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**Title:** Corticosterone accelerates atherosclerosis in the apolipoprotein E-deficient mouse

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Running title: The glucocorticoid corticosterone accelerates atherosclerosis

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**Summary**

Chronic stress is an important risk factor for atherosclerosis, which is a chief process in the development of cardiovascular disease. Increased circulating levels of corticosterone have been documented in several animal models of chronic stress. However, it remains to be established whether corticosterone is sufficient to exacerbate atherosclerosis. To test this hypothesis, apolipoprotein E (ApoE)-deficient mice were fed a high-fat diet for 13 weeks with exposure to either corticosterone or vehicle in the drinking water (CORT and Con). Corticosterone treatment significantly increased atherosclerotic plaque area at the aortic root. Such exacerbation of atherosclerosis was accompanied by significantly lower levels of circulating white blood cells and serum interleukin-1β (IL-1β), and significantly elevated serum concentrations of total cholesterol, low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL) and small dense low-density lipoprotein (sd-LDL) in CORT mice when compared to Con mice. These findings demonstrate that corticosterone is sufficient to exacerbate atherosclerosis *in vivo* despite its anti-inflammatory properties and that this marked pro-atherogenic phenotype is primarily associated with increased dyslipidaemia.

**Keywords**: stress, dyslipidaemia, cholesterol, Hypothalamic-pituitary-adrenal axis, apoE-deficient mice.

## Introduction

Atherosclerosis is a chief process in the development of cardiovascular disease (CVD), which is the leading cause of mortality in both developed and developing countries [1]. Several modifiable risk factors have been associated to CVD [2-4] with stress being an important contributor to atherosclerosis [5]. Indeed, it has been documented that chronic and/or repeated exposure to stressful stimuli leads to endothelial dysfunction and atherosclerosis [6]. Stressful conditions are known to activate the hypothalamic-pituitary-adrenal axis (HPA axis) inducing increases in circulating catecholamines and glucocorticoids; thus the development of CVD may result from certain pathophysiological effects of these hormones. In that sense, several studies have documented a correlative link between synthetic, as well as endogenous glucocorticoids, and atherosclerosis. Chronic synthetic glucocorticoid treatment (e.g. prednisone) is commonly prescribed in autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) and was found to be associated with atherosclerosis progression in both RA and SLE patients [7-10]. Unfortunately, the stage of disease progression in RA and SLE patients [8] coupled with the synthetic nature of

glucocorticoids used, which have higher affinities for glucocorticoid receptors [11, 12] and longer half-life [13], are important confounding factors in assessing a potential role of endogenous, or natural, glucocorticoids in atherosclerosis. Accelerated atherosclerosis has also been reported in patients with Cushing's Syndrome (CS), a hormonal disorder caused by excessive levels of the endogenous glucocorticoid cortisol [14]. However, several other important CVD risk factors such as visceral obesity, hypertension, insulin resistance and dyslipidaemia are commonly observed in patients with CS [15, 16] and are also confounding factors in establishing a causal relationship between natural glucocorticoids and atherosclerosis. Therefore, it remains to be established whether chronic exposure to elevated levels of natural glucocorticoids is sufficient to induce or accelerate atherosclerosis.

Several animal models of chronic stress or depression including forced swimming, restraint, and psychosocial stress have been shown to increase circulating levels of corticosterone [17-20]. Other studies have demonstrated that corticosterone administration is a valid model to study chronic stress in animals [20-22]. Therefore, in the present study we hypothesized that chronic administration of low levels of the natural glucocorticoid

corticosterone exacerbates atherosclerosis in Apolipoprotein E (ApoE)-deficient mouse, an animal model frequently used in atherosclerosis research due to the development of plaques of similar type and distribution in comparison to humans [23, 24].

## Materials and Methods

### *Animals and diet*

Male ApoE-deficient (B6.KOR/StmSlc-*Apoe*$^{shl}$) mice (6 weeks old) from the Japan SLC, Inc (Shizuoka, Japan) were housed individually and maintained in light- (12:12-h light-dark cycle) and temperature-controlled quarters (22°C) [25, 26].Mice were acclimated to the animal facilities while being fed normal chow for one week. High-fat diet feeding (21% fat; Oriental Yeast, Tokyo, Japan) was then initiated at 7 weeks of age [27, 28]. At this point mice were randomly assigned into control or corticosterone groups and monitored until sacrifice. The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments at Waseda University, Japan.

### *Corticosterone treatment*

The corticosterone group (CORT: n=8) was treated with corticosterone dissolved in drinking water for 13 weeks. Corticosterone was first dissolved in 30% (w/v) 2-hydroxypropyl-β-cyclodextrin (HBC) and then diluted to 25 μg/ml with distilled water. The final concentration of HBC in the drinking water was 0.6%. The control group of mice (Con: n=8) was treated with vehicle (i.e. 0.6% HBC in drinking water). The period of 13 weeks of intervention was used because previous studies have documented that ApoE-deficient mice develop atherosclerotic plaques after 12 weeks of exposure to high-fat diet [27]. Water and food intake were monitored throughout the experiment. Based on daily water intake, the dose of corticosterone was ~177 μg/day, or 8.4 mg/kg body weight, in the beginning of the study. This dose was previously shown to restore low physiological levels of corticosterone in adrenalectomized male mice [29], and was 1/5 or less of doses used in previous chronic studies [20-22]. Despite the weight gain in animals of both groups during the study, water intake was unaffected. As a result the daily corticosterone dose was continuously reduced down to ~3.8mg/kg at the last week of intervention (i.e. ½ of the initial dose). This treatment did not elicit any clear behavioral changes that could be observed during the daily monitoring of animals.

*Tissue Preparation and Quantification of Atherosclerosis*

Tissue preparation was performed as previously described [30]. Briefly, once mice were anesthetized with sevoflurane, blood was collected with and without heparin from the axillary artery for leukocyte and monocyte cell counting and serum lipid profiling. Mice were then perfused with PBS, and then the heart was harvested and fixed with 4% paraformaldehyde. Animals were then sacrificed by cervical dislocation and the gastrocnemius muscle was collected and weighted. Processing of fixed tissue then proceeded with exposure to sequential increasing grades of sucrose/PBS (10%, 15% and 20%), before samples were quickly frozen in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan). Atherosclerotic plaque quantification was performed as previously described [30]. Basically, cross sections of 6μm of the aortic root were stained with freshly prepared Oil Red O working solution, rinsed with distilled water and counterstained with Mayer's hematoxylin. Sections were then rinsed again with distilled water and mounted into slides with glycerol-based mounting medium. Images were acquired with a digital camera (Nikon, Tokyo, Japan) coupled to a Nikon TE2000

microscope. Areas of atherosclerotic plaques in five sections (at least 60μm apart) from each mouse were analyzed and quantified with Image J (NIH, Bethesda, MD, USA) and the average of those was considered representative of that animal and used for further analysis.

*Total and differential white blood cell count and serum lipid profile*

Total and differential white blood cells were counted in each heparinized blood sample using a SF-3000 (Sysmex, Kobe, Japan). The concentrations of total cholesterol, triglycerides (TG), chylomicron (CM), high-density lipoprotein (HDL), low-density lipoprotein (LDL), small dense low-density lipoprotein (sd-LDL), and very-low-density lipoprotein (VLDL) were analyzed in 60μL of serum as previously described [31] using a HPLC based on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides. This analysis was performed at Skylight Biotech Inc. (Akita, Japan).

*Measurement of serum interleukin-1β, interleukin-6 and CCL2*

Serum concentrations of interleukin-1β (IL-1β), interleukin-6 (IL-6) and monocyte chemotactic protein-1(MCP-1/CCL2) were measured by enzyme linked immunosorbent assay (ELISA) (Quantikine® R&D Systems, Minneapolis, MN, USA). All assays were performed in a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer instructions.

*Statistical analysis*

All data are presented as mean ± SEM. Values were compared by unpaired t-tests performed with the StatView software (SAS Institute, Cary, NC). A value of $p<0.05$ was considered statistically significant.

**Results**

Both CORT and Con mice had approximately doubled their body weight (BW) by the end of the study, with no significant difference in either BW or food intake between groups (Figures 1A, B). However, chronic low dose corticosterone treatment led to a significant reduction in gastrocnemius muscle weight ($P<0.01$) (Figure 1C). This

finding demonstrates that increased levels of the natural glucocorticoid corticosterone causes skeletal muscle atrophy similarly to the use of synthetic glucocorticoids (e.g. dexamethasone) [32]. Atherosclerotic plaque lesions at the aortic root were 77% larger in CORT mice compared with Con mice denoting a profound exacerbation of atherosclerosis by corticosterone ($P<0.05$) (Figure 1D, E). Of note, several variables suggest that the significant impact of corticosterone on the development of atherosclerosis was not linked to increased systemic inflammation. First, total circulating white blood cell and neutrophil numbers in CORT mice were 29% and 24% lower than in Con mice, respectively ($P<0.05$, Figure 2A). Second, no significant differences were observed in lymphocyte, monocyte, eosinophils or basophils between groups (Figure 2A). Third, IL-1β serum levels in CORT mice were 55% lower than the ones observed in Con mice ($P<0.05$), while IL-6 and CCL2 levels were similar between groups (Figure 2B). On the other hand, mice chronically treated with corticosterone were severely dyslipidaemic. Although no differences were observed between groups in relation to triglycerides, chylomicron and HDL levels, significantly elevated serum levels of total cholesterol, LDL, VLDL and sd-LDL were present in CORT mice in

comparison to Con mice (i.e. by 51%, 35%, 67% and 32%, respectively, $P<0.05$, Figure 3).

## Discussion

The findings presented here clearly demonstrate that chronic low dose corticosterone treatment exacerbates atherosclerosis in association with dyslipidaemia, despite having systemic anti-inflammatory effects. Although epidemiological studies have previously linked increased stress levels with the development of CVD [5], it is difficult to isolate the precise contribution of stress *per se* from its influence on other CVD risk factors, such as the ones associated with environmental factors (e.g. eating habits). A more causal link between stress and atherosclerosis was first established by Kumari et al., who observed that ApoE-deficient mice exposed to chronic stress for 12 weeks presented 3-fold increases in atherosclerosis in association with ~10-fold elevation in circulating corticosterone levels [33]. Nevertheless, it remained to be established whether the natural glucocorticoid corticosterone was sufficient to induce an atherogenic phenotype and what systemic effects would be associated with this phenomenon. To address these

questions and minimize adverse effects of chronic glucocorticoid administration, we opted to study ApoE-deficient mice fed a high-fat diet with or without exposure to a low dose of corticosterone in the drinking water for 13 weeks. In spite of the limitation of not assessing circulating levels of corticosterone during the study, we believe our model induced much lower increases in serum corticosterone than observed by Kumari et al. [33] for a couple of reasons. First, our initial dose of ~8.4mg/kg of body weight was previously shown to restore circulating corticosterone to only low physiological levels in adrenalectomized male mice [29]. Second, the required high-fat diet content (i.e. 21%) to induce atherosclerotic plaque formation in ApoE-deficient mice within the 13 week time frame [27] caused drastic body weight increases in both CORT and Con mice, without altering water consumption. Therefore, the daily corticosterone dose administered in the drinking water was ~3.8mg/kg of body weight at the end of the intervention. It is important to note that endogenous glucocorticoid production tends to decrease secondary to a suppression of HPA axis upon exogenous glucocorticoid treatment [34]. In our study, measurement of endogenous glucocorticoid levels was not feasible due to insufficient volume of blood in these mice. Despite this limitation,

increased atherosclerosis was observed in CORT mice (i.e. 80%) suggesting a role for corticosterone in accelerating atherosclerosis.

Obesity is widely recognized as an important risk factor for CVD [2, 3] that leads initially to local adipose tissue inflammation and then systemic low-grade inflammation [35-38]. In fact, systemic low-grade inflammation is a common feature of not only obesity, but also type 2 diabetes and cardiovascular disease [3, 39, 40]. CORT mice likely gained more fat mass than Con mice because only the CORT group presented significant muscle atrophy, despite increasing body weight to the same extent as Con mice throughout the intervention. Regardless, there was no evident exacerbation of low-grade systemic inflammation in CORT mice, as circulating white blood cells and IL-1β were significantly reduced by corticosterone. These findings support a predominant anti-inflammatory effect of corticosterone in mice. It is important to note, however, that an exacerbated inflammatory state could be present at the atherosclerotic plaques of CORT mice. In that case, our data suggests that this phenomenon would be resulting from the atherosclerotic process itself, rather than being a secondary effect of obesity and low-grade systemic inflammation. Actually, the effective use of glucocorticoids to

control inflammation in autoimmune diseases such as RA and SLE, despite being associated with exacerbation of atherosclerosis [7-10], provides further circumstantial evidence for this notion.

Dyslipidaemia is another critical risk factor for CVD [41]. Here, we found that corticosterone caused significant increases in total cholesterol, LDL, VLDL and sd-VLDL cholesterol, which are easily absorbed into the vascular endothelium, and are susceptible to oxidative modifications, thereby enhancing atherosclerosis [42]. Although the mechanisms by which corticosterone interferes in cholesterol metabolism is beyond the scope of this study, synthetic glucocorticoid treatment has been shown to increase the expression of the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) both *in vivo* and *in vitro* [43-45]. HMG-CoA reductase is a key regulatory enzyme in the conversion of HMG-CoA to mevalonic acid, which is a precursor for cholesterol synthesis [46]. Competitive inhibitors of HMG-CoA reductase (e.g., Statins) increase the expression of LDL receptors in the liver, which in turn decreases serum total cholesterol and LDL cholesterol concentrations [47, 48]. Altogether, these observations suggest that corticosterone exacerbates atherosclerosis by primarily

upregulating circulating levels of total cholesterol and pro-atherogenic lipoproteins. Future studies including co-treatment of ApoE-deficient mice with corticosterone and inhibitors of cholesterol biosynthesis should provide new insights into this notion. Further, investigations combining pharmacological blockage of glucocorticoid receptor and either stress stimuli or corticosterone treatment are still desired to provide further insights into the mechanisms of action of glucocorticoids in the development of atherosclerosis.

In summary, exposure to low levels of corticosterone for 13 weeks significantly exacerbated atherosclerosis in ApoE-deficient mice. This exacerbated phenotype was most likely driven by dyslipidaemia, without indications of increased systemic inflammation.

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**Conflict of interests**

None.

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This study was conducted in the Consolidated Research Institute for Advanced Science and Medical Care.

**Figure legends**

Figure 1. Atherosclerotic plaque lesions in control mice (Con) and mice treated with corticosterone (CORT). A) Body weight progression during 13 weeks of intervention. B) Weekly food intake. C) Gatrocnemius muscle weight. D) Representative Oil Red O stained sections with respective enlarged pictures of atherosclerotic lesions (reference bar=100μm). E) Quantification of atherosclerotic lesion area. Values represent mean ±

SE. ***P<0.001, *P<0.05.

Figure 2. Circulating white blood cell numbers and serum cytokine levels in control mice (Con) and mice treated with corticosterone (CORT). A) Circulating white blood cell, neutrophil, lymphocyte, monocyte, eosinophil and basophil number. B) Serum IL-1β, IL-6 and CCL2 concentration. Values represent mean ± SE. *P<0.05.

Figure. 3. Serum cholesterol and lipid protein profile of control mice (Con) and mice treated with corticosterone (CORT). Concentrations of total cholesterol (Total), high-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), small dense low-density lipoprotein (sd-VLDL), triglyceride (TG), and chylomicron (CM) were analyzed by a HPLC based on-line dual enzymatic method. Values represent mean ± SE. *P<0.05.

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