Glycyrrhetinic acid attenuates vascular smooth muscle vasodilatory function in healthy humans

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ABSTRACT
Abnormal glucocorticoid metabolism contributes to vascular dysfunction and cardiovascular disease. Cortisol activation of vascular mineralocorticoid and glucocorticoid receptors is regulated by two types of 11\(\beta\)-HSD (11\(\beta\)-hydroxysteroid dehydrogenase), namely 11\(\beta\)-HSD2 and 11\(\beta\)-HSD1 (type 2 and type 1 11\(\beta\)-HSD respectively). We hypothesized that inhibition of 11\(\beta\)-HSD would attenuate vascular function in healthy humans. A total of 15 healthy subjects were treated with the selective 11\(\beta\)-HSD inhibitor GA (glycyrrhetinic acid) or matching placebo in a randomized double-blinded cross-over trial. 11\(\beta\)-HSD activity was assessed by the urinary cortisol/cortisone ratio, and vascular function was measured using strain-gauge plethysmography. Endothelial function was measured through incremental brachial artery administration of methacholine (0.3–10 \(\mu\)g/min) and vascular smooth muscle function with incremental verapamil (10–300 \(\mu\)g/min). GA increased the 24-h urinary cortisol/cortisone ratio compared with placebo (\(P_\text{=0.008}\)). GA tended to reduce the FBF (forearm blood flow) response to methacholine (\(P_\text{=0.09}\)) and significantly reduced the FBF response to verapamil compared with placebo (\(P_\text{=0.04}\)). MAP (mean arterial pressure) did not differ between the study conditions. 11\(\beta\)-HSD inhibition attenuated vascular smooth muscle vasodilatory function in healthy humans. Disturbances in cortisol activity resulting from 11\(\beta\)-HSD inactivation is therefore a second plausible mechanism for mineralocorticoid-mediated hypertension in humans.

INTRODUCTION
Abnormal intravascular cortisol metabolism is increasingly appreciated as a contributor to vascular dysfunction and cardiovascular disease. In addition to its role as an activating ligand for the GR (glucocorticoid receptor), cortisol activates the MR (mineralocorticoid receptor) equipotently as aldosterone [1]. The interaction of cortisol with both the GR and MR is regulated by the enzyme 11\(\beta\)-HSD (11\(\beta\)-hydroxysteroid dehydrogenase) [2]. 11\(\beta\)-HSD has two isoforms: type 1, a bi-directional reductase-dehydrogenase that predominantly reduces inactive cortisone to active cortisol and modulates cortisol-mediated activation of the GR [3]; and type 2, a unidirectional dehydrogenase that converts cortisol into cortisone and regulates cortisol-mediated MR activation [4].

Abnormal activation of both of these receptors has been shown to cause hypertension and has been demonstrated in vascular endothelial cells and VSMCs (vascular smooth muscle cells) [5]. Pure GR activation has been shown to cause hypertension in animal models [6], whereas dysfunctional 11\(\beta\)-HSD2 with MR activation causes hypertension and hypokalaemia [7,8].

Key words: glycyrrhetinic acid, 11\(\beta\)-hydroxysteroid dehydrogenase 2 (11\(\beta\)-HSD2), hypertension, mineralocorticoid receptor, vascular function, vascular smooth muscle.

Abbreviations: BP, blood pressure; FBF, forearm blood flow; GA, glycyrrhetinic acid; GR, glucocorticoid receptor; 11\(\beta\)-HSD, 11\(\beta\)-hydroxysteroid dehydrogenase; MAP, mean arterial pressure; MR, mineralocorticoid receptor; SNP, sodium nitroprusside; VSMC, vascular smooth muscle cell.

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The clinical relevance of this system is made clear by SAMe (syndrome of apparent mineralocorticoid excess), an autosomal-recessive form of severe hypertension, hypokalaemia and metabolic acidosis in the absence of elevated aldosterone levels [8]. Natural liquorice and its extracts inhibit both forms of 11β-HSD, cause hypertension and augment vascular smooth muscle contractility in humans [9,10].

The effect of 11β-HSD2 inhibition on arterial vasodilatory function remains untested in humans. This is an important question because clinical states of 11β-HSD inactivation may contribute to cardiovascular risk by impairing arterial homoeostatic mechanisms and advancing the complications of hypertension [11]. The active component of natural liquorice GA (glycyrrhetic acid) and its derivative carbenoxolone are potent inhibitors of 11β-HSD2 [4]. Human studies with GA have shown a dose–response relationship with small increases in BP (blood pressure) observed with as little as 75 mg of GA/day in some [12], but not all [13], studies. Therefore we tested the hypothesis that 11β-HSD inhibition with GA would impair arterial vasodilatory function in healthy humans.

MATERIALS AND METHODS

Subjects

Healthy volunteers were recruited via newspaper advertisement and provided written informed consent. All subjects underwent screening, consisting of a medical history, physical examination and laboratory studies to obtain values for complete blood cell count, serum electrolytes, creatinine and total cholesterol. Subjects with hypertension, a history of tobacco use, total cholesterol greater than the 75th percentile for age and gender, cardiovascular disease or other chronic medical conditions were excluded.

The protocol was approved by the Human Research Committee of Brigham and Women’s Hospital.

Study design

The effect of 11β-HSD2 inhibition on endothelium-dependent and endothelium-independent vasodilation was studied using a randomized double-blinded placebo-controlled cross-over design. All subjects were studied in the morning in the post-absorptive state, fasting after the previous midnight. Subjects were randomized to receive either GA (130 mg orally once daily; MAFCO) to inhibit 11β-HSD2 activity or matching placebo for 14 days before and on the morning of each vascular function study [14]. After a 2-week washout period, subjects then crossed over and received the other medication for 14 days before the second study day. Female participants underwent vascular testing during the same menstrual phase at each visit. COS (cyclo-oxygenase) inhibitors, alcohol and caffeine were prohibited for 24 h before the study morning. Subjects collected urine in the 24 h prior to presentation to the vascular laboratory. On the morning of each study, an indwelling brachial artery catheter was inserted. After a minimum of 30 min following catheter insertion, basal FBF (forearm blood flow) was measured. Thereafter endothelium-dependent vasodilation was assessed by measuring the FBF response to incremental intra-arterial doses of methacholine chloride (0.3, 1.0, 3.0 and 10.0 μg/min) infused at a flow rate of 0.388 ml/min. Endothelium-independent vasodilation was assessed by measuring the FBF response to incremental intra-arterial doses of verapamil chloride (3, 10, 30 and 100 μg/min) infused at a flow rate of 0.388 ml/min. Verapamil infusion was begun once pre-methacholine infusion basal flow rates were re-established. Each dose was administered for 6 min, and FBF measurements were made during the last 2 min of the infusion. The vascular research laboratory was quiet, dimly lit and temperature-controlled at 23℃.

Biochemical assays

Plasma renin activity and cortisol were measured by RIA. Aldosterone was measured by microassay. Urinary electrolytes were measured using a flame spectrophotometer. Urinary cortisol and cortisone were measured by LC-MS/MS (liquid chromatography-tandem MS method using a triple-quadrupole mass spectrometer (API 2000; Applied Biosystems) [15]. Serum aldosterone was measured by solid-phase RIA using the Coat-A-Count method (Diagnostic). Serum cortisol was assayed by Access Cortisol (Beckman Coulter).

Haemodynamic measurements

Bilateral FBF was measured by venous occlusion mercury-in-silastic strain-gauge plethysmography (D.E. Hokanson) using established methods [16,17]. Hand circulation was excluded during data acquisition using wrist cuffs inflated to 200 mmHg. A venous occlusion pressure of 50 mmHg was generated by cuffs placed on each arm above the elbow for each measurement of FBF. FBF is reported as ml·100 ml−1 of tissue·min−1. Arterial BP was measured using the brachial artery cannula. The cannula was attached to a pressure transducer contiguous with an amplifier on a Hokanson AI-6 physiological recorder. Heart rate was determined using the RR interval of a continuous ECG monitor.

Statistical analyses

Descriptive measures are reported as means ± S.D. and experimental measures as means ± S.E.M. Basal FBF, urine tests and blood analyses were compared using paired two-tailed Student’s t tests (or non-parametric if appropriate). Two-way repeated-measures ANOVA was performed to compare the dose–response curves during GA and placebo treatment, using the absolute
increase in blood flow from the basal flow rate. Statistical significance was accepted at the 95% confidence level ($P < 0.05$).

RESULTS

A total of 15 healthy subjects were recruited for the protocol (Table 1). The subjects, including seven women, were normotensive, and had normal cholesterol levels, normal potassium levels and normal renal function.

At the end of the two treatment periods, there was no significant difference in MAP (mean arterial pressure) for the GA and placebo treatments ($95 \pm 7$ compared with $92 \pm 8$ mmHg respectively). GA treatment significantly reduced ($P = 0.01$) serum potassium concentrations compared with placebo (Table 2). Similarly, GA treatment significantly reduced ($P = 0.009$) plasma aldosterone concentrations compared with placebo (Table 2). Plasma sodium, creatinine, glucose, cortisol and plasma renin activity did not differ between treatments (Table 2). 24-h Urine analysis revealed that treatment with GA significantly increased ($P = 0.04$) urinary free cortisol excretion by 49% compared with placebo treatment (Table 2). Conversely, GA treatment significantly reduced ($P = 0.01$) 24-h urinary free cortisone levels by 20% compared with placebo treatment. As a result, the cortisol/cortisone ratio increased significantly ($P = 0.008$) (Table 2). Urinary volume, sodium, potassium and chloride levels did not differ between each condition (Table 2).

Baseline FBF was not significantly different after GA treatment compared with placebo treatment ($1.87 \pm 0.26$ and $1.34 \pm 0.20$ ml·100 ml$^{-1}$·min$^{-1}$ respectively). The FBF response to incremental doses of methacholine chloride tended to be 20% less after GA treatment compared with placebo treatment, but did not reach statistical significance ($P = 0.09$) (Figure 1). The FBF response to incremental doses of verapamil was significantly reduced ($P = 0.04$) by 30% during GA treatment compared with placebo treatment, indicating impaired endothelium-independent vasodilation (Figure 2).

DISCUSSION

The primary finding of the present study is that 2 weeks of 11β-HSD inhibition resulted in attenuated vascular smooth muscle vasodilatory function in healthy subjects. This observation confirms the functional significance of human vascular 11β-HSD that has been demonstrated in vitro, and suggests that augmented intravascular glucocorticoid activity in states of 11β-HSD inactivation may be an important contributor to vascular dysfunction and possibly cardiovascular disease. Novel analytical

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo</th>
<th>GA</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n) (male/female)</td>
<td>8/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>$27 \pm 7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (mmol/l)</td>
<td>$1.0 \pm 0.2$</td>
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</tr>
<tr>
<td>Sodium (mmol/l)</td>
<td>$140 \pm 1$</td>
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<td></td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>$4.0 \pm 0.2$</td>
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<td></td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>$87 \pm 6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>$167 \pm 28$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>$92 \pm 8$</td>
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<td></td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>$26.5 \pm 4.3$</td>
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</table>

Table 1 Baseline characteristics of the subjects

<table>
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<tr>
<th>Parameter</th>
<th>Placebo</th>
<th>GA</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mmol/l)</td>
<td>$139 \pm 2$</td>
<td>$139 \pm 4$</td>
<td>0.36</td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>$4.0 \pm 0.2$</td>
<td>$3.9 \pm 0.3$</td>
<td>0.01</td>
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<tr>
<td>Creatinine (mmol/l)</td>
<td>$0.9 \pm 0.2$</td>
<td>$0.8 \pm 0.2$</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>$90 \pm 29$</td>
<td>$91 \pm 28$</td>
<td>0.91</td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>$6.3 \pm 5.4$</td>
<td>$3.5 \pm 1.2$</td>
<td>0.009</td>
</tr>
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<td>Plasma renin activity (ng·ml$^{-1}$·h$^{-1}$)</td>
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<td>$0.70 \pm 0.38$</td>
<td>0.14</td>
</tr>
<tr>
<td>Cortisol ($\mu$g/dl)</td>
<td>$18.7 \pm 9.0$</td>
<td>$18.9 \pm 7.5$</td>
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</tr>
<tr>
<td>Urine volume (ml)</td>
<td>$1715 \pm 867$</td>
<td>$1553 \pm 990$</td>
<td>0.4</td>
</tr>
<tr>
<td>Cortisol ($\mu$g/24 h)</td>
<td>$20.1 \pm 11.5$</td>
<td>$30.0 \pm 22.7$</td>
<td>0.04</td>
</tr>
<tr>
<td>Cortisone ($\mu$g/24 h)</td>
<td>$46.1 \pm 19.4$</td>
<td>$36.9 \pm 19.9$</td>
<td>0.01</td>
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<tr>
<td>Cortisol/cortisone ratio</td>
<td>$0.44 \pm 0.18$</td>
<td>$0.81 \pm 0.45$</td>
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<td>Sodium (mmol/l)</td>
<td>$98.6 \pm 48.6$</td>
<td>$97.8 \pm 49.9$</td>
<td>0.9</td>
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<tr>
<td>Potassium (mmol/l)</td>
<td>$40.6 \pm 17.8$</td>
<td>$37.4 \pm 14.4$</td>
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<tr>
<td>Chloride (mmol/l)</td>
<td>$1715 \pm 867$</td>
<td>$1553 \pm 990$</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 2 Laboratory analysis at 14 days of treatment in the two groups
methods defining MR activation may be needed to diagnose cortisol-related MR-induced hypertension in settings of normal aldosterone concentrations.

**GA, 11β-HSD inhibition and BP**

In humans, a standard marker of renal 11β-HSD2 activity is the urinary cortisol/cortisone ratio. Al Sharef et al. [19] reported a median urinary cortisol/cortisone ratio of 0.41 in a healthy population of 92 subjects, nearly identical with our subjects at 0.44. Successful inhibition of renal 11β-HSD2 by GA in our present study was demonstrated by the increased urinary cortisol/cortisone ratio at the end of the GA treatment period (0.81) compared with the placebo period. Increased MR activation in our subjects is supported by the reduction in plasma potassium and suppression of plasma aldosterone levels at the end of the GA treatment period. These results are consistent with previous reports of liquorice administration [20,21]. In our cohort, there was no change in BP attributable to GA treatment. Previous investigators have observed either an increase [12] or no change [13,22] in BP with GA administration. Review of that work suggests that a second perturbation of BP homoeostasis may be necessary for a hypertensive effect of 11β-HSD inhibition. For example, in a cohort treated with glycyrrhizin for 4 weeks, only those with plasma renin activity above 1.5 ng·ml⁻¹·h⁻¹ had increases in their BP [23]. Mean plasma renin activity in our present cohort was 0.73 ± 0.59 ng·ml⁻¹·h⁻¹, below that threshold. In addition, inhibition of 11β-HSD was found to cause hypertension in subjects with renal disease at baseline, but not in patients with normal renal function [13]. The normal renal function of our subjects may have blunted the hypertensive effect of GA therapy.

**Vascular glucocorticoid metabolism and action**

VSMCs and endothelial cells contain both 11β-HSD isoforms and both MR and GR receptors [5]. In rodents, antisense mRNA inhibition of 11β-HSD1 attenuates the conversion of 11-dehydrocorticosterone into corticosterone and decreases the VSMC contractile response to phenylephrine [24]. In contrast, addition of corticosterone to cell culture with specific inhibition of 11β-HSD2 augments the contractile response to phenylephrine when compared with adding corticosterone alone [24]. Aortic rings from 11β-HSD2-knockout mice have reduced noradrenaline (norepinephrine)-mediated vasoconstriction, attenuated endothelium-dependent vasodilation in response to acetylcholine, and diminished vascular smooth muscle vasodilation to the NO donor 3’-morpholinosydnonimine [25]. In aortic rings from Sprague-Dawley rats, inhibition of both 11β-HSD isoforms with carbenoxolone has the same pattern of greater phenylephrine-mediated vasoconstriction, implicating 11β-HSD2 as the dominant isoform in modulating VSMC function when both are inhibited [26]. Previous studies have made it clear that 11β-HSD2 is expressed in human VSMCs and participates importantly in the regulation of the MR [27,28]. As would be expected from rodent models, inactivation of 11β-HSD with GA in VSMCs significantly increases MR activation in response to added cortisol [27]. MR activation increases intracellular calcium concentrations, from both intracellular and extracellular sources [29], and would be expected to reduce the response to calcium channel blockade as observed in our subjects. 11β-HSD2-knockout mice have attenuated endothelium-dependent and endothelium-independent vasodilation and augmented contraction to phenylephrine, whereas 11β-HSD1-knockout mice maintain normal vascular responsiveness [25].

**Vascular glucocorticoid action in humans**

Few studies have examined the impact of increased cortisol activity on vascular function in humans. van Uum et al. [30] found that the acute infusion of cortisol, with or without GA treatment, did not change forearm vascular resistance. Walker et al. [10] examined the effect of cortisol infusion before and after 7 days of 11β-HSD inhibition with carbenoxolone. Although basal FBF rates were unaffected, carbenoxolone potentiated noradrenaline-induced forearm vasoconstriction, consistent with acquired VSMC dysfunction [10]. In our hands, 2 weeks of 11β-HSD inhibition significantly attenuated the vasodilatory FBF response to verapamil, extending the VSMC abnormality to attenuated vasodilation.

One must consider the possibility that cortisol-mediated GR activation in addition to MR activation may, in part, contribute to our present findings that GA administration impairs vascular function [25,27,31]. High-dose cortisol administration has a hypertensive effect in the presence of MR antagonism in healthy volunteers, and cortivazol, a pure GR agonist, increases BP in experimental animals [6,32]. Moreover, Mangos et al. [33] have shown that administration of 80 mg of...
cortisol daily for 5 days impaired acetylcholine-mediated vasodilation, but not SNP (sodium nitroprusside)-mediated vasodilation [33]. However, high-dose glucocorticoid administration causes non-vascular effects that independently attenuate vascular function, such as hyperglycaemia, inflammation (white blood cell count increases) and increased aldosterone levels, none of which were observed in our present study. Plasma cortisol levels in our cohort were lower than in subjects treated with 40 mg of cortisol, a dose that does not cause hypertension [32]. Most revealingly, administration of the GR-specific glucocorticoid dexamethasone by Mangos et al. [34] did not cause endothelial or vascular smooth muscle dysfunction [34]. Thus, with the low levels of plasma cortisol in our subjects, the experimental findings of Mangos et al. [33,34], demonstrating endothelial but not vascular smooth muscle dysfunction with the MR and GR ligand cortisol and no evidence of endothelial dysfunction with the specific GR-ligand dexamethasone, we would surmise that cortisol-mediated GR activation does not contribute significantly to our findings.

Assessing endothelial function in the setting of vascular smooth muscle dysfunction

In the present study, during GA administration, the response to methacholine at the peak dose fell by an average of 23% or 4.8 ml·100 ml−1·min−1 compared with a fall of 33% or 5.7 ml·100 ml−1·min−1 during verapamil infusion. Thus the more pronounced defect is in vascular smooth muscle function and we are left unable to comment on whether NO bioavailability changes. Indeed, when studied, most animal and human investigations reveal a vascular smooth muscle defect, supporting our findings and making less clear the role of endothelial dysfunction with 11β-HSD inhibition [24,35,36]. Incorporation of SNP as a third infusion may have provided further insight into the mechanism of VSMC dysfunction, but this was not done in the present investigation.

Experimental considerations

The washout period was chosen on the basis of work by Ploeger et al. [37]; however, we cannot prove that all traces of GA were washed out by that time point. We believe that this period was sufficient for an analysis because order of medication administration did not indicate any variation in outcome.

Summary

GA-mediated inhibition of 11β-HSD deranges glucocorticoid metabolism in healthy subjects and attenuates vascular smooth muscle vasodilatory function in the absence of a change in BP. These results suggest that non-aldosterone-mediated activation of vascular MRs may contribute to vascular dysfunction in states of 11β-HSD deficiency.

AUTHOR CONTRIBUTION

Piotr Sobieszczynyk participated in data collection, analysis and writing of the manuscript; Barry Borlaug and Heather Gornik assisted with data collection and provided editing assistance prior to submission; Wesley Knauf helped with data collection; and Joshua Beckman contributed to the study conception, data collection, analysis and editing assistance prior to submission.

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