Thrombospondin-1 is a glucocorticoid responsive protein in humans

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Abstract

Objective: Thrombospondin-1 (TSP1) is a matricellular protein whose gene expression has previously been shown to increase acutely after exposure to dexamethasone in vitro. The aim of this study was to determine if TSP1 is altered by acute and chronic states of glucocorticoid excess in human subjects.

Design and methods: Three studies have been undertaken to assess the difference or change in TSP1 in response to altered glucocorticoid activity: i) an acute interventional study assessed the effects of a single 4 mg dose of dexamethasone in 20 healthy volunteers; ii) a cross-sectional study compared plasma TSP1 in 20 healthy volunteers and eight patients with Cushing’s syndrome; iii) an interventional study assessed the effect on plasma TSP1 of an increase in hydrocortisone dose from 20 mg/day to 30 mg/day for 7 days in 16 patients with secondary adrenal insufficiency.

Results: In healthy volunteers, 4 mg dexamethasone significantly increased peripheral blood mononuclear cell (PBMC) TSP1 mRNA levels (P < 0.0001) and plasma TSP1 concentrations (P < 0.0001), peaking at 12 h. Median (interquartile range) plasma TSP1 was higher in Cushing’s, 638 (535–756) ng/ml, than in healthy volunteers, 272 (237–336) ng/ml (P < 0.0001). Plasma TSP1 > 400 ng/ml diagnosed Cushing’s syndrome with sensitivity of 100% and specificity of 85%. The higher hydrocortisone dose increased plasma TSP1 from 139 (86–199) to 256 (133–516) ng/ml, (P < 0.01) in patients with secondary adrenal insufficiency. Conclusions: TSP1 is a glucocorticoid responsive protein in humans. Further research is required to determine if plasma TSP1 has a role as a glucocorticoid biomarker.

Introduction

Cortisone was first synthesized in sufficient quantities for clinical use in the late 1940s (1). Glucocorticoids are now widely prescribed to treat a diverse range of inflammatory and autoimmune diseases. In a recent study, nearly 1% of the general population was taking oral glucocorticoids long-term (2). Glucocorticoids are also prescribed as a physiological replacement in patients with primary and secondary adrenal insufficiency. In this setting, the therapeutic aim is to mimic the normal daily production and secretion of cortisol.

Typical hydrocortisone replacement regimens have been excessive (3), with a supraphysiologic rise in serum cortisol soon after the dose and subphysiologic concentrations at other times of the day (4, 5). Researchers have found a significant increase in the mortality of hypopituitary patients treated with ≥25–30 mg of hydrocortisone per day (6, 7). When used in pharmacological doses (> 5 mg/day of prednisolone or equivalent), glucocorticoids are potent anti-inflammatory agents and also have anti-neoplastic and immunsuppressive properties (8).
Subjects and methods

The study was approved by the Metro South Human Research Ethics Committee, Brisbane and Southern Adelaide Clinical Human Research Ethics Committee, Adelaide, and written informed consent was obtained from each participant.

Subjects and study design

Healthy volunteers ▶ Twenty healthy volunteers, ten male and ten female, (median age 26 years, range 21–49, median BMI 23.5 kg/m², range 16.0–36.8 kg/m²) participated in the study. On the control day, venous blood was sampled at 0800, 1200 and 1600 h in a non-fasting state for serum cortisol, plasma adrenocorticotropin (ACTH) and plasma TSP1 and for PBMC TSP1 mRNA. On a subsequent day, separated by at least 48 h from the basal sampling, each participant took 4 mg of dexamethasone orally at midnight, followed by repeat venous sampling at 0800, 1200 and 1600 h for plasma TSP1 and for PBMC TSP1 mRNA.

Patients with Cushing’s syndrome ▶ Eight patients (median age 45 years, range 35–71, median BMI 30.4 kg/m², range 21.7–51.3 kg/m²) with Cushing’s syndrome (Cushing’s disease, n=6 females and 1 male and functional adrenocortical carcinoma, n=1 female; Table 1) had a plasma sample drawn between 0800 and 1000 h in a non-fasting state. The patients with Cushing’s syndrome were significantly older compared to the healthy volunteers (P<0.0001), but BMI was not significantly different between the groups (P=0.10). All patients with Cushing’s disease had clinical features of cortisol excess and at least two abnormal diagnostic tests (elevated 24-h urinary-free cortisol, elevated late night salivary cortisol or failure to suppress serum cortisol to <50 nmol/l after 1 mg dexamethasone), in conjunction with a normal or elevated plasma ACTH (23). Cushing’s disease was confirmed by inferior petrosal sinus sampling with positive central: peripheral gradient in patients 1, 2 and 8. In the other four patients, Cushing’s disease was diagnosed based on positive magnetic resonance imaging of the pituitary gland, suppression of serum cortisol to <50% of baseline with high dose (8 mg) dexamethasone and plasma ACTH stimulation >50% with 1 μg/kg human corticotropin-releasing hormone. Patient 4 had cyclical Cushing’s that was histologically verified as a corticotrope adenoma at trans-sphenoidal surgery. The patient with adrenocortical carcinoma had a severe catabolic Cushing’s syndrome; and if plasma TSP1 in patients with secondary adrenal insufficiency is adequately sensitive to detect a small change in the hydrocortisone dose.

Glucocorticoids in excessive doses can result in serious side effects such as weight gain, diabetes, muscle weakness, osteoporosis, bruising, striae, cataracts and hypertension (8), while evidence for a glucocorticoid dose-dependent increase in mortality has been demonstrated in patients with rheumatoid arthritis (9) and Crohn’s disease (10).

Current approaches to individualize glucocorticoid replacement therapy include weight-based dosing (5), but ‘clinical judgment’ is still considered the main method of dose titration (11). Glucocorticoid dosing would be improved if a biomarker of glucocorticoid activity was available (12). A biomarker would ideally have a relatively long half-life, be sufficiently sensitive to detect changes within the usual glucocorticoid dose range and have a relationship with the physiologic effects of glucocorticoids. Furthermore, such a biomarker would reflect glucocorticoid activity and effect at the cellular level rather than simple circulating concentrations (13).

Thrombospondin-1 (TSP1) is a matricellular protein, first discovered in platelets (14). It is widely expressed in diverse tissues such as endothelial cells, monocytes and macrophages, smooth muscle cells, fibroblasts and adipocytes (15). TSP1 is readily measurable in human plasma and has a half-life of 9 h (16). Plasma TSP1 is unaffected by glucocorticoid effector.

The inhibitory effect of cortisol on endometrial angiogenesis was blocked by small interfering RNA against TSP1 (22), implying that TSP1 may be a downstream glucocorticoid effector.

The hypothesis is that TSP1 is a glucocorticoid responsive protein, which increases after acute and chronic upregulation of glucocorticoid activity. We have undertaken a series of linked clinical studies to assess if plasma TSP1 and/or TSP1 mRNA expression in PBMC in healthy volunteers are acutely upregulated by dexamethasone; if plasma TSP1 is elevated in patients with Cushing’s syndrome; and if plasma TSP1 in patients with secondary adrenal insufficiency is adequately sensitive to detect a small change in the hydrocortisone dose.
phenotype with markedly elevated serum cortisol, 24-h urinary-free cortisol 40 times the upper limit of normal and undetectable plasma ACTH.

Patients with secondary adrenal insufficiency ▶ Plasma was available from 16 of 17 patients with secondary adrenal insufficiency (eight male and eight female, median age 56 years, range 22–87), who participated in a study investigating the effects of an increase in the hydrocortisone dose on cardiovascular risk markers and insulin sensitivity (24). Samples were collected fasting ~2 h after the morning hydrocortisone dose when on a standard dose of ≤20 mg/day (median 16 mg/day, range 10–20 mg) and 1 week after hydrocortisone was increased to 30 mg/day.

The study of patients with secondary adrenal insufficiency was registered as a clinical trial: Australian New Zealand Clinical Trials Registry number, ACTRN12612000234819.

Laboratory analysis

PBMC isolation ▶ Ten milliliter of blood collected in EDTA was gently layered under 7 ml of Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) and centrifuged at 500 g for 20 min without brake. The interphase containing PBMCs was collected using a transfer pipette and transferred to a fresh tube. Cells were washed twice by resuspending up to 50 ml with PBS and spinning at 500 g for 5 min. Cells were resuspended up to 25 ml with PBS and spun at 200 g for 10 min to remove platelets.

Gene expression analysis ▶ Gene expression was measured by quantitative RT-PCR (qRT-PCR) and standardized against the expression of cyclophilin as previously described (25). Briefly, total RNA was extracted using RNA Mini Kit (Ambion Life Technologies, Victoria, Australia) according to the manufacturer’s instructions. cDNA was synthesized from 800 ng total RNA using a cDNA synthesis kit (Bioline, NSW, Australia), and RT-PCR was performed using the SYBR Hi-ROX kit (Bioline) on a 7900HT Fast Real-time PCR system (Ambion Life Technologies). Primer sequences are available on request.

Plasma TSP1 determination ▶ Plasma TSP1 was determined by an in-house indirect sandwich ELISA. Briefly, plates were coated overnight at 4 °C with human anti-TSP1 antibody (1 μg/ml) (MAB3074, R&D Systems, Minneapolis, MN, USA) in 0.05 M carbonate/bicarbonate buffer (pH 9.6). Following washing, plates were blocked with 1% (w/v) fraction V, IgG-free BSA buffer (blocking buffer) (Life Technologies) for 1 h at room temperature and then washed. Recombinant human TSP1 (3074-TH-050, R&D Systems) was serially diluted in blocking buffer to produce a standard ranging from 15.6 ng/ml to 1000 ng/ml, and plasma samples were diluted at 1:4 in blocking buffer. Samples were incubated for 2 h at room temperature and washed, and biotinylated human anti-TSP1 antibody (0.1 μg/ml) (BAF3074, R&D Systems) diluted in blocking buffer was added for a further 2 h at room temperature. Following washing, Poly-HRP Streptavidin conjugate (Thermo Scientific, Victoria, Australia) diluted 1:20 000 in blocking buffer was added to each well and incubated for 30 min at room temperature. Plates were washed, and peroxidase substrate 3,3',5,5'-Tetramethylbenzidine solution (Cell Signaling, Danvers, MA, USA) was added and incubated at room temperature for 20 min. The reaction was terminated by the addition of stop solution (Cell Signaling), and the optical density (OD) was measured at 450 nm. The assay sensitivity was 7.8 ng/ml, with an interassay coefficient of variation

Table 1 Characteristics of patients with Cushing’s syndrome.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>24 h urinary-free cortisol (×ULN)</th>
<th>BMI (kg/m²)</th>
<th>Imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>M</td>
<td>4.6</td>
<td>21.7</td>
<td>3 mm pituitary microadenoma</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>F</td>
<td>1.9</td>
<td>32.2</td>
<td>9 mm pituitary microadenoma</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>F</td>
<td>40</td>
<td>23.9</td>
<td>7.4 cm adrenal carcinoma with liver metastases</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>F</td>
<td>1.6</td>
<td>39.0</td>
<td>7 mm pituitary microadenoma</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>F</td>
<td>16.7</td>
<td>44.7</td>
<td>8 mm pituitary microadenoma</td>
</tr>
<tr>
<td>6</td>
<td>39</td>
<td>F</td>
<td>1.8</td>
<td>30.4</td>
<td>15 mm pituitary macroadenoma</td>
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<tr>
<td>7</td>
<td>45</td>
<td>F</td>
<td>3.5</td>
<td>23.1</td>
<td>6 mm pituitary microadenoma</td>
</tr>
<tr>
<td>8</td>
<td>62</td>
<td>F</td>
<td>1.1</td>
<td>51.3</td>
<td>Normal MRI*</td>
</tr>
</tbody>
</table>

ULN, upper limit of normal; MRI, magnetic resonance imaging. *Positive dexamethasone-suppressed corticotropin-releasing hormone stimulation test (40) and intravenous dexamethasone suppression test (41). Inferior petrosal sinus sampling is consistent with Cushing’s disease.
(CV) of 3–7% and intraassay CV of 3–4%. There was no measurable cross reactivity with TSP2. Samples from the same individual were run on the same assay.

**Statistical methods**

In the analysis of the PBMC TSP1 mRNA and plasma TSP1 response to dexamethasone, the data did not satisfy parametric assumptions, so log transformation was undertaken prior to statistical analysis. Two-way repeated measures ANOVA was performed to assess the effect of treatment (dexamethasone vs control) and time. Post-hoc analyses were undertaken using Sidak’s multiple comparisons test. Data for the change in TSP1 mRNA expression are shown as individual data points on a log scale (Fig. 1). Data for the change in plasma TSP1 are shown as geometric mean (±S.E.M.) after back transformation of the log-transformed data (Fig. 2). Correlations between plasma TSP1 and serum cortisol at each individual time point and area under the curve (AUC) and between mean plasma TSP1 (across 0800, 1200 and 1600 h) and age and BMI in the healthy volunteers were analyzed using the Spearman’s coefficient. Morning plasma TSP1 (expressed as median (interquartile range)) in the healthy volunteers was compared to the patients with Cushing’s syndrome using the Mann–Whitney U test. Receiver operating characteristic (ROC) curve analysis was employed to determine the optimal cut-off point for the diagnosis of Cushing’s syndrome. The effect on morning plasma TSP1 of an increase in maintenance hydrocortisone dose from ≤20 to 30 mg for 1 week in the patients with secondary adrenal insufficiency was assessed using the Wilcoxon signed rank test. Data analysis was performed using GraphPad Prism version 6 (GraphPad, La Jolla, CA USA). P<0.05 was considered statistically significant.

**Results**

**Basal TSP1 mRNA expression and plasma TSP1 in healthy volunteers**

Among the healthy volunteers, basal TSP1 mRNA levels on the control day varied ~ 100-fold among individuals (Fig. 1). There was no correlation between the plasma concentration of TSP1 and serum cortisol at any individual time point on the control day (data not shown). The correlation between the AUC for plasma TSP1 and AUC cortisol was 0.44, approaching statistical significance (P=0.054). Plasma TSP1 did not display a significant change over time (Fig. 2). There was no difference in plasma TSP1 between males (median (interquartile range) 321 (253–375) ng/ml) and females (median (interquartile range) 264 (218–321) ng/ml; P=0.26). There was no significant correlation between mean plasma TSP1 and age (r=0.31, P=0.18) or BMI (r=0.30, P=0.19).
Effect of dexamethasone on TSP1 mRNA and plasma TSP1

Following dexamethasone administration, cortisol and ACTH were appropriately suppressed at all three time points, confirming significant ongoing exogenous glucocorticoid activity throughout the sampling period (data not shown). Dexamethasone caused a significant increase in TSP1 mRNA levels in PBMC (P<0.0001). Post-hoc analysis showed a significant treatment effect at each time point (P<0.0001; Fig. 1). There was also a significant increase in plasma TSP1 concentration following dexamethasone (P<0.0001), time×treatment interaction (P<0.05). Post-hoc analysis showed a significant effect maximal at 1200 h, 12 h after the dexamethasone dose (P<0.0001), which was maintained at 1600 h (P<0.05; Fig. 2).

Plasma TSP1 in patients with Cushing’s syndrome

Median (interquartile range) plasma TSP1 was significantly higher in the patients with Cushing’s syndrome (638 (535–756) ng/ml) than in the healthy volunteers (272 (237–336) ng/ml) (P<0.001, Fig. 3a). The area under the ROC curve of 0.94 (95% CI 0.85–1.03) was significantly >0.5 (P<0.001, Fig. 3b).

The optimal diagnostic threshold for plasma TSP1 was 402.5 ng/ml. Rounding this to 400 ng/ml, the sensitivity for plasma TSP1 as a screening test for Cushing’s syndrome was 100% with a specificity of 85%. There was no relationship between the severity of hypercortisolism (as determined by 24-h urinary-free cortisol) and plasma TSP1 concentration (r = −0.33, P = 0.43).

Plasma TSP1 in patients with secondary adrenal insufficiency

Increasing the patients’ usual maintenance doses of hydrocortisone from ≤20 to 30 mg daily for 1 week was associated with a significant rise in median plasma TSP1, from 139 (86–199) ng/ml to 256 (133–516) ng/ml (P<0.01; Fig. 4).

Discussion

The results of this study demonstrate that TSP1 is a glucocorticoid responsive protein in human subjects. Both TSP1 mRNA and plasma concentration were acutely upregulated by dexamethasone in healthy volunteers. Chronic endogenous glucocorticoid excess in patients with Cushing’s syndrome was associated with an increase in plasma TSP1. Moreover, plasma TSP1 in patients with secondary adrenal insufficiency increased after a small change in the glucocorticoid replacement dose for 1 week. These data suggest TSP1 warrants further investigation as a potential biomarker of glucocorticoid activity.

Dexamethasone is a potent glucocorticoid with a prolonged duration of action. A single 4 mg dose of dexamethasone increased PBMC TSP1 gene expression after 8 h, which remained elevated for at least 16 h. In plasma, the stimulatory effect of dexamethasone on TSP1 concentrations was maximal at 12 h after the dose and remained significantly elevated at 16 h. These data are
consistent with previous in vitro assessments of the effects of dexamethasone on TSP1 in a number of different human cell types including PBMCs (20), endometrial cells (22) and osteoblasts (21). However, the current study is the first to demonstrate that administering dexamethasone to humans in vivo increases PBMC TSP1 mRNA levels and circulating plasma TSP1 concentrations. The data in patients with Cushing’s syndrome indicate that plasma TSP1 concentrations are also increased during chronic endogenous glucocorticoid excess. Thus, a second patient model suggested that TSP1 secretion is upregulated by glucocorticoids in human subjects. Furthermore, the sensitivity and specificity of TSP1 for Cushing’s syndrome was broadly similar to other screening tests (23). Although we acknowledge the sample size is small and the plasma TSP1 was not related to disease severity, three patients with Cushing’s syndrome had mild glucocorticoid excess (Table 1), a setting in which other diagnostic tests such as 24-h urinary-free cortisol have reduced sensitivity (26). While further studies of larger cohorts are clearly needed, TSP1 could potentially provide adjuvant information to the standard diagnostic tests for Cushing’s syndrome, particularly to distinguish between simple obesity and mild Cushing’s syndrome, where the greatest diagnostic difficulty lies.

TSP1 has multiple actions. It regulates cellular events involved in tissue repair, activation of transforming growth factor beta-1 (TGFβ1) (27), attenuates vasodilatation via an effect on nitric oxide synthase (28) and is positively correlated with BMI and negatively with insulin sensitivity (29). As all of these actions of TSP1 are also effects of glucocorticoids (8), we hypothesize that TSP1 may be an effector of glucocorticoid activity. TSP1 has been shown to be elevated in patients with peripheral vascular disease (30) and in patients with diabetes and coronary artery disease (18). This raises the question as to whether elevated TSP1 might be a risk factor for cardiovascular disease (31), which is the leading cause of morbidity and mortality in patients with endogenous or exogenous glucocorticoid excess (6, 7, 32, 33, 34, 35).

For a glucocorticoid-responsive protein to have clinical efficacy as a biomarker, physiologically relevant changes in glucocorticoid activity must be detected in an individual patient, and the biomarker should reflect glucocorticoid activity at the cellular level. Glucocorticoids have complex biology (36). They predominantly exert their effect via glucocorticoid receptors (GRs), which have multiple polymorphisms and isoforms that influence ligand-receptor binding and, consequently, downstream effects (37). The ideal glucocorticoid biomarker would also account for local tissue and intracellular generation of glucocorticoids via 11β-hydroxysteroid dehydrogenase type 1 (38) and expression of the efflux transporter P-glycoprotein (13). Plasma TSP1 concentrations were significantly increased in patients with secondary adrenal insufficiency 1 week after a small increase in the hydrocortisone dose; however, the extent of the response was variable between individuals. The variation in individual TSP1 response may indicate a greater change in intracellular glucocorticoid activity, but this requires confirmation in future studies.

Our data and another recent study (30) have shown a skewed distribution of plasma TSP1; the biological significance of higher plasma TSP1 concentrations in otherwise healthy people is unknown. It is acknowledged that factors other than glucocorticoid activity must contribute to plasma TSP1, but currently the regulation of circulating TSP1 is incompletely understood. Further research is required to define a reference range for plasma TSP1 in a larger cohort of healthy subjects across a broader age range than in the current study. It is likely that plasma TSP1 does not simply reflect serum cortisol concentration as there was no obvious diurnal change in healthy volunteers across the three sampling points. However, other clinically used biomarkers in the field of

**Figure 4**

Morning plasma TSP1 concentrations in patients with secondary adrenal insufficiency (n = 16) at baseline on ≤20 mg hydrocortisone per day (closed circle) and after 1 week on 30 mg hydrocortisone per day (open circle). *P < 0.01 compared to when taking ≤20 mg hydrocortisone per day.
endocrinology such as HbA1c and insulin-like growth factor 1 (IGF1) provide an estimate of integrated serum glucose or growth hormone (GH), respectively, without directly reflecting hour by hour fluctuations in concentration.

We did not find a significant correlation between urinary-free cortisol excretion and plasma TSP1 in patients with Cushing’s syndrome. However, in the healthy volunteers, the positive correlation between integrated plasma TSP1 and serum cortisol almost reached statistical significance. There are several possible explanations for these results. First, the sample size with Cushing’s syndrome may be too small to show a significant correlation. Second, the participant with the most severe Cushingoid phenotype had metastatic adrenocortical carcinoma, which may alter the relationship between glucocorticoid activity and plasma TSP1. Finally, the relationship between cortisol and TSP1 may not be linear. It could potentially be similar to the log-linear relationship between GH and IGF1, with IGF1 plateaueing at higher GH concentrations (39).

For TSP1 or any other potential glucocorticoid biomarker to have clinical utility, a therapeutic or target range needs to be identified. The variability in baseline TSP1 mRNA expression in PBMC indicates that this would not be a sensitive indicator of an individual’s glucocorticoid activity (Fig. 1). However, the inter-individual variability of plasma TSP1 was much less than that for mRNA (Fig. 3a). We plan a more detailed analysis of day profiles of plasma TSP1 in normal individuals and patients with Cushing’s syndrome, primary and secondary adrenal insufficiency, including determination of whether TSP1 changes after acute hydrocortisone. Prospective data assessing whether plasma TSP1 is associated with adverse outcomes is required, followed by studies examining whether glucocorticoid dose adjustment to a TSP1 target range results in the improvement in long-term morbidity and mortality in patients on glucocorticoids.

The study has some limitations. Although the blood sampling and sample processing was consistent across all subjects and both centers, it is possible that some platelet activation occurred, which can result in elevated concentrations (16). However, the current results are comparable to the plasma concentrations reported in other recent studies using an ELISA in healthy individuals (30) and lower than people with ischemic heart disease and diabetes (18). The number of patients with Cushing’s syndrome is small and the group is heterogeneous, containing a patient with adrenocortical carcinoma and a patient with cyclical Cushing’s disease. Despite this, a highly significant difference was observed. Finally, study (iii) did not include a longitudinal control group to exclude the possibility that a significant increase in plasma TSP1 might have occurred in the absence of a change in glucocorticoid dose.

In summary, TSP1 mRNA expression in PBMC and TSP1 peripheral plasma concentrations is significantly upregulated in healthy human subjects by a single 4 mg dose of the potent glucocorticoid dexamethasone. Plasma TSP1 is elevated in patients with endogenous glucocorticoid excess. An increase in the maintenance hydrocortisone dose in patients with secondary adrenal insufficiency from ≤20 to 30 mg daily for 1 week results in a significant increase in plasma TSP1. We conclude that plasma TSP1 is a glucocorticoid responsive protein that should be studied further to determine its clinical utility as a biomarker of glucocorticoid activity. TSP1 could potentially play a role in the diagnosis of Cushing’s syndrome and optimization of glucocorticoid replacement therapy.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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