Endogenous natriuretic factors: atrial natriuretic hormone and digitalis-like substance in Cushing's syndrome

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RECEIVED 3 October 1990

ABSTRACT

In order to investigate the effect of chronic hypercortisolaemia on endogenous natriuretic factors (atrial natriuretic hormone (ANH) and the Na⁺/K⁺ pump inhibitor) digitalis-like substance (DLS), and their relation to hypertension, 28 patients with pituitary- or adrenal-dependent Cushing's syndrome and six patients on high-dose prednisone treatment were studied. Plasma ANH levels were increased in patients with Cushing's syndrome (36.0±1.4 (S.E.M.) ng/l) compared with those in healthy controls (28.6±1.3 ng/l, P<0.01). In prednisone-treated patients, ANH levels (43.8±4.5 ng/l) were higher than those in patients with Cushing's syndrome and in controls (P<0.05 and P<0.01 respectively). DLS measured by radioimmunoassay and binding of [³H]ouabain to erythrocytes was not altered in patients with hypercortisolaemia. Slightly decreased DLS activity in the erythrocyte ⁸⁶Rb uptake inhibition assay was found in patients with Cushing's syndrome (52.9±2.7%) compared with that in controls (60.9±1.8%, P<0.02). With the exception of cortisol (r=0.52, P<0.01), none of the other factors determined correlated with the mean arterial pressure in patients with Cushing's syndrome.

Thus, a chronic excess of endogenous and exogenous glucocorticoids increases plasma levels of ANH, but does not substantially influence DLS activity or plasma levels. Neither natriuretic factor is directly related to hypertension in Cushing's syndrome. Journal of Endocrinology (1991) 129, 453-458

INTRODUCTION

Cushing's syndrome is a disease in which arterial hypertension is frequently detected, with a prevalence of 80-100% (Grekin & Gross, 1983). The pathogenesis of hypertension in Cushing's syndrome has not been fully elucidated, but chronic hypersecretion of glucocorticoids seems to be the main factor. This effect is not related to mineralocorticoid activity (Whitworth, 1987). Glucocorticoids increase pressor responses to angiotensin II and noradrenaline (Yagii & Krakoff, 1988), which is reversed by anti-glucocorticoid treatment (Grünfeld & Eloy, 1988). Glucocorticoids are known to stimulate the Na⁺/K⁺ pump (Na⁺/K⁺-ATPase) activity (Sinha, Rodriguez, Hogan & Klahr, 1981). In Cushing's syndrome, chronic hypercortisolaemia leads to an increase in Na⁺/K⁺ pump activity of erythrocytes (Wambach, Schmulling & Kaufmann 1985) or leucocytes (Ng, Evans & Burke, 1988). On the other hand, glucocorticoids increase atrial natriuretic hormone (ANH) gene expression and ANH content in rat heart muscle (Wiegand, Day, Rodi et al. 1987). Increased plasma concentrations of ANH have recently been reported in subjects receiving dexamethasone treatment (Saxenhofer, Angst, Weidemann et al. 1988) and in patients with Cushing's syndrome (Yamaji, Ishibashi, Yamada et al. 1988).

There is considerable evidence in support of the existence of an endogenous Na⁺/K⁺ pump inhibitor, which may be considered as an endogenous digitalis-like substance (DLS) (de Wardener, 1985; Graves, 1986). The chemical structure of this compound is still unknown, but its properties have been widely characterized (Crabos, Grichois, Guicheney et al. 1987; Tamura, Lam & Inagami, 1988; Goto, Yamada, Ishii et al. 1988; Hamlyn, Harris & Ludens, 1989). Among other effects, DLS inhibits Na⁺/K⁺-ATPase activity, inhibits erythrocyte [³H]ouabain binding and ⁸⁶Rb uptake, and cross-reacts with digoxin antibodies in radioimmunoassay. It has been shown to exhibit diuretic, natriuretic and pressor activity, and to be probably involved in the pathogenesis of various forms of arterial hypertension (de Wardener, 1985; Graves, 1986; Haddy, 1987). There have been no reports of the detection of DLS in patients with chronic hypercortisolaemia.
The aim of the present study was to measure plasma content of DLS and its activity in patients with hypercortisolism due to Cushing's syndrome or glucocorticoid treatment, as well as to relate DLS to plasma levels of ANH in those patients. The possible role of both natriuretic factors in the pathophysiology of hypertension in Cushing's syndrome was also evaluated.

PATIENTS AND METHODS

Twenty-eight patients with untreated or unsuccessfully treated Cushing's syndrome were studied (25 female/3 male; mean age 39.4, range 19–59 years). The diagnosis was established according to the clinical findings, increased levels and lack of circadian rhythm of serum cortisol and increased excretion of glucocorticoid metabolites in urine. The dexamethasone suppression test, repeated adrenocorticotropic (ACTH) measurements, as well as computerized tomography were used to differentiate between pituitary- and adrenal-dependent Cushing's syndrome. The diagnosis of pituitary microadenoma was established in 20 patients, whereas the remaining eight subjects suffered from adrenal adenoma. Arterial hypertension, defined as being when the systolic blood pressure was over 160 mmHg and/or the diastolic blood pressure was over 90 mmHg (measured at least three times on 2 different days), was detected in 26 out of 28 patients (93%). Other forms of secondary hypertension were excluded. Six patients with severe Graves' ophthalmopathy, treated with a high dose of prednisone (45–90 mg/day) for 3–12 weeks, were also studied (6 female; mean age 45.5, range 33–57 years). All these patients were euthyroid at the time of study and had no other disorders. The control group comprised 34 normotensive healthy subjects (28 female/6 male; mean age 37.8, range 23–60 years). Informed consent was obtained from each patient and healthy subject.

All the subjects studied remained on a standard hospital diet, with normal electrolyte supplementation (Na⁺, 120, and K⁺, 80 mmol/day). In most of the patients no medication was used before the study, and necessary treatment for hypertension (β-adrenoceptor blocking agents, Ca²⁺-channel blocking agents, diuretics) was discontinued at least 1 week before the study. After night bed-rest, fasting blood samples were obtained for (numbers of patients with Cushing's syndrome (P) and controls (C) are given in parentheses): plasma ANH (P, n = 24; C, n = 16), DLS immunoreactivity (P, n = 21; C, n = 13), ⁸⁶Rb by uptake erythrocytes (P, n = 12; C, n = 28) and [¹²⁵I]ouabain binding (P, n = 21; C, n = 13). An appropriate balance of sex and age between patient and control groups was maintained in all subgroups studied. In the patients on the prednisone treatment (n = 6), all the above estimations, except ⁸⁶Rb uptake, were performed. Blood samples were also obtained from all the subjects for determination of serum Na⁺, K⁺, supine plasma renin activity (PRA) and aldosterone. The second blood sample was taken after 3 h in an upright position and 40 mg i.v. furosemide for stimulated PRA and aldosterone estimation. Supine blood pressure, for estimation of mean arterial pressure (MAP), was measured with a mercury sphygmomanometer before the blood samples were withdrawn. In patients with Cushing's syndrome, the mean 24-h cortisol levels (mean of the measurements at 06.00, 12.00, 18.00 and 24.00 h), ACTH concentration and excretion of 17-hydroxycorticoids (17-OHCS) were also determined.

Plasma ANH was measured after extraction using a radioimmunnoassay (RIA). ANH was extracted from plasma on Sep-Pak C₁₈ mini-columns (Waters Associates, Milford, MA, U.S.A.). Plasma (1 ml), diluted to 3 ml with 4% (v/v) acetic acid, was passed through the methanol-activated columns. After washing with 0.9% (w/v) NaCl, ANH was eluted using 4 ml 86% (v/v) ethanol in 4% acetic acid. The recovery of ANH from plasma was approximately 90%; thus the results were not corrected for recovery. The reagents used for RIA were purchased from Amersham International plc, Amersham, Bucks, U.K. The between-assay coefficient of variation was 15%.

DLS immunoreactivity and inhibition of uptake of ⁸⁶Rb and [¹²⁵I] ouabain binding by erythrocytes were measured with the use of extracted plasma samples. Plasma (2 ml) was passed through preactivated Sep-Pak C₁₈ columns. After washing with distilled water, the extract was eluted with 3 ml methanol and evaporated at 37°C under a gentle stream of nitrogen. A similar method of DLS extraction was shown to eliminate salts and proteins, which may interfere in the DLS assays (Balzan, Ghione, Clerico & Montali, 1986). The recovery of synthetic digoxin was above 95%.

DLS immunoreactivity was determined with the use of a commercial kit RIANEN Digoxin ¹²⁵I (New England Nuclear, North Billerica, MA, U.S.A.). The specific antibodies supplied in that kit did not cross-react significantly with the endogenous steroids (below 0.02% for cortisol) and are widely used to detect DLS immunoreactivity. For RIA of DLS immunoreactivity, plasma extracts were resuspended in an assay buffer to obtain a fourfold concentration.

The digoxin-sensitive ⁸⁶Rb uptake by erythrocytes was measured as previously described by Soszynski, Slowinska-Srzednicka & Zgliczynski (1990), with some modifications. To 0.2 ml packed human erythrocytes in a Krebs–Ringer potassium-free buffer solution, 1 mCi ⁸⁶RbCl (Amersham) and 0.4 ml
resuspended plasma extract were added. Another batch of tubes also contained 1 mmol digoxin/l. After 1 h of incubation at 37 °C and subsequent alternate washing and centrifuging, the $^{86}$Rb radioactivity remaining in the erythrocytes was measured in a scintillation gamma counter. The within- and between-assay coefficients of variation of the method were 5.3% and 12.0% respectively. The plasma Na$^+$/K$^+$ pump inhibitory activity was calculated as a percentage of the total digoxin-sensitive $^{86}$Rb uptake by erythrocytes.

Erythrocyte [H]ouabain binding assay was performed according to the method of Hopp, Lasker, Grossman et al. (1986), with some modifications. To 0.3 ml fresh human erythrocytes (haematocrit 20–30%), diluted in 0.01 mol phosphate buffer/l with 1 mmol MgCl$_2$/l and 0.15 mmol NaCl/l, pH = 7.5) was added 0.1 ml [H]ouabain solution (Amersham; final concentration 52.8 nmol/l) and 0.2 ml resuspended plasma extract. Another set of tubes contained ouabain, dissolved in assay buffer to a final concentration ranging from 2.2 to 430 nmol/l, instead of plasma extracts. In this assay, plasma extracts were resuspended in a buffer to obtain twofold concentration. After 1 h of incubation at 37 °C, 2 ml ice-cold buffer was added to each tube to stop the reaction. After centrifugation, the supernatant was discarded. Two additional washings and centrifugations were performed, and 0.4 ml 10% (v/v) perchloric acid was added to the erythrocyte pellet in order to extract bound [H]ouabain. After centrifugation, the supernatant was dissolved in the scintillation fluid and the radioactivity was measured in a scintillation β-radiation counter. The results of [H]ouabain binding are expressed as ouabain equivalents (nmol/l), calculated from the displacement curve.

Plasma concentrations of ACTH were measured by an immunoradiometric method (Hodgkinson, Allolio, Landon & Lowry, 1984) (normal, below 80 ng/l), cortisol was measured fluorimetrically (Steenburg & Thomasson, 1964) (normal range, 0.19–0.69 mmol/l) and 17-OHCS was measured by the Silber–Porter method (Silber & Porter 1954) (normal range, 6.0–19.0 mmol/24 h). PRA and aldosterone were determined with the use of commercially available kits (ANGIO I and ALDO, Chemapol, Prague, Czechoslovakia).

The results were statistically analysed by one-way analysis of variance (ANOVA) when three groups were compared, with a subsequent Fisher least significant difference test, or with Student’s t-test, when two groups were compared. Correlations between the data were calculated by a linear regression method and Spearman non-parametric method. Because results obtained in both methods were similar, only coefficients revealed in the former method are shown. P values below 0.05 were considered significant, with the use of two-tailed tests. Values are expressed as means ± S.E.M., unless otherwise stated.

RESULTS

In all the patients with Cushing’s syndrome the mean 24-h cortisol level was 1.06 ± 0.08 μmol/l, and the urinary 17-OHCS excretion was 37.5 ± 2.8 μmol/24 h. A mean plasma level of ACTH of 112.9 ± 11.6 ng/l was found in patients with pituitary-dependent Cushing’s syndrome, whereas in patients with adrenal adenomas the ACTH concentration was 20.4 ± 3.3 ng/l. Concentrations of serum electrolytes were as follows: patients with Cushing’s syndrome, Na$^+$ 149 ± 0.5 and K$^+$ 3.7 ± 0.2 mmol/l, prednisone-treated subjects, 139 ± 2.7 and 3.9 ± 0.3 mmol/l, and healthy controls, 138 ± 0.8 and 4.2 ± 1.4 mmol/l respectively. MAP mean value was markedly ($P<0.01$) increased in patients with Cushing’s syndrome (117.9 ± 6.8 mmHg) in comparison with prednisone-treated subjects (96.1 ± 4.3 mmHg) and healthy controls (92.3 ± 1.4 mmHg).

The mean plasma level of ANH in the patients with Cushing’s syndrome was 36.0 ± 1.4 ng/l, which was significantly higher ($P<0.01$) than that of healthy controls (28.6 ± 1.3 ng/l). In the prednisone-treated subjects, ANH levels were further increased to 43.8 ± 4.5 ng/l, which was significantly higher than in patients with Cushing’s syndrome and controls ($P<0.05$ and $P<0.01$ respectively). DLS-immuno-reactivity levels and [H]ouabain binding inhibition were not significantly different in the groups under study (ANOVA: $F=2.18$ and $F=0.15$ respectively), with respective values as follows: patients with Cushing’s syndrome: 49.2 ± 4.8 mg/l and 31.7 ± 8.7 nmol ouabain equivalents/l, prednisone-treated subjects: 30.3 ± 2.9 mg/l and 38.2 ± 17.6 nmol/l, and controls: 51.9 ± 7.0 mg/l and 39.4 ± 12.5 nmol/l. $^{86}$Rb uptake by erythrocytes was significantly ($P<0.02$) less inhibited by the plasma extracts of patients with Cushing’s syndrome: 52.9 ± 2.7% than by plasma extracts from controls, 60.9 ± 1.8%.

The PRA and aldosterone data are presented in Table 1. In the patients with Cushing’s syndrome PRA values were higher than those in controls, but a significant difference was revealed only in the case of supine PRA. Other PRA results and aldosterone levels were not significantly different in the three groups.

Some of the results revealed in the correlation analysis, performed in the patients with Cushing’s syndrome, are shown in Table 2. Levels of ANH were correlated negatively with the stimulated PRA and $^{86}$Rb uptake inhibition by plasma extracts. A positive
TABLE 1. Plasma renin activity (PRA) and aldosterone concentrations in patients with Cushing’s syndrome, prednisone-treated subjects and in healthy controls. Supine PRA and aldosterone were determined after overnight supine position. Stimulated PRA and aldosterone were determined after 40 mg i.v. furosemide and 3 h in upright position.

<table>
<thead>
<tr>
<th>Group</th>
<th>PRA (µg/h)</th>
<th>Aldosterone (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supine</td>
<td>Stimulated</td>
</tr>
<tr>
<td>Cushing’s syndrome</td>
<td>0.89 ± 0.19</td>
<td>4.0 ± 0.84</td>
</tr>
<tr>
<td>Prednisone-treated</td>
<td>0.54 ± 0.11</td>
<td>2.23 ± 0.36</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0.45 ± 0.08</td>
<td>2.75 ± 0.41</td>
</tr>
</tbody>
</table>

*P < 0.05 vs healthy controls (ANOVA and Fisher LSD test).

TABLE 2. Correlation analysis in Cushing’s syndrome (the numbers of patients are given in parentheses)

<table>
<thead>
<tr>
<th>ANH</th>
<th>DLS-IR</th>
<th>[3H]Ouabain</th>
<th>86Rb</th>
<th>Cortisol</th>
<th>MAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supine</td>
<td>-0.13</td>
<td>-0.16</td>
<td>-0.61</td>
<td>0.22</td>
<td>0.17</td>
</tr>
<tr>
<td>Stimulated</td>
<td>-0.46*</td>
<td>0.07</td>
<td>0.39</td>
<td>-0.05</td>
<td>0.23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aldosterone</th>
<th>[3H]Ouabain</th>
<th>86Rb</th>
<th>Cortisol</th>
<th>MAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supine</td>
<td>-0.18</td>
<td>-0.09</td>
<td>-0.14</td>
<td>0.12</td>
</tr>
<tr>
<td>Stimulated</td>
<td>-0.12</td>
<td>0.08</td>
<td>-0.45</td>
<td>0.24</td>
</tr>
<tr>
<td>17-OHCS</td>
<td>0.05</td>
<td>-0.24</td>
<td>-0.54</td>
<td>0.47</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01.

ANH, atrial natriuretic hormone; DLS-IR, digitalis-like substance immunoreactivity; [3H]Ouabain, [3H]Ouabain binding by erythrocytes; 86Rb, 86Rb uptake by erythrocytes; MAP, mean arterial pressure; PRA and aldosterone supine, plasma renin activity and aldosterone determination after overnight supine position; PRA and aldosterone stimulated, determined after 40 mg i.v. furosemide and 3 h in upright position; 17-OHCS, 17-hydroxycorticoid excretion.

correlation was found to exist between [3H]Ouabain binding and 86Rb uptake, but [3H]Ouabain binding and DLS immunoreactivity were not correlated with other data. In contrast, 86Rb uptake inhibition values correlated positively with the stimulated PRA and negatively with cortisol levels. With the exception of cortisol concentrations, none of the other factors studied was correlated with MAP recordings. Other correlations noted are shown in Table 2.

**DISCUSSION**

The results obtained in the present study indicate that, in contrast to the increased ANH levels, plasma DLS measured indirectly by means of digoxin RIA, radio-receptor and biological methods is not substantially altered in patients with hypercortisolamine due to Cushing’s syndrome or prednisone treatment.

Two main causes of the increased ANH levels in Cushing’s syndrome may exist: an increased plasma volume or a direct glucocorticoid effect. In hypercortisolamine states, plasma renin substrate is increased, PRA is normal or slightly increased and mineralocorticoid levels are usually normal (Krakoff, Nicolis & Amsel, 1975; Saruta, Suzuki, Handa et al. 1986; Connell, Whitworth, Davies et al. 1987), which has been confirmed, in part, in our study. These observations disprove the possible volume overload theory in hypercortisolamine patients. However, the negative correlation between ANH and stimulated PRA in Cushing’s syndrome, noted in the present study, may suggest some relationship between those factors. On the other hand, glucocorticoids were shown to stimulate ANH secretion from the heart (Wiegand et al. 1987) and to increase plasma ANH levels in healthy subjects (Connell et al. 1987; Saxenhofer et al. 1988) and steroid-treated patients (Yamaji et al. 1988), similarly to our results. Thus, it seems more likely that chronic hypercortisolamine itself is the cause of the increased plasma ANH in Cushing’s syndrome.

Increased ouabain-sensitive 86Rb uptake in erythrocytes of patients with endogenous and exogenous
glucocorticoid excess has been reported (Wambach et al. 1985). Ng, Evans & Burke (1987) found a lowered ouabain-sensitive 22Na efflux rate in leucocytes, which reflects the activity of Na+/K+ pump, in adrenocortical insufficiency. The same authors observed an increased 22Na efflux rate in Cushing’s syndrome, which was corrected after successful treatment (Ng et al. 1988). Increased Na+/K+ pump activity is thought to depend directly on the hypersecretion of glucocorticoids, since these hormones stimulate Na+/K+ -ATPase in vitro (Ng et al. 1987) and in vivo (Kaji, Thakkar & Kahn, 1981; Sinha et al. 1981).

In the present study we found that the plasma extracts from patients with Cushing’s syndrome inhibited 86Rb uptake, which is believed to reflect Na+/K+ pump activity, to a smaller degree than healthy subjects. The fact that the patients’ hypercortisolaemic sera did not stimulate the Na+/K+ pump in our bioassay is not surprising, because glucocorticoids exert no effect in vitro on enucleated cells, such as erythrocytes (Kaji et al. 1981), or on the isolated Na+/K-ATPase (Sinha et al. 1981). Since in the present experimental protocol the glucocorticoid effect was excluded, it was demonstrated that the Na+/K+ pump inhibitory activity was slightly decreased in the patients studied. Thus, depressed DLS in addition to hypercortisolaemia may be the cause of the increased Na+/K+ -ATPase activity in Cushing’s syndrome. Inverse correlations of serum DLS with cortisol in the patients indicate that the observed lower DLS levels may have resulted from a direct action of glucocorticoids. Although, 86Rb uptake and [3H]ouabain binding was correlated, only 86Rb uptake inhibitory activity was significantly lower in patients with Cushing’s syndrome. Among the three DLS assays performed, this method is most reliable and sensitive. Therefore, it was possible to show a difference in sodium pump inhibitory activity which, however, is relatively small and may not be of any major biological relevance.

DLS-immunoreactivity levels were also similar in the three groups under study, and were not correlated with the results of other DLS assays, which has also been reported by others (Yamada, Goto, Ishii et al. 1988). The results of DLS immunoreactivity and [3H]ouabain binding, similar in the hypercortisolaemic patients and healthy subjects, support the findings of others that the supposed endogenous DLS is not a cortisol or other circulating glucocorticoid derivative (Schreiber, Stepan, Pribyl & Starka, 1981; Lau & Valdes, 1988).

The pathogenesis of hypertension in the hypercortisolaemia is unclear, and probably multiple factors play a role in its development (Whitworth 1987; Fraser, Davies & Connell, 1989). Besides the effects of cortisol on salt retention and on increasing vascular reactivity, the lower activity of such depressive substances as prostaglandin E₂ or kallikrein may contribute to hypertension in Cushing’s syndrome (Saruta et al. 1986).

The endogenous Na+/K+ pump inhibitor, DLS, has been found to be increased in various forms of experimental mineralocorticoid-induced hypertension (Kojima, 1984; Yamaji, Ishibashi, Sekihara et al. 1986), volume expansion (Gruber, Metzler, Robinson et al. 1985) and in human arterial hypertension, mainly with low renin activity (Haddy & Pannani, 1985; Soszynski et al. 1990). Normal, or even lower, DLS values in patients with hypercortisolaemia are in accordance with the above observations, since the hypertension in Cushing’s syndrome may be classified as normal/high renin (Fraser et al. 1989). The ANH role in essential or secondary hypertension development is not clear. Increased plasma ANH levels have been reported in certain forms of secondary hypertension, similar to those described above in the case of DLS (Espiner & Nichols, 1987). In Cushing’s syndrome, increased ANH levels probably have no important effect on blood pressure, despite the known hypotensive properties of ANH (Espiner & Nichols, 1987), since ANH levels were increased in hypertensive patients with Cushing’s syndrome and in normotensive subjects on prednisone treatment. The MAP recordings significantly correlated only with serum cortisol levels in our patients, which additionally indicates a close relationship between this hormone and high blood pressure.

In conclusion, chronic endogenous or exogenous glucocorticoid excess increases plasma ANH levels, but does not significantly influence Na+/K+ pump inhibitor, i.e. DLS activity. In contrast to cortisol, none of the natriuretic factors is directly related to hypertension in Cushing’s syndrome.

ACKNOWLEDGEMENTS

This work was supported by the UNPTiW grant CPBR 11-6.

REFERENCES

