Platelet angiotensin II binding and plasma renin concentration, plasma renin substrate and plasma angiotensin II in human pregnancy

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SUMMARY
1. The results are presented of a cross-sectional study of 25 non-pregnant and 125 pregnant/postnatal women in whom platelet angiotensin II binding and plasma angiotensin II, plasma renin concentration and plasma renin substrate were measured.

2. Platelet angiotensin II binding was significantly lower in the first-trimester patients as compared with the non-pregnant women ($P<0.001$). Specific binding remained low in the second and third trimesters, and in those patients studied 24 h after delivery. However, higher values, approximating to the non-pregnant level, were found 6 weeks postnatally ($n=25$ for each group).

3. Plasma angiotensin II, plasma renin concentration and plasma renin substrate increased in pregnancy, with the increase becoming statistically significant as compared with the non-pregnant women in the second trimester. Maximal median values of plasma angiotensin II and plasma renin substrate were found in the third trimester, but maximal median values of plasma renin concentration were found in the second trimester. The concentrations of all three hormones fell after delivery.

4. There was an inverse correlation between platelet angiotensin II binding and simultaneously measured endogenous levels of plasma angiotensin II ($P<0.02$) and plasma renin substrate ($P<0.05$) in the 25 non-pregnant subjects. These findings support the concept of the angiotensin II receptor concentration being regulated by the plasma angiotensin II level. There was no correlation between platelet angiotensin II binding and plasma renin concentration.

5. In pregnancy, no correlation between platelet angiotensin II binding and plasma angiotensin II, plasma renin concentration or plasma renin substrate was found. This suggests that any regulation by plasma angiotensin II may not operate in pregnancy, when reduced binding concentrations are present. No statistically significant correlation was found at either 24 h or 6 weeks after delivery.

Key words: angiotensin II, platelet membrane glycoproteins, pregnancy, renin–angiotensin system.

Abbreviations: ANG I, ANG II and ANG III, angiotensins I, II and III, respectively; $K_d$, dissociation constant; PRC, plasma renin concentration; PRS, plasma renin substrate.

INTRODUCTION
Receptors for the potent vasoconstrictor angiotensin II (ANG II) with high affinity and specificity have been found in a variety of vascular tissues [1–3]. Studies using animals have found that both the concentration of ANG II receptors in vascular smooth muscle and the pressor response to the hormone are modulated by the circulating ANG II levels [2, 4], although this effect is not invariable [5]. Increased levels of the peptide result in reduced receptor numbers and a decreased vasoconstrictor effect of ANG II, whereas low levels have the opposite effect.

Vascular smooth muscle tissue in man is inaccessible and thus difficult to study directly. Platelets have many of the structural and biochemical characteristics of smooth muscle cells, and similarities between catecholamine-induced changes in both platelet behaviour and vascular tone have been described [6]. A correlation between aspects of platelet behaviour and changes in forearm blood flow in response to cold stimulation has also been noted [7]. Specific ANG II binding sites, with many of the characteristics of receptors, have been reported on human platelets [8–10]. In non-pregnant subjects, changes in human platelet ANG II binding capacity have been shown to correlate inversely with changes in plasma ANG II levels induced by alterations in sodium intake [11, 12]. The consensus of studies reporting changes in the renin–angiotensin system in pregnancy show increases in...
plasma ANG II, plasma renin concentration (PRC) and plasma renin substrate (PRS) (for a review, see [13]). There is a progressive diminution in the ANG plasma renin substrate (PRS) (for a review, see [13]).

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Between platelet ANG with non-pregnant subjects [16]. This suggests to us an ANG 11-induced down-regulation of platelet ANG binding in pregnancy.

The purpose of this cross-sectional study was to determine whether there was an inverse correlation between platelet ANG II binding and simultaneously measured endogenous plasma ANG II levels in non-pregnant subjects, and secondly, whether any such correlation still pertained in pregnancy when ANG II binding was reduced.

MATERIALS AND METHODS

Materials

\[^{125}\text{I}^{\text{I}}\text{Asp}^{\text{I}}, \text{Ile}^{\text{I}}\text{ANG II}(2000 \text{ Ci/mmol})\], obtained from Amersham International plc (Amersham, Bucks, U.K.), was stored in Tris-buffered saline (50 mmol/l Tris–HCl/154 mmol NaCl, pH 7.35) at a concentration of 0.3 mmol/l. Unlabelled ANG II was obtained from Ciba–Geigy Ltd, Basle, Switzerland. Apyrase (Sigma Chemical Co., St Louis, MO, U.S.A.) was used as a 250 units/ml stock solution in Tris-buffered saline (50 mmol/l Tris–HCl/154 mmol NaCl, pH 7.35) at a concentration of 0.3 mmol/l. All these materials were stored at −20°C. Medium 199 (Flow Laboratories, Irving, Ayrshire, U.K.) was supplemented to give a solution containing (in mmol/l): Na⁺ 145.5, K⁺ 3.8, Ca²⁺ 2.54, Mg²⁺ 1.18, SO₄⁻ 1.18, HCO₃⁻ 24.9, Cl⁻ 128, PO₄⁻ 1.18, glucose 11.1, ethylenediaminetetra-acetate 5, and bovine serum albumin 0.5% (w/v); pH 7.4.

Patients and subjects

A total of 150 subjects were studied in a cross-sectional study, with 25 women in each group. Their ages and, where appropriate, gestational ages are summarized in Table 1. No woman was known to be suffering from renal, metabolic or cardiovascular disease. No patient was taking any medication except iron supplementation. All subjects were receiving sodium intake ad libitum and had normal serum urea, creatinine and electrolyte estimations.

Venepuncture was performed via the antecubital fossa using a 19-gauge needle. Samples were taken in the morning, from non-fasted subjects, who were in the semi-prone position. Venous blood (17.2 ml) was collected into polystyrene tubes containing 3.13% (w/v) trisodium citrate (2 ml) and 2.5 mmol/l acetylsalicylic acid (0.8 ml). An additional 5 ml of blood was collected for serum urea, creatinine and electrolyte estimation, and 10 ml of blood was collected into polystyrene tubes containing 125 mmol/l ethylenediaminetetra-acetate (250 µl) and 25 mmol/l 0-phenanthroline (250 µl) for plasma ANG II, PRC and PRS estimation.

An additional 34.4 ml of blood was taken from 43 non-pregnant subjects for platelet ANG II binding characterization studies.

Platelet preparation and platelet ANG II binding site assay

An initial platelet count was made (Ariane-5 platelet counter; ABX, Montpellier, France). Platelet-rich plasma was prepared by centrifugation (160 g, 20 min, 4°C). The supernatant platelet-rich plasma was filtered through 20 µm nylon gauze (Henry Simon, Stockport, U.K.) before being adjusted to pH 6.4 with 0.1 mol/l citric acid; apyrase (1 unit/ml) was added. The platelets were pelleted by centrifugation (400 g, 20 min, 4°C) and resuspended in modified Medium 199. The platelet concentration in a small portion of the suspension was measured and the volume of the medium was adjusted to give a platelet concentration of 10⁷/µl. Leucocyte and erythrocyte contamination was checked using a Minos STE counter (ABX, Montpellier, France).

Minor modifications were made to a competitive radioimmunoassay used to determine binding, which has been previously described in detail [9]. In brief, platelets were incubated in modified Medium 199 with 300 pmol/l \[^{125}\text{I}^{\text{I}}\text{ANG II}, \text{with and without an excess of unlabelled ANG II (25 µmol/l). Cells were incubated for 90 min at 26°C,\n
Table 1. Details of patients

The cross-sectional study consisted of 25 nulliparous, non-pregnant women and 125 normotensive women in or after their first pregnancy. Those studied after delivery had all been delivered vaginally. Values for age and gestational age are means ± SEM and those for platelet count are medians with quartiles in parentheses.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Gestational age (weeks)</th>
<th>10⁻³ × Platelet count (µl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-pregnant</td>
<td>27.0 ± 0.9</td>
<td>—</td>
<td>220 (174–274)</td>
</tr>
<tr>
<td>First trimester</td>
<td>20.5 ± 0.7</td>
<td>10.0 ± 0.3</td>
<td>198 (177–238)</td>
</tr>
<tr>
<td>Second trimester</td>
<td>24.1 ± 0.8</td>
<td>18.4 ± 0.8</td>
<td>183 (160–209)</td>
</tr>
<tr>
<td>Third trimester</td>
<td>24.1 ± 0.8</td>
<td>33.6 ± 0.9</td>
<td>170 (149–194)</td>
</tr>
<tr>
<td>24 h postnatal</td>
<td>25.3 ± 1.0</td>
<td>—</td>
<td>167 (157–232)</td>
</tr>
<tr>
<td>6 weeks postnatal</td>
<td>25.0 ± 1.0</td>
<td>—</td>
<td>243 (205–271)</td>
</tr>
</tbody>
</table>
Human platelet angiotensin II binding in pregnancy

then separated from the medium by centrifugation through a mixture of phthalate esters (specific gravity 1.03). The radioactivity of the platelets (bound) and medium (free) was counted using a LKB Wallac 1275 mini-gamma counter. Non-specific binding was defined as radioactivity not displaced by saturating unlabelled ANG II added with tracer. All binding studies presented here were corrected for non-specific binding. The characterization studies used have been described previously [8-10].

Non-specific binding represented less than 1% of the added tracer and ranged from 10-40% of total binding. This appeared to be dependent on the age of the tracer, presumably reflecting degradation. However, specific binding did not appear to be affected by the age of the tracer. In the platelet ANG II binding studies interassay variation was 11% and values obtained from four individuals on 5 successive days showed a mean variation of 29%.

Laboratory methods and statistics

PRC, PRS and plasma ANG II were measured by radioimmunoassay [17-19].

Scatchard analysis was used in calculating the equilibrium dissociation constant (Kd). Mean values ± SEM or medians are quoted as appropriate. The data relating to platelet ANG II binding were not normally distributed; non-parametric statistical methods have thus been used throughout. In group comparisons of binding and hormone concentrations, the Mann–Whitney U-test was used. The effect of pregnancy was assessed by using the non-parametric Kruskal–Wallis analysis of variance. Correlations between binding concentrations and hormone levels were assessed using Spearman correlation coefficients (r).

RESULTS

Recovery of platelets from whole blood was 52.7 ± 1.0%. There were no significant differences in platelet recovery in any group when compared with the non-pregnant control group (P = 0.2–0.6). Contamination with leucocytes was <1% (1-5 x 10/μl) and no erythrocytes were detected. A diminution in platelet count occurred during pregnancy (Table I), with the platelet distribution width and mean platelet volume both increasing. Kruskal–Wallis analysis of variance demonstrated a significant effect on pregnancy for all three parameters (P<0.001 in each case). However, there was no significant correlation between any of the platelet parameters and platelet ANG II binding.

Binding studies

In the characterization studies performed using non-pregnant subjects, platelet ANG II binding was found to be of high affinity, specific, saturable, reversible, time-, temperature- and pH-dependent, and linear with cell concentration. The values of Kd obtained from competition and from saturation studies were 1.4 ± 0.7 x 10^-10 mol/l and 2.6 ± 0.9 x 10^-10 mol/l, respectively. A representative Scatchard plot is illustrated in Fig. 1. Competition curves using various angiotensin analogues as the unlabelled hormone, to determine ligand specificity, indicated that des-[Asp]angiotensin II (ANG III) was equipotent with ANG II, followed by [Sar1,Ala8]angiotensin II, with angiotensin I (ANG I) the least potent peptide (Fig. 2).

The median value of platelet ANG II binding was 9.0 fmol/10^9 cells (Fig. 3) in the non-pregnant group, with marked individual variability being found. There was significantly lower binding in the first-trimester group (2.7 fmol/10^9 cells, P<0.001), with an apparent reduction in variability. In four women no ANG II binding could be detected at this time, an observation never made in non-pregnant women. (Values of binding below 0.5 fmol/10^9 cells were deemed to be below the limit of detection of the assay and have been coded as zero.) Platelet ANG II binding remained low in the groups of second-trimester women (3.3 fmol/10^9 cells), third-trimester women (2.3 fmol/10^9 cells) and in those studied 24 h after delivery.
Platelet ANG II binding in pregnancy. Specific ANG II binding is shown, with median values indicated by horizontal bars. The fall in ANG II binding was statistically significant by the first trimester (P<0.001), but levels had returned to non-pregnant values at 6 weeks after delivery.

In pregnant women, there is a diminution in pressor sensitivity to ANG II which is not seen in relation to noradrenaline [14, 20]. Moreover, an increased pressor sensitivity to ANG II has been shown to antedate the development of pregnancy-induced hypertension [15]. In studies of non-pregnant animals, manipulation of the renin-angiotensin system via altered sodium intake produces altered vascular sensitivity to ANG II, which corresponds to changes in vascular smooth muscle ANG II receptor concentrations [2, 14]. In pregnancy, studies of animal ANG II receptor concentrations in smooth muscle provide conflicting results, reduced levels of ANG II binding being found in the rabbit [21] but not in the rat [22]. It has been suggested that the resistance in pregnancy to the pressor action of ANG II that is observed in humans [14] does not occur in certain species [23]. In any case pregnancy-induced hypertension appears to be confined to humans. There is thus a need for an accessible model of vascular smooth muscle in pregnant women, in order to study ANG II receptor changes.
In non-pregnant women, platelets may well be such a model. The values of platelet ANG II binding found in the non-pregnant control group are of a similar order to those previously reported, as are those of $K_d$ [8, 9]. We have previously reported changes in the human menstrual cycle, with both platelet ANG II binding and variability in binding diminishing in the luteal phase of the cycle [24]. The higher variability of platelet ANG II binding in this study relative to previous reports [8] may result from all the subjects being female at days 5–9 of the menstrual cycle. Our findings confirm previous studies [8–10] in that platelet ANG II binding was specific, saturable, reversible and of high affinity, although as yet no correlation between binding site density and a target-cell response has been demonstrated. The demonstration in this study of an inverse correlation between plasma ANG II and simultaneously measured platelet ANG II binding in non-pregnant control subjects lends support to the contention that platelet ANG II binding site density is down-regulated by the plasma ANG II level [11, 12].

In pregnancy, we have previously reported lower levels of platelet ANG II binding in patients in the third trimester of pregnancy, as compared with non-pregnant subjects [16]. Furthermore, our present results suggest that the fall in platelet ANG II binding in pregnancy parallels the diminution in pressor responsiveness to infused ANG II, that also occurs in early pregnancy [15], and which would be expected if platelets reflect changes in vascular smooth muscle.

Changes in receptor occupancy are unlikely to account for the changes in platelet ANG II binding. In non-pregnant subjects, ANG II infusion has no effect on platelet ANG II binding [11] and preliminary results from an ongoing study of the effect of ANG II infusion in pregnant women suggest no effect on platelet ANG II binding. (P. N. Baker, F. Broughton Pipkin & E. M. Symonds, unpublished work). Changes in platelet ANG II binding due to synthesis or degradation of new receptors seems unlikely as platelets possess no nuclei and have minimal protein synthesis capacity. However, it is possible that altered platelet ANG II binding results from exposure or concealment of presynthesized receptors on the cell membrane or stored within the cell. The down-regulation of insulin receptors on hepatocytes has been related to this type of receptor mechanism [25] and our data are consistent with this model.

The lower levels of platelet ANG II binding in pregnancy, with many women having no detectable binding, make characterization studies difficult. We were able to perform competitive studies in only five normotensive subjects in the third trimester of their pregnancy, who had high levels of binding (median 8.0 fmol/10⁹ cells). Scatchard analysis indicated that the binding sites were of high affinity, with a $K_d$ of $2.5 \pm 0.7 \times 10^{-10}$ mol/l, which did not differ significantly from that of the non-pregnant subjects. Three of these patients subsequently developed pregnancy-induced hypertension, and they may thus be unrepresentative of the normotensive pregnant population.

Platelets undergo pregnancy-induced changes in morphology and behaviour, for example mean platelet volume and platelet distribution width increase [26], as does platelet aggregation [27]. Values of platelet ANG II binding were expressed per cell; however, although there was a significant increase in mean platelet volume in pregnancy, the maximal increase was of the order of 6%, so that the results would be similar if expressed per unit of membrane protein. It is possible that pregnancy-induced platelet changes per se, may be responsible for altered platelet ANG II binding, which may thus not reflect ANG II binding to vascular smooth muscle. The hypothesis that platelet and vascular muscle ANG II binding behave in a similar fashion is difficult to validate, but we plan to perform parallel binding studies in sheep.

If the levels of platelet ANG II binding in the pregnant women do reflect vascular smooth muscle receptors, these results suggest that their regulation may differ from that in the non-pregnant state. For although in pregnancy there was both a marked increase in plasma ANG II concentration and a diminution in platelet ANG II binding, the inverse correlation between the two parameters, demonstrated in the non-pregnant subjects, was not found. Interestingly, ANG II infusion studies have suggested that after the first trimester, pressor sensitivity to ANG II is also independent of endogenous plasma ANG II concentration [28].

There have been no other studies of ANG II binding in human pregnancy. Studies using animals suggest several possible regulatory influences upon ANG II receptor concentration. Changes in rat and rabbit uterine ANG II receptors in the ovarian cycle have been found to be directly proportional to changes in the serum levels of oestradiol [29]. Furthermore, oestradiol infusions caused a progressive increase in uterine ANG II receptor concentration [29]. Alternatively, the divergent cations,
Mn$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$ [3], and mineralocorticoid infusions [30] have been found to increase rat mesenteric artery ANG II receptor concentrations. Platelet intracellular free Ca$^{2+}$ concentrations have been reported to rise slowly during human pregnancy, the increase only reaching statistical significance in the third trimester [31]. Whether any of these factors regulate human platelet ANG II binding site concentration remains to be studied, and we are currently measuring the platelet intracellular free Ca$^{2+}$ concentration of pregnant subjects, in conjunction with platelet binding, to determine whether there is any correlation between the two.

In the postnatal patients, a rapid fall in plasma ANG II, PRC and PRS, evident at 24 h after delivery, was accompanied by a more gradual return to non-pregnant levels of platelet ANG II binding, confirming the dissociation between the two. The period of 6 weeks after delivery may not have been long enough to re-establish the inverse correlation between plasma ANG II and platelet ANG II binding. A longitudinal study has been commenced which will follow subjects until 3 months postnatally. The longitudinal study will also determine whether similar results are found when a cohort of women are followed through pregnancy.

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REFERENCES