Endothelial Glucocorticoid Receptor Suppresses Atherogenesis—Brief Report

Julie E. Goodwin,* Xinbo Zhang, Noemi Rotllan, Yan Feng, Han Zhou, Carlos Fernández-Hernando, Jun Yu, William C. Sessa

Objective—The purpose of this study was to determine the role of the endothelial glucocorticoid receptor in the pathogenesis of atherosclerosis.

Approach and Results—Control mice and mice lacking the endothelial glucocorticoid receptor were bred onto an Apoe knockout background and subjected to high-fat diet feeding for 12 weeks. Assessment of body weight and total cholesterol and triglycerides before and after the diet revealed no differences between the 2 groups of mice. However, mice lacking the endothelial glucocorticoid receptor developed more severe atherosclerotic lesions in the aorta, brachiocephalic artery, and aortic sinus, as well as a heightened inflammatory milieu as evidenced by increased macrophage recruitment in the lesions.

Conclusions—These data suggest that the endothelial glucocorticoid receptor is important for tonic inhibition of inflammation and limitation of atherosclerosis progression in this model. (Arterioscler Thromb Vasc Biol. 2015;35:779-782. DOI: 10.1161/ATVBAHA.114.304525.)

Key Words: atherosclerosis ■ endothelium ■ glucocorticoid receptor

The glucocorticoid receptor (GR) is a nuclear hormone receptor that is expressed ubiquitously in most cell types and is important in many states of health and disease. Recent work has demonstrated that tissue-specific loss of this receptor can produce profound phenotypes.1-4 The role of glucocorticoids in cardiovascular disease is complex. For example, the stress response, which is elevated in chronic conditions such as atherosclerosis, hypertension, and the metabolic syndrome, has been implicated in the heightened vulnerability to disease found in these conditions by activating the hypothalamic-pituitary-adrenal axis and increasing production of circulating endogenous steroid.5,6 Conversely, acute high-dose corticosteroids during the progression of atherosclerosis and plays a role in limiting the progression of atherosclerosis.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Littermate Apoe+/GR-/- (Apoe+) and Apoe-/- GR-/- Tie1-Cre (double knock-out [DKO]) mice were fed a high-fat diet for 12 weeks as described.9 Previously, we have documented the endothelial specificity of the deleted GR in several studies.1,3 Both groups of mice had similar weights (Figure 1A), triglycerides (Figure 1B), cholesterol levels (Figure 1C), and corticosterone levels (Figure 1D) at baseline and after feeding mice with high-fat diet. Similar corticosterone levels were indicative that there was neither derangement in the hypothalamic–pituitary–adrenal axis in DKO animals nor evidence of heightened stress in these animals. These results are in agreement with previously published work.1

At the completion of the feeding period, mice were euthanized, perfused, and the extent of atherosclerosis in multiple vessels was examined. Aortas were stained en face with oil red

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O and the percentage of total aortic neutral lipids deposition quantified. As shown in Figure 1E and quantified in Figure 1F, DKO mice showed significantly greater lesion areas than did Apoe−/− mice. There were no differences noted in the anatomic distribution of the lesions when suprarenal and infrarenal aortas were analyzed separately (Figure I in the online-only Data Supplement). Similar results were obtained in cross sections of brachiocephalic arteries (Figure 1G and 1H). To determine whether the increased lesion size in the DKO mice was associated with heightened inflammation, tissue macrophages (via CD68 labeling) were assessed immunohistochemically. As shown in Figure 2A and quantified in Figure 2B, DKO animals showed greater macrophage accumulation in the atherosclerotic lesions of brachiocephalic arteries, indicating a heightened inflammatory state. As additional evidence that inflammation was increased in the DKO animals, staining for vascular cell adhesion molecule was also performed in these vessels. DKO mice also showed increased vascular cell adhesion molecule-positive staining in the atherosclerotic lesions (Figure II in the online-only Data Supplement).

We also examined aortic root lesions in hearts from Apoe−/− and DKO mice. Hearts were prepared and sectioned as described above to allow visualization of the aortic sinus lesions and representative sections are shown in Figure 2C. Quantification of oil red O staining/lesion area (Figure 2D) and total lesion area (Figure 2E) showed more extensive lesions in DKO mice than in Apoe−/− mice. Macrophage infiltration was also assessed in aortic root lesions via CD68 staining. As shown in Figure 2C and quantified in Figure 2F, DKO mice had increased absolute macrophage area. In addition, lesions in the coronary ostiae were identified in 3 of 6 DKO mice but in none of the Apoe−/− mice (Figure III in the online-only Data Supplement).

In a separate series of experiments, mice were fed the Paigen diet to examine the effect of diet-induced, toll-like receptor 4-dependent inflammation on lesion progression.10,11 Although this diet has been studied in this context previously, it is important to note that it may have other effects that were not assessed in our study. Importantly, only 6 of 16 DKO mice survived this diet, whereas 12 of 13 Apoe−/− mice survived (Figure IV in the online-only Data Supplement). There were no differences in body weight between the groups; however, DKO mice exhibited higher corticosterone levels (Figure VA and VB in the online-only Data Supplement). Atherosclerotic lesions in the aorta and brachiocephalic artery were also significantly more diseased than in Apoe−/− mice. DKO mice had statistically more infrarenal lesion than in Apoe−/− mice although no differences were observed in the distribution of suprarenal lesions (Figures VI and VII in the online-only Data Supplement). Collectively, these data imply that endothelial GR is critical for the atheroprotective actions of endogenous corticosterone.
Discussion

The major finding of this study is that loss of the endothelial GR results in a more severe atherosclerotic phenotype in Apoe−/− mice. These results are striking because they demonstrate that elimination of the receptor for the endogenous ligand, corticosterone, in a single-cell type, namely the endothelium, is sufficient to produce this dramatic phenotype. These data support the importance of the permissive actions of endogenous corticosterone via endothelial GR in reducing vascular inflammation and highlights the possibility of tissue-specific manipulation of local glucocorticoid metabolism as a potential therapy for cardiovascular disease. Isozymes of 11β-hydroxysteroid dehydrogenase (11βHSD) are responsible for local regulation of the access of glucocorticoids to their receptors, with the enzyme 11βHSD1 responsible for the conversion of the inactive cortisol to cortisone (or corticosterone in mouse) and 11βHSD2 converting the active corticosteroid (corticosterone) to cortisol. Both enzymes are known to exist in the endothelium. Previous studies have shown that 11βHSD1 knockout attenuates atherosclerosis, whereas 11βHSD2 deficiency accelerates atherosclerosis indeed suggesting that local glucocorticoid metabolism is the key to understanding endogenous steroid effects in the context of cardiovascular disease. It is interesting to note that both of these studies used mice globally deficient for the enzymes, whereas we achieved a similar magnitude of enhanced atherosclerosis by altering the endogenous steroid milieu in the endothelium only. Our data in mice fed the Paigen diet are somewhat unexpected given that most of the DKO animals die during the feeding period, a phenomenon that is not often seen in mouse models of atherosclerosis. However, the cause of death was not directly assessed. We suspect that the higher corticosterone levels observed in the DKO animals fed the Paigen diet are a marker of increased stress and may have contributed to mortality. These results suggest that the endothelial GR is profoundly important for maintaining vascular homeostasis and that its loss in combination with a second hit, such as the inflammatory diet used here, accelerates disease. Collectively, these data indicate that both local glucocorticoid metabolism and tissue-specific glucocorticoid interactions are likely important for mediating the complex interplay between endogenous steroids and cardiovascular disease.

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Disclosures

None.
It is well appreciated that endothelial dysfunction is one of the first steps in the development of many cardiovascular diseases, including atherosclerosis. The role of steroids in the pathophysiology of cardiovascular disease is not as clear-cut and published studies exist which demonstrate evidence for both beneficial and detrimental effects of exogenous steroids. Our study directly assesses the role of the endothelial glucocorticoid receptor in the pathogenesis of atherosclerosis. Here, we show that loss of endothelial glucocorticoid receptor results in markedly more severe atherosclerotic lesions both in mice fed a standard Western diet and mice fed the more inflammatory Paigen diet. These data support an important role for endothelial glucocorticoid receptor in suppressing inflammation and also highlight the importance of tissue-specific glucocorticoid metabolism in the pathogenesis of cardiovascular disease. This concept is novel, and provides an explanation for the well-documented permissive role of endogenous cortisol.
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Materials and Methods

Animals
Mice lacking the endothelial glucocorticoid receptor (GR) were generated as previously described\(^1\) and bred to Apo E knockout mice on a C57BL/6 background. The mice were backcrossed for more than 10 generations. These mice were fully congenic according to microsatellite analysis done through the RADIL facility in Columbia, Missouri. Atherosclerosis was induced by feeding the mice a high fat diet containing 1.25% cholesterol (Research Diets, D12108). In some experiments, mice were fed the Paigen diet containing of 1.25% cholesterol and 0.5% Na cholate.\(^2\) Male mice were used for feeding experiments. Feeding commenced at 8 weeks of age and continued to 20-22 weeks of age and body weight was tracked during this time period. All experiments were approved by the Institutional Animal Care Use Committee of Yale University.

Lipid Analysis
Mice were fasted for 12-15 hours and blood was collected by retro-orbital venous puncture. Whole blood was spun down and plasma stored at -80° C. Total cholesterol and triglyceride levels were measured enzymatically by kits from Wako and Sigma, respectively, according to the manufacturer's instructions.

Corticosterone Measurements
Blood samples were obtained in the morning (8-10 AM) and measured by ELISA (Assay Designs) according to the manufacturer's instructions.

Atherosclerotic Lesion Analysis
At the completion of high-fat diet feeding mice were anesthetized and euthanized. Mouse hearts were perfused with PBS and then 4% paraformaldehyde (PFA) and the hearts, aortas and brachiocephalic arteries were dissected out using a dissecting microscope and maintained in PFA overnight. Whole aortas were stained with Oil Red O (Sigma) to quantify lesion area. Oil Red O stock solution (35 ml, 0.2% weight/volume in methanol) was mixed with 10 ml 1 M NaOH and filtered. Aortas were briefly rinsed in 78% methanol, incubated in Oil Red O for 45 minutes and then destained in 78% methanol for 5 minutes and mounted on microscopic slides. Brachiocephalic arteries and hearts were mounted in OCT. Brachiocephalic arteries were sectioned 100 µm distal to the bifurcation and hearts were sectioned beginning from the aortic sinus and proceeding inferiorly. These frozen sections were stained with various preparations including hematoxylin and eosin, Oil Red O and Trichrome. Lipid staining and lesion size were quantified by averaging six sections from the same mouse using the IMAGE J program.

Immunostaining
Frozen sections were stained for immunofluorescence as previously described.\(^3\) Primary antibodies used included CD68 (Dako) and VCAM (Santa Cruz). Six sections for each mouse were quantified using the IMAGE J program.
Statistical Analysis
GraphPad software was used for statistical analysis. Since some data sets were small, data were tested for use by both a parametric test (Student’s t-test) and a non-parametric test (Mann Whitney test) for single comparisons. In all cases both tests gave similar results. P values from the Student’s t test are reported. For multiple comparisons, data were again tested with both parametric (1-way ANOVA) and non-parametric tests (Kruskall Wallis with Dunns post-test). These statistical analyses again were in agreement with each other. P values from the 1-way ANOVA are reported. A p value <0.05 was considered statistically significant.

References
Supplementary Figure Legends

I: No differences in the distribution of (A) suprarenal or (B) infrarenal aortic atherosclerotic lesions in high-fat diet-fed mice.

II: VCAM staining (A) Representative images of VCAM staining in brachiocephalic arteries of Apoe-/- and DKO mice fed a high fat-diet. (B) Quantification of VCAM-positive staining in atherosclerotic lesions from both groups. *p<0.05

III: Oil Red O staining of coronary ostiae lesions in high fat diet-fed DKO mice. The aortic sinus is shown on the left and the inset containing the coronary ostia is magnified on the right for three separate mice in whom lesions were found. No coronary ostiae lesions were found in high fat diet-fed Apoe -/- mice.

IV: Kaplan Meier curve for mice fed the Paigen diet during the feeding period. Only 6/16 DKO mice as compared to 12/13 Apoe -/- mice survived until the end of the feeding period (p<0.01).

V: Evidence of systemic inflammation in mice fed the Paigen diet. (A) Weight gain is blunted in Apoe -/- (n=12) and DKO (n=6) mice at the conclusion of timed dietary feeding. (B) DKO mice (n=6), but not Apoe -/- mice (n=12) show higher levels of the endogenous steroid, corticosterone at the conclusion of the diet. *p<0.05.

VI: More severe aortic atherosclerotic lesions in DKO animals fed the Paigen diet. (A) Representative Oil Red O staining. (B) Apoe -/- (n=9) and DKO (n=6) mouse aortas were stained with Oil Red O after 14 weeks of Paigen diet feeding. Quantification of (C) suprarenal and (D) infrarenal lesions. *p<0.05.

VII: More severe brachiocephalic atherosclerotic lesions in DKO animals fed the Paigen diet. (A) Representative staining of brachiocephalic arteries. (B) Atherosclerotic lesion was quantified in Apoe -/- (n=12) and DKO (n=6) mice. P<0.05.
A

**DAPI**  **VCAM**  **Merge**

<table>
<thead>
<tr>
<th>Apoe -/-</th>
<th>DKO</th>
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<tbody>
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<td><img src="Apoe_-_/-_DAPI.png" alt="Image" /></td>
<td><img src="Apoe_-_/-_VCAM.png" alt="Image" /></td>
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<td><img src="DKO_DAPI.png" alt="Image" /></td>
<td><img src="DKO_VCAM.png" alt="Image" /></td>
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B

**VCAM-positive area (mm²)**

![Graph](Graph.png)

* $p < 0.05$
We weeks of Paigen diet

Survival (%)

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Weeks of Paigen diet

* Apoe -/-

DKO
**A**

Body weight (g)

- **Baseline**
  - Apoe -/-: [Value]
  - DKO: [Value]

- **After Paigen diet**
  - Apoe -/-: [Value]
  - DKO: [Value]

**B**

Corticosterone (ng/ml)

- **Baseline**
  - Apoe -/-: [Value]
  - DKO: [Value]

- **After Paigen diet**
  - Apoe -/-: [Value]
  - DKO: [Value]
Apoе−/− vs DKO

B

lesion size/lumen size (%)

Apoe −− DKO

*