Activation of Liver-X-Receptor α But Not Liver-X-Receptor β Protects Against Myocardial Ischemia/Reperfusion Injury

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Background—Liver-X-receptors, LXRα (NR1H3) and LXRβ (NR1H2), encode 2 different but highly homologous isoforms of transcription factors belonging to the nuclear receptor superfamily. Whether LXRα and LXRβ subtypes have discrete roles in the regulation of cardiac physiology/pathology is unknown. We determine the role of each LXR subtype in myocardial ischemia/reperfusion (MI/R) injury.

Methods and Results—Mice (wild type; those genetically depleted of LXRα, LXRβ, or both; and those overexpressing LXRα or LXRβ by in vivo intramyocardial adenoviral vector) were subjected to MI/R injury. Both LXRα and LXRβ were detected in wild-type mouse heart. LXRα, but not LXRβ, was significantly upregulated after MI/R. Dual activation of LXRα and LXRβ by natural and synthetic agonists reduced myocardial infarction and improved contractile function after MI/R. Mechanistically, LXR activation inhibited MI/R-induced oxidative stress and nitritative stress, attenuated endoplasmic reticulum stress and mitochondrial dysfunction, and reduced cardiomyocyte apoptosis in ischemic/reperfused myocardium. The aforementioned cardioprotective effects of LXR agonists were impaired in the setting of cardiac-specific gene silencing of LXRα, but not LXRβ subtype. Moreover, LXRα/β double-knockout and LXRα-knockout mice, but not LXRβ-knockout mice, increased MI/R injury, exacerbated MI/R-induced oxidative/nitrative stress, and aggravated endoplasmic reticulum stress and mitochondrial dysfunction. Furthermore, cardiac LXRα, not LXRβ, overexpression via adenoviral transfection suppressed MI/R injury.

Conclusions—Our study provides the first direct evidence that the LXRα, but not LXRβ, subtype is a novel endogenous cardiac protective receptor against MI/R injury. Drug development strategies specifically targeting LXRα may be beneficial in treating ischemic heart disease. (Circ Heart Fail. 2014;7:1032-1041.)

Key Words: apoposis • myocardium • receptors, cytoplasmic and nuclear • reperfusion injury

A cute myocardial infarction is a leading cause of morbidity and mortality worldwide.1 Myocardial ischemia/reperfusion (MI/R) injury, sustained by ischemic myocardium after current reperfusion therapies (including thrombolysis, coronary angioplasty, and coronary bypass surgery), represents an important clinical problem with significant morbidity and mortality.2 Novel pharmacological or molecular interventions mitigating reperfusion injury, adjunctive to current reperfusion therapies, are in great need.

Clinical Perspective on p 1041

Nuclear hormone receptors are a family of transcription regulators involved in diverse physiological functions, such as cell proliferation, apoptosis, tumorigenesis, and angiogenesis. Several members of this superfamily are expressed in the cardiovascular system, pivotally regulating cardiovascular function.3-6 Liver-X-receptors (LXRs) are ligand-activated transcriptional factors belonging to the nuclear receptor superfamily. The 2 known receptor subtypes, LXRα (NR1H3) and LXRβ (NR1H2), exhibit different expression patterns and may perform different functional roles.7 In contrast to LXRβ, a subtype ubiquitously expressed in all cell types, LXRα is selectively expressed in metabolically active tissues (such as liver, kidney, adipose, and intestine).7 As such, LXRα is traditionally viewed as a metabolic regulatory factor. Recent evidence suggests that LXRα/LXRβ are both expressed in the cardiovascular system. The importance of LXRs in vascular cells is well documented, and recent evidence implicates different regulatory functions of LXRs and LXRβ subtypes in the pathogenesis of vascular diseases both in vitro and in vivo.8-10 LXRs are also detected in cardiomyocytes and play important roles in several cardiac diseases.11-13 However, the
specific roles of individual LXR subtypes in the regulation of cardiac physiology/pathology are at present unknown. The question of whether LXRα and LXRβ have discrete roles in cardiac physiology/pathology is particularly important because current strategies for LXR-targeted drugs are focused on development of tissue- and subtype-selective ligands.

In this study, we determined the regulatory role of 2 different LXR isoforms in the pathogenesis of MI/R-induced myocardial injury, using mice genetically depleted of LXRs (LXRα, LXRβ, or both) and in vivo intramyocardial adenovirus-mediated LXRα or LXRβ overexpression.

**Methods**

This investigation conformed to the National Institutes of Health Guidelines on the Use of Laboratory Animals and was approved by the Institute’s Animal Ethics Committee. Experiments were performed on wild-type (WT) C57BL/6 and LXR-deficient (LXRα-, LXRβ-, or LXRαβ-double-knockout) male mice. Pharmacological experiments used LXR endogenous agonist 22(R)-hydroxycholesterol [22(R)-HC] and synthetic agonist GW3965.14,15 In the acute MI/R protocol, reperfusion commenced for 24 hours after 30 minutes of ischemia. Mice were randomly assigned to the following groups: sham, vehicle, 22(R)-HC (20 mg/kg), or GW3965 (20 mg/kg) by intraperitoneal injection 15 minutes before reperfusion. To observe the long-term cardioprotective effect of LXRαβ dual agonists, mice were randomly assigned to one of the following groups: sham, vehicle, 22(R)-HC (20 mg/kg), or GW3965 (20 mg/kg) by intraperitoneal injection 15 minutes before reperfusion and daily after reperfusion for 4 weeks. Other experiments were designed for MI/R outcome determination. LXRαβ-endogenous agonist was achieved by adenoviral-encoded LXRαβ transfection. In vivo cardiac gene overexpression was achieved by adenoviral-encoded LXRα or LXRβ transfection. An expanded Methods appears in the Data Supplement.

**Statistical Analysis**

All values in the text and figures are presented as the mean±SEM of independent experiments from given n sizes. Statistical analysis was performed with Mann–Whitney test for 2-group comparisons. Statistical significance of multiple treatments was determined by Kruskal–Wallis tests followed by Dunn post hoc test. Probabilities of 0.05 or less were considered to be statistically significant (2 tailed).

**Results**

Both LXRα and LXRβ Are Expressed in Adult Heart Tissue, But LXRα Is Selectively Upregulated by Ischemia/Reperfusion

Both LXRα and LXRβ subtypes were detected in cardiac tissue, as demonstrated by both Western blot (Figure 1A) and real-time polymerase chain reaction (Figure 1B), albeit to lesser degree in comparison with those in the liver. More importantly, endogenous LXRα protein level significantly increased after reperfusion in the ischemic risk area, whereas LXRβ expression remained mostly unaffected (Figure 1C and 1D).

![Figure 1. Expression of liver-X-receptors (LXRs) in heart tissue from control and mice subject to myocardial ischemia/reperfusion (MI/R).](image)
LXRα/β Dual Agonists Inhibit MI/R-Induced Myocardial Apoptosis, Infarct Size, and Cardiac Dysfunction

To determine whether post MI/R upregulation of LXRα mediates myocardial reperfusion injury or acts as a defensive prosurvival signal, mice were treated with vehicle, 22(R)-HC, or GW3965, and MI/R injury determinants were assessed. Treatment with LXR agonists had no significant effect on heart rate or mean arterial blood pressure (Figure I in the Data Supplement). Compared with vehicle, administration of either 22(R)-HC or GW3965 significantly reduced the number of apoptotic nuclei and caspase-3 activity (Figure 2A), decreased infarct size (from 40.2±1.0% in vehicle group to 15.7±1.2% [P=0.01] and 13.0±0.6% [P=0.001] in treated group, Figure 2B), and improved echocardiographic measurements of left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (65.3±4.7% and 34.3±2.8% in the 22(R)-HC group, 60.7±2.6% and 29.2±2.4% in the GW3965 group, versus 39.5±2.1% and 20.7±1.6% in the vehicle group; LVEF: P=0.001, P=0.002 versus vehicle; left ventricular fractional shortening: P=0.002, P=0.04 versus vehicle, respectively; Figure 2C). These data suggest that LXR activation plays an important cardioprotective role in acute MI/R injury.

To determine further the long-term cardioprotective effect of LXR agonists, mice received agonists 15 minutes before reperfusion and then daily after reperfusion for 4 weeks. Viable myocardial metabolism was assessed by fluorodeoxyglucose (18F) micro-positron emission tomography/computed tomography. Cardiac function was determined by echocardiography 4 weeks after reperfusion. MI/R markedly reduced mean myocardial standardized uptake values of fluorodeoxyglucose (18F). LXR agonists-treated mice manifested significantly increased fluorodeoxyglucose (18F) uptake compared with vehicle (2.6±0.2 in the 22(R)-HC group, 2.4±0.2 in the GW3965 group, versus 1.2±0.2 in the vehicle group, P=0.01 and P=0.04 respectively; Figure 3A and 3B). Furthermore, 22(R)-HC or GW3965 treatment significantly increased echocardiographic-measured LVEF compared with vehicle (53.7±4.7% in the 22(R)-HC-treated group or 52.7±5.6% in the GW3965-treated group, compared with 32.8±3.5% in the vehicle group, P=0.01 and P=0.02, respectively; Figure 3C).

**Figure 2.** Liver-X-receptor (LXR)α/β dual agonists inhibited acute myocardial ischemia/reperfusion (MI/R)-induced myocardial apoptosis, infarct size, and cardiac dysfunction. A, Myocardial apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labeling (TUNEL [green], apoptotic nuclei; α-actin [red], myocytes; and 4',6-diamidino-2-phenylindole [DAPI; blue], total nuclei [n=6–8, bar=25 μm, *P=0.02, †P=0.003 vs vehicle]) and caspase-3 activation (n=8–10, *P=0.0006, †P=0.002 vs vehicle). B, Myocardial infarction was determined by Evans blue/ TTC (2,3,5-triphenyltetrazolium chloride) double-staining (n=8–10, *P=0.01, †P=0.001 vs vehicle). C, Cardiac function was determined by echocardiography (n=8–10; ejection fraction: *P=0.001, †P=0.002 vs vehicle; fractional shortening: *P=0.002, †P=0.04 vs vehicle). 22(R)-HC indicates 22(R)-hydroxycholesterol; AAR, area at risk; LVEF, left ventricular ejection fraction; and LVFS, left ventricular fractional shortening.
LXRα/β Dual Agonists Inhibit the Endoplasmic Reticulum Stress- and Mitochondrial-Mediated Apoptosis Pathway

To determine the cellular mechanism by which LXR agonists reduce apoptosis and MI/R injury, the effects of LXR activation on caspase-12 (index of endoplasmic reticulum [ER] stress pathway), caspase-9 (mitochondrial pathway), and caspase-8 (death receptor pathway) activities were analyzed. As expected, all 3 caspases were activated after MI/R. However, administration of LXR agonists significantly inhibited caspase-12 and caspase-9 activity without altering caspase-8 activity (Figure 4A). Moreover, the cleaved caspase-12, CCAAT/enhancer-binding protein homologous protein, cleaved caspase-9, and cytosolic cytochrome c levels were markedly increased in the ischemic/reperfused heart, and significantly inhibited by 22(R)-HC or GW3965 treatment (Figure 4B–4E). Taken together, these results suggest that LXR activation reduces postischemic myocardial apoptosis primarily by inhibiting the apoptotic pathways mediated by ER stress and mitochondria.

LXRα/β Dual Agonists Attenuate Oxidative/Nitrative Stress in Ischemic/Reperfused Myocardium

To determine further the molecular mechanisms underlying the ER- and mitochondrial-mediated protective actions of LXR agonists, we investigated the effects of LXR activation on oxidative/nitrative stress in the ischemic/reperfused myocardium. Both 22(R)-HC and GW3965 attenuated MI/R-induced reactive oxygen species production (Figure 5A and 5B). Furthermore, both LXR agonists inhibited the expression of gp91phox subunit of the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (Figure 5C). Moreover, LXR agonists significantly reduced tissue nitrotyrosine content (a well-accepted footprint of in vivo nitrative stress, Figure 5D and 5E) and inhibited inducible nitric oxide synthase expression (Figure 5F). Collectively, these results demonstrate that LXR activation attenuated oxidative/nitrative stress after MI/R and subsequently inhibited the apoptotic pathways mediated by ER stress and mitochondria.

Cardioprotective Effects of LXRα/β Dual Agonist Are Impaired in Cardiac-Specific LXRα-Knockdown But Not LXRβ-Knockdown Mice

The aforementioned experimental results (MI/R upregulates LXRα but not LXRβ, and nonselective LXR agonists protect against MI/R injury) suggest that LXRα might be the cardioprotective LXR isotype. To obtain direct evidence supporting this notion, cardiac-specific gene silencing of either LXRα or LXRβ was performed before MI/R by in vivo intramyocardial small interfering RNA transfection (Figure IIA in the Data Supplement). The cardioprotective effects of GW3965 were blunted when endogenous cardiac LXRα was knocked out of the mouse genome.
down (Figure IIB and IIC in the Data Supplement). However, GW3965 administration in cardiac LXRβ-knockdown mice remained effective in reducing MI/R induced myocardial apoptosis, decreasing infarct size, and enhancing LVEF (Figure IIB and IIC in the Data Supplement). Finally, knockdown of LXRα, but not LXRβ, mitigated inhibition of caspase-9 and caspase-12 activation (Figure IIIA in the Data Supplement) and the antioxidative/antinitrative effects of GW3965 (Figure IIIB and IIIC in the Data Supplement).

These results demonstrate that the cardioprotective effects of GW3965 may be primarily mediated by the LXRα-receptor subtype. Collectively, these results demonstrate that LXRα subtype activation attenuated oxidative/nitrative stress after MI/R and subsequently inhibited ER- and mitochondrial-mediated apoptosis.

**LXRα/β Double-Knockout and LXRα-Knockout, But Not LXRβ-Knockout, Increase MI/R Injury**

To confirm further the role of the LXRα subtype in the MI/R injury, the effects of LXRα-, LXRβ-, and LXRα/β double-knockout on MI/R injury was investigated. The expression of LXRα and LXRβ subtypes in WT, LXRα-, LXRβ-, and LXRα/β double-knockout mice was measured by Western blot and real-time polymerase chain reaction. LXRα/β dual agonists inhibited endoplasmic reticulum stress- and the mitochondrial-mediated apoptosis pathway. Caspase-12, caspase-9, and caspase-8 activation (A) were measured by cleavage of specific substrates from sham-operated and mice subjected to myocardial ischemia/reperfusion (MI/R). Relative AFC (7-amino-4-trifluoromethyl coumarin) fluorescence is fold-increase activity compared with sham (n=6–8; caspase-12: *P=0.02, §§P=0.002 vs vehicle; caspase-9: †P=0.007, ||P=0.007 vs vehicle; caspase-8: ‡P>0.99, #P=0.96 vs vehicle). Expressions of caspase-12 (B), CCAAT/enhancer-binding protein homologous protein (CHOP; C), caspase-9 (D), and cytosolic cytochrome c (E) proteins in ischemic/reperfused myocardial tissue were determined by Western blot. GAPDH level served as loading control for total protein expression (n=5–6; full length caspase-12: *P=0.003, ‡P=0.03 vs vehicle; cleaved caspase-12: †P=0.01, §§P=0.01 vs vehicle; CHOP: *P=0.008, †P=0.006 vs vehicle; procaspase-9: *P=0.003, ‡P=0.02 vs vehicle; cleaved caspase-9: †P=0.003, §§P=0.04 vs vehicle; Cyt-C: *P=0.02, ‡P=0.002 vs vehicle). 22(R)-HC indicates 22(R)-hydroxycholesterol; and Cyt-C, cytochrome c.

**Cardioprotective Effects of LXRα/β Dual Agonist Are Lost in LXRα/β Double-Knockout and LXRα-Knockout But Not in LXRβ-Knockout Mice**

To obtain more direct evidence supporting cardioprotective effects of LXRα subtype, we determined the effects of LXRα-knockout, LXRβ-knockout, and LXRα/β double-knockout on MI/R and subsequently inhibited ER- and mitochondrial-mediated apoptosis.
LXR agonist-mediated cardioprotection. The antiapoptotic (Figure 8A), infarct sparing (Figure 8B), functional improving (Figure 8C), antioxidative (Figure IV A in the Data Supplement), and antinitrative (Figure IVB in the Data Supplement) effects of LXRα/β dual agonist were abrogated in LXRα/β double-knockout and LXRα−knockout mice, but not in LXRβ−knockout mice.

Cardiac Overexpression of LXRα, But Not LXRβ, Suppresses MI/R Injury

In a final attempt to obtain additional direct evidence supporting LXRα subtype activation being cardioprotective against MI/R injury, either cardiac LXRα or LXRβ overexpression was performed before MI/R by in vivo intramyocardial adenoviral-encoded LXRα or LXRβ (Ad.LXRα or Ad.LXRβ) transfection, respectively. Adenoviral-encoded LacZ (Ad. LacZ) transfection served as control (Figure VA in the Data Supplement). Compared with Ad.LacZ transfection controls, Ad.LXRα overexpression mice exhibited decreased apoptosis, infarct size, and cardiac dysfunction after MI/R (Figure VB and VC in the Data Supplement). However, no significant cardioprotective effects were observed in the cardiac Ad.LXRβ transfection group (Figure VB and VC in the Data Supplement). These data further demonstrated that LXRα, not LXRβ, is the protective subtype against MI/R injury.

Discussion

Our study provides new insights into understanding the discrete role of 2 different LXR subtypes in the heart. The novel contributions include the following: (1) Both LXRα and LXRβ subtypes were detected in adult cardiac tissue, but LXRα is selectively upregulated by ischemia/reperfusion; (2) LXRα/β dual agonists inhibited MI/R-induced infarct size and cardiac dysfunction via inhibition of oxidative/nitrative stress, and subsequently reducing the apoptotic pathways mediated by ER stress and mitochondria; (3) The cardioprotective effects of LXRα/β dual agonist were impaired in the setting of cardiac-specific gene silencing via in vivo intramyocardial transfection of small interfering RNA of LXRα, but not LXRβ, subtype; (4) LXRα/β double-knockout and LXRα-knockout, but not LXRβ-knockout, increased MI/R injury, exacerbated MI/R-induced oxidative/nitrative stress, and ER stress and mitochondrial dysfunction; (5) LXRα/β double-knockout and LXRα-knockout, but not LXRβ-knockout, completely blocked the cardioprotective effects of LXR agonists; and (6) Cardiac LXRα, not LXRβ, overexpression via adenoviral transfection suppressed MI/R injury. In summation, we present the first direct evidence that the LXRα, but not LXRβ, subtype is a novel endogenous cardiac protective receptor against MI/R injury.
LXRs, orphan nuclear hormone receptors originally cloned in liver tissue, function as cholesterol sensors with well-recognized function in regulating cholesterol homeostasis. Two different but highly homologous LXRx isoforms, LXRxα (NR1H3) and LXRxβ (NR1H2), have been discovered separately. The human LXRxα gene is located on chromosome 11p11.2, whereas the human LXRxβ gene is located on chromosome 19q13.3. LXRxα expression predominates in metabolically active tissues such as the liver, kidney, adipose, and intestines, whereas LXRxβ is more ubiquitously expressed with particularly high levels in the developing brain, suggesting differential physiological function regulation for these receptors. Although LXRxα and LXRxβ presence in the cardiovascular system had been identified, little information is available regarding their individual roles in the myocardium. To our knowledge, this study is the first to investigate the specific roles of 2 individual LXRx subtypes in the cardiac physiology/pathology. The results of our study contribute to the growing body of data emphasizing that the distinctively isoform-specific functions of LXRx are mediated by 2 different receptors, by demonstrating that LXRxα, a subtype with previously unappreciated cardiovascular significance, but not LXRxβ, a subtype known to be ubiquitously expressed (including heart), is a novel endogenous cardiac protective receptor against MI/R injury. This conclusion is supported by the following novel evidence. First, the expression of LXRxα (but not LXRxβ) was significantly increased in the heart subjected to MI/R. Second, MI/R injury was significantly increased in LXRxα/β double-knockout and LXRxα-knockout, but not in LXRxβ-knockout mice. Third, genetic ablation (knockout or cardiac-specific knockdown) of LXRxα, but not LXRxβ, blocked the cardio-protective effect of LXRxα/β dual agonists. Fourth, cardiac-specific LXRxα, but not LXRxβ, overexpression suppressed MI/R injury. Our current observations enrich our understanding of the different biological functions of LXRxα and

Figure 6. Liver-X-receptor (LXRα/β) double-knockout (KO) and LXRx-KO mice were more susceptible to myocardial ischemia/reperfusion (MI/R) injury. A, LXRxα and LXRxβ expression in cardiac tissue from wild-type (WT), LXRxα-KO, LXRxβ-KO, and LXRxα/β double-KO mice were determined by Western blot and real-time polymerase chain reaction. B, Myocardial apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labeling (TUNEL [green], apoptotic nuclei; α-actin [red], myocytes; and 4’,6-diamidino-2-phenylindole [DAPI; blue], total nuclei. [n=5, bar=25 μm; *P=0.03, †P>0.99, ‡P=0.04 vs WT]) and caspase-3 activation (n=5–6; *P=0.02, †P>0.99, ‡P=0.03 vs WT). C, MI/R injury was assessed by myocardial infarction size determined by Evans blue/TTC (2,3,5-triphenyltetrazolium chloride) double-staining (n=6; *P=0.007, †P>0.99, ‡P=0.03 vs WT), and left ventricular dysfunction determined by echocardiography (n=6; *P=0.03, †P>0.99, ‡P=0.04 vs WT). D, Cardiac function was determined by left ventricular viable myocardium assessed via fluorodeoxyglucose (18F) (18F-FDG) uptake (mean standardized uptake values [SUV]) by micro-positron emission tomography/computed tomography (n=5–6; *P=0.04, †P>0.99, ‡P=0.01 vs WT), and left ventricular performance determined by echocardiography (n=6; *P=0.04, †P>0.99, ‡P=0.02 vs WT). AAR indicates area at risk; ECHO, echocardiography; and LVEF, left ventricular ejection fraction.
LXRβ in different tissues. The LXRα subtype is the isoform responsible for hepatic lipogenesis, with a dominant role in limiting atherosclerosis in vivo. The LXRβ subtype regulates lipogenesis and cholesterol efflux in skeletal muscle, with antithrombotic effects in human platelets.

Such data concerning tissue- and isoform-specific effects of LXRs are of practical interest in the development of tissue- or isoform-specific LXR pharmacological modulators for treating various pathologies.

To provide mechanistic insight into how LXR activation protected heart against MI/R injury, we investigated the effect of LXR activation on the apoptosis pathway, a significant contributor to myocardial cell death after ischemia/reperfusion injury. Our study demonstrated that during MI/R, all 3 apoptosis pathways (the extrinsic, intrinsic, and ER stress-specific apoptotic pathways) were activated, as shown by the activation of different caspase markers (caspase-8, caspase-9, and caspase-12, respectively). LXR activation attenuated the activation of markers of ER stress (cleaved caspase-12 and CCAAT/-enhancer-binding protein homologous protein) and mitochondrial stress (cleaved caspase-9 and cytochrome c release) without altering cleaved caspase-8 protein levels (the extrinsic apoptotic marker) and its activity. To define the role of the different LXR isoforms in the regulation of cardiomyocyte apoptosis, LXRα-knockout, LXRβ-knockout, and LXRαβ-doublenull knockout mice were subject to MI/R. We observed that, compared with WT mice, ER stress- and mitochondrial-mediated apoptosis was exacerbated after MI/R in LXRαβ-doublenull knockout and LXRα-knockout, but not LXRβ-knockout mice. Furthermore, the ER- and mitochondrial-protective effects of LXR agonist were lost in the setting of genetic ablation of LXRα, but not LXRβ subtype. Thus, LXRα but not LXRβ inhibited the apoptotic pathways mediated by ER stress and mitochondria. Because oxidative/nitrative stress is causatively related to ER stress and mitochondrial dysfunction, we further determined the role of the different LXR isoforms in the regulation of oxidative/nitrative stress. We demonstrated that activation of LXRα, but not LXRβ, significantly decreased myocardial NADPH oxidase expression, attenuated superoxide generation, and reduced both inducible nitric oxide synthase expression and tissue nitrotyrosine content in ischemic/reperfusion myocardium. Taken together, our results demonstrated LXRα subtype protected against MI/R injury via mechanisms inhibiting oxidative/nitrative stresses, subsequently reducing ER- and mitochondrial-mediated apoptosis.

The involvement of LXR in regulating the apoptotic process has been reported in several cell and tissue types. Interestingly, LXR activation was shown to be antiapoptotic in several cell types, and proapoptotic in others. For example, LXR activation inhibited apoptosis in endothelial cells, intestine, and lung tissue but facilitated apoptosis in tumor cells, such as breast, colorectal, and prostate carcinomas. Thus, the role of LXR in inhibiting or promoting apoptosis may be dependent on cell and tissue type.

Heretofore, several members of the nuclear hormone receptor superfamily have reputed involvement in MI/R injury pathophysiology. Peroxisome proliferator-activated receptors (PPAR-α [NR1C1], -β [NR1C2], and -γ [NR1C3]), estrogen receptor (NR3A), and androgen receptor (NR3C4) have been proposed as the endogenous protective receptors against myocardial apoptosis and MI/R injury, while activation of nuclear receptor farnesoid-X-receptor (NR1H4) and...
Nur77 (NR4A1) exacerbates myocardial apoptosis and MI/R injury. Our study adds novel evidence that LXRα (NR1H3) acts as an endogenous protective factor against MI/R injury. Given the distinct regulatory roles of these nuclear receptors in myocardial apoptosis and MI/R injury, it is conceivable that potential regulatory cross talk among them might maintain the delicate homeostatic balance between cellular death and survival in the heart. Of particular interest is a human macrophage study demonstrating PPARγ activation directly increased LXRα expression via PPAR binding to an LXRα promoter site. In a study investigating adipocytes, activated LXRα promoted PPARγ gene expression by directly binding the LXR response element motif in the PPARγ promoter in a positive-feedback loop, such that these 2 nuclear hormone receptors reinforced each other’s expression. Characterization of the molecular cross talk between these nuclear receptor pathways is requisite for better design of novel therapeutics ameliorating MI/R injury.

Conclusions

In summary, our study provides the first evidence that LXRα, a LXR subtype traditionally viewed as a metabolic regulatory factor, has much stronger cardiac phenotype than ubiquitously expressed LXR subtype, LXRβ. LXRα (but not LXRβ) subtype is a powerful cardiac protection receptor against MI/R injury, via mechanisms (at least in part) inhibiting oxidative/nitrative stresses, and subsequently reducing ER- and mitochondrial-mediated apoptosis. LXRα subtype, therefore, represents a potentially attractive molecular target for the treatment of ischemic heart disease.

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Disclosures

None.

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opment strategies specifically targeting LXR for the treatment of ischemic heart disease.α

data body emphasizing the distinctively isoform-specific functions of LXR in cardiac physiology/pathology. LXR subsequently reduce endoplasmic reticulum– and mitochondrial-mediated apoptosis. These results contribute to the growing subtype protects against myocardial ischemia/reperfusion injury via mechanisms inhibiting oxidative/nitrative stresses that

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Reagents and Antibodies

Synthetic LXR ligand 3-[3-[N-(2-Chloro-3-trifluoromethylbenzyl)-(2,2-diphenylethyl)amino]propyloxy]phenylacetic acid hydrochloride (GW3965) was kindly donated by Jon Collins (GlaxoSmithKline, Research Triangle Park, NC). Natural LXR ligand 22(R)-hydroxycholesterol [22(R)-HC], 2,3,5-triphenyltetrazolium chloride (TTC), and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). Dihydroethidium (DHE) and TRIzol Reagent were from Life Technologies (Carlsbad, CA). Utilized antibodies were obtained from the following sources: mouse monoclonal antibody against LXRα (ab41902) and rabbit polyclonal antibody against LXRβ (ab28479) were from Abcam (Cambridge, UK); rabbit anti-mouse nitrotyrosine antibody (06-284) was from Millipore (Billerica, MA); mouse anti-α-actin antibody (Clone 5C5, A2172) was from Sigma-Aldrich; rabbit anti-caspase-12 (#2202), mouse anti-caspase-9 (C9, #9508), mouse anti-CCAAT/enhancer-binding protein homologous protein (CHOP, L63F7, #2895), rabbit anti-cytochrome c (#4272), rabbit anti-voltage-dependent anion channel (VDAC, #4866), and rabbit anti-GAPDH (14C10, #2118) were from Cell Signaling Technology (Beverly, MA); Alexa Fluor® 555 goat anti-mouse IgG antibody (A21422) and Alexa Fluor® 488 goat anti-rat IgG antibody (A-11006) were from Life Technologies; IRDye 800CW goat anti-mouse (926-32210) and anti-rabbit IgG (926-32211) secondary antibodies were from LI-COR Biosciences (Lincoln, NE).

Animals and In-Vivo Experimental Protocols

This investigation conforms to the National Institutes of Health Guidelines on the Use of Laboratory Animals, and was approved by the Institute’s Animal Ethics Committee. LXRα-deficient, LXRβ-deficient, LXRα/β-deficient, and wild-type (WT) C57BL/6 male mice (22-25 g) were obtained from the Jackson Laboratories (Bar Harbor, ME), and were housed at 25±5°C, adherent to a 12 hour light-dark cycle. For the acute myocardial ischemia/reperfusion (MI/R) protocol, reperfusion commenced
for 24 hours following 30 minutes of ischemia, and mice were randomly assigned to the following groups: sham, vehicle, 22(R)-HC (20 mg/kg), or GW3965 (20 mg/kg) by intraperitoneal injection 15 minutes before reperfusion. Pharmacologic dosages were chosen based upon our pilot study data and the published literature.\textsuperscript{1-3} LXRα/β dual agonists at these doses effectively invoked LXR activity without inducing observable hemodynamic changes during in-vivo animal studies.\textsuperscript{1-3} To observe the long-term cardioprotective effect of LXRα/β dual agonists, mice were randomly assigned to the following groups: sham, vehicle, 22(R)-HC (20 mg/kg), or GW3965 (20 mg/kg) by intraperitoneal injection 15 minutes before reperfusion, and daily following reperfusion for 4 weeks. Other experiments were designed for MI/R outcome determination in LXRα-, β-, and α/β double-deficiency mice and their WT littermates.

**Surgical Generation of MI/R Model**

MI/R procedures were performed utilizing a novel method as described in our previous studies.\textsuperscript{4-7} In brief, the heart was manually exposed without intubation via a small thoracic incision, and a slipknot was tied around the left anterior descending coronary artery 2-3 mm from its origin with a 6-0 silk suture. Sham-operated animals were subjected to identical surgical procedures, except that the suture passed beneath the left anterior descending coronary artery was not tied. After 30 minutes of ischemia, the slipknot was released, and myocardial reperfusion commenced. After recovery from surgery, mice were returned to standard animal housing conditions. Surgical mortality of this MI method is very low (<6%), and no difference was observed between any groups investigated.

**In-Vivo siRNA-Mediated Cardiac-Specific Gene Silencing**

In-vivo knockdown of cardiac-specific LXRα or LXRβ expression was achieved by intramyocardial delivery of siRNA.\textsuperscript{8} FlexiTube HP GenomeWide siRNA targeting mouse LXRα (4 siRNAs in FlexiTube Gene Solution for Mm_Nr1h3; Cat. No.SI00208355, SI02692942, SI02715601, and SI02740339) and LXRβ (4 siRNAs in FlexiTube Gene Solution for Mm_Nr1h2; Cat. No. SI00185227,
SI00185234, SI00185241, and SI02735439) were purchased from Qiagen (Hilden, Germany). AllStars Negative Control siRNA (Qiagen, Cat. No. 1027280) served as negative control. This non-silencing control has no homology to any known mammalian gene, and has been validated using Affymetrix GeneChips (Qiagen). FlexiTube LXRα siRNAs, FlexiTube LXRβ siRNAs, or AllStars Negative Control siRNA were complexed with in-vivo Jet-PEI Delivery Reagent (Polyplus-transfection, Illkirch, France) in 5% glucose per manufacturer’s recommendations. Mice were anesthetized with 2% isoflurane, and the heart was exposed via left thoracotomy at the fifth intercostal space. Mouse-specific LXR siRNA or negative control (20 µl; 1 µg/g) was delivered via three separate intramyocardial injections (32.5-gauge needle), temporarily blanching the left ventricular free wall. Our pilot experiments demonstrated cardiac LXR expression reached nadir (~25-30% of control levels) 48 hours after siRNA injection. MI/R protocol was therefore performed 48 hours after intramyocardial siRNA delivery.

**In-Vivo Adenoviral-Mediated Cardiac Gene Overexpression**

The adenoviral-mediated gene delivery was as previously described.⁹ Ad.LXRα, Ad.LXRβ (adenoviruses containing the LXRα or β gene) and Ad.LacZ (negative control) were generated following the instructions of ViraPower Adenoviral Expression System (Life Technologies) per manufacturer protocol. The full-length cDNAs for LXRα or LXRβ was cloned into the pENTR1A vector (Life Technologies) and transferred into the pAd/CMV/V5-DEST vector (Life Technologies) via Gateway LR Clonase II enzyme mix per manufacturer protocol (Life Technologies). The viral titer was determined via Adeno-X Rapid Titer Kit (Clontech Laboratories, Mountain View, CA). The mice were anesthetized with 2% isoflurane, and the heart was exposed via a left thoracotomy at the fifth intercostal space. Adenovirus (5×10⁹ IFU/ml) was administered by direct injection into the left ventricular free wall (three sites, 10 µl/site, 32.5-gauge needle). Three days after adenoviral injection, the mice were subjected to coronary artery occlusion and reperfusion as described above. Myocardial LXR expression was analyzed before the MI/R procedures.
Hemodynamic measurements

Blood pressure and heart rate in conscious mice were measured by a tail-cuff system (BP-2010, Softron, Tokyo, Japan), as described previously. Before study initiation, the animals were adapted to the apparatus for at least 5 days. Multiple measurements generated an average value for each animal, and the mean results of each group were calculated from average values.

Western Blot Analysis

Proteins were prepared per standard protocol, and protein lysate concentrations were determined via Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). To prepare the mitochondrial or cytosolic fractions, protein lysate was collected via Mitochondria Isolation Kit (Thermo Scientific). Equal quantities of proteins (30-50 µg/lane) were submitted to 10 or 12% SDS-PAGE, dependent upon the target proteins, electrotransferred onto nitrocellulose membranes, and incubated with primary antibodies against LXRα (1:1000), LXRβ (1:1000), caspase-12 (1:1000), CHOP (1:1000), caspase-9 (1:1000), and cytochrome c (1:1000). GAPDH and VDAC levels were utilized as loading controls for total and mitochondrial protein expression, respectively. After incubation with the corresponding second antibodies, protein bands were detected by an Odyssey® IR scanner (LI-COR Biosciences, Lincoln, NE). Quantitation was performed via Quantity One 4.4.0 software (Bio-Rad, Hercules, CA).

Real-Time Quantitative PCR

Total RNA was isolated from tissues and cardiomyocytes with TRIzol Reagent and purified with Qiagen’s RNeasy Mini Kit (Qiagen). Reverse transcription was performed by Omniscript RT Kit (Qiagen). The resultant cDNA was amplified by SYBR® Premix Ex Taq™ Perfect Real Time Kit (Takara BIO, Otsu, Japan). The PCR reaction was directly monitored by The LightCycler® 480 Real-Time PCR System (Roche Applied Science, Indianapolis, IN). Utilized SYBR Green real-time PCR
primers were as follows: mouse LXRα (GenBank Accession No. NM001177730 and NM013839), forward 5’-GCTCATTGCCATCAGCATC–3’ and reverse 5’-AGCATCCGTGGGAACATCA-3’; mouse LXRβ (GenBank Accession No. NM009473 and XM_001002072), forward 5’-TGCCAGGGTTCTTGCAAGTGTT-3’ and reverse 5’-AACGTGATGCATTCTGTCTCGT-3’; mouse NADPH oxidase gp91phox subunit (GenBank Accession No. NM_007807.4), forward 5’-TGATCCTGCTGCCAGTGTC-3’ and reverse 5’-GTGAGGTTCCTGTCCAGTTGTCTTC-3’; mouse inducible nitric oxide synthase (iNOS) (GenBank Accession No. NM_010927.3), forward 5’-CAAGCTGAACCTGAGCGAGGA-3’ and reverse 5’-TTTACTCAGTGCCAGAAGCTGGA-3’; mouse GAPDH (GenBank Accession No. BC083149), forward 5’-TGACACGTCAGGCCAGGCTG-3’ and reverse 5’-CTCTGAGCTGACGTGATGG-3’. Real-time PCR data were represented as Ct values, defined as the crossing threshold of PCR, obtained via LightCycler 480 Data Analysis software. Relative mRNA levels of the sample mRNA expression were calculated as described, and expressed as 2 ^ -\Delta\Delta C_t. 

**In-situ Detection of Apoptosis in Heart Tissue**

Myocardial apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) technique via a Fluorescein In Situ Cell Death Detection Kit (Roche Diagnostics) as described previously. Apoptotic nuclei were detected by green fluorescein staining, cardiomyocytes were identified by anti-α-actin antibody, and total cardiomyocyte nuclei were DAPI labeled. A commercial kit (Roche Diagnostics) was utilized for immunohistochemical detection and quantification of apoptosis (brown staining nuclei). Results were expressed as the percentage of apoptotic cells among the total cell population.

**Detection of Caspase Activities in Heart Tissue**

Cardiac caspase-3 activity was measured via caspase-3 Colorimetric Assay Kit (Millipore) as previously described. Briefly, 100 µg of total protein from tissues was loaded and incubated with
25 μg Ac-DEVD-pNA (a colorimetric-specific substrate) at 37°C for 1.5 hours. pNA cleaved from DEVD by caspase-3 was quantified by a microplate reader (BioTek, Winooski, VT) at 405 nm. Changes in caspase activity in the MI/R tissue samples were calculated against the mean value of caspase activity from sham experiment tissue, and expressed as pmol pNA/mg protein. Cardiac activation of caspase-8, caspase-9, and caspase-12 was evaluated utilizing respective caspase Fluorometric Assay Kits (BioVision, Mountain View, CA). Briefly, 100 μg of total protein from tissues per assay and a final concentration of 50 μM of AFC-conjugated with substrates specific for caspase-8, -9, and -12 (IEDT, LEHD, and ATAD) respectively was loaded. Samples were read by a fluorimeter equipped with a 400-nm excitation and a 505-nm emission filter. The activities of caspase-8, -9, and -12 were compared to sham control.

**Determination of Myocardial Infarct Size**

Myocardial infarct size was determined by Evans blue-TTC double staining methods as previously described.4-7 Briefly, the ligature around the coronary artery was re-tied after reperfusion, and 0.2 ml 2% Evans blue dye was injected into the left ventricular cavity. The dye was circulated and uniformly distributed, except in the cardiac regions previously perfused by the occluded coronary artery area-at-risk (AAR). The heart was quickly excised, frozen at -20°C, and sliced into 1 mm thick sections perpendicular to the long axis of the heart. Slices were incubated individually using a 24-well culture plate in 1% TTC solution (pH 7.4) at 37°C for 10 minutes, and photographed digitally. The Evan’s blue-stained area (area not at risk, ANAR), TTC-stained area, and TTC-negative staining area (infarcted myocardium) were measured via computer-based image analyzer SigmaScan Pro 5.0 (Systat Software, Chicago, IL). Myocardial infarct size was expressed as a percentage of the infarct area (I) over AAR (I/AAR); AAR size was expressed as the percentage of AAR over total left ventricular area (AAR/AAR+ANAR), as previously described.4-7 The myocardial infarct size was expressed as a percentage of infarct area over AAR.
Echocardiographic Measurements

In-vivo cardiac function was determined by echocardiography 24 hours and 4 weeks after reperfusion.4-7 Mice were anesthetized with 1.5% isoflurane. Two-dimensional echocardiographic views of the mid-ventricular short axis were obtained at the level of the papillary muscle tips below the mitral valve (Vevo 770, VisualSonic, Toronto, Canada). Left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF) were calculated as previously described.4-7

Fluorodeoxyglucose (18F) Micro-Positron Emission Tomography/Computed Tomography (18F-FDG Micro-PET/CT) Scanning and Analysis

Detection of viable myocardium was performed via micro-PET/CT scanning as previously described.12, 13 The animals were anesthetized with 2% isoflurane in O2 gas, and received 18F-FDG injection (0.1 ml, single intravenous tail vein injection with activity of 10 MBq13). Immediately awakened after injection, the animals were returned to the anesthesia cage. Two hours after administration of the tracer injection, animals were anesthetized with isoflurane, placed prone on the PET/CT scanner bed near the central field of view, and received continuous anesthesia for the study duration. Inveon Acquisition Workplace (IAW) was utilized for the scanning process. Ten-minute static PET scans were acquired and images were reconstructed by a 3-Dimensional Ordered Subsets Expectation Maximum (OSEM3D) algorithm followed by MAP (Maximization/Maximum a Posteriori) or FastMAP provided by IAW. The 3 Dimensional Regions of Interest (3D ROIs) were drawn over the heart guided by CT images and tracer uptake was measured by Inveon Research Workplace (IRW) 3.0 software. Individual quantification of 18F-FDG uptake in each animal was calculated. Mean standardized uptake values (SUV) were determined by dividing the relevant ROI activity by the ratio of the injected dose to body weight as follows:

\[
SUV = \frac{\text{ROI activity (MBq/g)}}{\frac{\text{injected dose (MBq)}}{\text{body weight (g)}}}
\]

Measurement of Oxidative Stress Generation in Heart Tissue
Myocardial reactive oxygen species generation was measured by confocal microscope via in-situ DHE stain or lucigenin-enhanced chemiluminescence. For DHE stain, unfixed frozen cross-sections (5 μm) were incubated with DHE (5 μmol/L) at 37°C for 30 minutes in a humidified chamber protected from light, followed by 5 minutes of PBS washing to remove nonintercalated ethidium bromide molecules. Images were obtained and analyzed via Leica laser scanning confocal microscope (Leica TCS SP5 II). NADPH oxidase activity within the heart homogenates was measured by lucigenin-enhanced chemiluminescence via luminometer, as previously described. The lucigenin concentration in the final reaction mixture was 0.25 mmol/L, and NADPH-dependent superoxide production was expressed as relative light units (RLU) per mg per second (RLU • mg⁻¹ • s⁻¹).

**Determination of Nitrative Stress Generation in Heart Tissue**

Myocardial nitrative stress was assessed by nitrotyrosine content, a footprint of in-vivo peroxynitrite formation, by both immunostaining and ELISA analysis. For immunostaining, paraffin-embedded slices were stained with primary antibody against nitrotyrosine (1:100), and then immunostained by Vectastain ABC kit (Vector Laboratories, Burlingame, CA; 1:200). For ELISA, cardiac tissue nitrotyrosine content was quantified by nitrotyrosine ELISA Kit (Abnova, Taiwan). Results were presented as nanomoles/g protein.

**Analyses of Lipid Profiles in Heart Tissue**

Analyses of lipid profiles was performed via an Agilent 6890 gas chromatography instrument (Santa Clara, CA) with electron Ionization detection as previously described.
Supplemental Results

**LXRα activation alters fatty acid profiles in heart tissue in murine MI/R model.**

It has been reported that LXR activation in heart leads to alteration of fatty acid composition in favor of accumulation of monosaturates. We thus further investigated the effect of LXR activation on the fatty acid profiles in our MI/R murine models. As shown in the Supplementary Table 1, treatment of GW3965 caused a shift in cardiac fatty acid composition in favor of unsaturates in heart tissue in WT mice, as evidenced by increased ratio of unsaturated fatty acid (including 16:1n9, 16:1n7, 18:1n7 and 20:3n6) and decreased saturated fatty acid (including 16:0 and 18:0). These effects were impaired in LXRα or LXRα/β double KO mice, but not in LXRβ KO mice. Thus, activation of LXRα, but not LXRβ, altered the type of fatty acids in favor of unsaturates.
Supplementary Table 1: Effects of LXR agonist GW3965 on triglyceride fatty acid profile in the ventricles of WT and LXRs KO mice.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>WT (24h)</th>
<th>WT + GW (24h)</th>
<th>WT (4 weeks)</th>
<th>WT + GW (4 weeks)</th>
<th>LXRα KO (24h)</th>
<th>LXRα KO + GW (24h)</th>
<th>LXRβ KO (24h)</th>
<th>LXRβ KO + GW (24h)</th>
<th>LXRα/β KO (24h)</th>
<th>LXRα/β KO + GW (24h)</th>
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<tr>
<td>14:0</td>
<td>0.1 ± 0.01</td>
<td>0.2 ± 0.006*</td>
<td>0.05 ± 0.02</td>
<td>0.2 ± 0.01†</td>
<td>0.2 ± 0.06</td>
<td>0.2 ± 0.004</td>
<td>0.1 ± 0.02</td>
<td>0.2 ± 0.03</td>
<td>0.2 ± 0.03</td>
<td>0.1 ± 0.02</td>
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<tr>
<td>16:0</td>
<td>14.2 ± 0.9</td>
<td>13.3 ± 0.3</td>
<td>16.3 ± 0.5</td>
<td>14.7 ± 0.2†</td>
<td>13.8 ± 0.8</td>
<td>13.6 ± 0.2</td>
<td>14.1 ± 0.1</td>
<td>14.4 ± 0.4</td>
<td>16.4 ± 0.6</td>
<td>16.9 ± 0.9</td>
</tr>
<tr>
<td>16:1n9</td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.02</td>
<td>0.1 ± 0.005</td>
<td>0.2 ± 0.01†</td>
<td>0.2 ± 0.04</td>
<td>0.2 ± 0.04</td>
<td>0.06 ± 0.002</td>
<td>0.08 ± 0.008</td>
<td>0.1 ± 0.02</td>
<td>0.1 ± 0.01</td>
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<tr>
<td>16:1n7</td>
<td>0.2 ± 0.02</td>
<td>0.4 ± 0.05*</td>
<td>0.2 ± 0.007</td>
<td>0.5 ± 0.02†</td>
<td>0.6 ± 0.2</td>
<td>0.3 ± 0.05</td>
<td>0.2 ± 0.02</td>
<td>0.3 ± 0.03†</td>
<td>0.2 ± 0.02</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>18:0</td>
<td>17.6 ± 1.2</td>
<td>16.0 ± 0.5</td>
<td>20.7 ± 1.2</td>
<td>16.4 ± 0.3†</td>
<td>18.3 ± 3.4</td>
<td>16.3 ± 0.6</td>
<td>18.2 ± 0.3</td>
<td>19.4 ± 0.6</td>
<td>20.4 ± 1.2</td>
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<tr>
<td>18:1n9</td>
<td>8.9 ± 0.3</td>
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<td>7.6 ± 0.2</td>
<td>8.7 ± 0.4</td>
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<td>5.8 ± 0.4</td>
<td>9.0 ± 0.8</td>
<td>9.1 ± 0.8</td>
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<tr>
<td>18:2n6</td>
<td>19.7 ± 0.6</td>
<td>18.6 ± 0.9</td>
<td>17.6 ± 0.4</td>
<td>17.2 ± 0.6</td>
<td>19.1 ± 1.4</td>
<td>20.4 ± 1.1</td>
<td>16.8 ± 0.6</td>
<td>14.1 ± 0.9</td>
<td>18.1 ± 1.3</td>
<td>17.5 ± 1.9</td>
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<tr>
<td>20:0</td>
<td>0.3 ± 0.03</td>
<td>0.3 ± 0.009</td>
<td>0.5 ± 0.03</td>
<td>0.3 ± 0.01†</td>
<td>0.5 ± 0.09</td>
<td>0.3 ± 0.07</td>
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<td>0.4 ± 0.04</td>
<td>0.4 ± 0.02</td>
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<tr>
<td>20:3n6</td>
<td>0.6 ± 0.02</td>
<td>0.8 ± 0.04*</td>
<td>0.5 ± 0.01</td>
<td>0.7 ± 0.02†</td>
<td>0.5 ± 0.04</td>
<td>0.6 ± 0.02</td>
<td>0.5 ± 0.02</td>
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<td>0.5 ± 0.04</td>
<td>0.6 ± 0.06</td>
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<td>7.3 ± 0.2</td>
<td>4.1 ± 1.0</td>
<td>6.2 ± 0.3</td>
<td>7.1 ± 0.3</td>
<td>6.9 ± 0.4</td>
<td>6.5 ± 0.3</td>
<td>6.7 ± 0.4</td>
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<tr>
<td>22:5n3</td>
<td>1.4 ± 0.03</td>
<td>1.8 ± 0.04</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.06</td>
<td>1.1 ± 0.2</td>
<td>1.7 ± 0.03</td>
<td>1.7 ± 0.05</td>
<td>1.8 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.6 ± 0.09</td>
</tr>
<tr>
<td>22:6n3</td>
<td>29.5 ± 2.1</td>
<td>31.2 ± 1.2</td>
<td>27.3 ± 1.5</td>
<td>29.5 ± 0.7</td>
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<td>25.4 ± 1.8</td>
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</tbody>
</table>

Values (means ± SEM) represent relative distribution (%) of different fatty acids in the triglyceride fraction isolated from MI/R murine ventricles. For the acute MI/R protocol, reperfusion commenced for 24 hours following 30 minutes of ischemia, and mice were randomly assigned to receive GW3965 (20 mg/kg) or vehicle 15 minutes before reperfusion. To observe the long-term cardioprotective effect of LXR activation, mice were randomly assigned to receive GW3965 (20 mg/kg) or vehicle 15 minutes before reperfusion, and daily following reperfusion for 4 weeks [n = 6-10 in each group; *P<0.05 vs. WT (24h) group, †P< 0.05 vs. WT (4 weeks) group, ‡P< 0.05 vs. LXRβ KO (24h) group]. Abbreviations: MI/R, myocardial ischemia/reperfusion; WT, wild type; KO, knockout; GW, GW3965.
Supplemental Figures and Figure Legends

Figure S1. LXRα/β dual agonists induced no observable hemodynamic changes in myocardial ischemia/reperfusion (MI/R) models.

Changes in heart rate and mean arterial pressure in each group (n=6-12) during the experiment.

Abbreviations: 22(R)-HC, 22(R)-hydroxycholesterol; HR, heart rate; MBP, mean arterial blood pressure.
Figure S2. Cardioprotective effects of LXRα/β dual agonist were lost in cardiac-specific LXRα–knockdown (KD) mice. A. Intramyocardial delivery of siRNA against LXRα or LXRβ effectively eliminated the expression of LXRα or LXRβ, respectively, in left ventricle. Time-courses changes in LXRα or LXRβ expression by real-time PCR after siRNA delivery are shown. Results were expressed as percentages of the control siRNA (n=4-5; LXRα: *P=0.55, †P=0.003, ‡P=0.01 vs. the control siRNA group; LXRβ: *P=0.39, †P=0.0007, ‡P=0.060 vs. the control siRNA group). B-C. Effect of cardiac-specific-KD of LXRα or LXRβ by in-vivo siRNA transfection upon MI/R injury. MI/R procedures were performed 48 hours after intramyocardial siRNA delivery. GW3965 (20mg/kg) was administered 15 minutes before reperfusion. B. MI/R injury was assessed by myocardial infarction size as determined by Evans blue/TTC double-staining (n=7-10; *P=0.0004, †P=0.006, ‡P>0.99, §P=0.002 vs. vehicle), and left ventricular dysfunction was determined by echocardiography (n=7-10; *P=0.002, †P=0.02, ‡P>0.99, §P=0.048 vs. vehicle). C. Myocardial apoptosis was determined by TUNEL labeling (n=6-8; Bar=25 μm; *P=0.006, †P=0.002, ‡P>0.99, §P=0.04 vs. vehicle) and caspase-3 activation (n=8-10; *P=0.0004, †P=0.002, ‡P>0.99, §P=0.002 vs. vehicle). Abbreviations: MI/R, myocardial ischemia/reperfusion; AAR, area at risk; LVEF, left ventricular ejection fraction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
Figure S3. Anti-oxidative/nitrative and anti-apoptosis effects of LXRα/β dual agonist were lost in LXRα-knockdown (KD) mice. A-C. Effect of LXRα or LXRβ gene KD upon MI/R injury. GW3965 (20mg/kg) was injected 15 minutes before reperfusion. A. Caspase-9 and caspase-12 activity was measured by fluorometric assay (n=6-8; caspase-12: *P=0.03, †P=0.04, ⊥P>0.99, **P=0.04 vs. vehicle; caspase-9: †P=0.004, §P=0.01, #P>0.99, ##P=0.04 vs. vehicle). B. Myocardial oxidative stress was measured utilizing confocal microscope with in-situ dihydroethidium stain (n=5-6; Bar=25 μm), NADPH oxidase activity by lucigenin-enhanced chemiluminescence (n=6-8; *P=0.001, †P=0.03, ‡P=0.03 vs. vehicle), and NADPH oxidase gp91phox gene expression by real-time PCR (n=5-6; *P=0.009, †P=0.04, ‡P>0.99, §P=0.05 vs. vehicle). Results were normalized against GAPDH and converted to fold induction relative to vehicle group. C. Myocardial nitrative stress was assessed by nitrotyrosine content determined by immunohistochemistry (n=5-6; Bar=25 μm) and ELISA assay (n=6-8; *P=0.007, †P=0.01, ‡P>0.99, §P=0.04 vs. vehicle). The gene expression of iNOS was determined by real-time PCR (n=5-6; *P=0.02, †P=0.03, ‡P>0.99, §P=0.048 vs. vehicle). Results were normalized against GAPDH and converted to fold induction relative to vehicle group. Abbreviations: MI/R, myocardial ischemia/reperfusion; RLU, relative light units; iNOS, inducible nitric oxide synthase.
Figure S4. Anti-oxidative and anti-nitrative effects of LXRα/β dual agonist were lost in LXRα/β double- and LXRα-knockout (KO) mice. A. Myocardial oxidative stress was measured utilizing confocal microscope with in-situ dihydroethidium stain (n=5-6 hearts per group, Bar=25 μm). NADPH oxidase activity was assessed by lucigenin-enhanced chemiluminescence (n=5-6; *P=0.01, †P=0.047, ‡P=0.01, §P=0.02 vs. vehicle). NADPH oxidase gp91phox gene expression was determined by real-time PCR (n=5-6; *P=0.02, †P=0.02, ‡P=0.006, §P=0.005 vs. vehicle). Results were normalized against GAPDH and converted to fold induction relative to vehicle group. B. Myocardial nitrative stress was assessed by nitrotyrosine content determined by immunohistochemistry (n=5-6 hearts per group, Bar=25 μm) and ELISA assay (n=5-6; *P=0.006, †P=0.04 ‡P=0.03, §P=0.04 vs. vehicle). The gene expression of iNOS was determined by real-time PCR (n=5-6; *P=0.008, †P=0.02, ‡P=0.03, §P=0.007 vs. vehicle). Results were normalized against GAPDH and converted to fold induction relative to vehicle group. Abbreviations: WT, wild type; RLU, relative light units; iNOS, inducible nitric oxide synthase.
Figure S5. Cardiac LXRα overexpression inhibited MI/R-induced myocardial apoptosis, infarct size, and cardiac dysfunction. A. Cardiac LXRα and LXRβ alteration after adenovirus-mediated cardiac-specific gene delivery. LXR protein and gene alteration detected by Western blot and real-time quantitative PCR from mouse left ventricular tissues. Adenovirus-encoded LacZ (Ad.LacZ) treatment served as control. Results were normalized against GAPDH and converted to fold induction relative to Ad.LacZ group (n=4; LXRα: *P=0.03 vs. Ad.LacZ; LXRβ: *P=0.03 vs. Ad.LacZ). B-C. Effect of LXRα or LXRβ gene overexpression upon MI/R injury. B. Myocardial apoptosis was determined by immunohistochemical staining of TUNEL (n=5-6, Bar=25 μm; *P=0.04, †P>0.99, vs. Ad.LacZ) and caspase-3 activation (n=5-6; *P=0.03, †P>0.99, vs. Ad.LacZ). C. MI/R injury was assessed by myocardial infarction size as determined by Evans blue/TTC double-staining (n=6; *P=0.001, †P=0.66, vs. Ad.LacZ), and left ventricular dysfunction was determined by echocardiography (n=6; *P=0.02, †P>0.99, vs. Ad.LacZ). Abbreviations: Ad., adenoviral-encoded; MI/R, myocardial ischemia/reperfusion; AAR, area at risk; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; LVEF, left ventricular ejection fraction.
Supplemental References


