and flushed with saline for 60 s. The LCA was reoccluded and Evans blue dye was infused in retrograde fashion to mark the area at risk (AAR). The heart was then cut into six slices. The slices were incubated in 1% triphenyl tetrazolium chloride (TTC; pH 7.4) for 15 min. For each section, the AAR (Evans blue-negative tissue) and infarct area (TTC-negative tissue) were traced and then measured by planimetry. The extent of myocardial infarction was calculated as the total area of infarction divided by the AAR for that slice (23).

Data analysis. All data represent the mean of eight performed experiments. Data are shown as means ± SD. Statistical significance was determined in multiple comparisons among independent groups of data, in which ANOVA and the F test indicated the presence of significant differences. A P value <0.05 was considered significant.

RESULTS

LOX-1 expression during myocardial I/R. The expression of LOX-1 (mRNA and protein determined by RT-PCR and Western blot, respectively) was markedly increased during I/R. Administration of the neutralizing antibody to LOX-1 significantly attenuated the expression of LOX-1 (P < 0.01). In contrast to the LOX-1 antibody, administration of nonspecific IgG had no effect (Fig. 1).

The expression of LOX-1 was confirmed in rat myocardial tissues by immunostaining, which showed LOX-1 expression to be increased in I/R myocardium. Immunopositivity for LOX-1 was mainly identified in the endocardium and in the subendocardial areas of myocardium. Again, the use of LOX-1 antibody re-
duced LOX-1 staining induced by I/R, whereas nonspecific IgG had no effect (Fig. 2).

**LOX-1 and expression of MMP-1 and adhesion molecules during I/R.** Expression of MMP-1 and adhesion molecules (P-selectin, ICAM-1, and VCAM-1) was markedly increased during I/R. Administration of the neutralizing antibody to LOX-1 reduced the expression of MMP-1 and all adhesion molecules. In contrast to LOX-1 antibody, the use of nonspecific IgG had no effect (Fig. 3).

Expression of MMP-1 and adhesion molecules was confirmed in rat I/R myocardial tissues by immunostaining. Immunopositivity for MMP-1 and adhesion molecules was mainly identified in the endocardium and in the subendocardial areas of myocardium, similar to that for LOX-1. Again, the use of LOX-1 antibody reduced the expression of MMP-1 and adhesion molecules, whereas the nonspecific IgG had no effect (Fig. 4).

**LOX-1 and recruitment of leukocytes.** To examine the relative significance of adhesion molecule upregulation, staining for leukocytes was performed in myocardial tissues from I/R area in different groups of rat hearts. The number of leukocytes located in or outside the blood vessels in the AAR was markedly increased during I/R (P < 0.01). LOX-1 antibody reduced leukocyte recruitment despite I/R, whereas nonspecific IgG had no effect (Fig. 5).

**LOX-1 and p38 MAPK during I/R.** The activity of p38 MAPK was also increased during I/R (P < 0.01 vs. sham control). Administration of the neutralizing antibody to LOX-1 inhibited p38 MAPK activation (P < 0.01 vs. I/R group). In contrast to LOX-1 antibody, the use of nonspecific IgG had no effect (Fig. 6). Importantly, I/R alone or the use of LOX-1 antibody had no effect on p38 MAPK protein levels.

**LOX-1 expression and infarct size.** Hearts in the sham control group did not reveal any area of necrosis. The AAR was similar in all I/R groups. About 45% of AAR in the I/R group (saline-treated rats) was necrotic, and antibody to LOX-1 decreased the infarct size by 55% (P < 0.01). In contrast, nonspecific IgG had no effect on infarct size (Fig. 7).

**DISCUSSION**

In this study, we show that LOX-1 gene expression is upregulated in the I/R myocardium of rats. The up-
regulation of LOX-1 is associated with myocardial injury, p38 MAPK activation, and expression of MMP-1 and adhesion molecules. The administration of LOX-1 antibody markedly reduces I/R-induced myocardial injury and the expression of LOX-1, MMP-1, and adhesion molecules. Finally, use of LOX-1 antibody reduces activation of p38 MAPK.

**LOX-1 expression during I/R.** Recent studies (5, 6) show that ox-LDL levels are increased in plasma and myocardial tissues from patients with myocardial ischemia. Ox-LDL decreases constitutive nitric oxide synthase activity and induces free radical generation, platelet aggregation, vasoconstriction, and apoptosis (3, 15, 21). The actions of ox-LDL are thought to be mediated by its scavenger receptors expressed in macrophages and smooth muscle cells (18). Recent reports (1, 14, 16, 20–22, 26, 28–30, 33) indicate that the activation of LOX-1 is responsible for ox-LDL-induced injury to endothelial cells.

The expression of LOX-1 is regulated by free radicals, cytokines, shear stress, angiotensin II, and ox-LDL itself (16, 20–22, 26, 28, 30, 33). It is well known that myocardial I/R causes release of cytokines and free radicals (11, 30, 44). Therefore, it is possible that the release of cytokines and free radicals during reperfusion stimulates LOX-1 gene expression. In this study, we demonstrate that LOX-1 gene expression is increased in the myocardium of rats subjected to I/R. Direct evidence for the role of LOX-1 came from studies in which LOX-1 antibody blocked LOX-1 expression and reduced myocardial I/R injury.

**LOX-1 and MMP-1 expression during I/R.** MMPs are an endogenous family of enzymes that are responsible for cardiac remodeling (31, 39). Increased myocardial MMP activity and expression have been identified in human and animal models of heart failure (37, 41). Recent studies (32) using rodent models have suggested a functional role of myocardial MMPs in remodeling after myocardial infarction.

A recent study (4) found that MMP levels were increased in the coronary effluent and peaked within the first minute of reperfusion after 20 min of ischemia. The release of MMP increased with increasing duration of ischemia and correlated negatively with the recovery of mechanical function during reperfusion. The use of MMP antibody and the inhibitors of MMPs...
doxycycline and o-phenanthroline improved, whereas exogenous MMP worsened, the recovery of mechanical function during reperfusion. Another study (9) found that serum MMP-1 levels were increased in patients with acute myocardial infarction with successful reperfusion. In the present study, we found that MMP-1 expression was significantly increased during I/R in the rat. The expression of MMP-1 appears to be associated with myocardial I/R injury because the LOX-1 antibody markedly attenuated MMP-1 expression and reduced myocardial infarct size.

**LOX-1 and adhesion molecule expression and leukocyte recruitment during I/R.** Several investigators (2, 36) have suggested a role for leukocytes, specifically polymorphonuclear neutrophils, in mediating functional damage to endothelial and myocardial cells during acute and late reperfusion. Experimental studies (10, 11, 44) have demonstrated that leukocytes release free radicals and proteolytic enzymes that damage the myocardium. For this role to be manifested, leukocytes must first adhere to the postcapillary coronary venules. Leukocytes are first decelerated by an interaction of selectins with their ligands, whereas leukocyte β2 integrins and endothelial ICAMs provide firm adhesion (42). Leukocyte adhesion is caused by acute endothelial activation, which takes place within seconds through translocation of stored P-selectin and VCAM-1 (13). Other studies (11, 13, 42) have shown that antibodies to various adhesion molecules reduce I/R injury. In the present study, we found that I/R upregulates the expression of a number of adhesion molecules. LOX-1 expression seems to play a critical role in this process because LOX-1 antibody markedly attenuated the expression of these adhesion molecules and subsequent leukocyte recruitment.

**LOX-1 and MAPK activation during I/R.** In previous studies (19, 21), we have shown that LOX-1 mediates ox-LDL-induced apoptosis and expression of various adhesion molecules on endothelial cells. In this process, activation of p42/44 MAPK and nuclear factor-κB plays a critical signaling role. In another study (12), we showed that LOX-1 induces apoptosis of cultured rat cardiac myocytes through p38 MAPK activation. It is well known that intracellular protein kinases are involved in I/R injury. In the present study, we demonstrate that p38 MAPK is activated during I/R. More importantly, we found that LOX-1 antibody inhibits p38 MAPK activation. These observations taken together suggest that p38 MAPK activation plays an important signaling role in the expression of MMP-1 and adhesion molecules. LOX-1 expression may be critical in this pathway because the use of LOX-1 antibody inhibited p38 MAPK activity and simultaneously reduced the expression of MMP-1 and adhesion molecules.

**LOX-1 and infarct size during I/R.** It is possible that the release of cytokines and free radicals during I/R oxidizes LDL, which upregulates LOX-1 gene expression. Interaction between ox-LDL and its receptor LOX-1 augments myocardial injury initiated during I/R. Experimental studies have demonstrated that ox-LDL induces ultrastructural abnormalities in cardiac myocytes and decreases myocardial contractility in isolated perfused rat heart (8). A recent study (6) found that ox-LDL is localized in the venticles of hearts from patients with coronary heart disease. Ox-LDL was present in the left and right ventricular walls from coronary heart disease patients compared with patients with dilated cardiomyopathy or controls without heart disease. The accumulation of ox-LDL was higher in the left than in the right ventricle. Positive immunoreactivity for ox-LDL was present mainly in the endocardium and the subendocardial areas of the ventricles. These findings provide basis for LOX-1 expression in the I/R myocardium, which was mainly upregulated in the endocardium and the subendocardial region of the left ventricle. Ox-LDL acting on LOX-1 could induce free radical generation resulting in lipid peroxidation and apoptosis that further increase infarct size and worsen cardiac function. This concept is proven by the use of functional LOX-1 blocking antibody, which reduced infarct size by >50%. In contrast, nonspecific anti-goat IgG had no protective effect. These observations provide a new insight into the genesis of myocardial I/R injury.

In summary, this study shows that I/R increases LOX-1 gene expression that contributes to myocardial injury. Expression of MMP-1 and adhesion molecules seems to play a role in LOX-1-mediated myocardial injury. Inhibition of LOX-1 expression and activation may be a potential target for therapy of I/R injury.

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