Thiol Oxidative Stress Induced by Metabolic Disorders Amplifies Macrophage Chemotactic Responses and Accelerates Atherogenesis and Kidney Injury in LDL Receptor–Deficient Mice

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Background—Strengthening the macrophage glutathione redox buffer reduces macrophage content and decreases the severity of atherosclerotic lesions in LDL receptor–deficient (LDLR−/−) mice, but the underlying mechanisms were not clear. This study examined the effect of metabolic stress on the thiol redox state, chemotactic activity in vivo, and the recruitment of macrophages into atherosclerotic lesions and kidneys of LDL-R−/− mice in response to mild, moderate, and severe metabolic stress.

Methods and Results—Reduced glutathione (GSH) and glutathione disulfide (GSSG) levels in peritoneal macrophages isolated from mildly, moderately, and severe metabolically-stressed LDL-R−/− mice were measured by HPLC, and the glutathione reduction potential (Eh) was calculated. Macrophage Eh correlated with the macrophage content in both atherosclerotic (r²=0.346, P=0.004) and renal lesions (r²=0.480, P=0.001) in these mice as well as the extent of both atherosclerosis (r²=0.414, P=0.001) and kidney injury (r²=0.480, P=0.001). Compared to LDL-R−/− mice exposed to mild metabolic stress, macrophage recruitment into MCP-1–loaded Matrigel plugs injected into LDL-R−/− mice increased 2.6-fold in moderately metabolically-stressed mice and 9.8-fold in severely metabolically-stressed mice. The macrophage Eh was a strong predictor of macrophage chemotaxis (r²=0.554, P<0.001).

Conclusion—Thiol oxidative stress enhances macrophage recruitment into vascular and renal lesions by increasing the responsiveness of macrophages to chemotaxatnts. This novel mechanism contributes at least in part to accelerated atherosclerosis and kidney injury associated with dyslipidemia and diabetes in mice. (Arterioscler Thromb Vasc Biol. 2009;29:1779-1786.)

Key Words: glutathione ■ macrophage recruitment ■ metabolic stress ■ atherosclerosis ■ inflammation

Metabolic disorders such as hypercholesterolemia and diabetes are strongly associated with both macro- and microvascular diseases, a common feature of which is the recruitment of blood monocyte–derived macrophages to sites of vascular injury. Whereas most studies exploring the mechanisms underlying atherosclerosis and other vascular pathologies have focused on the impact of a dysregulated metabolism on the vasculature itself, a number of more recent studies suggest that metabolic disorders may also directly impact monocytes and alter their functionalities in ways that promote and accelerate the disease process. Phenotypic abnormalities in blood monocytes of diabetic patients have been reported, including altered metabolism,1–3 phagocytosis,4,5 and cytokine release.6–8 Furthermore, peritoneal macrophages isolated from either atherosclerosis-prone mice or diabetic mice show altered cytokine and chemokine responses compared with macrophages from healthy control mice.9,10 However, it is not yet well-understood to what extent monocyte dysfunction induced by metabolic diseases contributes to macrophage recruitment and vascular diseases such as atherosclerosis.

The recruitment of blood monocyte–derived macrophages into the vessel wall is considered one of the earliest events in the onset of atherosclerosis.11,12 The mechanisms that trigger macrophage recruitment are not fully understood but prolonged retention and subsequent modification of LDL may play a critical role.13–15 Modified LDL, particularly oxidatively-modified LDL, stimulates vascular cells and mac-
rophones to secrete a vast array of inflammatory molecules, including chemokines, which in turn promote and sustain a continuous influx of macrophages into the vasculature.\textsuperscript{12,16} Studies in transgenic and knockout mice revealed that at least 2 major chemokine/chemokine receptor systems are involved in the recruitment of macrophages into atherosclerotic lesions, MCP-1/CC2 and the receptor CCR2, and RANTES/CC5 and the receptor CCR5. Deficiencies in either the macrophage chemoattractant MCP-1 or its receptor CCR2 reduced the severity of atherosclerosis in different mouse models of atherosclerosis, and the reduction in lesion size was accompanied by a reduction in macrophage accumulation.\textsuperscript{17-19} Inhibiting the effect of RANTES in high-cholesterol diet–fed LDL-R\textsuperscript{−/−} mice with Met-RANTES, a RANTES antagonist, also reduces macrophage infiltration and inhibited lesion formation.\textsuperscript{20} The reduction in these lesions’ macrophage content was associated with a more stable plaque phenotype. Deficiency in the RANTES receptors CCR5 in ApoE\textsuperscript{−/−} mice showed similar effects,\textsuperscript{21} but other studies suggest that CCR5 contributes primarily to the later stages of lesion development.\textsuperscript{22,23}

Although the individual contributions of these chemotactants and their receptors to macrophage recruitment and atherogenesis appear to change throughout the maturation of atherosclerotic lesions, these studies suggest that macrophage recruitment can be accelerated either by increasing the chemotactic signals originating from the vasculature or by sensitizing monocytes to these signals, for example by increasing chemokine receptor expression levels or activity. Here we examined the hypothesis that metabolic stress promotes macrophage recruitment and atherogenesis by increasing the responsiveness of circulating blood monocytes to chemoattractants, specifically to MCP-1. We compared normolipidemic, hypercholesterolemic, and dyslipidemic diabetic LDL-R\textsuperscript{−/−} mice and showed that with each increase in the level of metabolic stress macrophage recruitment into the vasculature and kidneys increased, and the development of vascular and renal lesions accelerated accordingly. Increased monocyte responsiveness to chemoattractants appeared to account at least in part for the observed increase in macrophage recruitment as MCP-1–loaded Matrigel plugs implanted into these mice also showed enhanced macrophage accumulation in response to each increase in the level of metabolic stress, even though the concentration of chemoattractant was identical in each implant. We provide evidence that increased cellular thiol oxidation induced by metabolic stress sensitizes macrophages to MCP-1–induced chemotaxis and that thiol oxidative stress contributes to the enhanced recruitment of macrophages to sites of tissue injury in metabolically-challenged mice.

Materials and Methods

Animals

Female LDL-R\textsuperscript{−/−} mice (B6.129S7-Ldr\textsuperscript{m1Hko/J}, stock no. 002207) on a C57BL/6J background and C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, Me). After 1 week on a maintenance diet (MD, AIN-93G, BioServ), mice were randomized into 3 groups and subjected to either mild (MD), moderate (HFD), or severe metabolic stress (STZ+HFD). Mice in the STZ+HFD group were rendered diabetic with intraperitoneal injection of streptozotocin (STZ; 60 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) dissolved in citrate buffer (50 mmol/L; pH=4.5) for 5 consecutive days and, after a 2-day rest, again for 2 consecutive days. Mice in the MD and HFD groups received a comparable volume of citrate buffer. To induce hypercholesterolemia, mice in the HFD and STZ+HFD group were fed a diet supplemented with fat (21% wt/wt) and cholesterol (0.15% wt/wt; AIN-76A, BioServ) beginning at 3 weeks after the first injection, for a total of 12 weeks. The remaining animals (MD group) received MD for 12 weeks. All studies were performed with the approval of the UTHSCSA Institutional Animal Care and Use Committee.

Analysis of Atherosclerosis

After mice were euthanatized, the right atrium was removed and hearts and aortas were perfused with PBS through the left ventricle. Hearts were embedded in OCT and frozen on dry ice. Aortas were fixed overnight with 4% paraformaldehyde in PBS, dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and then placed in 4% paraformaldehyde. After 2 days, they were embedded in paraffin and cut into 4-μm sections and stained with Masson’s trichrome or hematoxylin and eosin. HPLC analysis revealed that the animals were rendered diabetic with intraperitoneal injection of streptozotocin (STZ; 60 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) dissolved in citrate buffer (50 mmol/L; pH=4.5) for 5 consecutive days and, after a 2-day rest, again for 2 consecutive days. Mice in the MD and HFD groups received a comparable volume of citrate buffer. To induce hypercholesterolemia, mice in the HFD and STZ+HFD group were fed a diet supplemented with fat (21% wt/wt) and cholesterol (0.15% wt/wt; AIN-76A, BioServ) beginning at 3 weeks after the first injection, for a total of 12 weeks. The remaining animals (MD group) received MD for 12 weeks. All studies were performed with the approval of the UTHSCSA Institutional Animal Care and Use Committee.

Analysis of Kidney Injury

Kidneys were frozen in OCT, and 5 cross cryosections separated by an interval of 50 μm were stained with ORO or Masson’s Trichrome and were processed for immunohistochemical analysis of macrophage content with anti-CD68 antibodies (Serotech). Of these animals, the remaining animals (MD group) received MD for 12 weeks. All studies were performed with the approval of the UTHSCSA Institutional Animal Care and Use Committee.

Blood and Urine Analysis

Mice were fasted overnight before glucose and lipid measurements. Glucose was measured biweekly using a Contour meter (Bayer). For measurements of white blood cell (WBC) and monocyte counts, blood was obtained by retro-orbital bleed after 11 weeks of diet feeding, and differential blood cell counts were performed by the Department of Laboratory Animal Resources at UTHSCSA on a VetScan HM II Analyzer (Abaxis). After peritoneal macrophages were harvested, blood was drawn by cardiac puncture. Plasma cholesterol and triglyceride levels were determined using enzymatic assay kits (Wako Chemicals). Lipoprotein cholesterol distributions were analyzed in individual plasma samples (50 μL) for 8 mice in each group. Plasma samples were separated by size exclusion chromatography on tandem superose 6 to 10/300GL columns. Serum amyloid A (Biosource), leptin, and insulin levels (Chrysal Chem) were measured by ELISA. Urine creatinine and albumin concentrations were measured by ELISA (Exocell).

Macrophage Glutathione/Glutathione Disulfide Analysis

Resident peritoneal cells were harvested by lavage, plated, and after 3 hours nonadherent cells were removed with multiple washing. Adherent cells were cultured overnight before cell harvest. Glutathione and glutathione disulfide levels were measured by HPLC and values were normalized to cell DNA levels as described elsewhere. The glutathione reduction potential \(E_{A}^{0}\) was calculated using the
The mean macrophage volume was estimated at 1.4×10⁶ μL.¹¹

Statistics
Data were analyzed using ANOVA (SPSS 16.0). Data were tested for use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed by using the Least Significant Difference method. All data are presented as mean ± SE. Results were considered statistically significant at the P<0.05 level.

Results
Metabolic Stress in LDL-R⁻/⁻ Mice Promotes the Oxidation of the Glutathione Redox State in Macrophages and Accelerates Atherosclerotic Lesion Formation
To examine the effect of metabolic stress on the thiol redox state of monocyte-derived macrophages, we determined the GSH/GSSG ratio in peritoneal macrophages isolated from LDL-R⁻/⁻ mice that were exposed for 12 weeks to either mild (MD), moderate (HFD), or severe metabolic stress (STZ-induced hyperglycemia plus HFD). With increasing metabolic stress, these mice showed a progressive decrease in their macrophage GSH/GSSG ratios, indicating a progressive increase in intracellular (thiol) oxidative stress (Figure 1A). As expected, increasing metabolic stress increased plasma cholesterol levels (Table) and accelerated atherosclerotic lesion formation in both the aortic arch and descending aorta of LDL-R⁻/⁻ mice (Figure 1B). Lesion size showed a strong correlation with plasma cholesterol levels in these animals (r² = 0.635, P<0.001, Figure 1C). HFD feeding increased cholesterol in both the IDL/LDL and the VLDL fractions, whereas STZ treatment before HFD feeding resulted in only a minor, statistically not significant (P=0.19) further increase in plasma total cholesterol (Table), primarily as VLDL cholesterol (supplemental Figure 1, available online at http://atvb.ahajournals.org). Both plasma triglycerides and glucose levels were also increased by the STZ treatment, but the differences did not reach statistical significance.

Surprisingly, insulin levels were similar in nondiabetic and diabetic mice (Table). However, it is unlikely that β-cells recovered from the STZ treatment, as fasting glucose levels peaked 4 weeks after the first STZ injection (ie, 1 week after initiating HFD feeding) and plateaued thereafter. A more likely explanation for the similar insulin levels is that the depletion of β-cells was incomplete. Injecting higher doses of STZ resulted in slightly higher fasting glucose levels in this mouse model, but the survival rate of the HFD-fed animals dropped precipitously at these higher STZ doses. Also, mice were fasted for 15 hours to obtain true fasting glucose levels, and this extended fasting period likely contributed to the low insulin levels we observed in the nondiabetic mice.

To evaluate the relationship between thiol oxidative stress in macrophages induced by low, modest, or severe metabolic stress and atherosclerotic lesion formation, we calculated the glutathione reduction potential (Eh) based on the cellular GSH and GSSG concentrations we determined in peritoneal macrophages isolated from each animal. The correlation between the macrophage glutathione reduction potential and aortic lesion size was statistically highly significant (r² = 0.414, P=0.001; Figure 1D), whereas plasma triglyceride levels (r² = 0.345, P=0.003) and plasma glucose concentrations (r² = 0.252, P=0.011) were both poorer predictors of atherosclerotic lesion formation.

With increasing metabolic stress we also observed an acceleration in atherosclerotic lesion formation in the aortic...
root (Figure 2A, supplemental Figure II). Increased lesion area was paralleled by an increase in macrophage content in the vessel wall (Figure 2B, supplemental Figure II). Importantly, the macrophage glutathione reduction potential was a strong predictor of macrophage content in the lesions (expressed as mm²: \( r^2 = 0.346, P = 0.004 \); expressed as % lesion area: \( r^2 = 0.683, P < 0.001 \)), suggesting that thiol oxidative stress in blood monocytes promotes macrophage recruitment into the vascular wall.

Han et al reported that increasing extracellular cholesterol levels by adding native LDL to THP-1 monocytes increases their chemotactic response to MCP-1. The increase in macrophage recruitment in these mice was not the result of increased blood monocyte counts. Although the number of WBCs appeared to increase with increasing metabolic stress, we did not observe any statistically significant differences in blood monocyte counts between mildly (MD), moderately (HFD), or severely metabolically-stressed (STZ+HFD) mice (Table). This suggests that the increase in macrophage recruitment in these mice was not the result of increased blood monocyte counts.

### Hyperglycemia in Dyslipidemic LDL-R\(^{-/-}\) Mice Accelerates Glomerular Lipid Deposition, Macrophage Accumulation, and Kidney Injury

Next, we examined the kidneys of these mice to determine whether the apparent relationship between the macrophage glutathione reduction potential, macrophage recruitment, and the severity of vascular lesions also extended to other sites of tissue injury. The kidneys of LDL-R\(^{-/-}\) mice exposed to low, modest, or severe metabolic stress showed progressive glomerular lipid deposition (Figure 3A, supplemental Figure III), which was accompanied by increased macrophage recruitment into the glomeruli (Figure 3B, supplemental Figure III). Like in the vasculature, we found a highly significant correlation between the macrophage glutathione reduction potential and macrophage accumulation in the kidney (\( r^2 = 0.481, P = 0.001 \), supplemental Figure IV). Increased renal lipid deposition and macrophage accumulation was accompanied by increased fibrosis (supplemental Figure III). Interestingly, we also observed a significant correlation between macrophage glutathione reduction potential and renal lipid accumulation in LDL-R\(^{-/-}\) mice exposed to mild, modest, or severe metabolic stress (\( r^2 = 0.526, P < 0.001 \), supplemental Figure V). Of note, mice from the STZ+HFD group showed a significant increase in the urinary albumin/creatinine ratio (Table), indicating that the extent of kidney injury in these severely metabolically-stressed diabetic mice had resulted in the loss of renal function.
Metabolic Stress Enhances Macrophage Chemotactic Activity In Vivo

To examine whether metabolic stress changes the responsiveness of macrophages to chemoattractants, we injected mildly (MD), moderately (HFD), or severely metabolically-stressed (STZ/HFD) mice with Matrigel plugs loaded with either vehicle or MCP-1. After 3 days, the plugs were removed and macrophage recruitment into these plugs was quantified. Increasing the level of metabolic stress in LDL-R⁻/⁻ mice by feeding a HFD increased macrophage recruitment into MCP-1–loaded Matrigel plugs 2.6-fold (Figure 4A). No macrophages were detected in vehicle-loaded plugs removed from the opposite flank of either MD-or HFD-fed mice. When we further increased metabolic stress by inducing hyperglycemia in LDL-R⁻/⁻ mice before HFD feeding, we observed an additional 3.8-fold increase in macrophage recruitment. Even though the MCP-1 concentration in the Matrigel injected into all 3 groups of mice was identical, 9.8-fold more macrophages were recruited into MCP-1–loaded Matrigel plugs isolated from severely metabolically-stressed diabetic LDL-R⁻/⁻ mice than into plugs removed from MD-fed LDL-R⁻/⁻ mice (Figure 4A). In contrast to mildly (MD) and moderately metabolically-stressed LDL-R⁻/⁻ mice (HFD), we also detected small numbers of macrophages in vehicle-loaded plugs from severely metabolically-stressed LDL-R⁻/⁻ mice (HFD), which were not observed in MD-fed LDL-R⁻/⁻ mice (Figure 4A, STZ/HFD, numbers in parentheses). This indicates that the sensitivity of macrophages in these animals was increased to such an extent that the cells even responded to residual chemotactic factors present in the growth factor-poor Matrigel plugs. When we measured GSH and GSSG levels and calculated the glutathione reduction potential of the peritoneal macrophages, we again found a highly significant correlation (r²=0.554, P<0.001) between the macrophage glutathione reduction potential and the number of macrophages recruited into the MCP-1–loaded Matrigel plugs.
Discussion

In this study we examined whether metabolic stress induced by hypercholesterolemia alone or hyperlipidemia plus hyperglycemia accelerates atherosclerosis and renal injury by promoting thiol oxidative stress in macrophages and increasing the recruitment of macrophages to sites of tissue injury. We found that with each additional level of metabolic stress, macrophage accumulation increased in both atherosclerotic lesions and in kidneys of LDL-R<sup>−/−</sup> mice. Analysis of the macrophage glutathione reduction potential (E<sub>H</sub>) revealed that metabolic stress promotes intracellular thiol oxidation and that oxidation of the macrophage glutathione reduction state correlates with both accelerated macrophage recruitment and increased lesion severity. Macrophage recruitment into MCP-1-loaded Matrigel plugs implanted in these mice was also accelerated with each additional level of metabolic stress, suggesting that increased cellular thiol oxidation induced by metabolic stress amplifies macrophage responses to chemoattractant signals. These studies identified a novel thiol-dependent mechanism that contributes to macrophage recruitment and the development of atherosclerotic lesions.

Macrophages are continually recruited into atherosclerotic lesions, and macrophage accumulation appears to increase in proportion to lesion size. Although the rate of macrophage recruitment depends on (1) the nature of chemoattractants released by the injured tissue and (2) the size of chemoattractant gradient, our data suggest that there is a third key factor determining the extent of macrophage accumulation and thus lesion size: the intensity of the monocytes’ response to a chemoattractant signal. The increase in chemoattractant activity we observed in response to increased metabolic stress could not be fully accounted for by a single cardiovascular risk factor (eg, plasma cholesterol, triglycerides, or glucose levels), suggesting that the overall metabolic state rather than any individual metabolite determines the monocyte responsiveness to chemoattractants. Monocytes appear to act as sensors...
of metabolic stress and their chemotactic activity may reflect cardiovascular risk, a concept, if confirmed, that would have important diagnostic and therapeutic implications.

Changes in the cellular redox environment not only initiate (or inhibit) individual signaling pathways but dictate the fate of a cell with regard to function, differentiation, proliferation, and survival. The cellular glutathione reduction potential serves as a key indicator of a cell’s redox environment. Previously, we showed that increased expression of macrophage glutathione reductase activity reduces the severity of atherosclerosis in LDL-R−/− mice, providing evidence that the glutathione redox state in macrophages plays a critical role in the development and progression of atherosclerotic lesions.

The strong correlations we observed in this study between the macrophage glutathione reduction potential (Eh) and macrophage chemotactic activity in vivo, macrophage accumulation in vascular and renal lesions, and the severity of atherosclerotic lesions and renal injury, confirm that the level of thiol oxidation in macrophages appears to be a critical determinant for both the extent of macrophage recruitment and the rate of lesion development. Our in vivo chemotaxis assay demonstrated that macrophage chemotactic activity increased with increasing levels of metabolic stress, even though the concentration of chemotactant (ie, MCP-1), was identical in all Matrigel plugs. Two mechanisms could have contributed to this increase in macrophage recruitment: increased numbers of blood monocytes or increased responsiveness of monocytes to chemoattractants. We observed no statistically significant increases in blood monocyte counts in metabolically-stressed mice, indicating that chronic metabolic stress rendered monocyte-derived macrophages hyperresponsive to MCP-1. Increased cell surface expression of the MCP-1 receptor CCR2 could account for the detected increase in macrophage responsiveness to MCP-1. In support of this hypothesis, Han et al reported that CCR2 transcript levels are elevated 2-fold in mice, providing evidence that chronic metabolic stress renders monocyte-derived macrophages hyperresponsive to MCP-1. Increased cell surface expression of the MCP-1 receptor CCR2 could account for the detected increase in macrophage responsiveness to MCP-1. In support of this hypothesis, Han et al reported that CCR2 transcript levels are elevated 2-fold in mice, providing evidence that chronic metabolic stress renders monocyte-derived macrophages hyperresponsive to MCP-1.

In summary, these studies provide evidence that increased macrophage responsiveness to chemoattractants induced by thiol oxidative stress is a novel mechanism contributing to increased macrophage recruitment and accelerated atherosclerosis and renal injury associated with metabolic disorders. Our data underscore the critical role of the macrophage glutathione redox state in regulating macrophage chemotaxis and identify the glutathione-dependent antioxidant system in monocytes as a potential therapeutic target for the prevention and treatment of atherosclerosis.

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Disclosures

None.

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Supplemental Figure I: Lipoprotein profiles and Aortic Lesions in LDL-R−/− Mice Exposed to Either Mild (MD), Moderate (HFD) or Severe Metabolic Stress (STZ+HFD). (A) Mean cholesterol content in lipoprotein fractions in LDL-R−/− mice after 12 weeks on MD (○), HFD (●), or after STZ-treatment followed by HFD (STZ+HFD, ●) for 12 weeks. Results shown are means of plasma samples from 8 mice per group.
Supplemental Figure II: Aortic Lesions in LDL-R⁻/⁻ Mice Exposed to Either Mild (MD), Moderate (HFD) or Severe Metabolic Stress (STZ+HFD). Representative images of sections in the aortic root from LDL-R⁻/⁻ mice after 12 weeks on MD, HFD, or after STZ-treatment followed by HFD for 12 weeks (STZ+HFD) stained with ORO and adjacent sections stained with a macrophage-specific antiserum directed against CD68. Magnification: 100X
Supplemental Figure III: Lipid Deposition, Macrophage Accumulation and Fibrosis in LDL-R⁻⁻ Mice Exposed to Either Mild (MD), Moderate (HFD) or Severe Metabolic Stress (STZ+HFD). Representative images of sections from kidneys stained with ORO and adjacent sections stained with either a macrophage-specific antiserum directed against CD68 (red arrows identify macrophages) or Mason-Trichrome (M-T). Magnification: 1000X
Supplemental Figure IV: Correlation of Glomerular Macrophage Accumulation with Macrophage Glutathione Reduction Potentials of LDL-R⁻/⁻ Mice Exposed to Either Mild (MD), Moderate (HFD) or Severe Metabolic Stress (STZ+HFD).

Correlation between the glutathione reduction potential ($E_h$) of peritoneal macrophages and macrophage accumulation ($r^2=0.481, P=0.001$) in the kidneys of mildly (MD, ○, n=6), modestly (HFD, ◆, n=7) and severely metabolically stressed LDL-R⁻/⁻ mice (STZ+HFD, ▲, n=8).
Supplemental Figure V: Correlation of Glomerular Lipid Deposition with Macrophage Glutathione Reduction Potentials of LDL-R<sup>-/-</sup> Mice Exposed to Either Mild (MD), Moderate (HFD) or Severe Metabolic Stress (STZ+HFD). Correlation between the glutathione reduction potential ($E_h$) of peritoneal macrophages and renal lipid accumulation ($r^2=0.526$, $P<0.001$) in the kidneys of mildly (MD, O, n=6), modestly (HFD, ◆, n=7) and severely metabolically stressed LDL-R<sup>-/-</sup> mice (STZ+HFD, ▲, n=8).