Activation of Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Induces Apoptosis in Cultured Neonatal Rat Cardiac Myocytes

Eri Iwai-Kanai, MD; Koji Hasegawa, MD, PhD; Tatsuya Sawamura, MD, PhD; Masatoshi Fujita, MD, PhD; Tetsuhiko Yanazume, MD; Shinya Toyokuni, MD, PhD; Souichi Adachi, MD, PhD; Yasuki Kihara, MD, PhD; Shigetake Sasayama, MD, PhD

Background—Lectin-like oxidized LDL receptor-1 (LOX-1) was originally identified as a receptor expressed predominantly in endothelial cells. LOX-1 can also be expressed in other cell types, and the activation of the LOX-1 pathway has been implicated in apoptosis. There have been no reports, however, about LOX-1 expression in cardiac myocytes or regulation of myocardial cell apoptosis by LOX-1.

Methods and Results—In primary cardiac myocytes from neonatal rats, immunohistochemical analyses using a specific monoclonal antibody against LOX-1 demonstrated that LOX-1 expression was markedly induced by stimulation with norepinephrine and endothelin-1. LOX-1 expression was upregulated in cardiac myocytes as well as in vessel walls of failing rat hearts in vivo. In the presence of a low concentration of oxidized LDL that did not induce apoptosis by itself, artificial overexpression of LOX-1 in cardiac myocytes in culture resulted in apoptosis. LOX-1 overexpression induced activation of p38 mitogen-activated protein kinase (MAPK) and oxidative stress in cardiac myocytes, as demonstrated by an increase in positive immunostaining for 8-hydroxy-2'-deoxyguanosine. Inhibition of p38 MAPK by cotransfection of a dominant-negative form of MKK6 as well as by administration of a specific inhibitor, SB203580 or FR167653, almost completely blocked the induction of apoptosis by LOX-1 activation. Antioxidant catalase also blocked LOX-1–induced apoptosis as well as activation of p38 MAPK.

Conclusions—These findings demonstrate that LOX-1 expression in cardiac myocytes is induced by neurohormonal factors activated in heart failure and that LOX-1–dependent apoptosis in these cells requires p38 MAPK, a component of oxidant stress–sensitive signaling pathways. (Circulation. 2001;104:2948-2954.)

Key Words: lipoproteins ▪ apoptosis ▪ myocytes ▪ heart failure ▪ receptors

Lectin-like oxidized LDL (ox-LDL) receptor-1 (LOX-1) is a type II membrane protein that belongs to the C-type lectin family and was originally identified as a receptor for oxidatively modified LDL.1 LOX-1 is expressed in highly vascularized organs, such as the placenta and lung, and is expressed predominantly in vascular endothelial cells. LOX-1 expression can be induced in macrophages and smooth muscle cells as well as endothelial cells by various kinds of stimuli, including ox-LDL, angiotensin II, shear stress, and tumor necrosis factor-α.2 LOX-1 expression in hypertensive rats is upregulated in their vessels, which might imply a pathophysiological role for LOX-1 in hypertensive vascular remodeling.3 Recent studies suggest that the LOX-1 pathway can be activated by platelets and aged red blood cells as well as ox-LDL and that the activation of this pathway is involved in apoptosis.4,5 It is not known, however, whether LOX-1 is expressed in cardiac myocytes or whether it regulates apoptosis there.

Accumulating evidence suggests potential roles of myocardial cell apoptosis in the development of heart failure. We demonstrate here that LOX-1 expression can be induced in cardiac myocytes by stimulation with norepinephrine and endothelin-1, important neurohormonal factors activated in heart failure.8 LOX-1 expression in cardiac myocytes is also upregulated in failing rat hearts in vivo. In addition, we show that activation of LOX-1 induces myocardial cell apoptosis and that this apoptosis requires the oxidative stress–sensitive p38 mitogen-activated protein kinase (MAPK) pathway.

Methods

Immunocytochemistry

Primary ventricular cardiac myocytes were prepared as previously described.9,10 The cardiac myocytes were then grown on flask-style...
glass slides (Nalgen Nunc) and stimulated with endothelin-1 (10^{-7} \text{ mol/L}) or norepinephrine (10^{-5} \text{ mol/L}) or saline as a control for 24 hours. These agents were obtained from Sigma and were of the highest purity available. Then the cells were fixed with Bouin’s solution for 10 minutes at room temperature and subjected to immunocytochemistry for LOX-1 by the indirect immunoperoxidase method, as previously described.8,10 As the primary antibody, we used anti–LOX-1 monoclonal antibody at a dilution of 1:80.

For analysis of 8-hydroxy-2'-deoxyguanosine (8-OHdG), immunocytochemistry using the avidin-biotin complex method with alkaline phosphatase was performed as previously described.11 8-OHdG immunocytochemistry specimens were subjected to densitometric analysis. Quantification of immunological data (expressed as 8-OHdG Index) was performed as previously described.11 The following equation was used for the quantification of immunological data: 8-OHdG index = \sum[(X - \text{threshold}) \times \text{area (\mu m^2)})/\text{total cell number}, X > \text{threshold}, where X is the staining density indicated by a number between 0 and 150 on the gray scale.

Rat Model of Heart Failure

Male inbred Dahl salt-sensitive and salt-resistant rats obtained from Brookhaven National Laboratories (Upton, NY) were fed a 0.3% NaCl (low-salt) diet after weaning until the age of 6 weeks, after which they were fed an 8% NaCl (high-salt) diet for 11 weeks. On the day they were euthanized, left ventricles were subjected to immunocytochemistry with anti–LOX-1 monoclonal antibody. B, Dahl salt-resistant (A) or salt-sensitive (B and C) rats were kept on high-salt diet for 11 weeks, and left ventricles were subjected to immunocytochemical staining with anti–LOX-1 monoclonal antibody. C, We substituted primary antibody for normal mouse IgG.

Plasmids and Transfection

Cardiac myocytes were transfected with 2 \mu g of an expression vector encoding rat LOX-1 or \beta-galactosidase (\beta-gal) with Lipofectamine Plus (Gibco BRL) as described previously.9 MKK6AL, an expression vector encoding a dominant-negative mutant of MKK6, was provided by Dr J.R. Woodgett (University of Toronto, Toronto, Canada).12

Western Blotting

Activation of the MAPKs extracellular signal–regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase (JNK), and p38 MAPK was estimated by Western blot analysis using antibodies (all from New England Biolabs) that specifically recognize the phosphorylated, active forms of these enzymes, as previously described.13

Assessment of Apoptotic Cells

Terminal deoxynucleotidyl transferase–mediated nick end-labeling of fragmented nuclei (TUNEL assay) was performed as previously described.9,10 Individual nuclei were visualized at ×400 magnification for quantitative analysis. An average of 400 to 500 nuclei from random fields were analyzed on each slide. The apoptotic index (percentage of apoptotic nuclei) was calculated as (apoptotic nuclei/total nuclei) × 100. Sample indicators were concealed during scoring, and samples from ≥3 independent experiments were scored per group.

Nucleosomal ladder assay and cytofluorimetric analysis were performed as previously described.9,10

Statistical Analysis

Data are presented as mean ± SEM. Statistical comparisons were performed with unpaired 2-tailed Student’s t tests or ANOVA with Scheffé’s test when appropriate, with a value of P < 0.05 taken to indicate significance.

Results

LOX-1 Expression in Cardiac Myocytes Is Upregulated in Failing Hearts

To examine whether LOX-1 can be expressed in cardiac myocytes, we carried out immunocytochemical analysis using anti–LOX-1 monoclonal antibody. As shown in Figure 1A, positive brown signals indicating LOX-1 were observed in cardiac myocytes stimulated with norepinephrine (10^{-5} \text{ mol/L}, B) or endothelin-1 (10^{-7} \text{ mol/L}, C and D) for 24 hours and subjected to immunocytochemical staining with anti–LOX-1 monoclonal antibody. D, We substituted normal mouse IgG for primary antibody. B, Dahl salt-resistant (A) or salt-sensitive (B and C) rats were kept on high-salt diet for 11 weeks, and left ventricles were subjected to immunocytochemical staining with anti–LOX-1 monoclonal antibody. C, We substituted primary antibody for normal mouse IgG.
Artificial Overexpression of LOX-1 Induces Myocardial Cell Apoptosis

To examine the impact of LOX-1 activation on cardiac myocytes, we transfected an expression vector encoding LOX-1 or one encoding β-gal as a control into cardiac myocytes in culture. Western blot analysis using anti-LOX-1 monoclonal antibody confirmed that LOX-1 is expressed in cardiac myocytes transfected with a LOX-1 expression vector but not in cells transfected with a β-gal expression vector. In preliminary experiments, we incubated these cells in serum-free medium in the presence of various concentrations of ox-LDL for 48 hours. As shown in Figure 2, at higher concentrations (3×10⁻⁴ and 3×10⁻³ mg/mL), ox-LDL increased the number of TUNEL-positive nuclei to similar extents in both cardiac myocytes transfected with a LOX-1 expression vector and those transfected with a β-gal expression vector. In contrast, at the concentration of 3×10⁻⁶ mg/mL, the number of TUNEL-positive myocytes was significantly higher in cardiac myocytes transfected with a LOX-1 expression vector than in those transfected with a β-gal expression vector. To confirm that the increase of TUNEL-positivity in LOX-1-expressing cardiac myocytes is mediated specifically through the activation of LOX-1-dependent pathways, we examined the effect of anti–LOX-1 antibody. As shown in Figure 3, administration of anti–LOX-1 antibody dose-dependently decreased the TUNEL-positivity of cardiac myocytes transfected with a LOX-1 expression vector.

We next performed a series of experiments to further examine the induction of apoptosis by transfecting a LOX-1 expression vector or a β-gal expression vector into cardiac myocytes and incubating these cells with medium in the presence of 3×10⁻⁶ mg/mL of ox-LDL for 48 hours. Representative photographs of TUNEL staining are shown in Figure 4A. Artificial expression of LOX-1 markedly increased the number of cells with nuclei strongly stained brown. These cells may specifically reflect the presence of internucleosomal DNA fragmentation, because no such cells were found when we omitted the terminal deoxynucleotidyl transferase treatment. The cells transfected with a LOX-1 expression vector displayed small, condensed nuclei, cell shrinkage, and nuclear fragmentation, consistent with the morphological features of apoptosis. Figure 4B shows LOX-1-induced typical ladder formation in agarose gels, a hallmark of apoptosis. The reduction of mitochondrial transmembrane potential has been shown to precede DNA fragmentation in apoptosis. Figure 4C shows mitochondrial membrane potential and cell membrane permeability by cytofluorimetric analysis. Transfection of cardiac myocytes with a LOX-1 expression vector did not alter cell membrane permeability compared with transfection of a β-gal expression vector, as shown by the lack of increase in propidium iodide binding to DNA. Transfection with a LOX-1 expression vector, however, increased the number of cells with low JC-1 fluorescence, indicating that LOX-1 expression reduced the mito-
chondrial transmembrane potential. It was found that 25.6 ± 4.2% (n = 3) of LOX-1–transfected cells (lower left quadrant in LOX-1) and 6.0 ± 1.6% (n = 3) of β-gal–transfected cells (lower left quadrant in β-gal) had low JC-1 fluorescence (P < 0.01).

LOX-1 Pathway Induces Oxidative Stress and p38 MAPK Activation

To examine the mechanism of LOX-1–dependent apoptosis in cardiac myocytes, we performed immunocytochemistry using monoclonal antibody (N45.1) against 8-hydroxy-2′-deoxyguanosine (8-OHdG), a derivative of 2′-deoxyguanosine hydroxylated at the C-8 position, one of the most commonly used markers for evaluation of cellular oxidative stress.11 As shown in Figure 5, purple nuclear signals indicating the presence of 8-OHdG were observed in cardiac myocytes transfected with a LOX-1 expression vector (B). Substitution of normal mouse IgG for N45.1 abolished the staining (D). Few positive cells, however, were observed in cardiac myocytes transfected with a β-gal expression vector (A) and in myocytes transfected with a LOX-1 expression vector in the presence of antioxidant catalase (C, 10 U/mL). We performed quantitative determination of 8-OHdG immunostaining. The 8-OHdG Index in LOX-1–expressing cells (15 112 ± 1467, n = 4) was 26-fold higher than that in β-gal–expressing cells (574 ± 68, n = 4). Administration of catalase (10 U/mL) severely attenuated this increase (3347 ± 429, n = 4, P < 0.001).

We examined whether the MAPKs are activated by LOX-1 activation. Activation of ERK 1/2, JNK, and p38 MAPK was expressed as the ratio of phospho-MAPK to total MAPK immunoreactivity. As shown in Figure 6A, the activation states of ERK 1/2 and JNK were almost the same in LOX-1–expressing cells (lane 2) and in β-gal–expressing cells (lane 1). In contrast, dual phospho-p38 MAPK immunoreactivity was much higher in LOX-1–expressing cells.
(lane 2) than in β-gal–expressing cells (lane 1), whereas total p38 MAPK immunoreactivity was similar in these 2 types of cells.

**LOX-1–Mediated Apoptosis Requires Oxidant Stress–Sensitive p38 MAPK Pathway**

To assess whether p38 MAPK is involved in myocardial cell apoptosis induced by LOX-1 expression, we used 2 different inhibitors, SB203580 and FR167653, both of which have been shown to inhibit p38 MAPK selectively relative to other MAPK family members and several other kinases.14 As shown in Figure 6A, both SB203580 and FR167653 almost completely inhibited the activation of p38 MAPK in LOX-1–expressing cardiac myocytes. These agents, however, did not affect the phosphorylation status of ERK 1/2 or JNK. We then examined the effect of these 2 specific p38 MAPK inhibitors on LOX-1–induced myocardial cell apoptosis. Both SB203580 (3 and 10 μmol/L) and FR167653 (0.1 and 1 μmol/L) dose-dependently decreased the number of TUNEL-positive cardiac myocytes. These agents, however, did not affect the phosphorylation status of ERK 1/2 or JNK. We then examined the effect of these 2 specific p38 MAPK inhibitors on LOX-1–induced myocardial cell apoptosis. Both SB203580 (3 and 10 μmol/L) and FR167653 (0.1 and 1 μmol/L) dose-dependently decreased the number of TUNEL-positive cardiac myocytes. These agents, however, did not affect the phosphorylation status of ERK 1/2 or JNK. We then examined the effect of these 2 specific p38 MAPK inhibitors on LOX-1–induced myocardial cell apoptosis. Both SB203580 (3 and 10 μmol/L) and FR167653 (0.1 and 1 μmol/L) dose-dependently decreased the number of TUNEL-positive cardiac myocytes.

To better specify the role of p38 MAPK in induction of apoptosis by LOX-1, we used MKK6AL, an expression vector encoding a dominant-negative mutant of MKK6 (an active kinase positioned upstream from p38 MAPK). As shown in Figure 7A, cotransfection of MKK6AL resulted in complete loss of LOX-1–induced activation of p38 MAPK. As shown in Figure 7B, the cotransfection of MKK6AL almost completely inhibited the increase in the number of TUNEL-positive cardiac myocytes in cultures transfected with a LOX-1–expression vector (P<0.001). The mutant had no effect on the basal level of TUNEL positivity.

**Discussion**

The present study demonstrates for the first time that LOX-1 expression in cardiac myocytes is upregulated in failing rat hearts in vivo as well as by stimulation with endothelin-1 and norepinephrine in culture. Although LOX-1 is a membrane protein, immunohistochemistry showed cytoplasmic staining. One possible explanation for this discrepancy is the thickness of the sections (≈6 μm) relative to myocyte size (≈16 μm). A second possible explanation is diffusion of DAB products during the staining procedure. It is also possible, however, that cytoplasmic staining is not an artifact but rather represents an abnormal distribution of excessive protein that induces cell stress and apoptosis. Thus, further studies of the subcellular localization of LOX-1 in cardiac myocytes should be performed at the electron microscopic level.

It has been implied that endothelial dysfunction plays a pathophysiological role in patients with heart failure. Tumor necrosis factor-α and angiotensin II can induce expression of...
LOX-1 in endothelial cells, and the levels of these factors are increased in heart failure.\textsuperscript{2,4} Compatible with this, we showed that LOX-1 expression was upregulated in vessel walls as well as in cardiac myocytes of failing rat hearts in vivo. To clarify the role of LOX-1 in heart failure in vivo, studies of LOX-1 in various cell types will be required.

Although LOX-1 expression in cardiac myocytes is induced in heart failure, the physiological ligands that activate LOX-1 have not been identified. Because ox-LDL cannot readily pass through endothelium, cardiac myocytes may rarely encounter ox-LDL. LOX-1, however, not only binds ox-LDL but also actively binds various cells, including apoptotic cells and activated platelets.\textsuperscript{6} These findings suggest that the LOX-1 pathway could be activated in heart failure even if cardiac myocytes may rarely encounter ox-LDL.

Using primary neonatal rat cardiac myocytes, we showed that in the presence of a low concentration of ox-LDL, LOX-1 overexpression in cardiac myocytes resulted in an increase in the number of TUNEL-positive cells. Administration of anti-LOX-1 antibody decreased the proportion of TUNEL-positive nuclei in LOX-1–expressing cardiac myocytes, suggesting a critical role of the LOX-1–dependent pathway. LOX-1–mediated apoptosis in rat cardiac myocytes was demonstrated by 3 additional lines of evidence: (1) nucleosomal ladder formation, (2) morphological features of apoptosis, and (3) a decrease of mitochondrial transmembrane potential.\textsuperscript{9} These findings demonstrate that the activation of the LOX-1–dependent pathway in cardiac myocytes results in apoptosis.

Our results suggest that p38 MAPK–dependent pathways are required for myocardial cell apoptosis mediated by a LOX-1–dependent pathway. It has been reported that p38 MAPK is activated by reactive oxygen species in other cell types.\textsuperscript{12} We have also shown that catalase, which eliminates reactive oxygen species, blocked apoptosis as well as p38 MAPK activation induced by LOX-1 activation. These findings suggest that activation of LOX-1 produces reactive oxygen species, such as hydrogen peroxide, in cardiac myocytes and that the oxidant stress induced by these species activates the p38 MAPK–dependent pathway and induces apoptosis. At least 4 members of the p38 MAPK family have been identified, and the isoforms $\alpha$ (also called p38, CSBP, or RK) and $\beta$ are both expressed in heart tissue.\textsuperscript{15} Although these isoforms share $\approx 74\%$ sequence identity, they have been suggested to have opposing functions in cardiac myocytes. Apoptosis appears to be mediated by the $\alpha$-isoform, whereas the hypertrophic response is mediated by the $\beta$-isoform.\textsuperscript{15} The roles of $\alpha$- and $\beta$-isoforms of p38 MAPK in the process of LOX-1–mediated apoptosis should be further investigated.

Several possibilities should be taken into account when the data of this study are applied to the in vivo setting in the adult. First, because myocardial development is not complete at birth, differences may exist between neonatal and adult cardiac myocytes. Second, the biological properties of dissociated myocytes in culture studies that cannot precisely mimic the in vivo situation. Several lines of evidence, however, suggest that these factors accumulate within myocytes in vivo during heart failure.\textsuperscript{8} Also, the elevation of these factors in patients with heart failure continues for a long time. In addition, we showed that LOX-1 expression is actually induced in the ventricular myocardium of failing rat hearts. These data might provide novel insights into roles of LOX-1 in the development of heart failure. Although further studies are needed to elucidate the precise role of LOX-1 in the clinical setting, it would be particularly interesting to test whether endothelin-1 antagonists or $\beta$-adrenergic receptor blockers will decrease the cardiac expression of LOX-1 in failing hearts. Furthermore, development of a LOX-1 antagonist and its application to the treatment of heart failure as well as atherosclerosis would be worth attempting in the future.

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