Radioimmunoassay of plasma ouabain in healthy and pregnant individuals

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Abstract

Ouabain was recently isolated from human plasma, bovine hypothalamus and bovine adrenal in attempts to identify endogenous substances inhibiting the cell membrane sodium pump. A number of radioimmunoassays have been developed in order to study the clinical significance of ouabain. The results have been controversial with regard to the presence and chemical nature of plasma ouabain-like immunoreactivity. We have now measured ouabain in healthy and pregnant individuals using solid-phase extraction of plasma samples followed by a new radioimmunoassay with the extraordinary sensitivity of at least 2 fmol/tube (5 pmol/l). Plasma extracts, a previously isolated human plasma ouabain-like compound and bovine hypothalamic inhibitory factor displaced the tracer in parallel and eluted identically with ouabain in high-performance liquid chromatography. Plasma ouabain immunoreactivity was found to be much lower than reported previously: 12.6 ± 1.3 pmol/l in healthy men (mean ± s.e., n=20) and 9.4 ± 0.7 pmol/l in women (n=14). In pregnant women (n=28) plasma ouabain concentration was 16.3 ± 4.0 pmol/l during the first trimester, 18.8 ± 4.3 pmol/l during the second trimester and 24.3 ± 4.0 pmol/l during the third trimester (all P<0.01 compared with non-pregnant women). Plasma ouabain 3–5 days after the delivery was 13.6 ± 1.1 pmol/l (n=10, P<0.05–0.01 compared with second and third trimesters). The pregnancy-related changes in the plasma concentrations of ouabain resembled those of cortisol. Therefore cortisol was measured from the same plasma samples and a significant positive correlation was found (r=0.512, P=0.006). The similar profiles of plasma ouabain and cortisol during pregnancy and their rapid decreases postpartum are consistent with the adrenal cortical origin of ouabain and also show that the secretions of these hormones are possibly under the control of same factors. Journal of Endocrinology (2000) 165, 669–677

Introduction

The ubiquitous cell membrane enzyme Na⁺/K⁺-ATPase, the sodium pump, regulates the transmembrane sodium and potassium gradients. Inhibitors of the sodium pump such as digitalis glycosides have long been used in the treatment of heart failure and endogenous sodium pump inhibitors have been sought for decades. An endogenous ouabain-related compound has been isolated from human plasma (Hamlyn et al. 1991), bovine hypothalamus (Tymiak et al. 1993, Zhao et al. 1995, Kawamura et al. 1999) and bovine adrenals (Schneider et al. 1998). The isolated compounds were identical to plant-derived ouabain in several reverse phase HPLCs, mass spectrometry and hydrogen-1 nuclear magnetic resonance and also inhibited the sodium pump and increased the contractile force of atrial muscle. For some time it was believed that the endogenous compound was an isomer of ouabain (Tymiak et al. 1993, Zhao et al. 1995), but the isomerism was recently found to be caused by the artificial complexation of ouabain to borate from glassware used in the isolation process (Kawamura et al. 1999).

Normal pregnancy involves marked changes in endocrine functions in addition to the blood volume and vascular smooth muscle tone. Considering the observed adrenocortical origin of ouabain (Hamlyn et al. 1991, Schneider et al. 1998), the association of plasma ouabain with cortisol is of great interest, as the activation of the adrenal cortex during pregnancy is well known. Circulating endogenous cardiac glycoside-like compounds also link the sodium pump to vascular changes. In pregnant women, the lymphocyte sodium pump density and activity (Ang et al. 1990) and the erythrocyte sodium pump rate and activity (MacPhail et al. 1992, Yoshimura et al. 1993) have been found to be greater than in non-pregnant individuals. In contrast, the amount of the 2α subunit of ouabain (Tymiak et al. 1995, Zhao et al. 1995) has been reported to be much lower than in non-pregnant individuals. Whether plasma ouabain-like immunoreactivity is increased in pregnancy and linked to circulating cortisol is not known.
the sodium pump in the myometrium is smaller in pregnant than in non-pregnant women (Maxwell et al. 1998). There are also many studies showing that the circulating concentrations or activities of digitals- or ouabain-like compounds as measured by receptor assays or by immunoassays are greater in pregnant than in non-pregnant individuals (Graves et al. 1984, Valdes et al. 1985, Poston et al. 1989, Seely et al. 1992, Paci et al. 1996). However, the circulating concentrations of these compounds have been remarkably high and no detailed analyses concerning the nature of the biological activity or immunoreactivity measured as ouabain have been performed.

In order to study the secretion profile of ouabain and to understand the changes in blood pressure that occur during pregnancy, we decided to measure plasma concentrations of ouabain and cortisol in pregnant women. For this purpose we set up a new sensitive radioimmunoassay for ouabain and calibrated it with the previously isolated ouabain preparations of human plasma (Hamlyn et al. 1991) and bovine hypothalamus (Tymiak et al. 1993). We observed that plasma ouabain concentrations in healthy individuals were much lower than reported previously and that plasma ouabain and cortisol concentration profiles change in parallel during pregnancy, possibly reflecting a common regulation of these hormones.

Materials and Methods

Study participants

Informed consent was obtained from all the participants in the study. Blood samples were obtained from 14 non-pregnant female (mean age 32·7 years) and 20 male laboratory workers (43·2 years), and from 28 pregnant women (28·9 years) from the first (n=10), second (n=8) and third (n=10) trimesters (gestational weeks 1–13, 14–25 and 26–40 respectively). A blood sample was also taken from 10 women (25·5 years) at 3–5 days after delivery. All the blood samples were taken between 0900 h and 1200 h, into polypropylene or polystyrene test tubes containing EDTA as an anticoagulant. Plasma was stored at −20 °C. Umbilical cord plasma and amniotic fluid samples were collected from six mothers (30·2 years) after the delivery or amniotomy. Blood pressure was recorded by a sphygmomanometer with the person sitting after a 10-min rest. The pregnant women had normal pregnancies and delivered healthy babies.

Chemicals

Ouabain (G-strophantin) octahydrate, ouabagenin, digoxin, digitoxin and related steroids (aldosterone, cortisol, hydrocortisone, progesterone, β-estradiol, testosterone), bovine thyroglobulin and sodium cyanoborohydride were obtained from Sigma Chemical Company (St Louis, MO, USA). Freund’s incomplete and complete adjuvants were purchased from Difco Laboratories (Detroit, MI, USA), L-tyrosine and polyethylene glycol 6000 from Fluka AG (Buchs, Switzerland), radioiodinated (125I) ouabain derivative (70–80 MBq/nmol) from Biotop Ltd (Oulu, Finland), Sephadex G-50F from Pharmacia (Uppsala, Sweden). All other chemicals were obtained from E Merck AG (Darmstadt, Germany).

Reference compounds

Lyophilized preparations of the ouabain-like compound from human plasma (Hamlyn et al. 1991), approximately 1 pmol, and from bovine hypothalamus (Tymiak et al. 1993), 171 pmol, were generously supplied by Dr J M Hamlyn and Dr G T Haupert Jr respectively.

Radioimmunoassays

Ouabain was coupled to bovine thyroglobulin using the periodination and sodium cyanoborohydride method according to Masugi et al. (1986). The ouabain–thyroglobulin conjugate was purified by Sephadex G-50 gel filtration. The conjugate was emulsified in Freund’s complete adjuvant and injected subcutaneously at multiple sites on the backs of five rabbits (1 mg immunogen/rabbit). Boosters (0·5 mg/rabbit in Freund’s incomplete adjuvant) were given at monthly intervals. All five rabbits produced antisera against ouabain after the primary immunization. The antiserum ‘199’ at a final titer of 1·750 000, the ouabain tracer and ouabain standards or samples in duplicate were dissolved in the radioimmunoassay buffer (PBS with 0·1% gelatin and NaN3, pH 7·4) and each pipetted in 100 µl aliquots into polystyrene test tubes. After an overnight incubation at 6 °C the bound and free fractions were separated by double antibody precipitation in the presence of 8% polyethylene glycol, and the precipitates were counted for radioactivity. The sensitivity of the ouabain radioimmunoassay calculated at a level of 5% displacement of the tracer with antiserum 199 was 2 fmol/tube or 5 pmol/l (Fig. 1). The ouabain concentrations in normal human plasma extracts varied between 3 and 10 fmol/tube.

The within and between assay coefficients of variation were 3·8% and 13·4% at 15 pmol/l and 3·7% and 9·4% at 42 pmol/l respectively (n=10). All the samples from pregnant and postpartum women were assayed in the same assay. Of the compounds tested, ouabagenin showed the greatest cross-reactivity (52%): digoxin and digitoxin cross-reacted slightly (1·7%), and other less relative compounds (butaline 1%, dihydro-ouabain 0·7% and digoxigenin 0·5%). Naturally occurring steroids cross-reacted very little: cortisol 0·0005%, aldosterone 0·0008%, progesterone 0·00017%, estrone 0·00027%, β-estradiol 0·0006%, dehydroepiandrosterone 0·0003%, hydroxyandrostenedione 0·0001% and testosterone 0·0005% (Table 1).
the maximum intra-assay and interassay coefficients of variation are 4·1 and 9·0%, respectively. Cortisol was measured from the 27 plasma samples available to us at the time of the assay.

Solid-phase separation of plasma and amniotic fluid samples

Ouabain was extracted from 1–2 ml plasma or amniotic samples with Sep-Pak-Vac 500 mg C18 cartridges (Waters, Milford, MA, USA) using an automated Gilson 5100 Aspec system and polypropylene test tubes. Briefly, the cartridge, preconditioned with 2 ml 2-propanol and 4 ml 0·1% aqueous trifluoroacetic acid (TFA), was loaded with the plasma sample to which 0·2 ml 1 M HCl/1·6% glycine/ml was added. The cartridge was washed with 0·1% TFA. Ouabain was eluted with 2 ml 40% acetonitrile in 0·1% TFA and evaporated to dryness. The dried residue was reconstituted with 250 µl radioimmunoassay buffer (see above). The recoveries of ouabain added to plasma were 103 ± 2·5% at 43 pmol/l and 94 ± 1·3% at 130 pmol/l (mean ± s.e., n=6).

HPLC analysis of the reference materials and plasma extracts

Aliquots of human plasma ouabain-like compound (Hamlyn et al. 1991) and bovine hypothalamic inhibitory factor (Tymiak et al. 1993) and Sep-Pak extracts of plasma or amniotic fluid (see above) were dissolved in 0·1% TFA, centrifuged, filtered and subjected to reverse-phase HPLC using a Vydac C8 218TP column (0·46 × 15 cm, Hesperia, CA, USA). A 30-min linear gradient from 5 to 30% acetonitrile in 0·1% TFA was run at a flow rate of

| Table 1 Comparison of immunoassays developed for ouabain with respect to specificity (cross-reactivity was determined at 50% displacement of tracer on a weight basis), sensitivity (read at 95% binding level of tracer, or as mean for the zero calibrator – 2SD) and plasma ouabain (concentrations in healthy adults) |
|---|---|---|---|---|
| Assay method | Ouabagenin | Dihydro-ouabain | Digoxin | Digitoxin | Sensitivity (pmol/l) | Plasma ouabain (pmol/l) |
| Authors |  | Ouabagenin | Dihydro-ouabain | Digoxin | Digitoxin | Steroids* |
| Harris et al. (1991) | ELISA | 40 | 0·16 | 5·2 | 28 | ≤0·01 | 100 | 100–180 |
| Doris et al. (1994) | 3H-RIA | 56·2 | — | 4·5 | — | ≤0·025 | 80 | <80 |
| Gomez-Sanchez et al. (1994) | ELISA | 2·2 | 0·02 | <0·001 | 0·12 | ≤0·001 | 30 | 30–50 |
| Lewis et al. (1994) | ELISA | 77·2 | — | 1·25 | — | ≤0·07 | 60 | <60 |
| Naruse et al. (1994) | 3H-RIA | — | — | 9·2 | — | <0·01 | 20 | 20–60 |
| Worgall et al. (1996) | 3H-RIA | 88 | — | 0·06 | 0·18 | ≤0·1 | 20 | 30–100 |
| Balzan et al. (1997) | 3H-RIA | — | — | 3·6 | — | ≤0·06 | 10 | 15–30 |
| Ferrandi et al. (1997) | 3H-RIA | 21 | — | 0·4 | 9 | ≤0·02 | 20 | 50–750 |
| Harwood et al. (1997) | ELISA | 55 | 0·3 | 5·3 | 78 | ≤0·02 | 40 | 60–170 |
| Komiyama et al. (1997) | ELISA | 72 | 0·82 | 5·6 | 8·4 | ≤0·01 | 10 | 20–140 |
| Bernini et al. (1998) | ELISA* | — | — | 3·6 | 5 | ≤0·01 | 10 | 10–175 |
| Butt et al. (1998) | 3H-RIA | 4·0 | 2·0 | 0·4 | 0·6 | ≤0·016 | 60 | 400–800 |
| Present study | 125I-RIA | 52·2 | 0·7 | 1·7 | 0·6 | <0·001 | 5 | 10–20 |

* Aldosterone, cortisol, progesterone, β-estradiol, testosterone and other common steroids.

**Du Pont–New England Nuclear ELISA kit.

RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunoassay.
1 ml/min. Fractions of 1 ml were collected into polypropylene test tubes, dried in Speed Vac, reconstituted with 250–500 μl radioimmunoassay buffer as described before and subjected to the ouabain radioimmunoassay. In the reverse-phase HPLC, 90% of the ouabain-like compound (Hamlyn et al. 1991) and 78% of the bovine hypothalamic inhibitor (Tymiak et al. 1993) could be recovered.

**Statistical analyses**

The data are expressed as mean ± s.e. For comparison of the ouabain or cortisol concentrations and blood pressure recordings between various experimental groups, one-way ANOVA followed by Newman–Keul’s multiple comparison test was used. Spearman coefficients of rank correlation and linear correlation were calculated with immunoreactive plasma ouabain concentration as independent and systolic and diastolic blood pressure recordings or plasma cortisol as dependent variables.

**Results**

*Validation of the radioimmunoassay for use with human plasma samples*

The displacement curves of the bovine hypothalamic inhibitory factor and human plasma ouabain–like substance were parallel with that of plant–derived ouabain (Fig. 1). The apparent cross-reactivity of the hypothalamic inhibitory factor obtained from Dr Haupert (Tymiak et al. 1993) was 70% and that of plasma ouabain–like compound obtained from Dr Hamlyn (Hamlyn et al. 1991) was 52%. Endogenous immunoreactivity present in human male plasma Sep–Pak extracts displaced the tracer in parallel with the ouabain standard (Fig. 2). The Sep–Pak extracts of the reference preparations from bovine and human plasma origin eluted identically with plant ouabain in the reverse phase HPLC system (Fig. 3). The recovery of the added or endogenous materials in the HPLC run was 78–103%. When the ouabain–like compound isolated from human plasma was subjected to HPLC, a very small immunoreactive peak was found to elute after ouabain (Fig. 3B). Immunoreactive ouabain in Sep–Pak extracts of human pregnant plasma, umbilical cord plasma and amniotic fluid eluted in the reverse-phase HPLC system identically with plant ouabain (Fig. 4). These findings show that our radioimmunoassay is suitable for the measurement of endogenous ouabain.

*Plasma immunoreactive ouabain in reference and pregnant individuals*

The mean plasma ouabain concentrations measured by our radioimmunoassay were 12·6 ± 1·3 pmol/l in healthy men and 9·4 ± 0·7 pmol/l in women. The mean plasma ouabain concentrations in healthy pregnant women during the first, second and third trimesters were 16·3 ± 4·0, 18·8 ± 4·3 and 24·3 ± 4·0 pmol/l respectively; all these values are significantly greater than those in non-pregnant women (P < 0·01; Fig. 5). Plasma ouabain concentration during the second and third trimesters was also significantly greater than that during the first trimester (P < 0·05–0·01). After the delivery, plasma ouabain concentrations were 13·6 ± 1·1 pmol/l, significantly (P < 0·05) lower than those during the second and third trimesters. Amniotic fluid and umbilical cord plasma ouabain levels were 25·5 ± 4·1 and 22·0 ± 2·0 pmol/l respectively.

The mean systolic and diastolic blood pressures in healthy women were 127 ± 4 and 82 ± 1 mmHg respectively. In pregnant women the systolic blood pressure during the third trimester was significantly (P < 0·05) greater than that during the first or second trimesters (124 ± 3 mmHg compared with 110 ± 3 and 118 ± 5 mmHg respectively). The mean systolic and diastolic blood pressures in postpartum women were 125 ± 3 mmHg and 69 ± 5 mmHg. There was a significant rank correlation between plasma ouabain concentration and the systolic blood pressure in pregnant women (r = 0·434, P = 0·0269, n = 26; Fig. 6). The correlation between plasma ouabain and diastolic blood pressure in the same individuals was not significant (r = 0·08, P > 0·05).

The mean morning plasma cortisol concentrations were 183 ± 17 nmol/l during the first trimester, 214 ± 44 nmol/l during the second trimester, 278 ± 25 nmol/l during the third trimester and 146 ± 12 nmol/l
at 3–5 days after the delivery. The cortisol concentrations during the third trimester were significantly greater ($P<0.05$) than those during the first trimester or postpartum. There was a significant linear correlation between plasma ouabain and plasma cortisol concentrations in pregnant and postpartum women ($r=0.512$, $P=0.006$, $n=27$; Fig. 7). It should be stressed that our ouabain antiserum does not have any detectable cross-reaction with cortisol or the naturally occurring steroid hormones.

**Discussion**

In order to validate our ouabain radioimmunoassay for the analysis of human plasma samples, we compared the displacement curves of the human plasma endogenous ouabain-like compound (Hamlyn et al. 1991) and the bovine hypothalamic inhibitor (Tymiak et al. 1993) with commercial plant-derived ouabain. Both endogenous substances diluted in parallel with ouabain and their potencies compared with commercial plant ouabain were 52% and 70% respectively. Considering the inaccuracies inherent in weighing small amounts of natural materials (1 pmol and 171 pmol), both endogenous substances probably are equipotent with ouabain in our radioimmunoassay. In addition, we wanted to see if the endogenous materials were homogenous. Reverse-phase HPLCs of both endogenous substances were performed and demonstrated almost
complete recovery; in addition, the great majority (78–90%) of the immunoreactivity eluted identically with ouabain. However, a small broad immunoreactive peak eluted after ouabain in the human plasma preparation. This finding and the slightly lower than expected immunoreactivity of the human plasma and bovine hypothalamic preparations that we observed in this study may have been presumed to reflect the complexation of ouabain with borate from glassware that was recently shown to be possible (Kawamura et al. 1999); however, we did not use glassware in this study. Our radioimmunoassay is thus valid for the measurements of endogenous ouabain in biological fluids.

In the present study, the mean plasma ouabain concentrations in non-pregnant women were found to be 9.4 pmol/l and those in men were 12.4 pmol/l. Previously, an enzyme-linked immunosorbent-assay (ELISA) method gave plasma ouabain concentrations of 138 pmol/l (Hamlyn et al. 1991). In other studies even greater concentrations have been reported, but the identity of the immunoreactivity was not studied (Masugi et al. 1986, Harris et al. 1991, Gottlieb et al. 1992, Rossi et al. 1995). In a more recent radioimmunoassay study, plasma ouabain concentrations in healthy volunteers (sex not stated) were found to be 25 pmol/l and the immunoreactivity eluted in HPLC in the same way as ouabain (Balzan et al. 1997). In contrast, plasma ouabain concentrations less than 5 pmol/l have also been reported (Gomez-Sanchez et al. 1994, Doris et al. 1994, Lewis et al. 1994). A summary of the results of previous ouabain immunoassays was presented in Table 1. It is our opinion that the use of tritiated or enzyme-labeled tracers and the resulting low sensitivity of the previously used ouabain antisera are major reasons why greatly variable ouabain concentrations in human plasma have been reported. In addition, no attempts to compare the chemical and immunological properties of the endogenous ouabain-like substances with commercial ouabain have been made in any of the previous studies.

There are no previous studies reporting plasma immunoreactive ouabain concentrations in pregnant women. Using a radioreceptor assay, plasma ouabain concentrations in non-pregnant individuals were found to be 204 pmol/l; those in pregnant women were up to 2000 pmol/l (Paci

Figure 5 Plasma ouabain concentrations in non-pregnant women (CON, ○, n=14), and in pregnant women during gestational weeks 8–40 (●, n=28) and 3–5 days after the delivery (PP, △, n=10). There was a significant linear correlation between plasma ouabain and the duration of gestation (solid line). Prediction lines with 95% probability are shown (dotted lines).

Figure 6 Systolic blood pressure as a function of plasma ouabain concentration in pregnant women. There was a significant positive rank correlation between the parameters (solid line: r=0.434, P=0.0269, n=26).
Positive linear correlation between the parameters (solid line: \textit{reactive ouabain-like substance (SS like substance is much smaller than that of immuno-

observed that the amount of immunoreactive digitalis-

digitalis-like substances in human pregnant plasma and

et al. 1996). We have recently measured immunoreactive
digitalis-like immuno-

analyses of the chemical identity of the measured immu-

concentrations in pregnant women have varied from

to 930 pmol/l, but in none of these studies were

chemical identity of the measured immu-

et al. 1984, Valdes & Graves 1985, Poston et al. 1989, Seely et al. 1992, Paci et al. 1996). We have recently measured immunoreactive
digitalis-like substances in human pregnant plasma and

after solid-phase extraction and HPLC and

observed that the amount of immunoreactive digitalis-

like substance is much smaller than that of immuno-

reactive ouabain-like substance (S S \text{ Árnason, unpublished observation}).

Systolic but not diastolic blood pressure in our pregnant

women was significantly greater in the third trimester than

in the first or second trimesters. We wanted therefore to

know whether plasma ouabain concentrations correlated
to blood pressure during pregnancy, and we observed a

significant rank correlation between plasma ouabain con-

centration and systolic blood pressure but not between
diastolic blood pressure. We also performed a linear

correlation analysis between plasma ouabain and systolic

blood pressure, but this did not reach statistical signi-

ficance, indicating that the correlation is complex. It has

been proposed that plasma ouabain could interfere with

the sodium pump of vascular smooth muscle, resulting in

increased vascular tone and increased blood pressure

(Hamlyn & Manunta 1992, Blaustein 1996). Our findings

are consistent with this concept during pregnancy but not

after the delivery, when plasma ouabain decreased but

systolic blood pressure remained at increased values. The

significant correlation between plasma ouabain and systolic

blood pressure during pregnancy may still impart some
role for ouabain in regulating blood pressure, but other

factors have also to be taken into account.

In view of the observed adrenal origin of ouabain

(Hamlyn et al. 1991, Schneider et al. 1998) and the finding

that the stimulation of adrenal cells by adrenocorticotropic

hormone (ACTH) \textit{in vitro} increases release of ouabain to

the medium (Laredo et al. 1994), it was interesting to note

that there was a gradual increase in maternal plasma

ouabain during pregnancy and a rapid decrease within

3–5 days after the delivery in the present study. These

changes resemble those in maternal plasma total cortisol

centration (Brien & Dalrymple 1976, Cousins et al. 1983, Alloio et al. 1990). In our study, the mean plasma

total cortisol concentration was 48\% less in the postpartum

women than in the third trimester women; in a similar

study, a 25\% decrease was observed (Alloio et al. 1990).

The difference is evidently due to the fact that our

postpartum women were different from the third trimester

ones.

We detected in this study high concentrations of

ouabain in the amniotic fluid and cord plasma, indicating

an association of ouabain with the fetoplacental unit. The

significant positive linear correlation between the concen-

trations of ouabain and total cortisol in samples taken
during pregnancy and postpartum further strengthens the

proposal that ouabain and cortisol are under similar regu-

lation in these conditions. The fluctuations in maternal

cortisol have been accounted for by stimulation of adrenal
cortex by ACTH and corticotropin-releasing hormone

(Sasaki et al. 1989), an increase in cortisol-binding globulin,
or an antiglucocorticoid action of progesterone (Brien &


The potential roles of these factors in determining plasma

ouabain concentrations are not known.

In conclusion, we have developed a sensitive radio-

immunoassay for ouabain that detects equally well

plant-derived ouabain and the endogenous ouabain-like

substance previously isolated from human plasma and

bovine hypothalamus. Using the assay, we demonstrated

that plasma ouabain concentrations in healthy individuals

are much lower than reported previously and that plasma

ouabain in pregnant women increases towards the deliv-

ery, abruptly decreases after it and correlates with plasma

total cortisol, suggesting that the secretions of ouabain and

Figure 7 Plasma cortisol as a function of plasma ouabain in pregnant (●) and postpartum (○) women. There was a significant

positive linear correlation between the parameters (solid line:
$r = 0.512, P = 0.006, n = 27$). Prediction lines with 95\% probability

are shown (dotted lines).
cortisol during pregnancy may be regulated by the same factors.

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