# Cardiac 12/15 lipoxygenase–induced inflammation is involved in heart failure

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**To identify a novel target for the treatment of heart failure, we examined gene expression in the failing heart. Among the genes analyzed, *Alox15* encoding the protein 12/15 lipoxygenase (LOX) was markedly up-regulated in heart failure. To determine whether increased expression of 12/15-LOX causes heart failure, we established transgenic mice that overexpressed 12/15-LOX in cardiomyocytes. Echocardiography showed that *Alox15* transgenic mice developed systolic dysfunction. Cardiac fibrosis increased in *Alox15* transgenic mice with advancing age and was associated with the infiltration of macrophages. Consistent with these observations, cardiac expression of monocyte chemoattractant protein 1 (MCP-1) was up-regulated in *Alox15* transgenic mice compared with wild-type mice. Treatment with 12-hydroxy-eicosatetraenoic acid, a major metabolite of 12/15-LOX, increased MCP-1 expression in cardiac fibroblasts and endothelial cells but not in cardiomyocytes. Inhibition of MCP-1 reduced the infiltration of macrophages into the myocardium and prevented both systolic dysfunction and cardiac fibrosis in *Alox15* transgenic mice. Likewise, disruption of 12/15-LOX significantly reduced cardiac MCP-1 expression and macrophage infiltration, thereby improving systolic dysfunction induced by chronic pressure overload. Our results suggest that cardiac 12/15-LOX is involved in the development of heart failure and that inhibition of 12/15-LOX could be a novel treatment for this condition.**

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Abbreviations used: cDNA, complementary DNA; FS, fractional shortening; HETE, hydroxy-eicosatetraenoic acids; LOX, lipoxygenase; LVDd, left ventricular diastolic dimension; MCP-1, monocyte chemoattractant protein 1; mRNA, messenger RNA; TAC, transverse aortic constriction.

Heart failure is a clinical syndrome that is associated with various cardiovascular diseases such as hypertension and myocardial infarction (Libby and Braunwald, 2008). Comprehensive management using current therapeutic options can markedly reduce the morbidity and mortality of heart failure. Large-scale clinical trials of drugs targeting neurohormonal mechanisms, such as angiotensin-converting enzyme inhibitors and β blockers, have shown that such treatment is effective for reducing mortality in patients with heart failure (Garg and Yusuf, 1995; McMurray, 1999; Goldstein, 2002). However, heart failure is still one of the leading causes of death worldwide (Libby and Braunwald, 2008), so it is important to investigate the underlying mechanisms of this condition and develop more effective treatments.

Arachidonic acid is a free fatty acid that, when liberated from cell membranes, can be metabolized by cyclooxygenase, cytochrome p450, and lipoxygenase (LOX) to form biologically active products such as prostaglandins, leukotrienes, and hydroxy-eicosatetraenoic acids (HETEs;

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Kudo and Murakami, 2002). LOXs are a family of lipid-peroxidizing enzymes that oxidize free and esterified polyenolic fatty acids to form the corresponding hydroperoxy derivates (Kuhn and O'Donnell, 2006). The LOX enzymes are named according to the specific carbon atoms of arachidonic acid that are oxidized. Thus, 12/15-LOX is a member of the LOX family that catalyzes the step from arachidonic acid to 12(S)-HETE and 15(S)-HETE (Chen et al., 1994). 12/15-LOX was originally isolated from porcine leukocytes (Yokoyama et al., 1986), but its tissue distribution is now known to be relatively wide, including blood vessels, the brain, and the kidneys (Kuhn and O'Donnell, 2006). Several lines of evidence have suggested that 12/15-LOX may play an important role in the development of atherosclerosis, diabetes, and neurodegenerative disease (Natarajan and Nadler, 2004; Kuhn and O'Donnell, 2006). For example, disruption of the gene for 12/15-LOX in mice significantly reduces the onset of atherosclerosis (Cyrus et al., 1999, 2001; George et al., 2001), whereas an increase of 12/15-LOX expression in mice promotes monocyte–endothelial cell interactions that lead to atherogenesis (Hatley et al., 2003; Reilly et al., 2004; Bolick et al., 2005). Several studies have shown that monocyte 12/15-LOX mediates the oxidative modification of low-density lipoprotein (McNally et al., 1990; Sakashita et al., 1999; Zhu et al., 2003b). An increase of 12/15-LOX activity in vessel walls also contributes to atherogenesis by impairing the macrophage cholesterol efflux pathway (Nagelin et al., 2008). Interestingly, mice with deficiency of 12/15-LOX are resistant to the development of streptozotocin-induced diabetes (Bleich et al., 1999) and autoimmune diabetes (McDuffie et al., 2008). However, there is currently little evidence that 12/15-LOX has a role in heart failure.

In the present study, we showed that cardiac 12/15-LOX induces inflammation that is involved in heart failure. We found that 12/15-LOX expression was markedly increased in the failing heart. Increased expression of this enzyme up-regulates monocyte chemoattractant protein 1 (MCP-1) and promotes the infiltration of macrophages into the heart, thereby causing cardiac fibrosis and systolic dysfunction. Conversely, disruption of *Alox15* reduces cardiac MCP-1 expression and macrophage infiltration, thereby improving systolic dysfunction induced by chronic pressure overload. These findings suggest that inhibition of 12/15-LOX could be a novel treatment for heart failure.

## RESULTS

### Increased expression of 12/15-LOX causes heart failure

To clarify the molecular mechanisms of heart failure, we performed microarray analysis using cardiac tissue samples obtained from a hypertensive heart failure model (Dahl salt-sensitive rats). Approximately 300 genes showed significant changes of expression in failing hearts compared with control hearts. For example, fetal genes, such as the natriuretic peptide genes and the β-type myosin heavy chain gene, were up-regulated, whereas cardioprotective genes, such as heat shock proteins, were down-regulated (Table S1). Among the genes analyzed, *Alox15* encoding the protein 12/15-LOX was most markedly up-regulated in failing hearts compared with control hearts (Fig. 1 A). Northern blot analysis confirmed that the messenger RNA (mRNA) for this gene was strikingly elevated in heart failure (Fig. 1 B). Immunohistochemistry showed that expression of 12/15-LOX was specifically up-regulated in cardiomyocytes of failing hearts (Fig. 1 C).

To determine whether increased expression of 12/15-LOX could cause heart failure, we established *Alox15* transgenic mice in which expression of the murine *Alox15* gene was under the control of the α-cardiac myosin heavy chain promoter. We obtained two lines of transgenic mice, both of which showed an ∼10-fold increase in the myocardial expression of 12/15-LOX compared with their WT littermates (Fig. 2 A; and Fig. S1, A and B). Histological examination also indicated that the transgenic mice showed increased myocardial expression of 12/15-LOX (Fig. 2 B and Fig. S1 C). Consequently, production of 12(S)-HETE and 15(S)-HETE was significantly increased in the hearts of *Alox15* transgenic mice (Fig. 2 C). The left ventricular diastolic dimension (LVDd) was increased and left ventricular fractional shortening (FS) was decreased in *Alox15* transgenic mice from 26 wk of age compared with their WT littermates (Fig. 2 D). These changes observed in the transgenic animals showed further progression with aging (Fig. 2 D). Histological examination revealed that cardiac fibrosis was increased in *Alox15* transgenic mice and that this fibrosis also progressed with advancing age and was associated with infiltration of macrophages (Fig. 2, E and F). There was no difference in blood pressure between *Alox15* transgenic mice and their WT littermates at 16 or 48 wk of age (Fig. S1 D). The cardiac changes were similar in two independent lines of *Alox15* transgenic mice, suggesting that increased expression of 12/15-LOX might cause heart failure by inducing myocardial inflammation.

### 12/15-LOX induces cardiac inflammation

To investigate the mechanism by which cardiac infiltration of macrophages was increased in *Alox15* transgenic mice, we examined the expression of various proinflammatory cytokines that are thought to be macrophage chemoattractants by the ribonuclease protection assay. We found that cardiac expression of *Ccl2* (MCP-1) was significantly increased in *Alox15* transgenic mice compared with WT mice (Fig. 3 A). In vitro experiments demonstrated that treatment with 12(S)-HETE increased *Ccl2* expression by cardiac fibroblasts and endothelial cells (Fig. 3, B and C), whereas there was no effect when cardiomyocytes were treated with 12(S)-HETE (Fig. 3 D). Moreover, incubation of COS7 cells with 12(S)-HETE significantly increased the activity of nuclear factor κB, a transcription factor that regulates the induction of proinflammatory cytokines including MCP-1 (Fig. 3 E). In contrast, 12(S)-HETE did not affect the activity of this factor when cells were transfected with a reporter plasmid containing mutant κB binding sites (Fig. 3 E). These results suggest that increased production of 12(S)-HETE by cardiomyocytes

causes up-regulation of MCP-1 in other cells of the heart, thereby leading to accumulation of macrophages.

To investigate the relationship between up-regulation of MCP-1 and heart failure, we examined the effect of MCP-1 inhibition on cardiac dysfunction in *Alox15* transgenic mice. We injected an expression vector encoding mutant human MCP-1 with deletion of N-terminal amino acids (7ND plasmid; Egashira, 2003) or the empty vector (mock) into the thigh muscles of mice every 2 wk until 48 wk of age. The result was a significant increase in the blood level of 7ND and elevation of plasma human MCP-1 (Fig. 4 A and Fig. S2). This mutant MCP-1 binds to the MCP-1 receptor (chemokine receptor 2) and inhibits downstream signaling (Egashira, 2003). Consequently, injection of the 7ND plasmid has been reported to suppress MCP-1 activity in vivo and inhibit the development of atherosclerosis (Ni et al., 2001), as well as inhibiting cardiac remodelling after myocardial infarction (Hayashidani et al., 2003). In agreement with these results,

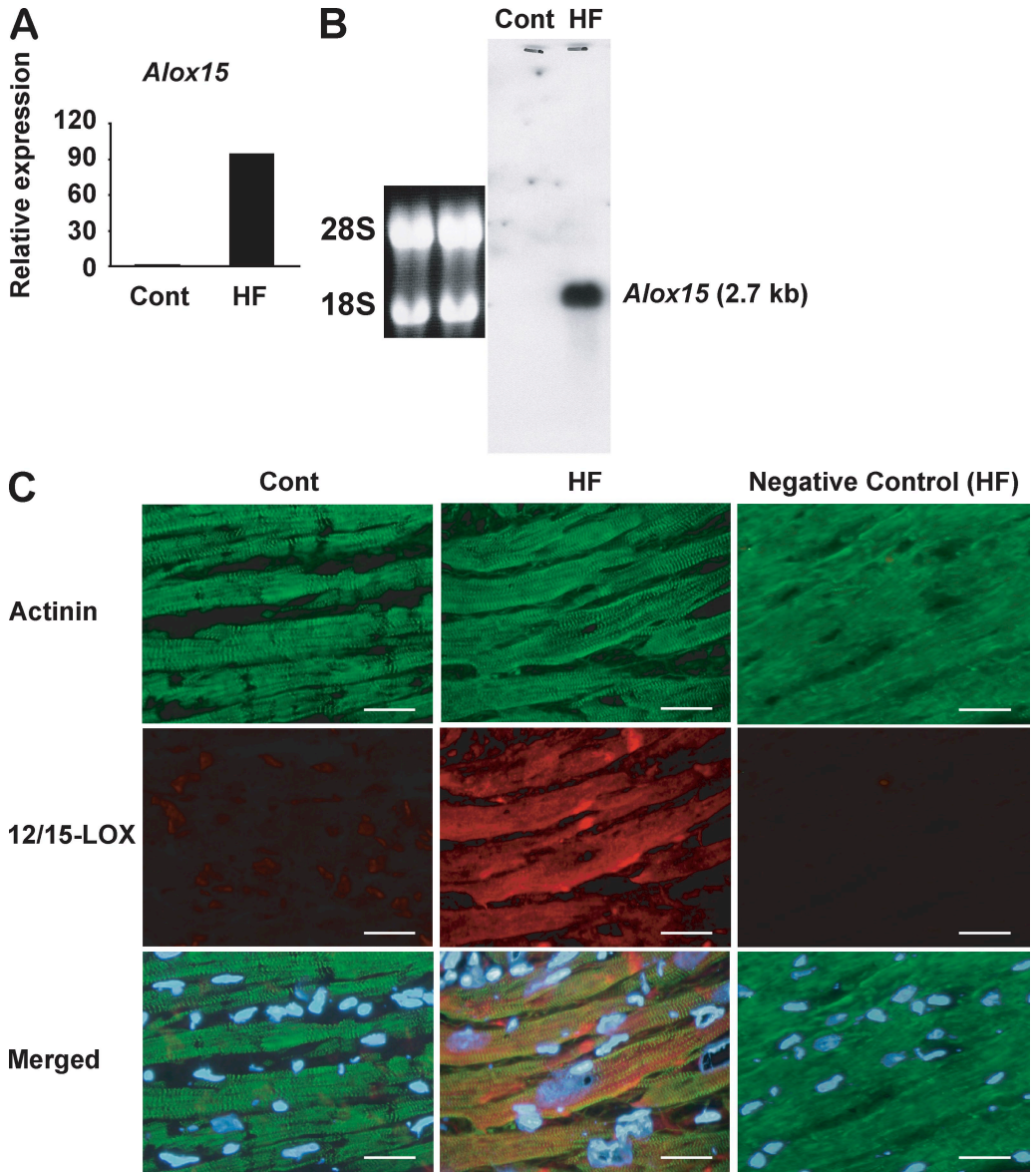


**Figure 1. Expression of 12/15-LOX is up-regulated in the failing heart.** (A) Dahl salt-sensitive rats were fed a low-sodium diet until the age of 6 wk and then a high-sodium diet (8% NaCl) throughout the experimental period. In this model, prominent cardiac hypertrophy developed and left ventricular systolic function was impaired by 17 wk of age. Rats fed a low-salt diet (0.3% NaCl) served as the control. The animals were sacrificed for gene chip analysis at 17 wk of age. Expression of *Alox15* was markedly up-regulated in failing hearts (HF) compared with control hearts (Cont). (B) Northern blot analysis confirmed that the expression of mRNA for *Alox15* was strikingly elevated in failing hearts. (C) Immunohistochemistry for 12/15-LOX in the heart at 17 wk of age. Expression of 12/15-LOX (red) was specifically up-regulated in cardiomyocytes (green) of failing hearts. Nuclei were stained with DAPI (blue). Bars, 20 µm. Normal rabbit serum was used as a negative control of polyclonal antibody against 12/15-LOX. Results in A are obtained from one experiment. Results in B and C are representative of three independent experiments.

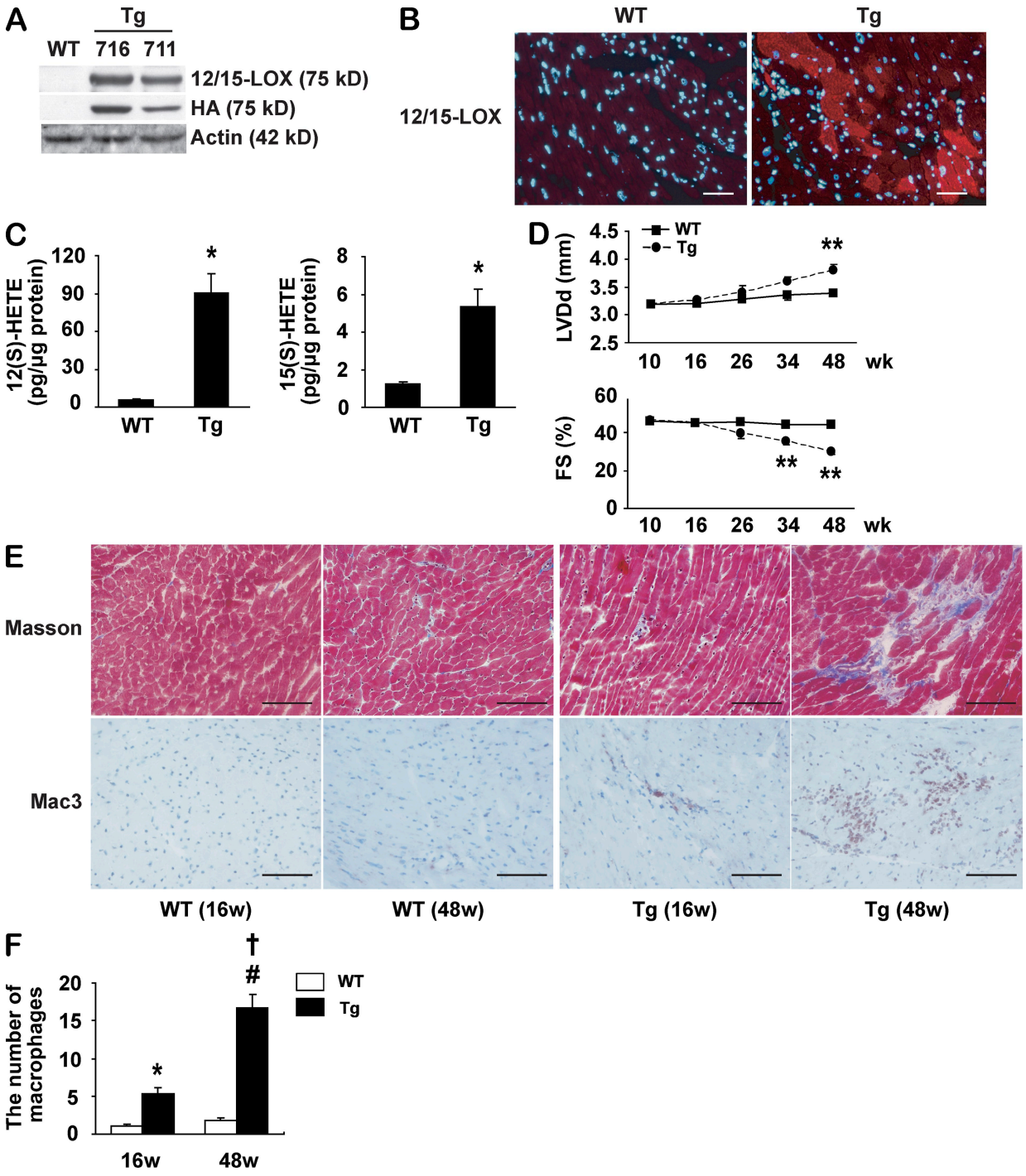


**Figure 2. Increased expression of 12/15-LOX causes heart failure.** (A) Western blot analysis of 12/15-LOX expression in the hearts of WT and *Alox15* transgenic (Tg) mice using anti–12/15-LOX antibody (12/15-LOX) or anti-HA antibody (HA). (B) Immunohistochemistry for 12/15-LOX (red) in the hearts of WT and *Alox15* transgenic mice. Nuclei were stained with DAPI (blue). Bars, 40 μm. Results in A and B are representative of three independent experiments. (C) 12/15(S)-HETE levels in the hearts of WT and *Alox15* transgenic mice. (D) Echocardiographic findings in WT and transgenic mice. The LVDd was increased and left ventricular FS was decreased in *Alox15* transgenic mice compared with their WT littermates. These changes observed in the transgenic animals showed progression with aging. *, $P < 0.05$; **, $P < 0.01$ versus WT. Results in C and D represent the mean ± SEM of three independent experiments. C, $n = 6$; D, $n = 14$. (E) Masson trichrome staining (top) and immunohistochemistry for Mac3 (bottom) in the hearts of WT and transgenic mice at the ages of 16 wk (16w) and 48 wk (48w). Cardiac fibrosis was increased in *Alox15* transgenic mice, and this fibrosis progressed with advancing age and was associated with infiltration of macrophages. Bars, 100 μm. Results are representative of three independent experiments. (F) The number of Mac3-positive cells in the hearts of WT and transgenic mice at the ages of 16 wk (16w) and 48 wk (48w). *, $P < 0.01$ versus WT (16w); #, $P < 0.01$ versus WT (48w); †, $P < 0.01$ versus transgenic (16w). Results represent the mean ± SEM of three independent experiments; $n = 7$.

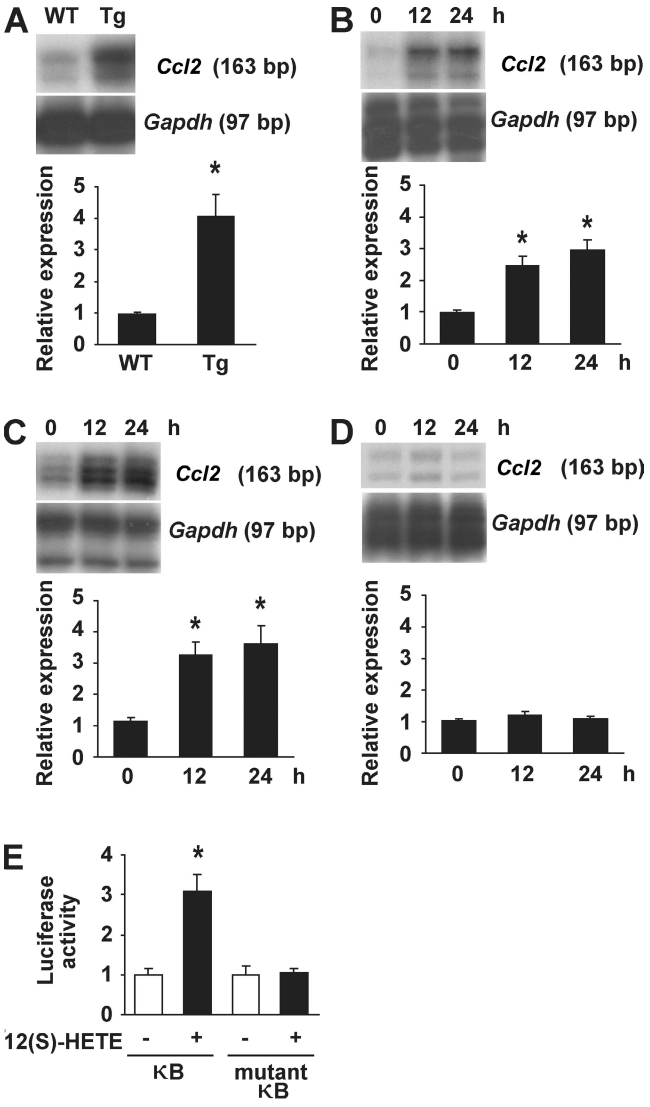


**Figure 3. 12/15-LOX up-regulates MCP-1 expression.** (A) Expression of *Ccl2* (MCP-1) was examined in the hearts of WT and *12/15-LOX* transgenic (Tg) mice by the ribonuclease protection assay. The graph indicates relative expression of *Ccl2*. Cardiac expression of *Ccl2* was significantly greater in *Alox15* transgenic mice than in WT mice. *, $P < 0.05$ versus WT. Results represent the mean ± SEM of three independent experiments; $n = 6$. (B–D) Cardiac fibroblasts (B), endothelial cells (C), and cardiomyocytes (D) were treated with $5 \times 10^{-7}$ M 12(S)-HETE for the indicated times (0–24 h), and expression of *Ccl2* was examined by the ribonuclease protection assay. Graphs display relative expression of *Ccl2*. Incubation with 12(S)-HETE increased *Ccl2* expression by cardiac fibroblasts and endothelial cells. *, $P < 0.01$ versus time 0. Results represent mean ± SEM of four independent experiments; $n = 4$ for B and C; $n = 7$ for D. (E) The luciferase reporter gene plasmid containing the κB binding site was transfected into COS7 cells, which were cultured in the absence or presence of $5 \times 10^{-7}$ M 12(S)-HETE. The luciferase assay was performed 12 h later. A reporter plasmid containing the mutant κB binding site was used as the negative control. Incubation of cells with 12(S)-HETE significantly increased the activity of nuclear factor κB. *, $P < 0.01$ versus 12(S)-HETE (−)/κB. Results represent the mean ± SEM of five independent experiments; $n = 6$.

histological examination and echocardiography demonstrated that injection of this plasmid reduced the myocardial infiltration of macrophages in *Alox15* transgenic mice, as well as preventing systolic dysfunction and left ventricular dilatation (Fig. 4, B and C). These results suggested that 12/15-LOX induces cardiac dysfunction by up-regulation of MCP-1 expression in the heart.

### Cardiac expression of 12/15-LOX is up-regulated during pressure overload

To further investigate the role of 12/15-LOX in heart failure, we examined its cardiac expression in WT mice with severe transverse aortic constriction (TAC). In this model, cardiac hypertrophy gradually progresses to reach a peak on day 7 after TAC and then decreases afterward (not depicted). FS was preserved until day 7 but was significantly decreased on day 14 along with left ventricular dilatation (Fig. 5 A). Cardiac expression of *Alox15* was significantly up-regulated after TAC (Fig. 5 B), and the production of both 12(S)-HETE and 15(S)-HETE was increased in the heart (Fig. 5 C). Histological examination demonstrated an increase in the expression of 12/15-LOX by cardiomyocytes after TAC (Fig. 6 A and Fig. S3).

We next created *Alox15*-deficient mice with TAC and compared them to WT TAC mice. The increase of 12(S)-HETE and 15(S)-HETE production after TAC was markedly attenuated by disruption of *Alox15* (Fig. 6). Disruption of *Alox15* also significantly improved systolic dysfunction and prevented left ventricular dilatation in the presence of chronic pressure overload without any change of blood pressure (Fig. 5 A and Fig. S4), indicating that 12/15-LOX has an important role in the induction of cardiac dysfunction by pressure overload. To examine whether *Alox15* deficiency could inhibit cardiac inflammation, we assessed the expression of *Ccl2* and a macrophage marker (Cd68) in the heart after TAC. Expression of both genes was increased by about threefold at 14 d after TAC. The increase of *Ccl2* and *Cd68* expression was significantly inhibited by disruption of *Alox15* (Fig. 6 C), suggesting that this gene has a crucial role in the development of heart failure by promoting cardiac inflammation.

## DISCUSSION

We demonstrated a crucial role of 12/15-LOX–induced inflammation in the development of heart failure. Activation of this enzyme has been shown to promote neuronal death, whereas inhibition of 12/15-LOX protects against brain damage caused by oxidative stress or ischemia by inhibiting neuronal death (Lebeau et al., 2004; Jin et al., 2008; Seiler et al., 2008). In contrast, treatment with 12(S)-HETE does not induce the apoptosis of cultured cardiomyocytes (unpublished data). Indeed, few apoptotic cardiomyocytes were detected in the hearts of *Alox15* transgenic mice even after the onset of systolic dysfunction (unpublished data). Instead, these mice showed an increase of macrophages infiltrating into the myocardium, which was associated with cardiac fibrosis and systolic dysfunction. Our findings suggested that MCP-1 may

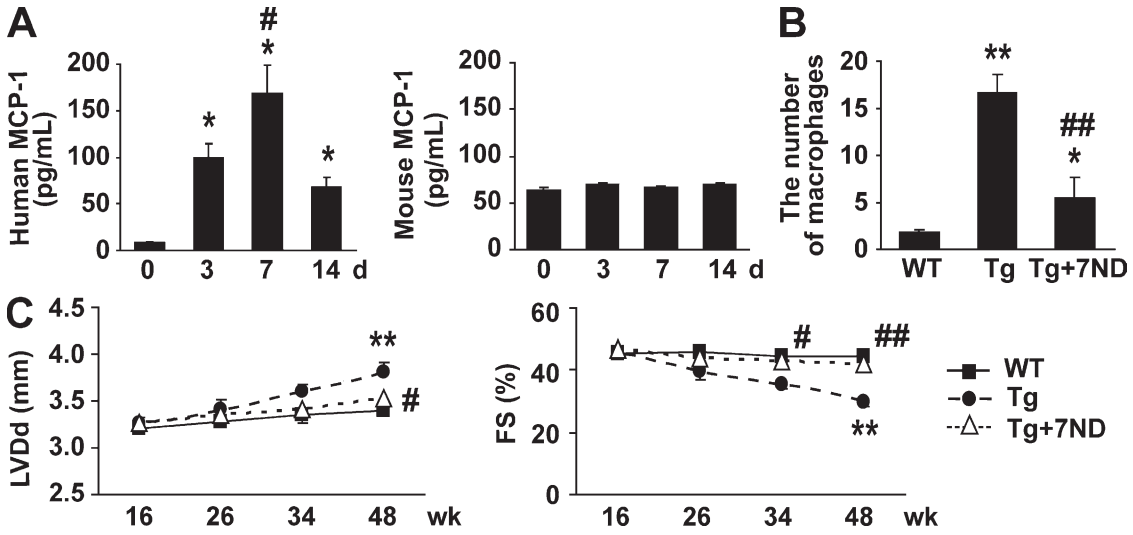


**Figure 4. Inhibition of MCP-1 prevents cardiac dysfunction in *Alox15* transgenic animals.** (A) The plasma levels of 7ND (human MCP-1) and murine MCP-1 were determined by ELISA at the indicated times after introduction of the 7ND expression vector. The plasma level of human MCP-1 was significantly increased after injection of the 7ND plasmid. *, $P < 0.01$ versus day 0; #, $P < 0.01$ versus day 3. Results represent the mean ± SEM of three independent experiments; $n = 5$. (B) Number of Mac3-positive cells in the hearts of WT mice, transgenic (Tg) mice, and transgenic mice treated with 7ND (Tg + 7ND). Injection of the 7ND plasmid reduced the myocardial infiltration of macrophages in *Alox15* transgenic mice. (C) Echocardiographic findings in WT mice, transgenic mice, and transgenic mice treated with 7ND (Tg + 7ND). Injection of the 7ND plasmid prevented systolic dysfunction and left ventricular dilatation in *Alox15* transgenic mice. *, $P < 0.05$; **, $P < 0.01$ versus WT; #, $P < 0.05$; ##, $P < 0.01$ versus transgenic. Results represent mean ± SEM of three independent experiments. B, $n = 7$; C, $n = 10–14$.

have a major role in promoting cardiac inflammation in *Alox15* transgenic mice because its inhibition almost completely abolished the accumulation of macrophages and prevented systolic dysfunction. We also showed that 12/15-LOX induces up-regulation of MCP-1 expression in the setting of pressure overload, thereby increasing cardiac inflammation and leading to systolic dysfunction. Consistent with our findings, inhibition of MCP-1 has been reported to attenuate

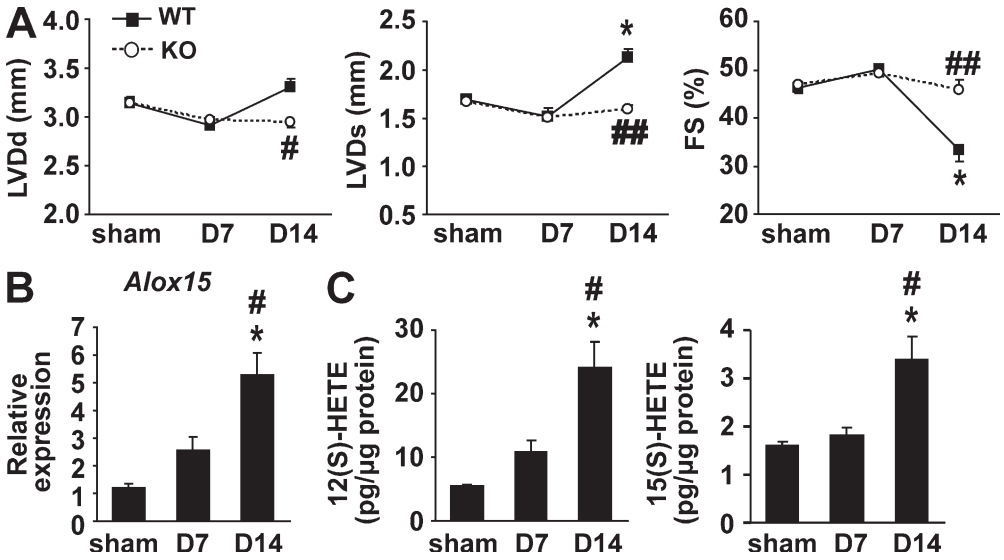


**Figure 5. Cardiac expression of 12/15-LOX is up-regulated during pressure overload.** (A) Echocardiographic findings in WT and *Alox15*-deficient (KO) mice on day 7 (D7) and day 14 (D14) after TAC surgery. FS was preserved until day 7 but was significantly decreased on day 14 along with left ventricular dilatation in WT mice. Disruption of *Alox15* (KO) significantly improved systolic dysfunction and prevented left ventricular dilatation caused by chronic pressure overload. LVDs, left ventricular systolic dimension; sham, sham operation. *, $P < 0.01$ versus sham; #, $P < 0.05$; ##, $P < 0.01$ versus WT. Results represent the mean ± SEM of three independent experiments; $n = 10$. (B and C) *Alox15* expression (B) and the 12/15(S)-HETE level (C) were examined in the hearts of WT mice on day 7 (D7) and day 14 (D14) after TAC surgery by real-time PCR and ELISA, respectively. Cardiac expression of 12/15-LOX was significantly up-regulated after TAC, and production of both 12(S)-HETE and 15(S)-HETE was increased in the heart. *, $P < 0.01$ versus sham; #, $P < 0.01$ versus D7. Results represent the mean ± SEM of three independent experiments; $n = 6$.

myocardial inflammation, fibrosis, and cardiac dysfunction induced by chronic pressure overload (Kuwahara et al., 2004). It has also been reported that transgenic animals with cardiac expression of MCP-1 develop myocardial fibrosis and systolic dysfunction (Kolattukudy et al., 1998). In agreement with our in vitro data, it has been reported that MCP-1 expression is up-regulated in vascular endothelial cells and fibroblasts by pressure overload (Kuwahara et al., 2004). Collectively, these

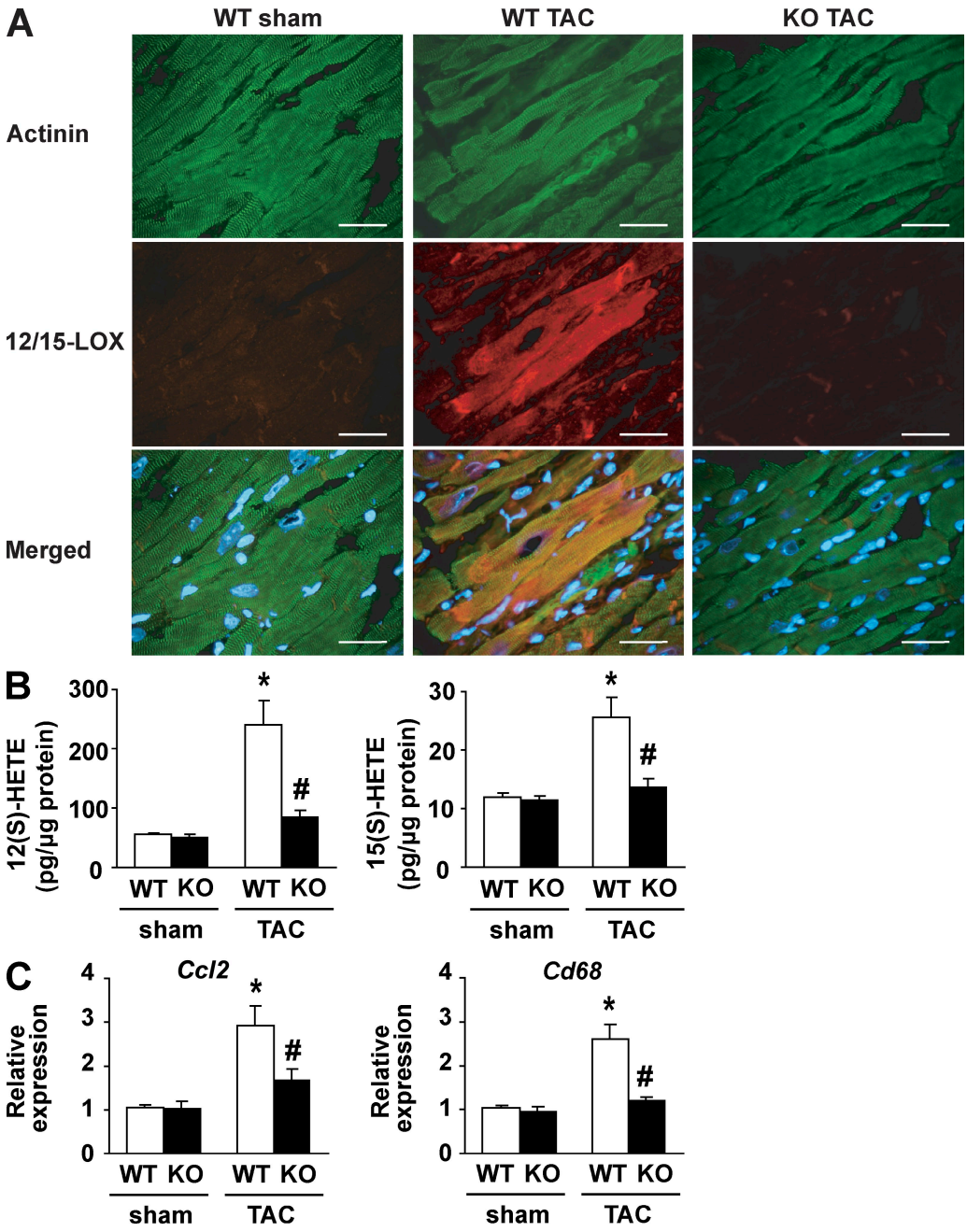


**Figure 6. Disruption of *Alox15* attenuates cardiac inflammation during pressure overload.** (A) Double immunostaining for 12/15-LOX (red) and actinin (green) in the hearts of sham-operated WT mice (WT sham), WT mice with TAC (WT TAC), and *Alox15*-deficient mice with TAC (KO TAC). Increased expression of 12/15-LOX was observed in cardiomyocytes after TAC in WT mice but not KO mice. Nuclei were stained with DAPI (blue). Bars, 20 µm. Results are representative of four independent experiments. (B) 12/15(S)-HETE levels were examined in the hearts of WT and *Alox15*-deficient (KO) mice after sham surgery or TAC. The increase of 12(S)-HETE and 15(S)-HETE production after TAC was markedly attenuated in *Alox15*-deficient mice (KO). *, $P < 0.01$ versus WT sham; #, $P < 0.01$ versus WT TAC. Results represent the mean ± SEM of three independent experiments; $n = 6–8$. (C) Expression of *Ccl2* (MCP-1) and *Cd68* was examined in the hearts of WT and *Alox15*-deficient (KO) mice after sham surgery or TAC. Expression of both genes was increased by about threefold at 14 d after TAC. This increase of expression was significantly inhibited by disruption of *Alox15*. *, $P < 0.01$ versus WT sham; #, $P < 0.01$ versus WT TAC. Results represent the mean ± SEM of three independent experiments; $n = 6–8$.

results indicate that chronic pressure overload increases the expression of 12/15-LOX, which then causes heart failure by promoting cardiac inflammation and fibrosis.

Target gene disruption or overexpression of 12/15-LOX in mice with a genetic background of apolipoprotein E or low-density lipoprotein receptor deficiency has shown that this enzyme may have a role in atherogenesis. The data indirectly support a role for 12/15-LOX in the oxidative modification of low-density lipoprotein. Consistent with our results, recent evidence suggests that 12/15-LOX plays a crucial role in the regulation of proinflammatory molecules and that this regulatory activity of 12/15-LOX may be important for linking 12/15-LOX activation to atherogenesis. For example, 12(S)-HETE increases the expression of MCP-1, interleukin 6, tumor necrosis factor α, and adhesion molecules by macrophages and vascular cells (Bolick et al., 2005, 2006; Wen et al., 2007, 2008; Dwarakanath et al., 2008), and these changes are partly mediated by activation of nuclear factor κB (Bolick et al., 2005, 2006; Dwarakanath et al., 2008). Disruption of 12/15-LOX has also been shown to attenuate airway allergic inflammation by modulating the expression of proinflammatory cytokines (Andersson et al., 2008).

The mechanism of 12/15-LOX activation in the failing heart is unclear. We previously demonstrated that mismatch between the number of capillaries and the size of cardiomyocytes occurs during the development of cardiac hypertrophy, leading to myocardial hypoxia and systolic dysfunction (Sano et al., 2007). Because exposure of cultured cardiomyocytes to hypoxia up-regulates 12/15-LOX expression (unpublished data), a hypoxic state might be one reason for the induction of 12/15-LOX in the failing heart. This concept is supported by previous results that hypoxia up-regulates 12/15-LOX expression in the lungs and the brain (Bernaudin et al., 2002; Zhu et al., 2003a). Moreover, we have found that 12/15-LOX expression is significantly up-regulated in the heart after myocardial infarction (unpublished data). There are putative binding elements for CCAAT/enhancer binding proteins and nuclear factor κB within the promoter region of the *Alox15* gene (unpublished data), and both of these molecules are known to be activated by hypoxia (Cummins and Taylor, 2005).

Inflammation has an important role in the pathogenesis and progression of many forms of heart failure, and biomarkers of inflammation have become the subject of intense investigation. In the Framingham Heart Study, an increase of C-reactive protein (as well as inflammatory cytokines such as interleukin 6 and tumor necrosis factor α) was found to identify asymptomatic older persons in the community with a high risk of developing heart failure in the future (Braunwald, 2008). Multivariate analysis has shown that an increase of C-reactive protein is an independent predictor of adverse outcomes in patients with acute or chronic heart failure (Anand et al., 2005), suggesting that heart failure is closely associated with systemic inflammation. Because metabolites of 12/15-LOX may have a role in vascular inflammation, insulin resistance, and renal dysfunction (Natarajan and Nadler, 2004; Kuhn and O'Donnell, 2006), activation of 12/15-LOX in the failing heart could induce systemic inflammation and have a detrimental effect on other inflammatory diseases such as atherosclerosis, metabolic syndrome, and nephropathy. Conversely, inhibition of 12/15-LOX could be an attractive new strategy for the treatment of heart failure, as well as various other inflammatory conditions.

## MATERIALS AND METHODS

**Animal models.** All of the experimental protocols were approved by Chiba University review board. Male Dahl salt-sensitive (DS) rats were purchased from SLC. The rats were fed a low-sodium diet until the age of 6 wk and then a high-sodium diet (8% NaCl) throughout the experimental period. In this model, marked cardiac hypertrophy developed and left ventricular systolic function was impaired at 17 wk of age. Accordingly, DS rats were sacrificed for gene chip analysis at 17 wk. All of the DS rats given a high-sodium diet showed signs of heart failure such as rapid and labored respiration and diffuse left ventricular hypokinesis on echocardiography at the time of sacrifice. Other DS rats were fed a low-salt diet (0.3% NaCl) as a control group.

We generated transgenic mice on a C57BL/6 background that expressed 12/15-LOX in cardiomyocytes under the control of the α–cardiac myosin heavy chain (α-MHC) promoter. A mouse *Alox15* complementary DNA (cDNA) fragment (gift from C.D. Funk, University of Pennsylvania, Philadelphia, PA) fused with the HA tag was subcloned into the α-MHC promoter vector. The transgene was identified by genomic PCR with transgene-specific oligonucleotide primers (5′-CCACACCAGAAATGACAGAC-3′ and 5′-GCGGGCAGGGAGACAAGTAG-3′) and by Southern blot analysis. Two independent lines of *Alox15* transgenic mice (lines 711 and 716) were obtained. The cardiac phenotype was similar in both lines of transgenic animals. WT littermates were used as the control for all experiments.

*Alox15*-deficient mice on a C57BL/6 background were purchased from The Jackson Laboratory. WT littermates served as a control for all experiments. TAC was performed as described previously (Sano et al., 2007) on 10–11-wk-old male mice. Sham-operated mice underwent the same procedure without aorta constriction.

An expression vector encoding mutant human MCP-1 with deletion of N-terminal amino acids (7ND plasmid) was prepared as described elsewhere (Hayashidani et al., 2003). Under anesthesia, mice received an injection of 100 μg of either the empty vector or the 7ND plasmid in PBS into the bilateral tibial muscles using a 27-gauge needle fitted with a plastic collar that limited muscle penetration to ∼5 mm. Injection was performed every 2 wk from 10 wk until 48 wk of age. To increase the efficiency of gene transfection, 100 μl of the myotoxic agent bupivacaine (0.25% wt/vol) was injected into the muscles 3 d before transfection. Transfection of 7ND leads to an increase of mutant MCP-1 in the blood, as indicated by elevation of its plasma concentration after 14 d. The circulating mutant MCP-1 binds to the receptor for MCP-1 (chemokine receptor 2) on target cells and effectively blocks MCP-1 signaling (Ni et al., 2001; Hayashidani et al., 2003).

**Physiological and histological analysis.** Echocardiography was performed with a Vevo 770 High Resolution Imaging System (Visual Sonics Inc.). To minimize variation of the data, the heart rate was ∼500-600 beats per minute when cardiac function was assessed. The peak systolic blood pressure was recorded by a photoelectric pulse devise (Blood Pressure Meter BP-98A; Softron Co. Ltd.) placed on the tails of unanesthetized mice. Under anesthesia, a micropressure transducer with an outer diameter of 0.42 mm (Samba 201 control unit and Samba Preclin 420 transducer; Samba Sensors AB) was introduced into the right carotid artery. Pressure signals were recorded with a MacLab 3.6/s data acquisition system (AD Instruments) at a sampling rate of 2,000 Hz. 4-μm frozen cross sections of the heart were fixed in 4% paraformaldehyde and subjected to Masson trichrome staining or immunohistochemistry for Mac3 (BD). Digital photographs were taken at 400× magnification of 25 random fields from each heart, and the number of Mac3-positive cells was counted in each field. The frozen cardiac cross sections

were also stained with antibodies for 12/15-LOX (Cayman Chemical) and actinin (Sigma-Aldrich).

**DNA chip analysis.** 10 μg of total RNA was extracted from the left ventricles of rats by the Li-Urea method and was used to synthesize biotin-labeled cRNA, which was then hybridized to a high-density oligonucleotide array (Gene Chip U34A array; Affymetrix) according to the previously published protocol (Ishii et al., 2000). The array contains probe sets for ∼8,800 genes and ESTs, which were selected from Build 34 of the UniGene Database (created from GenBank 107/dbEST 11/18/98). GeneChip 3.3 software (Affymetrix) was used to calculate the mean difference for each probe on the array, which showed the intensity of gene expression defined by Affymetrix using their algorithm. The mean difference has been shown to quantitatively reflect the abundance of a particular mRNA in a population. The data were deposited in GEO (GSM406556, GSM406557, and GSE16199).

**RNA analysis.** Total RNA was isolated from the hearts of mice with RNAZol-B (Molecular Research Center, Cincinnati, OH) and the ribonuclease protection assay (RiboQuant; BD) was performed according to the manufacturer's instructions. For Northern blot analysis, 30 μg of total RNA was separated on formaldehyde denaturing gel and transferred to a nylon membrane (GE Healthcare). Then the blot was hybridized with radiolabeled *Alox15* cDNA probe using Quickhyb hybridization solution (Agilent Technologies) according to the manufacturer's instructions. Rat *Alox15* cDNA fragment was a gift from T. Yoshimoto (Kanazawa University Graduate School of Medical Science, Kanazawa, Japan). Mouse *Alox12* cDNA fragment was a gift from C.D. Funk. Real-time PCR was performed using a LightCycler (Roche) with the Taqman Universal Probe Library and the Light Cycler Master (Roche) according to the manufacturer's instructions.

**Western blot analysis.** Whole cell lysates were prepared in lysis buffer (10 mM Tris-HCl, pH 8, 140 mM NaCl, 5 mM EDTA, 0.025% $NaN_3$, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 50 mM NaF, and 1 mM $Na2VO_3$). 40–50 μg of the lysates were resolved by SDS-PAGE (PAGE). Then proteins were transferred to a nitrocellulose membrane (GE Healthcare), which was incubated with the primary antibody, followed by anti–rabbit or anti–mouse immunoglobulin G conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories). Specific proteins were detected by using enhanced chemiluminescence (GE Healthcare). The primary antibodies used for Western blotting were as follows: anti-HA antibody (Santa Cruz Biotechnology, Inc.), anti–12/15-LOX antibody (Cayman Chemical), and anti-actin antibody (Sigma-Aldrich). ELISA was performed according to the manufacturer's instructions to examine the levels of 12(S)-HETE, 15(S)-HETE (Assay Designs), human MCP-1, and mouse MCP-1 (Invitrogen).

**Cell culture.** Neonatal Wistar rats were purchased from Takasugi Experimental Animal Supply. Cardiomyocytes and cardiac fibroblasts were prepared from these neonatal rats and cultured as described previously (Sano et al., 2007). Human umbilical vein endothelial cells (BioWhittaker; Lonza) were cultured according to the manufacturer's instructions.

**Luciferase assay.** 1 μg of the reporter gene plasmid was transfected into COS7 cells at 24 h before the luciferase assay. 0.1 μg of the control vector encoding *Renilla* luciferase was cotransfected as an internal control. The assay was performed using a dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. p55-A2-Luc (the luciferase reporter gene containing the κB binding site) was a gift from T. Fujita (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; Fujita et al., 1993).

**Statistical analysis.** Data are shown as the mean ± SEM. Multiple group comparison was performed by one-way ANOVA, followed by Bonferroni's test for comparison of means. Comparisons between two groups were done with the two-tailed unpaired Student's *t* test or two-way ANOVA. In all analyses, $P < 0.05$ was considered statistically significant.

**Online supplemental material.** Fig. S1 depicts *Alox15* transgenic animal data. Fig. S2 shows MCP-1 levels after treatment with 7ND. Fig. S3 shows a negative control of immunohistochemistry for 12/15-LOX. Fig. S4 shows blood pressure of *Alox15*-deficient mice. Table S1 summarizes the microarray data. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082596/DC1.

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