11β-HSD1 inhibition reduces atherosclerosis in mice by altering proinflammatory gene expression in the vasculature


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Luo MJ, Thieringer R, Springer MS, Wright SD, Hermanowski-Vosatka A, Plump A, Balkovec JM, Cheng K, Ding GJ, Kawka DW, Koo GC, Le Grand CB, Luo Q, Maletic MM, Malkowitz L, Shah K, Singer I, Waddell ST, Wu KK, Yuan J, Zhu J, Stepaniants S, Yang X, Lum PY, Wang I. 11β-HSD1 inhibition reduces atherosclerosis in mice by altering proinflammatory gene expression in the vasculature. *Physiol Genomics* 45: 47–57, 2013. First published November 20, 2012; doi:10.1152/physiolgenomics.00109.2012.—11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is implicated in the etiology of metabolic syndrome and attenuated atherosclerosis in ApoE−/− mice. Howev- er, the molecular mechanism underlying the atheroprotective effect was not clear. In this study, we tested whether and how 11β-HSD1 inhibition affects vascular inflammation, a major culprit for atherosclerosis and its associated complications. ApoE−/− mice were treated with an 11β-HSD1 inhibitor for various periods of time. Plasma lipids and aortic cholesterol accumulation were quantified. Several microarray studies were carried out to examine the effect of 11β-HSD1 inhibition on gene expression in atherosclerotic tissues. Our data suggest 11β-HSD1 inhibition can directly modulate atherosclerotic plaques and attenuate atherosclerosis independently of lipid lowering effects. We identified immune response genes as the category of mRNA most significantly suppressed by 11β-HSD1 inhibition. This anti-inflammatory effect was further confirmed in plaque macrophages and smooth muscle cells procured by laser capture microdissection. These findings in the vascular wall were corroborated by reduction in circulating MCP1 levels after 11β-HSD1 inhibition. Taken together, our data suggest 11β-HSD1 inhibition regulates proinflammatory gene expression in atherosclerotic tissues of ApoE−/− mice, and this effect may contribute to the attenuation of atherosclerosis in these animals.

STUDIES IN HUMANS AND ANIMAL models have implicated glucocorticoid excess in the etiology of metabolic syndrome (46), a constellation of risk factors for cardiovascular diseases, including obesity, insulin resistance, dyslipidemia, and hypertension. 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is an intracellular enzyme that acts primarily as a reductase to convert inactive glucocorticoids into active GCs in tissues. It thus potentiates local glucocorticoid activity (38, 40), and inhibition of 11β-HSD1 emerges as a promising approach for the treatment of this prevalent condition (43, 45). We previously reported that an 11β-HSD1 inhibitor, compound 544, ameliorated multiple facets of the metabolic syndrome and attenuated atherosclerotic plaque progression in ApoE−/− mice (22). However, the mechanism(s) underlying the atheroprotective effect of 11β-HSD1 inhibition was not entirely clear. While inhibition of 11β-HSD1 in those experiments led to some improvement of dyslipidemia, it appeared unlikely the modest reduction in plasma lipids could fully account for the robust antiatherosclerotic effect of the 11β-HSD1 inhibitor.

Atherosclerosis is a complex disease characterized by chronic low-grade inflammation. The immune system plays an integral role in atherosclerotic plaque initiation and progression (17, 29). 11β-HSD1 functions primarily as a reductase in activated human macrophages (40). Inhibition of 11β-HSD1 is thus expected to lower intracellular active glucocorticoids levels. Given the general perception that glucocorticoids are immune-suppressive agents (6), it was not clear how 11β-HSD1 inhibition would affect the inflammatory network in the atherosclerotic plaque. To answer this question, we first confirmed expression of 11β-HSD1 in mouse atherosclerotic plaque. To distinguish a direct vascular effect of the compound from indirect effects subsequent to plasma lipid changes, we identified a treatment regimen that lowered atherosclerosis burden in apolipoprotein E-deficient (ApoE−/−) mice without discernable changes in plasma lipids. A series of global gene expression profiling studies were carried out with atherosclerotic tissues from ApoE−/− mice treated with an 11β-HSD1 inhibitor for various intervals. Treatment led to suppression of multiple cellular pathways involved in atherosclerosis progression, most notably immune response and coagulation pathways, changes likely to contribute to the atheroprotective effect of 11β-HSD1 inhibition.

MATERIAL AND METHODS

11β-HSD1 inhibitor. Compound L-750 was administered in the feed at either 30 or 50 ppm as indicated. The average inhibition of 11β-HSD1 in the liver, as measured by the pharmacodynamic assay described previously (22), attained 100% at both doses at the time of euthanasia.

Animal husbandry. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee of Merck.

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Research Laboratories. Unless otherwise stated, all animals were maintained under controlled conditions of 25 ºC and 12 h light/dark cycles (7:00 AM/7:00 PM), with food and water available ad libitum.

*ApoE−/− mouse model.* For aortic cholesterol content measurement: C57BL/6 male ApoE−/− mice (The Jackson Laboratory) were weaned at 4 wk of age onto an atherogenic Western diet containing 21% fat and 0.15% cholesterol (88137, Harlan Teklad). At 16 wk of age, mice were separated into the following treatment groups: Western diet only (vehicle) or Western diet containing 50 ppm 11β-HSD1 inhibitor. After 8 wk of treatment, mice were euthanized, serum was collected, and aortas were dissected from the aortic root to the right renal artery for cholesterol extraction. Aortic lesions were quantified by measuring total cholesterol (TC), free cholesterol, and cholesterol ester incorporated into the vessel, as previously described (22). Samples were also collected from 16-wk-old mice on Western diet to establish baseline control levels of aortic lipid. Plasma TC, total triglyceride (TG), and monocyte chemotactic protein 1 (MCP1) levels were measured as previously described (22). For gene expression profiling studies (see Figs. 3–7), protocols of animal handling is essentially the same as described above with a few modifications as follows. Western diet treatment started when the animals were at 8 wk of age. The compound treatment group received Western diet containing 30 ppm of compound L-750 for the durations as indicated. This dosing regimen has led to consistent reduction in aortic cholesterol accumulation without changing plasma lipids. The animals were housed in a barrier facility.

*Cryostat serial sectioning and hematoxylin and eosin staining.* The RNase-free, optimal cutting temperature compound (OCT)-embedded, frozen brachiocephalic arteries were cryosectioned at 8 µm on a Bright Model OTF cryotome (Hacker Instruments, Fairfield, NJ) and transferred to adhesive slides with cryo-transfer tape according to the manufacturer’s instructions (Instrumedics, Hackensack, NJ). Four serial sections were mounted per slide in groups of four slides, kept frozen in the cryo-chamber, transported on dry ice, and stored at −80 °C, until laser capture microdissection (LCM). The fifth slide in the series had only one section per slide. These slides were stained with hematoxylin and eosin (H&E) according to standard procedure. The H&E images serve as a guide for LCM of sections on the adjacent slides.

*Immunohistochemistry.* Tissues were fixed for 4 h at 4 ºC in Nakane fixative and then were gradually infused with 20% sucrose for 5% glycerol overnight. The tissue was then placed in Tissue-Tek cryomolds (Sakura, Torrance, CA) filled with OCT, frozen in liquid nitrogen, and stored at −80 ºC. Frozen sections (5 µm) were cut and mounted on Instrumedics-coated slides. To block nonspecific labeling, sections were treated with 5% donkey serum in PBS for 20 min, then with a clarified solution of 5% nonfat dry milk for 30 min, and finally with Fc blocker (Accurate Chemical, Westbury, NY) for 20 min. Sections were labeled for 1 h with purified IgG primary antibodies or appropriate IgG controls (0.5–10 µg/ml). 11β-HSD1 and CD68 immunolocalization experiments were performed using the following antibodies: Rabbit anti-mouse HSD1 (generated in house against COOH-terminal peptide RKESVYVYDPLTIPILGGPR-KIMEFF) and rat anti-mouse CD68 antibody (cat. #MCA1957 GA; Serotec, Raleigh, NC). All nonimmune IgG controls and donkey serum were obtained from Jackson Laboratories (West Grove, PA). Slides were washed and incubated for 30 min with affinity purified F(ab)2, anti-rat, or anti-rabbit donkey IgG, conjugated with either Cy3 (red fluorescence), Cy2 (green fluorescence), or biotin (Jackson Laboratories) at 5 µg/ml. For double-labeling studies, two primary antibodies derived from different species, and corresponding species-specific fluorescent secondary antibodies were mixed together and incubated on each slide. Nonimmune IgG controls were also run in this fashion. Coverslips were mounted on the slides with Vectashield plus 4’,6-diamidino-2-phenylindole nuclear stain (Vector Laboratories, Burlingame, CA). Sections were photographed with an Evereest imaging system from Intelligent Imaging Innovations (Denver, CO).

*Immunoperoxidase ABC staining.* To block endogenous peroxidases, frozen serial sections were treated with 1% H2O2 in methanol for 5 min. To block nonspecific labeling, sections were treated with avidin/biotin blocking solution (cat #SP-2001, Vector Labs) for 20 min. The sections were further incubated for 20 min in 5% donkey serum in PBS, followed by incubation in Fc blocker for an additional 20 min. The slides were then stained with primary antibodies as described above, washed, and incubated with corresponding species-specific biotinylated secondary antibodies for 30 min. Sections were then treated with Vectastain ABC solution (Vector Labs), followed by diaminobenzidine/nickel enhancement. After washing and eosin counterstaining, the sections were dehydrated, and the samples were mounted on coverslips in Permound mounting solution (Fisher Scientific, Pittsburgh, PA). Immunoperoxidase stained slides were photographed on a CCD equipped Olympus Provis microscope.

*LCM.* To preserve RNA quality while visualizing the plaque and smooth muscle cell (SMC) samples under a microscope, we stained the cryostat sections with the HistoGene LCM Frozen Section Staining Kit (Arcturus, Mountian View, CA). The stained sections were air-dried in a chemical hood and then transferred to the microscope. The LCM was performed according to the Arcturus protocol. Briefly, an LCM cap with a transparent thermoplastic film was placed on the brachiocephalic artery section. Laser capture was carried out according to the following parameters: laser spot size at 15 µm, power at 70 mW, and duration at 700 µs. The LCM cap with the whole plaque or SMC was transferred to a CapSure HS Alignment tray. The captured cells were finally dissolved in 10 µl extraction buffer and incubated at 42ºC for 30 min. The cell extract was stored at −80 ºC. RNA was

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**Fig. 1.** 11β-hydroxy steroid dehydrogenase type 1 (11β-HSD1) inhibitor compound L-750 attenuated aortic cholesterol accumulation in apolipoprotein E-deficient (ApoE−/−) mice without lowering plasma total cholesterol (TC) and triglyceride (TG). A: chemical structure of compound L-750. B–D: treatment of ApoE−/− mice. At 16 wk of age compound L-750 was introduced at 50 ppm in feed (male mice), and the experiment continued for another 8 wk. The error bars represent SE. Baseline group, n = 9; vehicle group, n = 18; compound L-750 treatment group, n = 15. **P < 0.01 and *P < 0.05 compared with vehicle group. B: measurements of aortic TC, free cholesterol, and cholesterol ester. Measurements of plasma TC (C) and plasma TG (D)."
extracted with the Acturus PicoPure RNA isolation kit and quantitated with a Quant-iT RiboGreen RNA reagent and kit (Molecular Probes).

RNA amplification. We amplified 1 ng of RNA in triplicate with the Arcturus RiboAmp HS RNA amplification kit (cat. #K0205). Briefly, 1 ng of RNA was mixed with 200 ng of poly(dI-dC) as the RNA carrier. The first-strand cDNA was synthesized with addition of mRNA-specific first primers followed by synthesis of second-strand cDNA, which was driven by exogenous second primers. The double-stranded cDNA was transcribed in vitro to generate amplified antisense RNA. All of the aRNA was applied to the second round of RNA amplification. After in vitro transcription, the amplified antisense RNA was purified, quantified and stored at -80°C until needed for downstream RNA analysis.

RNA extraction, reverse transcription, and quantitative PCR. RNA from whole aorta and LCM atherosclerotic plaque tissues were extracted with Qiagen RNeasy kit (cat. #74181) according to manufacturer’s recommended protocol. Reverse transcription (RT) was carried out with a high-capacity cDNA archive kit (Applied Biosystems, cat. #4322171). For total RNA samples from whole aorta tissue, 500 ng RNA was used for each 100 µl RT reaction. For amplified RNA from LCM-captured samples, 2 µg two round amplified RNA was used for each 100 µl RT reaction. Quantitative PCR (Q-PCR) was then accomplished using the ABI Prism 7900 sequence detection system.

Expression level of each gene was normalized to internal control β-actin. The relative amount of each gene was then calculated by arbitrarily setting the value for the control group at 1.

Microarray study design and methodology. Microarray profiling was done as previously described (49). The two-color microarrays were scanned with the Agilent scanner and proprietary image acquisition software. Rigorous image quality control (QC) with proprietary image data mining of these clusters was performed using prior biological knowledge and known pathways, such as Ingenuity Pathway Analysis software. Rigorous image quality control (QC) with proprietary image data mining of these clusters was performed using prior biological knowledge and known pathways, such as Ingenuity Pathway Analysis (IPA) software. Rigorous image quality control (QC) with proprietary image data mining of these clusters was performed using prior biological knowledge and known pathways, such as Ingenuity Pathway Analysis (IPA) software.

Expression data were loaded into an Ingenuity knowledge database. Experimental QC was performed in MATLAB (Mathworks, Natick, MA, http://www.mathworks.com). At this point, spiked-in exogenous mRNA hybridizations were examined for a large number of known problems and attempts were made to explain any abnormal trends or outlier arrays. Expression data were loaded into Resolver (Rosetta’s proprietary software database), for transformation, normalization, and error modeling (47). Depending on the signature of interest, data from fluor-reversed pairs for each sample were split and the relevant data combined in silico (reratio) to serve as the reference pools. Next, one-dimensional and two-dimensional clustering and classifier analysis were used to get an overview of the experiment. Data mining of these clusters was performed using prior biological knowledge and known pathways, such as Ingenuity Pathway Analysis software.

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**A** High fat diet modulated signature genes in aorta

**B** HSD1 inhibitor treatment signature genes in aorta

**C** K-means clustering

**D**

![Network diagram showing gene interactions](image)
RESULTS

11β-HSD1 inhibitor attenuated aortic cholesterol accumulation in ApoE−/− mice in the absence of plasma lipid reduction. We previously reported that an 11β-HSD1 inhibitor, compound 544, significantly slowed progression of atherosclerosis in ApoE−/− mice concomitant with reduction in plasma lipids (22). To distinguish a direct vascular effect of the compound from the indirect effect subsequent of plasma lipid changes, we evaluated several structurally distinct inhibitors. Compound L-750 was found to have no discernable effects on plasma TG and TC when dosed at 50 ppm (Fig. 1). Interestingly, this treatment paradigm still led to significant reduction in plaque progression, as demonstrated by decreases of 65, 67, and 59% in the accumulation of aortic TC, free cholesterol, and cholesterol ester, respectively. Although we cannot rule out changes in plasma TC and TG during the course of the study, these data nonetheless suggest that reduction in plasma lipids are unlikely to be essential for attenuation of atherosclerosis following 11β-HSD1 inhibition.

Localization of 11β-HSD1 in mouse atherosclerotic plaque.
To support a potentially direct effect of the 11β-HSD1 inhibitors on vascular cells, we confirmed expression of 11β-HSD1 in the aorta at protein levels. Immunohistochemistry data confirm expression of 11β-HSD1 protein in vascular endothelial cells (EC) in aortas from wild-type animals (Fig. 2, A and C). In vessels with atherosclerotic plaques (Fig. 2, E and G), 11β-HSD1 protein was detected in all the major cell types in the vasculature, including EC, macrophage foam cells, and SMC. Immunofluorescent costaining with an anti-CD68 antibody showed colocalization of CD68 (green) and 11β-HSD1 (red) in macrophage foam cells (Fig. 2G).

Identification of gene signatures after 11β-HSD1 inhibition.
To investigate the effect of 11β-HSD1 inhibition on aortic gene expression, we carried out a series of studies in ApoE−/− mice (Fig. 3). These include a prophylactic study in ApoE−/− female mice (F-P) with a treatment time course, a prophylactic study in ApoE−/− male mice (M-P), and a therapeutic study in male ApoE−/− mice (M-T) (Fig. 3).

The goal of the time course study (F-P) was to assess timing for onset of gene expression changes following dietary or compound treatment. As shown in Fig. 4A, ANOVA identified a gene set of 1,298 sequences that were progressively modulated during high-fat diet (HFD) feeding in the vehicle-treated groups. This gene set is referred to as the HFD-modulated signature. Among the most statistically significant biological processes involved with the gene set were humoral defense mechanisms, wound healing, acute-phase response, immune response, and angiogenesis (data not shown). These data are consistent with a recent report from our laboratory that examined several models of experimental atherosclerosis (49). The compound L-750 treatment gene signature was obtained by ANOVA comparing the L-750-treated samples to their corresponding vehicle-treated samples at each time point. A gene set of 1,603 sequences was identified that was progressively modulated by L-750 treatment (Fig. 4B). Figure 4C shows a Venn diagram representing the comparison between HFD-modulated and L-750-treatment signatures. The 312 common sequences (Fig. 4C) contain 127 out of the 216 (59%) and 119 out of the 207 (57%) sequences in cluster 3 of both gene signatures shown in Fig. 4, A and B. Importantly, gene expression changes occurred as early as 10 days following HFD or compound treatment (cluster 3; Fig. 4, A and B). These analyses clearly indicated that similar pathways were affected by HFD and compound L-750 treatments.

After 8 wk of treatment, a more significant proportion of HFD modulated gene expression changes were reversed by compound L-750 treatment. To confirm the result, we subsequently performed an Ingenuity Pathway analysis. Figure 4D shows the gene network with the highest statistical significance. Remarkably, HFD-induced changes in 33 out of 35 genes in this network were reversed by compound L-750 treatment. Among these are a list of innate immunity genes implicated in atherogenesis, including CCL2 (MCP1), CCL3 (MIP1α), TLR2, TNFRS18, CD80, CD86, NOS2A (iNOS), and ARG1. These genes were upregulated by high-fat feeding but downregulated by compound L-750 treatment, indicating a significant suppression of vascular inflammation by 11β-HSD1 inhibition.

These observations in female mice were extended to male mice treated with either prophylactic or therapeutic protocols. Specifically, a significant proportion of gene expression changes during atherosclerotic lesion development was reversed by compound L-750 treatment (data not shown). To identify a common gene signature among the three studies, we performed a two-way ANOVA of all three data sets. Data from all compound L-750-treated samples were compared with data from their time-matched vehicle-treated samples combined in silico as reference pools. This analysis identified 1,900 sequences consistently modulated by compound L-750, including genes inhibited or induced by drug treatment (Fig. 5). Annotation of this common signature indicated that immune response was the most significant biological process inhibited by compound L-750 (Table 1). Biological processes were also identified for genes induced by compound L-750 treatment (Table 1). However, statistical significances of these changes were much lower.

The compound L-750 signature genes involved in immune response further segregated into three subcategories. These include genes that encode for receptors, signal transducers, and transcription factors in inflammatory signaling pathways (Table 2A), coagulation factors (Table 2B), and chemokines (Table 2C). Together, these data shed lights on how 11β-HSD1 inhibition may suppress vascular inflammation via modulating multiple...
cellular pathways. Relevance of these pathways to atherosclerosis will be discussed.

**Gene signatures in vascular cells procured by LCM.** The gene expression changes observed in whole aorta after 11\(\beta\)-HSD1 inhibition may be attributed to reduced inflammatory cell infiltration or to a dampened inflammatory state in the vascular cells. To distinguish between these possibilities, we employed LCM technique to selectively procure the atherosclerotic plaques (macrophages) and medial SMC from cross cryo-sections of the mouse brachiocephalic artery (41). Tissues were taken from mice at the 8 wk time point from the F-P study as described in Fig. 3. To increase the quality of the comparison between animals, we chose an anatomically definable arterial bed at the bifurcation of the brachiocephalic artery for the study (Fig. 6A). The plaque materials obtained were enriched in macrophages as indicated by CD68 expression. Remarkably, three proinflammatory genes identified from studies with whole aorta, namely MCP1, CXCL1, and IL6, were significantly downregulated in both the macrophages and SMC of compound L-750-treated mice (Fig. 6B). Note that the large error bars observed in some samples may be attributed to the RNA amplification procedure for the RNA samples procured by LCM. Nevertheless, these data suggest a dampened inflammatory state in the vascular cells in response to 11\(\beta\)-HSD1 inhibitor treatment.

Global gene expression profiling was also performed with the LCM-captured macrophages and SMC. Interestingly, compound signatures from macrophage and SMC samples showed significant overlap (Fig. 6C). Pathway analysis revealed that these overlapping genes are involved in processes such as cytokine-cytokine receptor interaction, immune cell chemotaxis, and the JAK-STAT cascade. The similarity between the compound gene

<table>
<thead>
<tr>
<th>Biological Functions Inhibited by L-750</th>
<th>P Value</th>
<th>Biological Functions Induced by L-750</th>
<th>P Value</th>
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<tr>
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<td>1.23E-11</td>
<td>Accumulation of alpha-amino acid</td>
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<td>Adhesion of blood cells</td>
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<td>Differentiation of connective tissue cells</td>
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<td>Concentration of r-glucose</td>
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<td>Metabolism of xenobiotic</td>
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<td>Modification of glutamine</td>
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<tr>
<td>Connective tissue disorders</td>
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<td>Synthesis of ketone body</td>
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<td>Inflammatory response</td>
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<td>Tubulation of endothelial cells</td>
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<td>Cell cycle</td>
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Greater significance was achieved for biological functions inhibited by L-750 treatment.
Table 2. Common signature genes suppressed by compound L-750 treatment in all three microarray studies with whole aortic tissues

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<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
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<th>M-P</th>
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<td>-1.33</td>
<td>-1.28</td>
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<td>-1.94</td>
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<td>fibrinogen gamma chain</td>
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<td>1.18</td>
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<td>plasminogen</td>
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<td>3.18</td>
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DISCUSSION

11β-HSD1 inhibitors are currently being developed by several pharmaceutical companies for the treatment of metabolic syndrome (45). Given the well-known anti-inflammatory actions of glucocorticoids (6) and the critical role of inflammation in vascular disease progression (17, 29), an outstanding question was whether 11β-HSD1 inhibition may adversely affect vascular function. In this report, we provide several lines of evidence to support a direct beneficial effect of 11β-HSD1 inhibition on the vasculature. Treatment with compound L-750 attenuated atherosclerosis in the absence of significant changes in plasma lipids (Fig. 1). These observations were corroborated by similar findings in 11β-HSD1 and ApoE double knockout mice (Ricardo Garcia, personal communication). Several microarray studies further suggest the 11β-HSD1 inhibitor modulates multiple cellular pathways in atherosclerotic plaques, resulting in suppression of vascular inflammation (Figs. 4–6).

The time course study in female ApoE−−/− mice suggests early onset of gene expression changes after commencement of 11β-HSD1 inhibitor treatment. Gene expression changes were evident 10 days after compound L-750 treatment and became more significant after 3 and 8 wk of treatment (Fig. 4B). These early gene expression changes elicited by 11β-HSD1 inhibition argue for a direct and favorable effect on the atherosclerotic plaque, compared with secondary effect to reduced plaque burden. This notion is further supported by the reduction in proinflammatory genes in LCM-procured plaque macrophages and medial SMC (Fig. 6B).

In the prophylactic study in female mice, we observed very robust gene signatures for both HFD feeding and compound signatures from whole aorta and LCM-procured vascular cells further suggests a dampened inflammatory state at the cellular level after compound L-750 treatment.

11β-HSD1 inhibition lowered circulating MCP1 levels after acute and chronic treatments. To test whether the anti-inflammatory effects of compound L-750 in the vasculature are reflected in circulating inflammatory proteins, we measured plasma levels of MCP1 in all three studies (Fig. 7). In the prophylactic time course study in female mice, a significant reduction in MCP1 protein levels was observed after 10 days of compound treatment compared with the vehicle group (Fig. 7A). This reduction was sustained after 3 wk and 8 wk of compound treatment (Fig. 7A). Similar effects were observed in male mice after 8 wk of prophylactic treatment (Fig. 7B) and 8 wk of therapeutic treatment (Fig. 7C).
A highlight is an Ingenuity inflammatory network of 35 genes. HFD-induced changes in 33 out of 35 genes in this network were reversed by compound L-750 treatment (Fig. 4D). However, because of the complex cross talk in the inflammatory network, these data alone provided limited insights into the molecular mechanism how 11β-HSD1 inhibition led to these gene expression changes. In an attempt to address this issue, we conducted two other studies in the ApoE−/− mice, namely the prophylactic study in males and the therapeutic study in males (Fig. 3). The goal of the therapeutic study was to assess compound effects on established lesions. We reasoned that by applying higher stringency in the data analysis, gene changes that are consistent across the sexes and across different treatment protocols would have greater chance to provide mechanistic clues for how the compound works. Indeed, this effort led to the identification of three classes of genes that are more likely to be the first tier responders to 11β-HSD1 inhibition (Table 2). These include genes that encode receptors, signal transducers, and transcription factors in inflammatory signaling pathways (Table 2A), coagulation factors (Table 2B), and chemokines (Table 2C).

Among the first class of compound signature genes (Table 2A), TLR1 (Toll-like receptor 1) and STAT1 (signal transducer and activator of transcription 1) are particularly worth noting.

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**Fig. 6.** Gene expression studies with plaque macrophages and medial SMC procured by laser capture microdissection (LCM). A: representative hematoxylin and eosin-stained section for LCM. The arrow indicates a histological marker for the bifurcation point that can be easily identified to help orient the sections. Atherosclerotic plaque (mostly macrophages) and the SMC layer directly underneath the plaque were captured by LCM. B: Q-PCR analysis of expression of a panel of proinflammatory genes in LCM-captured macrophages and SMC from the 2 treatment groups (n = 5). β-Actin serves as internal control. The error bars represent SE (n = 5). C: identification of compound L-750 gene signatures in Mac and SMC by microarray. Amplified samples from both vehicle- and compound L-750-treated mice were analyzed by Rosetta Agilent array, Ultra Low Input platform. Compound L-750 signatures were identified by Resolver with P < 0.001. The Venn diagram indicates a significant overlap of gene signatures from Mac and SMC. The 989 common sequences were uploaded into TGI Gene Set Annotator tool, and the biological processes returned with the most significant expectation values are shown.

**Fig. 7.** 11β-HSD1 inhibitor compound L-750 reduced circulating levels of MCP1 in ApoE−/− mice. A: treatment with compound L-750 in female ApoE−/− mice for 3 days, 10 days, 3 wk, 8 wk as indicated in Fig. 3. B: prophylactic treatment with compound L-750 in male ApoE−/− mice for 8 wk as indicated in Fig. 3. C: therapeutic treatment with compound L-750 in male ApoE−/− mice for 8 wk as indicated in Fig. 3. P values were calculated with Student’s t-test. *P < 0.05 and **P < 0.01 compared with respective vehicle treatment groups; ##P < 0.01 compared with baseline.

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L-750 treatment. A highlight is an Ingenuity inflammatory network of 35 genes. HFD-induced changes in 33 out of 35 genes in this network were reversed by compound L-750 treatment (Fig. 4D). However, because of the complex cross talk in the inflammatory network, these data alone provided limited insights into the molecular mechanism how 11β-HSD1 inhibition led to these gene expression changes. In an attempt to address this issue, we conducted two other studies in the ApoE−/− mice, namely the prophylactic study in males and the therapeutic study in males (Fig. 3). The goal of the therapeutic study was to assess compound effects on established lesions. We reasoned that by applying higher stringency in the data analysis, gene changes that are consistent across the sexes and across different treatment protocols would have greater chance to provide mechanistic clues for how the compound works. Indeed, this effort led to the identification of three classes of genes that are more likely to be the first tier responders to 11β-HSD1 inhibition (Table 2). These include genes that encode receptors, signal transducers, and transcription factors in inflammatory signaling pathways (Table 2A), coagulation factors (Table 2B), and chemokines (Table 2C).

Among the first class of compound signature genes (Table 2A), TLR1 (Toll-like receptor 1) and STAT1 (signal transducer and activator of transcription 1) are particularly worth noting.
TLR1 is a member of the Toll-like receptor (TLR) family, which plays a fundamental role in pathogen recognition and activation of innate immunity (36). STAT1 plays a critical role in macrophage polarization toward the M1 phenotype (27). Other than mediating IFNγ signaling, STAT1 also plays a role in TLR4 and IL6 signaling (37). Suppression of TLR1 and STAT1 may dampen the innate immune response in the atherosclerotic tissues after 11β-HSD1 inhibition. Consistent with these data, both TLR1 and STAT1 were also found to be suppressed in adipose tissue (44) and thioglycolate-elicited peritoneal macrophages (Ricardo Garcia, personal communication) by genetic deficiency of 11β-HSD1.

The second class of compound signature genes is enriched with coagulation factors, including factor I (fibrinogen), factor II (prothrombin), factor VII, and factor X (Table 2B). Interestingly, an intimate crosstalk between hemostasis and inflammation has been recognized in recent years (2, 10, 28). Numerous studies have documented that hemostasis is closely linked to the pathophysiology of atherogenesis (2). Coagulation proteins are implicated in processes such as the disruption of the endothelial barrier, oxidative stress, leukocyte recruitment, inflammation, migration, and proliferation of vascular smooth muscle cells. The TF-FVIIa complex, factor Xa, and thrombin mediate some of these actions by activation of G protein-coupled protease-activated receptors (PAR). Fibrinogen may exert its prothrombotic actions through mechanisms distinct from activation of PAR (2). Suppression of multiple coagulation factors in the vasculature by 11β-HSD1 inhibition thus may directly contribute to the anti-inflammatory and atheroprotective effect of this compound.

The third class of compound signature genes is enriched with chemokines, including MCP1 (CCL2) (Table 2C). Reduction of MCP1 was also confirmed in LCM-procured plaque macrophages and medial SMCs (Fig. 6). These observations were further substantiated by a decrease in circulating MCP1 levels after 11β-HSD1 inhibitor treatment (Fig. 7). MCP1 is a potent chemotactant that has been causally linked to atherosclerosis and its associated complications (35). Suppression of MCP1 expression is thus expected to contribute to the therapeutic benefit of 11β-HSD1 inhibitors.

11β-HSD1 catalyzes production of the active form of glucocorticoids in the cell. Our findings that 11β-HSD1 inhibition suppressed vascular inflammation would appear contradictory to the general perception of GCs as anti-inflammatory agents. These observations, however, are in step with older concepts of the role of GCs. Indeed, almost a century ago the prevailing concept of GC physiology was that these hormones are a necessary part of an organism’s coordinated response to stress or injury. GCs modulate multiple cellular pathways to prime the host defense mechanisms to ensure an efficient response to stressors, such as body injury, starvation, and infection (34). To integrate the multiple facets of GC function in host defense mechanisms, Sapolsky et al. (34) proposed a biphasic response model that includes both “permissive” and “suppressive” actions of GCs. Within normal diurnal variations, GCs exert permissive/stimulative actions on host defense mechanisms via activation of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). Under stress conditions, high levels of GCs suppress host defense mechanisms primarily via activation of GR (34, 50). In this integrated picture, the well-known anti-inflammatory effects of GCs serve to prevent stress-activated defense mechanisms from “overshooting” and thus threatening homeostasis.

At the gene expression level, studies with immune cells have uncovered both enhancing and suppressive functions of GC in the immune system (11, 18). GC have been shown to stimulate cytokine receptor expression (18, 34, 50) and have also been reported to increase cytokine secretion (4, 50). In humans, chronic exposure to high levels of GC is associated with hypercytokinemia and hypercoagulable state (26, 42). Our data confirm and extend these previous reports. We show that inhibition of intracellular production of active GC via treatment with L-750 suppressed or reduced gene expression of cytokine receptors, signal transducers, and transcription factors (Table 2A), coagulation factors (Table 2B), and lowered circulating levels of the proinflammatory cytokine MCP1 (Fig. 7).

GC are high-affinity ligands of MR. Activation of MR has been suggested to partly mediate the proinflammatory actions of GCs (12, 23, 34, 52). It is well established that activation of MR leads to increased oxidative stress, inflammation, and fibrosis in the kidney (3) and heart (19, 33, 48, 51). In recent years, a role of MR in atherosclerosis is being increasingly recognized (13). MR is expressed in vascular SMC (24), EC (5), and macrophages (32) and contributes to vascular inflammation, fibrosis, and remodeling (30). In the settings of heightened oxidative stress such as atherosclerosis, GC may play a significant role in activating MR, predisposing to development of inflammation (14–16). Indeed, deficiency of 11β-HSD2, the enzyme that inactivates intracellular GC (31), led to sustained activation of MR and exacerbated atherosclerosis in ApoE−/−mice (9). Consistently, we found that inhibition of 11β-HSD1, the enzyme that activates intracellular GC, led to suppression of vascular inflammation and attenuation of atherosclerosis in ApoE−/−mice. These observations share similarities with the reported effects of MR blockade in experimental atherosclerosis (9, 25, 39). It is conceivable that the anti-inflammatory effects observed with 11β-HSD1 inhibition may be partly attributed to suppression of GC-mediated MR activation. Future work will help delineate the extent of changes in MR signaling following 11β-HSD1 inhibition.

Compared with MR signaling, it is less well understood how perturbation of GR-mediated signaling may contribute to the atheroprotective effects of 11β-HSD1 inhibition. Interestingly, GR antagonist RU486 was reported to stimulate ATP-binding cassette transporter-A1 (ABCA1) expression and apolipoprotein-mediated cholesterol efflux from macrophages, a pivotal event in maintaining intracellular cholesterol levels and preventing the formation of macrophage-derived foam cells (1). Understanding the effect of RU486 on atherosclerosis will help shed light on potential contribution of GR in the pharmacologic effects of 11β-HSD1 inhibition in the vasculature.

11β-HSD1 has been implicated in feedback regulation of the hypothalamus-pituitary-adrenal (HPA) axis (21). Indeed 11β-HSD1 knockout mice showed evidence of HPA axis activation, including elevated plasma corticosterone and ACTH levels at the diurnal nadir (21). To understand potential impact of L-750 treatment on the HPA axis, we monitored circulating corticosterone levels in mice after 11 days of treatment with L-750 at levels 3–10 times greater than the current studies. No changes were observed in either basal or stress induced levels of corticosterone (data not shown). These data suggest that partial
pharmacologic inhibition of 11β-HSD1 is sufficient to drive beneficial effects without measurably altering HPA function. 11β-HSD1 may play a different role in regulating acute inflammation compared with its role in modulating low-grade chronic inflammation in the settings of atherosclerosis. 11β-HSD1 homozygous deficiency was reported to decrease macrophage phagocytosis and delayed resolution of inflammation in experimental peritonitis (20) and worsen acute inflammation and experimental arthritis in mice (7). These phenotypes may be partly attributed to activation of the HPA axis in the 11β-HSD1 knockout mice (53). During clinical development 11β-HSD1 inhibitors, it will be important to carefully examine the potential impact of pharmacologic inhibition of 11β-HSD1 on HPA axis activation and acute inflammation (8).

In conclusion, our study shows that chronic inhibition of 11β-HSD1 led to suppression of vascular inflammation. Comprehensive gene expression profiling studies identified several classes of genes that were consistently downregulated across both sexes and in both prophylactic and therapeutic treatments. These include signaling receptors/signal transducers/transcription factors, coagulation factors, and proinflammatory chemokines. These observations are consistent with the recent reports on the potentiating and proinflammatory actions of glucocorticoids. Importantly, these gene expression changes may directly contribute to the anti-inflammatory and atheroprotective effects of the 11β-HSD1 inhibitor. Together, these data lend strong support for clinical development of 11β-HSD1 inhibitors, it will be important to carefully examine the anti-inflammatory and atheroprotective effects of glucocorticoids, recent developments and mechanistic insights, Mol Cell Endocrinol 335: 2–13, 2011.


