Activin A and activin receptors in gestational tissue from preeclamptic pregnancies

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Abstract

Maternal serum activin A levels are elevated in women with preeclampsia. To explore whether this could be due, at least in part, to increased production by the gestational tissues, we have measured activin A in the serum of women with (n=23) or without preeclampsia (n=62) at 29–40 weeks of gestation and in placenta and fetal membranes from preterm preeclamptic (PT-PE, n=8), term preeclamptic (T-PE, n=10) and healthy term controls (T-C, n=10). We have also explored if there are associated changes in activin receptor Alk2, ActRII and ActRIIB in these tissues. The relative amounts of receptor proteins were measured by densitometry on Western blots and receptors and activin β_A subunit localised by immunohistochemistry in PT-PE, T-PE and T-C gestational tissues (n=8–10/group).

Maternal serum activin A levels were significantly elevated in women with preeclampsia (multiples of the normal median (MoM)=3·5, P<0·0001, Mann–Whitney U test) compared with healthy women (median MoM=1·0). Compared with control tissues, the activin A content was significantly higher in preeclamptic placentae (P=0·001 and P=0·0005 for PT-PE and T-PE respectively, Mann–Whitney U test), but significantly lower in the amnion (P=0·005 and P=0·014 for PT-PE and T-PE respectively) and choriodecidua (P=0·009 for T-PE). The maternal serum activin A level in women with preeclampsia was significantly correlated with elevated placental production (P=0·01, Pearson's correlation). Receptor Alk2 protein levels were significantly elevated in T-PE placentae (P=0·0006, Mann–Whitney U test), ActRIIB levels were significantly lower in PT-PE placentae (P=0·01) and ActRII levels were significantly lower in PT-PE choriodecidua (P=0·0002) compared with controls. There were no apparent differences in the distribution of the β_A subunit and receptors Alk2, ActRII and ActRIIB between control and preeclamptic tissues.

These findings suggest that elevated levels of activin A in the maternal circulation in association with preeclampsia are due, at least in part, to increased placental production, and that the regulation of activin synthesis in placenta and fetal membranes is differentially regulated. Further, the differences in activin receptor protein levels between preeclamptic and control placenta and choriodecidua suggest that activin A-induced regulation may be altered in preeclampsia.

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Introduction

Activins are dimeric glycoproteins belonging to the transforming growth factor β family that exert pleiotrophic effects on a wide variety of tissues (Massague 1990, Ying *et al.* 1997). Of the three activins – A, B and AB – that have been identified in human tissues, activin A is the predominant form in pregnancy (Qu & Thomas 1995, Fowler *et al.* 1998). The placenta and fetal membranes are the main sources of circulating activin A (Rabinovici *et al.* 1992, Petraglia *et al.* 1997, Keelan *et al.* 1999). In early and mid pregnancy, maternal serum activin A levels are stable and low, rising dramatically from approximately 24 weeks of gestation, reaching peak levels proximate to term (Petraglia *et al.* 1993*a*, Fowler *et al.* 1998, Schneider-Kolsky *et al.* 2000). In preeclampsia, maternal serum activin A levels are significantly higher than those observed in gestation-matched normal pregnancies (Muttukrishna *et al.* 1997, 2000*a*, D'Antona *et al.* 2000). However, whether this is due to increased production in the placenta, fetal membranes and/or decreased clearance remains unexplored.

Activin A signal transduction is dependent upon the formation of a ligand receptor complex involving both a type II and a type I activin receptor (Attisano *et al.* 1996). mRNA for the activin receptors Alk2, ActRII and ActRIIB has been identified in the human placenta (Peng *et al.* 1999) and for ActRII and ActRIIB in the fetal membranes (Petraglia *et al.* 1997) but receptor protein localisation in these tissues has not been reported. It is also unknown whether the distribution and levels of receptor mRNA or protein are altered in gestational tissues in preeclampsia.

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We undertook this study to determine if there were differences in activin A production in gestational tissue in association with preeclampsia and whether tissue content was related to maternal serum activin A levels. We also studied the distribution and relative levels of activin receptor Alk2, ActRII and ActRIIB proteins in gestational tissue from healthy and preeclamptic women to explore potential differences in local regulation by activin A.

Materials and Methods

Clinical details

The control group consisted of healthy women with a normal, uncomplicated singleton pregnancy delivered at term (37–40 weeks of gestation) by elective Caesarean section for either breech presentation or previous Caesarean section. The women with preeclampsia were also delivered prior to labour by Caesarean section, preterm (29–35 weeks of gestation) or at term. Preeclampsia was diagnosed according to International Society for the Study of Hypertension in Pregnancy criteria (Perry & Beevers 1994). There was no obvious intrauterine fetal growth restriction in any of the preeclamptic pregnancies, as defined by birth weight <10th percentile for gestation. In all cases, the gestational age was calculated either by the last certain menstrual period or by an early pregnancy ultrasound. The study was approved by the Monash Medical Centre Human Research and Ethics Committee and informed, written consent was obtained from each patient.

Activin A content in maternal serum and tissue lysates

Blood samples were collected from the antecubital vein at 29–40 weeks of gestation (control n=62, preeclampsia n=23), centrifuged immediately at 3000 g for 15 min at 4 °C and the serum stored at −20 °C. Placenta and reflected fetal membranes were collected within 30 min of delivery from term control (T-C, n=10), preterm preeclamptic (PT-PE, n=8) and term preeclamptic women (T-PE, n=10). The amnion was manually stripped from the choriodecidua, all tissues rinsed four to five times in sterile phosphate-buffered saline (PBS) solution, frozen in liquid nitrogen and stored at −80 °C. Frozen tissue was homogenised in cold sterile PBS with 0·05% Tween 20, centrifuged for 5 min at 13 000 r.p.m. and supernatants stored at −80 °C. Total activin A in maternal serum and tissue lysates were measured by a specific two site enzyme-linked immunosorbent assay (Knight *et al.* 1996), with minor modifications as described previously (Riley *et al.* 1998). Serum and placental tissue lysates were diluted 1:10 and lysates of the fetal membrane diluted 1:2 in assay diluent. The sensitivity of the assay was 72 pg/ml and the mean intra- and interassay coefficients of variation were 8% and 15% respectively.

Protein assay

The total protein concentration in tissue lysates was measured by the bicinchoninic acid method (Pierce, Rockford, IL, USA) calibrated against bovine serum albumin (BSA) standards ranging between 25 and 2000 µg/ml.

Activin receptor protein levels

Fifteen micrograms of solubilised tissue protein (T-C, PT-PE and T-PE, n=8–10 per group) were separated on 10% polyacrylamide gels by SDS-PAGE and transferred onto nitrocellulose membranes. Non-specific binding was blocked with 1% gelatine and 0·02% Tween 20 in Tris-buffered saline and blots were incubated with polyclonal goat anti-human activin receptor Alk2, ActRII and ActRIIB antibodies (0·3 µg/ml; R & D Systems, Minneapolis, MN, USA) for 1 h at room temperature. Immunoreactive protein was detected by applying horseradish peroxidase-conjugated rabbit anti-goat IgG (H+L; 1:2000; Zymed, San Francisco, CA, USA) for 1 h at room temperature followed by 4-chloro-1-napthol. Secretory phase endometrium served as a positive control. The relative amount of immunoreactive protein was measured by densitometry (BioRad, Hercules, CA, USA) performed on the Western blot. Transfer efficiencies were checked by Coomassie blue staining of transferred gels and immunoreactive protein levels were standardised against the 81 kDa BSA band of the prestained molecular weight marker (BioRad).

β_A subunit and activin receptor localisation

Placenta and fetal membranes (T-C, PT-PE and T-PE, n=8/group) were fixed in neutral buffered formalin for 12–16 h and embedded in paraffin. The endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in methanol for 15 min and non-specific binding blocked for 15 min with protein blocking agent (Dako, Carpinteria, CA, USA). Sections were incubated with primary antibodies (2 µg/ml monoclonal mouse anti-human activin β_A subunit from N P Groome, Oxford Brookes University, Oxford, Oxon, UK and 1·5 µg/ml activin receptor Alk2, ActRII and ActRIIB from R & D Systems) for 1 h at room temperature followed by incubation with biotinylated secondary antibodies (LSAB$^+$; Dako) for 15 min at room temperature. Immunostaining was detected by the addition of streptavidin–horseradish peroxidase conjugate and 3-amino 9-ethlcarbazole chromogen (Zymed). Sections were counterstained in haemotoxylin and mounted. For endothelial cell staining, serial sections of placenta were incubated with monoclonal mouse anti-human CD34 antibody (1:50; Serotec, Oxford, Oxon, UK) for 45 min at 37 °C followed by alkaline phosphatase-conjugated secondary antibodies

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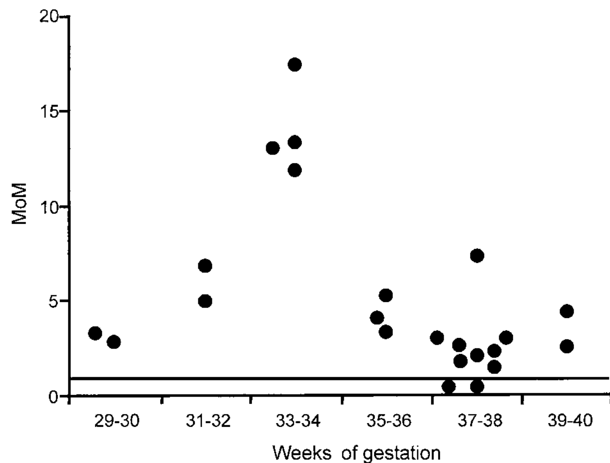

Figure 1 Total activin A levels in maternal serum of women with preeclampsia expressed as MoM. The horizontal line represents the median value of healthy pregnant women.

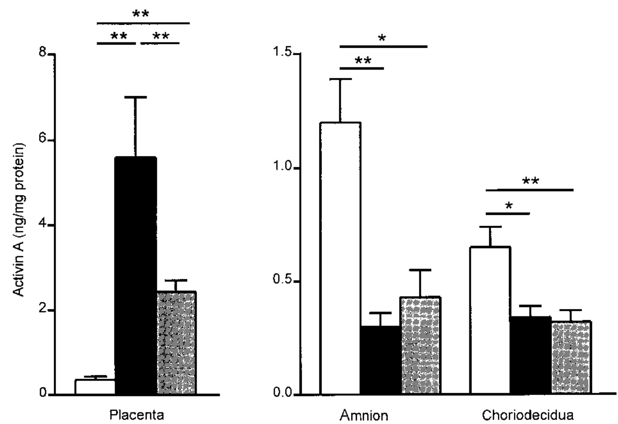

Figure 2 Mean ± S.E.M. total activin A concentrations in gestational tissue from healthy T-C (open bars), PT-PE (solid bars) and T-PE (shaded bars) women. *P<0·05, **P<0·01, Mann–Whitney U test, n=8–10/group.

(LSAB$^+$; Dako) for 15 min. Staining was visualised with Vector Blue (Vector Laboratories, Burlinghame, CA, USA). Negative and positive controls consisted of sections incubated with preimmune sera and secretory phase human endometrial biopsies respectively.

Statistical analyses

To correct for gestational-related changes, maternal serum activin A levels are expressed as multiples of the normal median (MoM) derived from the normal healthy controls. Differences in serum and tissue activin A content and receptor protein levels were assessed by the Mann–Whitney U test. Correlations between serum and tissue activin A content were assessed by Pearson's linear correlation coefficient (n=18). Significance was accorded when P<0·05.

Results

Maternal serum activin A levels in women with preeclampsia are shown in Fig. 1. Overall, activin A levels were significantly elevated in women with preeclampsia compared with healthy pregnant women. The median (95% confidence interval) activin A MoM values were 3·5 (2·6–5·1) vs 1·0 (0·9–1·1) for the preeclamptic women and control groups respectively (P<0·0001, Mann–Whitney U test).

Activin A was present in all placental, amnion and choriodecidual tissue lysates (Fig. 2). Placental total activin A content was significantly higher in PT-PE and T-PE tissue (P=0·001 and 0·0005 respectively, Mann–Whitney U test) compared with T-C placentae. Levels in PT-PE placentae were significantly higher compared with T-PE placentae (P=0·002). In the amnion, however, the activin A content was significantly lower in both preeclamptic groups compared with T-C (PT-PE, P=0·005; T-PE, P=0·014) with no significant difference between PT-PE and T-PE amnion. The choriodecidual activin A concentration was also significantly lower in T-PE tissue (P=0·009) compared with healthy women with no significant difference between the PT-PE and T-PE groups.

In 18 preeclamptic pregnancies where paired maternal serum and tissue samples were analysed (PT-PE, n=8; T-PE, n=10), a significant positive correlation was found between serum and placental activin A content (r=0·84 by Pearson's linear correlation, P=0·01).

The activin β_A subunit and receptor Alk2, ActRII and ActRIIB proteins were localised by immunohistochemistry in all sections of placenta and fetal membranes analysed. There were no apparent differences in the distribution of these proteins between healthy and preeclamptic tissue delivered at term or preterm (hence data for only the T-PE group are shown). The syncytiotrophoblast, cytotrophoblast and stromal cells stained positively for the β_A subunit in placentae with intense staining seen in the tissue of preeclamptic women compared with healthy controls (Fig. 3A and B). In the fetal membranes, the amniotic epithelial, fibroblast, reticular, chorionic trophoblast and adherent decidual cells showed positive staining for the β_A subunit (Fig. 3C and D). In positive control sections, the endometrial glandular epithelial cells stained intensely for the β_A subunit whereas staining was absent in the negative control sections (Fig. 3E and F). Activin receptor Alk2, ActRII and ActRIIB localised to similar cell types in the placenta and fetal membranes. Staining of low intensity was found in the cytotrophoblast cells of the placenta and sporadic staining was present in the syncytiotrophoblast layer (Fig. 3G and H). Intense staining for receptor Alk2, ActRII and ActRIIB was seen in the lining of fetal blood

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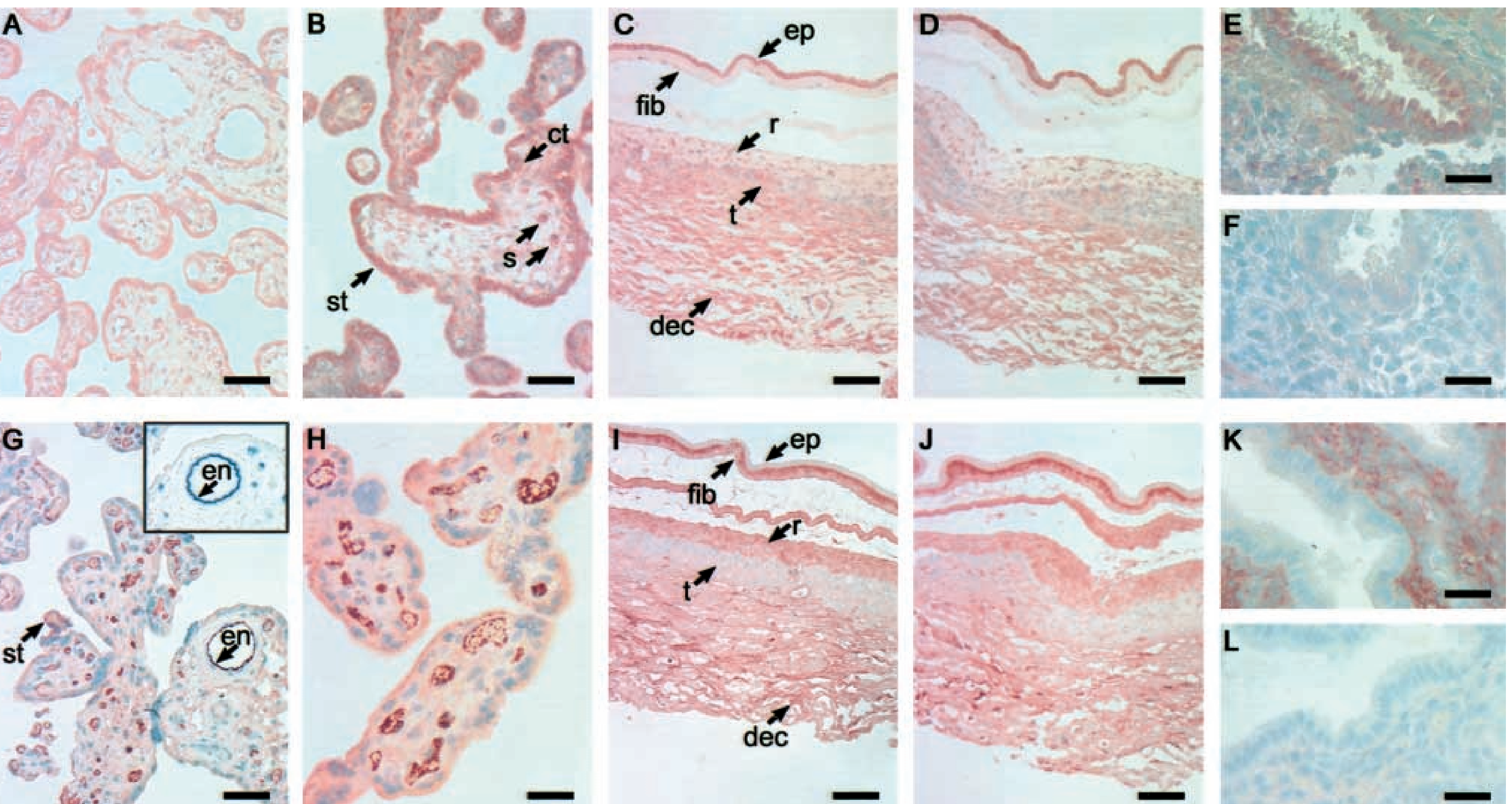

Figure 3 Localisation of activin β_A subunit and receptor Alk2. Panels A and B show activin β_A staining in healthy term and preeclamptic term placentae respectively. Panel B shows intense staining in syncytiotrophoblast (st) of preeclamptic placenta. Less intense staining was seen in cytotrophoblast (ct), and stroma (s) of healthy and preeclamptic placentae. Fetal membranes from healthy and preeclamptic pregnancies are shown in panels C and D respectively. β_A staining is present in amniotic epithelium (ep), fibroblast layer (fib), reticular layer (r), chorionic trophoblast (t) and adherent decidua (dec). Receptor Alk2 staining in healthy and preeclamptic placentae is seen mainly in the endothelium (en) of the placental vasculature (panels G and H respectively). The insert in panel G is a serial section with the vasculature stained with the endothelial marker CD34. Alk2 staining in healthy and preeclamptic membranes (panels I and J respectively) is similar to β_A except that the amniotic epithelium and chorionic trophoblast were mostly negative. Positive and negative control sections (endometrium) for β_A are shown in panels E and F and panels K and L for Alk2. Scale bars=50 µm except panels E, F, K and L where scale bars=25 µm.

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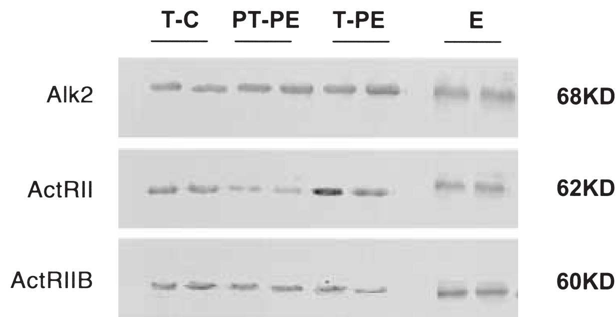

Figure 4 Western hybridisation; representative blots with placental, choriodecidual and amnion tissue protein (upper, mid and lower panels respectively) hybridised with activin receptor Alk2, ActRII and ActRIIB antibodies. Each blot contains proteins from T-C, PT-PE, T-PE and endometrium (E, positive control).

vessels of placental villi, and in serial placental sections these regions stained positively for the endothelial cell marker CD34 (Fig. 3G insert). In the fetal membranes, receptor Alk2, ActRII and ActRIIB proteins were detected in the fibroblast, reticular, chorionic trophoblast and adherent decidual cells and a few cells in the amniotic epithelium (Fig. 3I and J). The stroma of the positive control (endometrium) showed strong staining for activin receptors Alk2, ActRII and ActRIIB whereas staining was absent in the negative control sections (Fig. 3K and L).

Activin receptor proteins Alk2, ActRII and ActRIIB with estimated sizes of 68, 62 and 60 kDa respectively were detected in all healthy and preeclamptic gestational and endometrial tissues following Western hybridisation. Figure 4 shows representative blots probed with receptor Alk2, ActRII and ActRIIB antibodies. The relative amounts of activin receptor Alk2, ActRII and ActRIIB proteins in the placentae, amnion and choriodecidua from T-C, PT-PE and T-PE groups were compared by densitometry on Western blots except levels of receptor ActRII in amnion, where levels were too low for analysis (Fig. 5). Receptor Alk2 levels were significantly elevated in T-PE placenta (P=0·0006, Mann–Whitney U test) whereas levels of receptor ActRIIB were significantly lower in PT-PE placenta (P=0·01) compared with controls. There were no significant differences in receptor Alk2 or ActRIIB levels in PT-PE, T-PE and healthy term amnion tissues. In the choriodecidua, receptor ActRII levels were significantly lower in PT-PE compared with normal (P=0·0002).

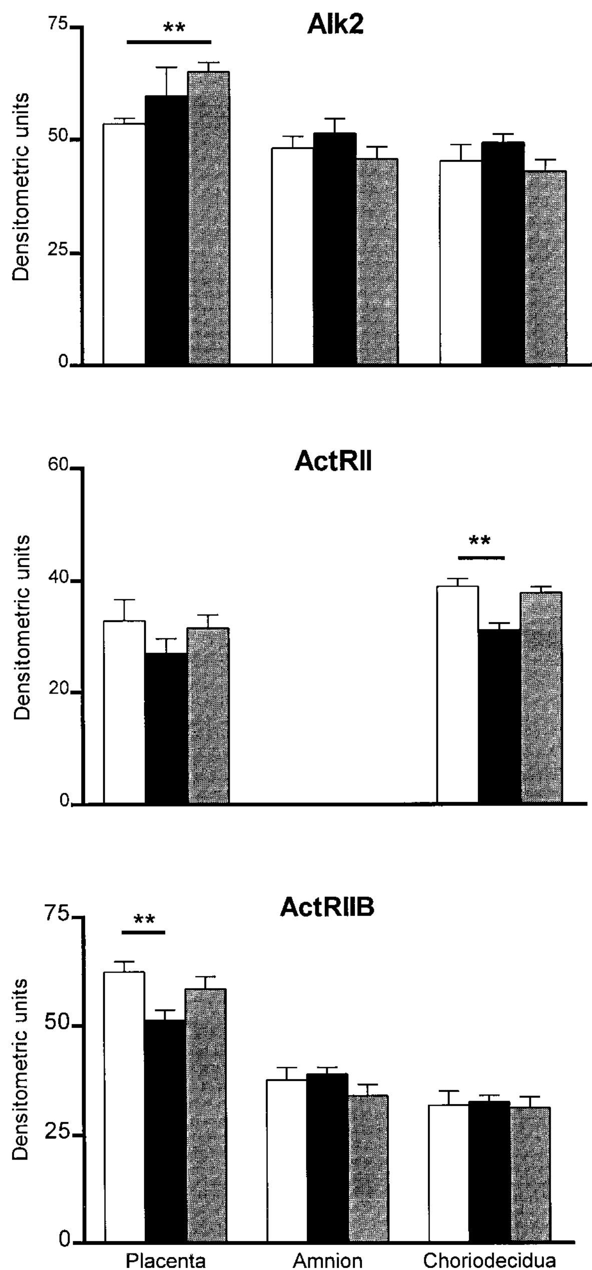

Figure 5 Semi-quantitation of activin receptor proteins; the relative receptor content in T-C (open bars), PT-PE (solid bars) and T-PE (shaded bars) tissue, expressed as the mean ± S.E.M. of densitometric analyses of Western blots is shown. $^{**}P \leq 0{\cdot}01$ by Mann–Whitney U test, n=8–10 per group.

Discussion

Several recent studies have shown that maternal serum activin A levels are significantly elevated in women with established preeclampsia (Muttukrishna *et al.* 1997, 2000*a*, Silver *et al.* 1999, D'Antona *et al.* 2000). It is also accepted that the placenta is the major source of circulating activin in normal pregnancy (Wallace & Healy 1996). In this study, we have shown that placental activin A production is increased in preeclampsia and that placental activin A content is highly correlated with maternal serum levels. Taken together, these data suggest that the elevated activin

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A levels observed in preeclampsia arise from the increased placental production.

However, preeclampsia is characterised by impaired renal function and we cannot exclude reduced renal clearance of activin A contributing to the increased circulating levels. On the other hand, the observation that maternal serum activin A levels are increased in early pregnancy, as early as 15 weeks of gestation, in women who are destined to develop preeclampsia (Muttukrishna *et al.* 2000*a*) and long before the onset of hypertension or renal involvement argues against impaired renal clearance as a major cause of the increased levels. Preeclampsia is also characterised by a systemic and intense inflammatory response (Redman *et al.* 1999) and it is therefore possible that activin A production by monocytes (Yu & Dolter 1997) may contribute to elevated circulating levels. Indeed, it has recently been shown that peripheral blood monocyte production of activin A is increased in pre-eclampsia (Muttukrishna *et al.* 2000*b*). However, we believe that it remains most likely that the majority of the increase in circulating activin A in preeclampsia is of placental origin, a belief supported by the correlation of serum activin A levels with placental activin A content.

The regulation of placental activin secretion and therefore the mechanisms underlying the increased production in preeclampsia remain poorly understood. The pro-inflammatory cytokines tumour necrosis factor-α (TNFα) and interleukin 1β (IL-1β) increase activin A synthesis in placental explant cultures derived from healthy pregnancies (Keelan *et al.* 1998). The observation that the preeclamptic placenta contains higher amounts of both cytokines (Conrad & Benyo 1997) therefore offers a possible mechanism. However, physiological concentrations of activin A inhibit TNFα production in term placental explants (Keelan *et al.* 2000), suggesting that other factors may be involved. An alternative mechanism for elevated placental activin A synthesis could be hypoxaemia resulting from reduced intervillous blood flow in preeclampsia (Conrad & Benyo 1997). Recent studies in the sheep have shown that acute foetoplacental hypoxia increases activin A levels and that levels fall rapidly with the return to normoxia (Jenkin *et al.* 2001). Whether this effect of hypoxia on activin A is direct or via other agents known to be hypoxia responsive such as TNFα, IL-1β (Benyo *et al.* 1997) or hypoxia-inducible transcription factors remains unclear and worthy of further investigation.

Intriguingly, while placental production of activin A in preeclampsia is elevated, the content in the amnion and choriodecidua was significantly lower than normal, suggesting differential regulation in these tissues. Interestingly, based on studies of inhibins and activins in maternal serum and amniotic fluid from normal and Down's syndrome pregnancies, it has been suggested previously that placental and chorion trophoblast production of the β_A subunit may be differentially regulated (Wallace *et al.* 1997, 1999), a suggestion supported by the findings here. Nonetheless, these findings remain surprising because amniotic fluid TNFα levels are elevated in preeclampsia (Wang & Walsh 1996) and TNFα increases activin A production from both normal amnion and choriodecidua *in vitro* (Keelan *et al.* 1998). Such studies of the fetal membranes derived from preeclamptic pregnancies would be worthwhile to explore whether differences in response to TNFα *in vitro* exist in comparison with normal tissue.

This study is the first to report the localisation of type I and type II activin receptor proteins in the placenta and fetal membranes and to explore if activin regulation in gestational tissue is altered in women with preeclampsia. Activin binds to ActRII or ActRIIB and the ligand receptor complex interacts with type I receptors Alk4 and possibly Alk2 leading to signal transduction (Attisano *et al.* 1996). *In situ* studies have localised activin receptor ActRII and ActRIIB mRNA to the syncytium and amniotic epithelium (Petraglia *et al.* 1994), consistent with the proposed roles for activin in both the regulation of placental secretion of various hormones, such as gonadotrophin hormone-releasing hormone, progesterone, human chorionic gonadotrophin (Petraglia *et al.* 1989, Song *et al.* 1996) and oestradiol (Ni *et al.* 2000), and in the onset of parturition, as suggested by inducing prostaglandin release by the fetal membranes (Petraglia *et al.* 1993*b*). However, we found only weak, sporadic receptor ActRIIB (also receptor Alk2 and ActRII) staining by immunohistochemistry in the trophoblast and amniotic epithelium which does not fully support a role for activin A in placental hormonogenesis or parturition *in vivo* in either healthy or preeclamptic women. This is consistent with the stable activin levels observed in the last weeks of pregnancy and during labour (Schneider-Kolsky *et al.* 2000). Of course, we have not studied the distribution of activin receptors in earlier pregnancy and an important role for activin in hormonogenesis cannot be excluded.

Most interestingly, we have found that all three activin receptor proteins are present in the endothelium of the placental vasculature. Type ActRII and ActRIIB mRNA have been shown to be expressed in adrenal capillary, pulmonary artery, aortic and human umbilical vein endothelial cells (HUVEC; McCarthy & Bicknell 1993). Activin A inhibits HUVEC proliferation (McCarthy & Bicknell 1993); however, activin mRNA expression is up-regulated in HUVECs in response to an atherogenic stimulus (de Waard *et al.* 1999) and activin A stabilises atherogenic plaques (Engelse *et al.* 1999). In contrast, follistatin, a specific activin-binding protein, stimulates HUVEC proliferation and angiogenesis (Kozian *et al.* 1997). However, follistatin levels in the maternal circulation are not significantly different in preeclamptic women (D'Antona *et al.* 2000). These observations are relevant here because preeclampsia is associated with systemic endothelial dysfunction (Redman *et al.* 1999), and it is possible that increased activin represents a

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response to endothelial injury, affording repair and stabilisation of a damaged endothelium. That activin is elevated in very early pregnancy prior to overt preeclampsia (Muttukrishna *et al.* 2000*a*) is still consistent with this hypothesis. In women who subsequently develop preeclampsia, there is some evidence of a hyperdynamic circulation in early pregnancy (Easterling & Bernadetti 1989) and it is possible that this induces early pregnancy endothelial dysfunction.

In conclusion, we have found that placental activin A production is elevated in women with preeclampsia and that it may largely explain the elevation in the maternal circulation. Activin A and its receptor proteins are present in a number of cell types in the placenta and fetal membranes with no apparent differences in their distribution between preeclamptic and healthy tissue. However, receptor levels are altered in women with preeclampsia, suggesting that the paracrine and autocrine regulation could be altered.

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