JCI The Journal of Clinical Investigation

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J Clin Invest. 1998;101(2):442-454. https://doi.org/10.1172/JCI119881.

Research Article

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Cellular Localization of AT_1 Receptor mRNA and Protein in Normal Placenta and Its Reduced Expression in Intrauterine Growth Restriction

Angiotensin II Stimulates the Release of Vasorelaxants

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Abstract

Angiotensin II (ANG II) is a potent vasoconstrictor and growth promoter. Quantitative receptor autoradiography using the nonselective radioligand [^{125}I]ANG II and subtype-selective competing compounds demonstrated the presence of both ANG II receptor $(AT)_1$ and AT_2 receptor recognition sites. In addition, a relatively small population of apparently non-AT_1/non-AT_2 sites was identified that may represent a novel high affinity ANG II recognition site in human placenta. Using placental membrane preparations, the AT_2 receptor antagonist PD123177 failed to compete for [^{3}H]ANG II binding at relevant concentrations, whereas the AT_1 receptor antagonist losartan competed in a monophasic manner for all the specific binding, suggesting that the non-AT_1/non-AT_2 recognition site identified using autoradiography may be a cytosolic binding site. AT_1 receptor binding was significantly reduced ($P < 0.02$) in intraeuterine growth restriction (IUGR) pregnancies. Western blot analysis confirmed this showing a reduction in AT_1 receptor protein. In situ hybridization and immunocytochemistry revealed that AT_1 receptor mRNA and protein were localized throughout pregnancy in the cytotrophoblast, syncytiotrophoblast, and extravillous trophoblast, as well as in or around the blood vessels of placental villi. The intensity of the hybridization signal for AT_1 receptor mRNA over the syncytium was reduced in IUGR. ANG II evoked a rapid and concentration-dependent release of NO in first trimester cytotrophoblast-like cells that was abolished by the inclusion of the competitive NOS inhibitor N^G-monomethyl-L-arginine. Neither losartan nor PD123177 alone significantly inhibited ANG II–evoked NO release, and when cells were stimulated with ANG II in the presence of losartan (10 μM) and PD123177 (10 μM) in combination, NO release was significantly inhibited ($P < 0.05$). These observations also suggest, for the first time, the existence of a cross-talk between AT_1 or AT_2 receptors in trophoblast and that the reduction in placental AT_1 receptors in IUGR may, in part, account for poor placental function in this disorder. (*J. Clin. Invest.* 1998. 101:442–454.)

Key words: angiotensin II • AT_1 receptor • nitric oxide • PTHrP • pregnancy • trophoblast • in situ hybridization • immunocytochemistry

Introduction

Successful placentation requires an orchestrated development of a low impedance utero-placental circulation and an adequate feto-placental circulation to meet the increasing demand of the growing fetus for oxygen and nutrients. Any disturbance in the balance between these circulations will alter intraplacental exchange. As the feto-placental vasculature lacks autonomic innervation (1), the control of this vascular bed must involve humoral mechanisms. The placenta is capable of producing both the potent vasoconstrictor angiotensin II (ANG II)[1] and the potent vasodilator and inhibitor of platelet aggregation nitric oxide (NO); the balance between these could control feto-placental hemodynamics, ensuring adequate placental blood flow, fetal nutrition, and oxygenation. Vascular insufficiency arising from abnormal placentation, as is evident by the reduction in umbilical blood flow, hypovascularity, and reduced placental villous development, may affect the fetus (2). This can lead to intrauterine growth restriction (IUGR), in which the risk of fetal death is an order of magnitude higher than in appropriately grown fetuses.

Angiotensinogen, as well as renin and ANG-converting enzyme, has been found in high concentrations in human placenta (3), supporting the concept of a local renin–ANG system. ANG I is converted to the biologically active octapeptide ANG II by ANG-converting enzyme (ACE) in the feto-pla-

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This work was presented in part at the Society for Gynecologic Investigation Annual Meeting, Chicago, IL, March 1995.

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Received for publication 30 December 1996 and accepted in revised form 20 November 1997.

J. Clin. Invest.

0021-9738/98/01/0442/13 $2.00
Volume 101, Number 2, January 1998, 442–454
http://www.jci.org

1. *Abbreviations used in this paper:* ACE, angiotensin-converting enzyme; ANG II, angiotensin II; AT, ANG II receptor; FGF-2, basic fibroblast growth factor 4; IRMA, immunoradiometric assay; IUGR, intrauterine growth restriction; NO, nitric oxide; NOS, nitric oxide synthase; PTHrP, PTH-related protein; SSC, standard saline citrate; TBS-T, Tris-buffered saline–Tween; VEGF, vascular endothelial growth factor.

cental bed (4) and has potent vasoconstrictor activity in the feto-placental circulation (5). In the ovine fetus, infusion of ANG II markedly reduces feto-placental perfusion and causes a redistribution of blood flow to the heart and brain at the expense of renal, hepatic, and gut perfusion (6). Experimentally induced hypoxemia stimulates the release of renin and subsequent generation of ANG II in the ovine fetus (7) and intrauterine growth restriction (IUGR) is associated with increased plasma renin activity (8) and fetal hypoxemia (9).

ANG II interacts with specific, high-affinity receptors on the surface of target cells to exert its biological effects. The genes encoding the AT_1 (10) and AT_2 (11) receptors have been cloned and the proteins share only a 32% overall amino acid sequence homology, although as is common, the transmembrane regions show a somewhat higher degree of homology. The occurrence and proportion of the AT_1 and AT_2 receptor subtypes vary significantly among different tissues and organs of the same species (12) and also in the same tissue and organs of different species (13). Experiments with the AT_1 receptor-selective antagonist losartan (previously designated DuP753) indicate that this subtype is responsible for the vast majority of the hemodynamic and cardiovascular effects of ANG II (14). The biological function of AT_2 receptor is not clear, although AT_2 receptor is abundantly expressed in fetal tissues (15), suggesting that the AT_2 receptor is associated with differentiation and development. In recent mouse studies, targeted disruption of the AT_2 receptor gene resulted in a significant increase in blood pressure and increased sensitivity to the pressor action of ANG II indicating that ANG II acts via the two receptors with opposing effects and that there may be functional interaction between the two receptor subtypes (16, 17). Indeed, Gelband et al. (18) have suggested the existence of a cross-talk between AT_1 and AT_2 receptors in catecholaminergic neurons.

The feto-placental unit is a source of vasorelaxants such as NO and parathyroid hormone-related protein (PTHrP), both potent vasorelaxants. Formation of NO is achieved by the conversion of L-arginine to L-citrulline by the enzyme NO synthase (NOS) in the presence of molecular oxygen, NADPH, and cofactors (for review see reference 19). Human placenta selectively expresses the endothelial NOS isoform that is present in human placental vasculature (20) and syncytiotrophoblast (21, 22). NO is released from trophoblast cells in culture (23), and exogenous NO relaxes umbilical (24) and chorionic plate (25) artery preparation in vitro. NO attenuates the actions of vasoconstrictors in the feto-placental circulation and inhibition, either of NO synthesis or its action on vascular smooth muscle, increases basal perfusion pressure in the isolated human placental cotyledon (26, 27, 28). Like NO, bioactive PTHrP was detected in human placenta (29, 30), and PTHrP was shown to be localized in the brush border of the syncytiotrophoblast and fetal vessels of placental villi (31). PTHrP causes a concentration-dependent vasodilator effect in the dually perfused placental cotyledons that have been preconstricted with a thromboxane mimetic U46619 (32).

This study demonstrates for the first time the cellular localization of receptor protein and mRNA encoding the AT_1 receptor in human placenta and that AT_1 receptors are reduced in severe IUGR placentae. In addition, it shows that ANG II can stimulate placental NO and PTHrP release, and it identifies an apparently non-AT_1/non-AT_2 high affinity ANG II recognition site in placental sections using receptor autoradiography.

Methods

Source of tissue

Human placental tissues were obtained from uncomplicated term pregnancies delivered by elective cesarean section for breech presentation or a recurring indication in otherwise uncomplicated pregnancies. Placental tissue was also obtained from pregnancies complicated by severe asymmetrical IUGR. Growth-restricted fetuses were prospectively identified using ultrasound biometry and diagnosed by the absence of umbilical artery end diastolic blood flow. Ethical committee approval was obtained from the South Birmingham Ethical Committee. After dissection, the tissue was wrapped in parafilm to prevent dehydration, rapidly frozen over dry ice, and then stored at −80°C until they were either sectioned for in situ hybridization or autoradiography or homogenized. Placental tissue was also collected in 10% formalin, paraffin embedded, and then sectioned for immunohistochemical studies.

Preparation of tissue sections

Frozen placental tissue was surrounded in embedding medium (OCT compound; Miles Inc., Kankakee, IL) before 20-μm sections were cut using a cryostat (−17°C) and thaw mounted onto gelatin-coated glass slides for receptor autoradiographic studies, and 10-μm sections were cut and thaw-mounted onto poly-L-lysine–coated (Sigma Chemical Co., Poole, UK) glass slides for in situ hybridization studies. Sections were stored (> 2 wk) at −80°C until used.

[^{125}I]ANG II autoradiography

Receptor autoradiography studies were performed as previously described (12). Slide-mounted placental sections (three/slide) were incubated in incubation buffer that contained 0.1 nM [^{125}I]-[5-L-isoleucine]ANG II ([^{125}I]ANG II, 2,000 Ci/mmol; Amersham International, Little Chalfont, UK) in the absence (total binding) or presence of competing compound (either 1.0 μM unlabeled ANG II, 1.0 μM DuP753, and 1.0 μM PD123177 or 1.0 μM DuP753 plus 1.0 μM PD123177) for 60 min at 25°C. Immediately after incubation, the tissue sections were washed in ice-cold incubation buffer and dipped in ice-cold distilled water to remove buffer salts. The sections were rapidly air dried and exposed to [^{3}H]-Hyperfilm (Amersham International) in x-ray cassettes together with [^{125}I]standards (Amersham International) for 10 d. Autoradiographic films were developed in Kodak LX 24 developer and fixed in Kodak Unifix.

Quantitative image analysis

After the development of autoradiographic film, the silver grain density was assessed using image analysis (MCID, Imaging Research Inc., St. Catherines, Ontario, Canada) from the three sections on each slide and a mean value generated that represented $n = 1$. The film was quantified by reference to [^{125}I]standards (fmol/mg tissue equivalent values for intact gray matter; Amersham International). An unpaired Student t test or ANOVA test followed by Dunnett's test, where appropriate, was applied to compare the changes in the mean values of silver grain density between the two groups.

[^{3}H]ANG II homogenate binding

Placental tissues were homogenized (Polytron setting 7, 10 s) in ice-cold incubation buffer and centrifuged (48,000 *g*, 4°C, 10 min). The pellet was gently resuspended in incubation buffer and recentrifuged (48,000 *g*, 4°C, 10 min). The binding homogenate was formed by gentle resuspension of the pellet in incubation buffer. The preparation of the binding homogenate was performed immediately before assay. For [^{3}H]ANG II binding, test tubes in duplicate contained 150 μl competing compound (or vehicle, incubation buffer) and 100 μl [^{3}H]ANG II (final concentration 80–120 pM). 250 μl homogenate was added to initiate binding which was allowed to proceed for 60 min at 25°C before termination by rapid filtration through prewet (0.1% vol/vol polyethyleneimine in incubation buffer) GF/B filters followed by

washing (4 × 4 s) with ice-cold incubation buffer. Radioactivity was assessed using a gamma counter.

Data analysis

Competition data were analyzed by computer-assisted iterative curve fitting according to the equation; $b = (B_{max}[L]^n)/ ([L]^n + [K]^n)$. Where, b = bound radioligand, B_{max} = maximum bound radioligand, K = IC_{50} of competing compound, L = concentration of competing compound and *n* = Hill number.

Western blot analysis

Protein was extracted from frozen placental tissue using high salt buffer (0.4 M KCl, 20 mM Hepes, pH 7.4, 1 mM DTT, 20% glycerol, 0.5 mg/ml bacitracin, 40 μg/ml PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin). Tissue homogenate was centrifuged at 12,000 rpm (3,000 *g*) at 4°C for 10 min. The supernatant was stored at −70°C until further use. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories Inc., Hercules, CA) using BSA as standard. The nonradioactive enhanced chemiluminescence Western blotting system (Amersham International) was used to detect AT_1 receptor in placental samples as previously described (33). In brief, 50 μg total protein was separated by electrophoresis on a polyacrylamide gel. The samples were loaded in a final volume of 25 μl with sample buffer containing 0.002% bromophenol blue and electrophoresed at 50V for ~2 h. A kaleidoscope protein marker (Bio-Rad Laboratories) was run alongside the samples. After electrophoresis the protein was transferred onto a nitrocellulose membrane in a cooling system (10°C) overnight at 36V. The filter was blocked to reduce nonspecific binding of the antibody using Tris-buffered saline Tween (TBS-T) containing 10% Marvel and 2.5% BSA for 4 h. After washing in TBS-T the membrane was incubated overnight at 4°C with the AT_1 receptor antibody at a dilution of 1:1,000. This high affinity rabbit polyclonal antibody was raised against a peptide corresponding to amino acids 306–359 of the human AT_1 receptor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA and Autogen Bioclear UK Ltd., Wiltshire, UK). The filter was washed and incubated with the secondary anti–rabbit antibody for 1.5 h at room temperature. After a final wash in TBS-T, the filter was incubated for 1 min at room temperature in detection reagent, immediately wrapped in saran wrap, and then exposed for periods of 30 s, 1 min, 5 min, and 10 min to an x-ray film.

In situ hybridization

A 45-base synthetic oligonucleotide probe complementary to the bases 1821–1866 of the rat AT_{1A} was synthesized and used to locate the mRNA that encode the receptor protein (34). This region of the AT_{1A} gene was chosen because it was found to be divergent from other homologous genes (10) and the probe was used previously to localize the AT_{1A} mRNA in human endometrium (12). The oligoprobe was 3′ end-labeled either with [^{35}S]dATP (for first trimester and term placentae) by terminal transferase using a commercially available kit (Du Pont, Stevenage, UK) or fluorescein-dUTP (for term placentae) by using terminal deoxynucleotidyl transferase using a oligo color kit (Amersham International) for nonradioactive in situ hybridization. The reaction protocol was optimized to produce a tail length with sufficient sensitivity without compromising the stringency of the specific probe sequence. In situ hybridization was performed as previously described using both radiolabeled (12) and fluorescein-dUTP labeled (33) probe. As a negative control, some sections were incubated in hybridization buffer containing labeled probe along with 100-fold excess of unlabeled probe. At the same time some sections that were pretreated with 100 μg/ml of RNAse A (Sigma Chemical Co.) for 1 h at 37°C were also incubated with labeled probe.

Immunocytochemical studies

Serial 3-μm sections of formalin-fixed, paraffin-embedded tissue from five normal term placentae were used for immunohistochemistry as previously described (12). Anti–human mouse monoclonal cytokeratin, CD31, and macrophage CD68 antibodies were all purchased from Dako Ltd. (Bucks, UK). Nonimmune goat serum (10% in PBS) was used as a diluent of the primary antibody (1:25) to reduce nonspecific staining. Amplification of the primary antibody reaction was achieved using a goat anti–rabbit secondary antibody (diluted 1:100 in PBS) for 30 min followed by a complex of streptavidin (DAKO Ltd.) and biotinylated peroxidase (DAKO Ltd.) for an additional 30 min. Finally, the binding was visualized by the addition of diaminobenzidine (Sigma Chemical Co.) and 0.01% hydrogen peroxide in PBS to form the insoluble antigen–antibody complex. Between each step the sections were washed in PBS for 5 min. All incubations of antiserum were performed at room temperature in a wet chamber mounted on a rocking tray that ensured movement of antiserum over the whole section. Sections were counterstained with Mayers hematoxylin, dehydrated, and mounted. To test the specificity of the immunohistochemical staining, the primary antibody was replaced with goat nonimmune serum in control experiments.

Preparation and stimulation of cells for NO release

Increasing legislative and moral controls have recently been imposed on the use of fetal tissues. A spontaneously transformed first trimester cytotrophoblast cell line was generated by repeated passaging of trophoblast obtained from chorionic villous sampling. This cell line provides a convenient model to investigate primary villous trophoblast (23). The cells were maintained in 75-cm^2 flasks in Ham F12/DME containing 15% (vol/vol) FCS, 1% L-glutamine, and 1% antibiotic mixture (10,000 IU penicillin and 10 μg streptomycin) at 37°C in 95% O_2 and 5% CO_2 at 95% humidity. Subconfluent monolayers were plated at a density of 300,000 cells/ml in 24-well culture plates and grown in complete culture media for 24–36 h. Before agonist stimulation, growth medium was removed and near confluent monolayers serum starved for 24 h. Stimulations were initiated by the addition of ANG II (Sigma Chemical Co.) in DME without FCS or ANG II in the presence of either the ANG II subtype-selective receptor antagonists or the nitric oxide synthesis inhibitor, N^G-monomethyl-L-arginine (L-NNA; Sigma Chemical Co.), or vehicle.

Measurement of NO release by chemiluminescence

For concentration dependence and time course experiments, stimulations were initiated, for the times indicated, in a final volume of 0.5 ml at 37°C. For experiments with antagonists, cells were pretreated for 30 min and then stimulated with ANG II in a final volume of 0.5 ml at 37°C for an additional 30 min. Reactions were terminated by removal of the supernatant which was subsequently stored at −80°C for NO analysis. Levels of NO were measured in the gas phase using a Sievers NOA 270B chemiluminescence analyzer (Sievers NOA, Boulder, CO) as previously described (23). The sensitivity of the NO assay was 1 pM. NO_3^- was not reduced in this system and was undetectable.

Tissue explant studies

Three to four small pieces of amnion or placenta (total weight of each ~ 50 mg) were placed on a sterile inset support and incubated for 2 h at 37°C in 2 ml culture medium consisting of DME supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), and streptomycin (50 U/ml). The media was replaced after 2 h and the explants were incubated with increasing concentrations of ANG II for a further 20 h at 37°C in a humidified 5% CO_2 incubator. The medium was removed and centrifuged at 1,000 *g* for 5 min to remove cells and the supernatant was stored at −80°C until assay. Protein concentrations of extracts were determined by a protein kit (Bio-Rad).

Assay for PTHrP

PTHrP was measured by immunoassay in conditioned media reconstituted in assay diluent (PBS, pH 7.4) containing 0.25% (wt/vol) Polypep (Sigma Chemical Co.), 0.01% (wt/vol) sodium azide, and 0.1% (vol/vol) Triton X-100. $PTHrP_{1–86}$ was measured by a two-site immunoradiometric assay (IRMA) with antibodies specific for resi-

dues 17–27 and 52–61 of PTHrP as previously described (35). The detection limits of the IRMA was 0.23 pM. Unpaired Student *t* test was applied to compare the changes in the mean values of PTHrP in response to increasing concentrations of ANG II.

Drugs

Losartan DuP753 (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole) and PD123177 (1-(4-amino-3-methylphenyl)-methyl-5-diphenyl-acetyl-4, 5, 6, 7-tetrahydro-1H-imidazo [4, 5-C] pyridine-6-carboxylic acid) were from DuPont Merck Pharmaceuticals (Wilmington, DE). ANG II and saralasin were purchased from Sigma Chemical Co.; [^{125}I]ANG II (2200 Ci/mmol) and [^{3}H]ANG II were purchased from Amersham International (Amersham International).

Results

[^{125}I]ANG II autoradiography

[^{125}I]ANG II labeled a saturable binding site in 20-μm sections of human placenta (Fig. 1). Specific binding (defined by the inclusion of unlabeled ANG II, 1.0 μM) was associated mainly with the AT_1 receptor since it was sensitive to the inclusion of losartan (Fig. 1). The specific losartan-sensitive binding represented mean (±SD) 80±9% of the total binding. In contrast, the AT_2 receptor–specific ligand competed for only 6±6% of the total binding (Fig. 2 *A*). In addition to losartan- and PD123177-sensitive specific [^{125}I]ANG II, an additional high affinity binding site was consistently detected that was insensitive to both losartan (1 μM) and PD 123177 (1 μM) and represented ~ 15±10% of the total binding.

Western blot and binding studies of AT_1 receptors in IUGR

AT_1 receptor binding was significantly reduced ($P < 0.02$) in IUGR pregnancies with reduced or absent end-diastolic flow velocity (Fig. 2 *A*). Western blot analysis confirmed the binding studies and showed quantitatively a marked reduction in AT_1 receptor protein in IUGR compared with normal placentae (Fig. 2 *B*). The anti-AT_1 receptor antibody recognized a major band of 60 kD and a minor band of ~ 45 kD. The antiserum was able to detect specific bands whose molecular size was consistent with the predicted molecular mass of the glycosylated AT_1 receptor based on the amino acid sequence.

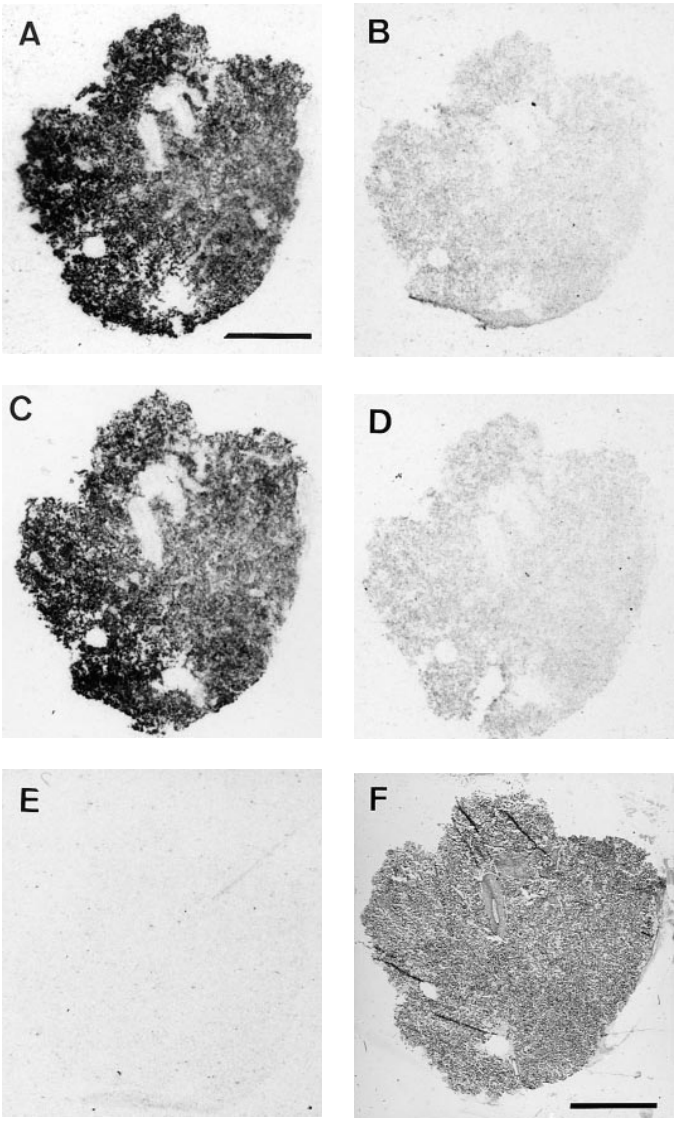

Figure 1. Autoradiograms of the distribution of [^{125}I]ANG II binding to adjacent sections (20 μm) of the human term placentae. Dark regions indicate high densities of labeled receptors. (*A*) Total binding; (*B*) binding in the presence of the AT_1 receptor-selective ligand DuP753 (1.0 μM); (*C*) binding in the presence of the AT_2 receptor-selective ligand PD123177 (1.0 μM); (*D*) binding in the presence of DuP753 (1.0 μM) plus PD123177 (1.0 μM); (*E*) binding in the presence of unlabeled ANG II (1.0 μM); (*F*) histological section of term placenta. The difference in density between *A* and *B* represents AT_1 receptor specific binding; difference in density between *A* and *C* represents AT_2 receptor specific binding; difference between *A* and *D* represents specific AT_1 plus AT_2 receptor density and the difference between *D* and *E* represents the non-AT_1/non-AT_2 novel ANG II recognition site. Bar, 30 mm.

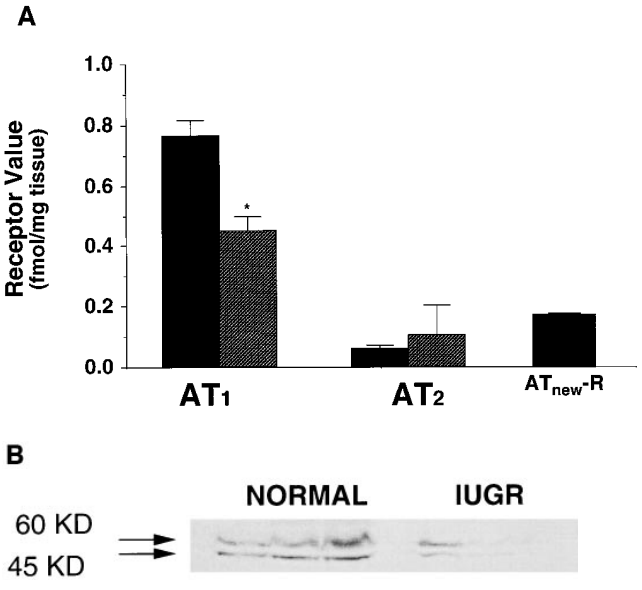

Figure 2. (*A*) The levels of specific [^{125}I]ANG II binding sites in human normal and IUGR placentae. Data represents the mean (±SEM) of AT_1-, AT_2-, and non-AT_1/non-AT_2–specific binding in specimens from normal (*filled column*; $n = 12$) and IUGR (*hatched column*; $^*P < 0.05$, $n = 7$) placentae. (*B*) Enhanced chemiluminescence Western immunoblotting of AT_1 receptor in normal and IUGR placental protein lysates. Equal amount of total protein for each sample was loaded from normal ($n = 3$) and IUGR ($n = 3$) placentae. The human AT_1 receptor antibody recognized a major band of 60 kD and a minor band corresponding to ~ 45 kD. Both these bands were markedly reduced in IUGR.

[^{3}H]ANG II homogenate binding to placental membranes

ANG II at nanomolar concentrations, competed in a monophasic manner for [^{3}H]ANG II binding to placental membranes (IC_{50} 4.8±0.64 nM; Fig. 3 *A*). Specific binding (defined by the inclusion of ANG II, 1.0 μM) represented ~ 95–100% of the total binding. Losartan also competed for [^{3}H]ANG II binding to placental membranes (IC_{50} 92.8±15.4 nM; Fig. 3 *A*) while PD123177 at concentrations up to 10 μM failed to compete for [^{3}H]ANG II binding (Fig. 3 *A*).

In the presence of 1.0 μM PD123177, there was no reduction in the total specific binding. Both ANG II and losartan competed for [^{3}H]ANG II binding in a monophasic manner (Fig. 3 *B*) completely displacing [^{3}H]ANG II binding at 1 and 10 μM, respectively. Hill coefficients tended towards unity (Hill coefficient ANG II 0.773±0.11; losartan 0.89±0.03) while IC_{50} values were 1.94±0.1 and 60.5±12.6 nM for ANG II and losartan, respectively.

Localization of mRNA encoding AT_1 receptor

In situ hybridization was performed in order to identify the site of expression of AT_1 receptor mRNA in first trimester ($n = 7$) and term placentae ($n = 10$). In first trimester (12 wk gestation) AT_1 receptor mRNA hybridization signal was localized in the cytotrophoblast (Fig. 4, *bold arrows*) and syncytiotrophoblast of the villi (Fig. 4, *A* and *B*). When tissue sections were incubated with ^{35}S-labeled probe along with 100-fold excess of unlabeled probe in the hybridization buffer, the hybridization signal was substantially lower under these conditions compared with hybridization produced by the labeled probe alone (Fig. 4, *C* and *D*).

At term, AT_1 receptor mRNA localization was performed using both radiolabeled and nonradioactive probes as the latter method ensured that the villous structures were more clearly distinguishable. AT_1 receptor mRNA was localized in the syncytiotrophoblast of the terminal villi (Fig. 5, *A* and *B*) but the endothelium of the villous capillary expressed no detectable AT_1 receptor mRNA hybridization signal (Fig. 5 *B*). The highest level of AT_1 mRNA was localized in the brush border of the syncytiotrophoblast (the specificity of the signal was negligible in RNAse pretreated sections; Fig. 5 *C*). Similarly, using radiolabeled probes, AT_1 receptor mRNA hybridization signal was detected in the syncytium of term placentae (Fig. 6, *A* and *B*). Weak AT_1 receptor mRNA expression was noted in the amnion, whereas the chorio-decidua showed strong hybridization signal for AT_1 receptor mRNA (Fig. 6, *G* and *H*). The specificity of the signal was further confirmed by incubating labeled probe along with 100-fold excess of unlabeled probe in the hybridization buffer which demonstrated that the hybridization signal was markedly lower under these

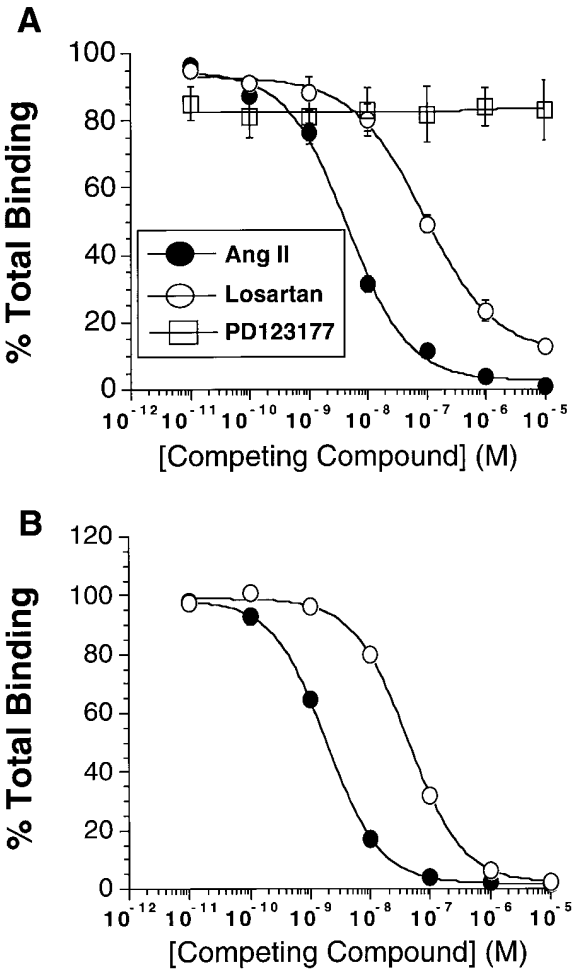

Figure 3. Ability of peptide and nonpeptide AT receptor ligands alone (*A*) and in the presence of 1 μM PD123177 (*B*) to compete for the binding of [^{3}H]ANG II to membranes prepared from the human placenta. Data fitted to a one-site model. The values represent the mean±SEM of three separate experiments.

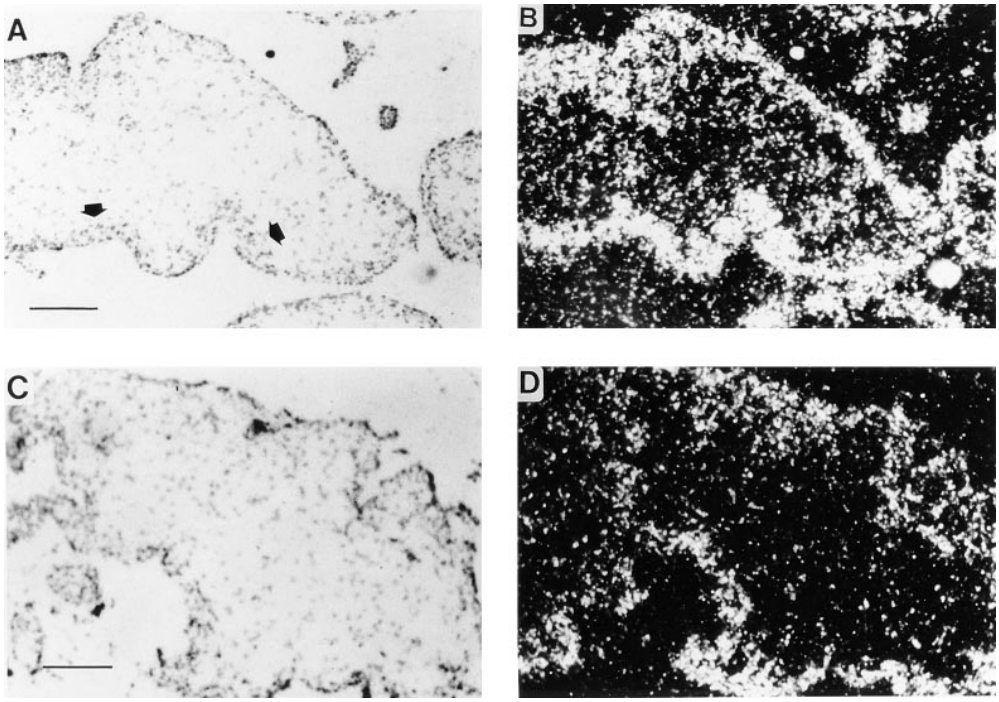

Figure 4. Localization of AT_1 receptor mRNA in human early gestational placental villi. Photomicrograph shows in bright field (*A*) intense signal in the cytotrophoblast (*bold arrows*) and syncytiotrophoblast of the villi in first trimester (12 wk gestation) placental section hybridized with ^{35}S-labeled oligonucleotide directed against AT_1 receptor. The signal is seen as silver grains in the same field visualized with dark field optics (*B*). The arrowhead indicates the trophoblasts lining the villi. When tissue sections were incubated with ^{35}S-labeled probe along with 100-fold excess of unlabeled probe in the hybridization buffer, the hybridization signal was substantially lower under these conditions (*C* and *D*) compared to hybridization produced by the labeled probe alone. Bar, 50 mm.

conditions compared with the signal produced by the labeled probe alone (Figs. 5 *D* and 6, *C* and *D*). In addition, pretreating with 100 μg/ml of RNAse A markedly reduced the hybridization signal (Fig. 6, *E* and *F*) confirming specificity of the AT_1 receptor probe.

The intensity of the hybridization signal for AT_1 receptor over the syncytium was reduced in IUGR (Fig. 7, *C* and *D*, $n = 6$) compared with normal (Fig. 7, *A* and *B*, $n = 10$) placentae. In the media of the stem vessels, AT_1 receptor mRNA signal was apparently increased in the IUGR cases (Fig. 7, *G* and *H*) as compared with normal placental vessels (Fig. 7, *E* and *F*).

Localization of AT_1 receptor protein

Intense immunostaining for AT_1 receptor was observed in the cytotrophoblast and syncytiotrophoblast of first trimester (12 wk gestation; Fig. 8, *A* and *B*) and in the CD68-positive Hofbauer (H) cells (Fig. 8 *B*) while weak AT_1 receptor immunoprotein was detected in the CD31-positive endothelial cells of

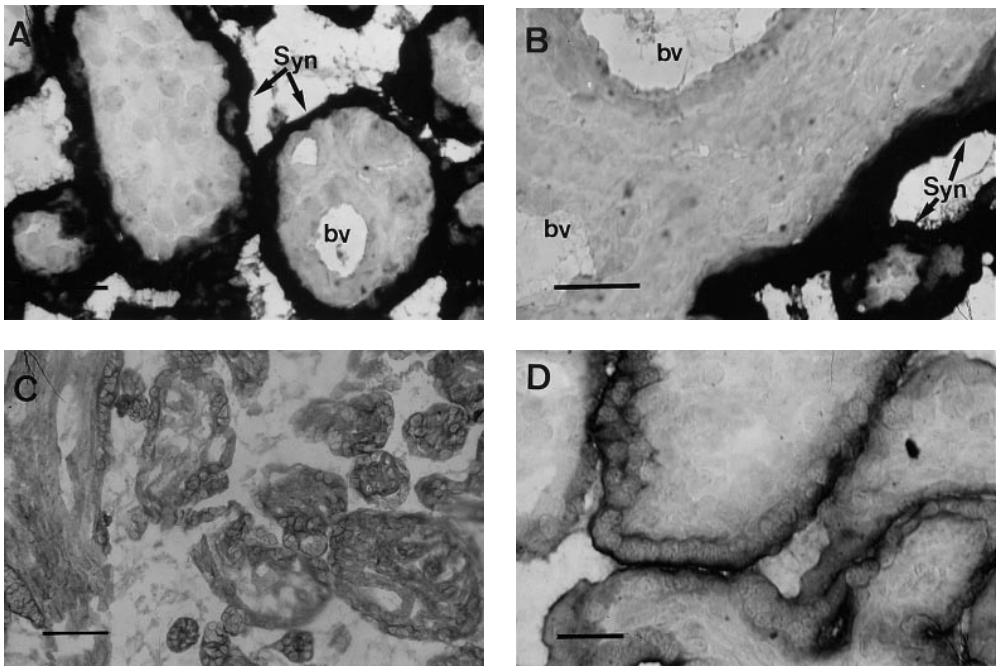

Figure 5. Localization of AT_1 receptor mRNA in human term placenta. *A* shows intense signal in the syncytiotrophoblast (*Syn*) of placental section hybridized with fluorescein-dUTP–labeled oligonucleotide directed against AT_1 receptor; *B* shows lack of expression of AT_1 mRNA in or around the fetal blood vessels (*bv*); *C* indicates RNAse-treated serial section showing no hybridization signals around blood vessels (*bv*); and *D* shows reduced hybridization signal after excess cold labeling. Bar, 50 mm.

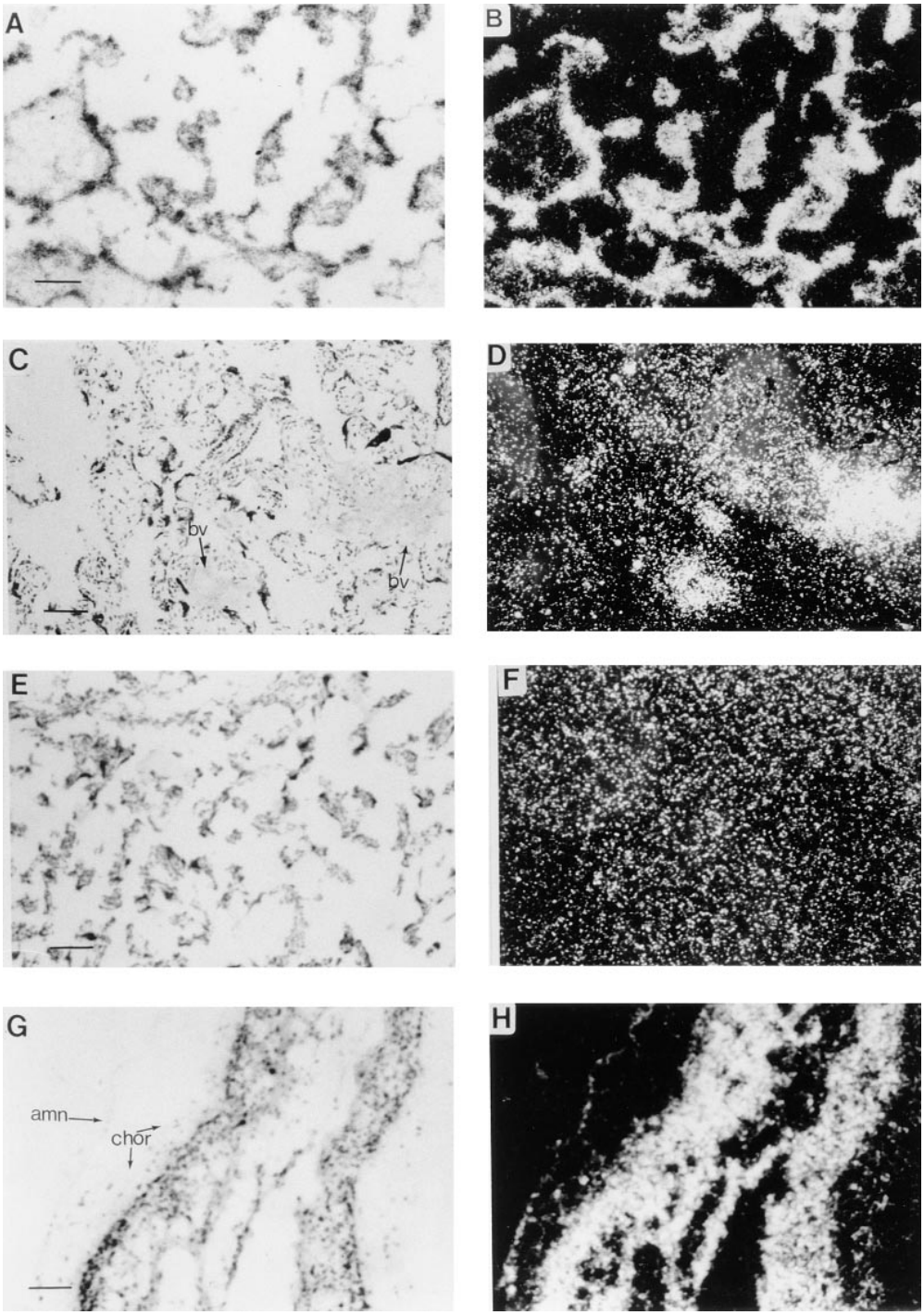

Figure 6. Localization of AT_1 receptor mRNA in term placental villi and fetal membranes. Photomicrograph shows in bright field (*A*) intense signal in the syncytium hybridized with ^{35}S-labeled oligonucleotide directed against AT_1 receptor. The signal is seen as silver grains in the same field visualized with dark field optics (*B*). When tissue sections were incubated with ^{35}S-labeled probe along with 100-fold excess of unlabeled probe in the hybridization buffer, the hybridization signal was substantially lower and negligible around the blood vessels (*bv*) under these conditions (*C* and *D*). *E* and *F* indicate RNAse-treated serial section showing weak hybridization signals. *G* and *H* show localization of AT_1 receptor mRNA in fetoplacental membranes. Weak signal was seen in the amnion (*amn*) and strong hybridization was detected in the choriodecidua (*chor*). Bar, 50 mm.

the villous capillary (Fig. 8, *A* and *B*). The decidual (Fig. 8 *C*) and cytokeratin-positive extravillous cells (Fig. 8 *D*) showed strong staining for AT_1 receptors. At term, AT_1 receptor immunoprotein was localized to the syncytium, endothelial cells of the villous capillary network, and to smooth muscle of placental blood vessels higher up the vascular tree (Fig. 8 *E*). The control section in which the primary anti-AT_{1A} receptor antibody was preabsorbed overnight with the synthetic peptide fragment (1:100) showed negligible staining (Fig. 8 *F*).

Effect of ANG II on nitric oxide production

Consistent with activation of a constitutive isoform of NOS, NO formation increased rapidly in response to 10 nM ANG II, exceeding basal release within 15 s, the earliest time point measured ($P < 0.01$, $n = 2$; Fig. 9 *A*) and reached a maximum within 2 min of stimulation ($P < 0.01$, $n = 2$). Thereafter, total NO levels plateaued but remained elevated. In contrast, addition of 10 ng/ml vascular endothelial growth factor (VEGF), a tyrosine kinase activator, caused a less rapid generation of NO

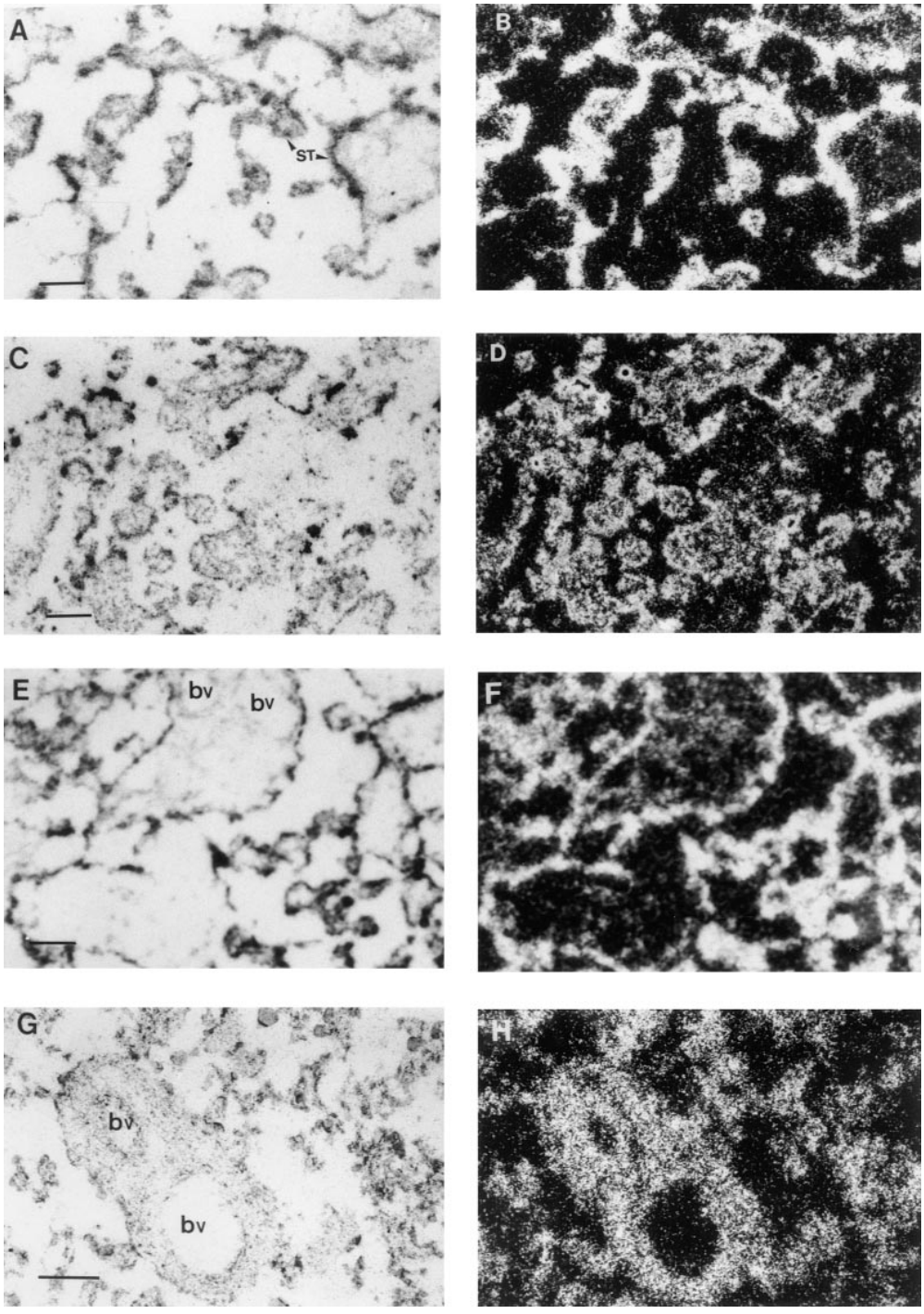

Figure 7. Localization of AT_1 receptor mRNA in normal and IUGR placentae. *A* and *B* show intense signal in the syncytium (*ST*, *bold arrows*) of normal placental section hybridized with ^{35}S-labeled oligonucleotide directed against AT_1 receptor. *C* and *D* show reduced expression of AT_1 mRNA in the syncytium of IUGR placentae. *E* and *F* show hybridization signal around fetal blood vessels (*bv*) in normal placental and *G* and *H* show increased signals around blood vessels in IUGR. Bar, 50 mm.

release and reached a maximum at 10 min of stimulation that was sustained over a 30-min time period (Fig. 9 *A*).

The concentration-dependency of ANG II–stimulated NO release from trophoblast cells over a 30-min period is shown in Fig. 9 *B*. The increase in total NO evoked by ANG II within the concentration range tested (0.1 nM–10 μM) was almost abolished by the inclusion of 0.1 mM of the competitive NOS inhibitor L-NNA at the time of stimulation (Fig. 9 *B*). Stimulation of the trophoblast cell line with 10 nM ANG II in the presence of external calcium evoked a significant increase in total NO with values typically 63.0±3.2% above basal ($P < 0.001$, $n = 3$). In contrast, when stimulations were conducted in the presence of low calcium buffer (150 nM), ANG II failed to stimulate a significant release of NO, indicating dependency on extracellular calcium ($P < 0.001$, $n = 3$).

To identify which AT receptor subtype was responsible for the ANG II–mediated NO release, the trophoblast cells were stimulated with either ANG II alone or in the presence of either the AT_1-specific receptor antagonist losartan (DuP753) or the AT_2-specific receptor antagonist PD123177 (alone and in

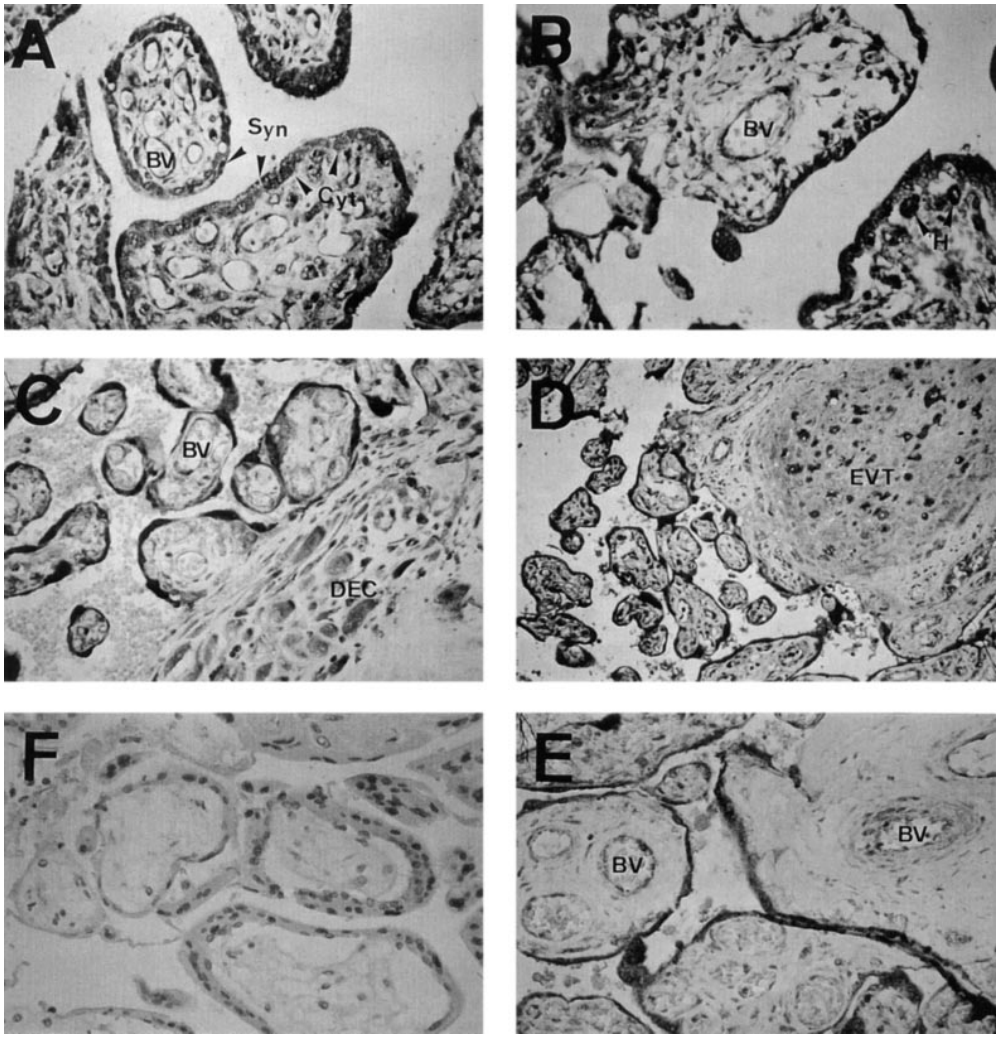

Figure 8. Immunolocalization of AT_1 receptor throughout pregnancy. *A* shows first trimester villi (12 wk), and *B* shows placental villi at 24 wk stained with anti-AT_{1A} receptor antibody raised against the synthetic peptide corresponding to amino acids 15–24 of the rat AT_{1A} receptors . Strong staining is seen in the cytotrophoblast shell (*A, Cyt arrowhead*) and weaker staining in syncytiotrophoblast layer (*A, Syn arrowhead*). *B* shows staining in Hofbauer cells within the villous mesenchyme (*B*, *H arrowhead*). *C–E* show term placenta. In *C*, *D*, and *E,* positive immunostaining was detected in the bilayer of syncytiotrophoblast and cytotrophoblast, in the endothelium of fetal blood vessels (*BV*), the decidual cells (*DEC*), and the extravillous trophoblast (*EVT*) in the maternal bed. *F* is control in which the primary anti-AT_{1A} receptor antibody was preabsorbed overnight with the synthetic peptide fragment (1:100). (*A, B, C, E, F*) ×284; (*D*) ×142 .

combination). ANG II (100 nM)–stimulated NO release was not prevented by either losartan or PD123177 alone or when combined (Fig. 10). ANG II–mediated NO release was inhibited when the concentration of the two antagonists (losartan and PD123177) were applied in combination and their concentrations were raised to 10 μM ($P < 0.05$; Fig. 10). ANG II stimulated NO release at a range of ANG II concentrations (0.1–10 μM), whereas at the higher concentrations of ANG II, losartan or PD123177 still failed to antagonize the NO release (Fig. 11, *A* and *B*). Although at nonphysiological concentrations of ANG II (1–10 μM), the further elevation of NO release was prevented by combined application of losartan and PD123177 (Fig. 11 *C*).

Placental explant studies

In vitro explants of amnion chorion and placenta ($n = 3$) cultured for 20 h in condition medium produced mean (±SD) 0.76±0.09, 2.51±0.22, and 0.16±0.01 fmol $PTHrP_{1–86}$ per mg protein, respectively. After 20 h incubation at 37°C, ANG II, in a concentration-dependent manner, stimulated the release of $PTHrP_{1–86}$ ($n = 3$) in condition medium from amnion (Fig. 12) and placental explants (Fig. 12). There was a twofold increase in placental PTHrP production by ANG II (10 nM) and from chorion, ANG II induced $PTHrP_{1–86}$ release was increased to 3.63±0.77 at 100 nM (data not shown).

Discussion

This study shows the localization of AT_1 receptor mRNA and protein in cytotrophoblast and syncytiotrophoblast in placental villi during early and late pregnancy using in situ hybridization and immunocytochemistry, respectively. It also demonstrates that AT_1 receptor mRNA and protein is reduced in IUGR placenta and shows that ANG II (100 nM) can stimulate vasorelaxants such as NO and PTHrP. It confirms the earlier work of Kingdom et al. (36) who states that human term placenta contains predominantly AT_1 receptor binding sites with low levels of AT_2 receptor binding sites. Although specific receptor binding sites for ANG II were identified in placental vascular smooth muscle homogenates (37) and AT_1 receptor binding sites in membrane homogenates of vascular tissue from human term placenta (36), a novel aspect of this study is that it shows that human trophoblast contains AT_1 receptors and that an apparently additional high affinity non-AT_1/non-AT_2 ANG II recognition site is identified in human placenta that may be responsible for ANG II–mediated NO

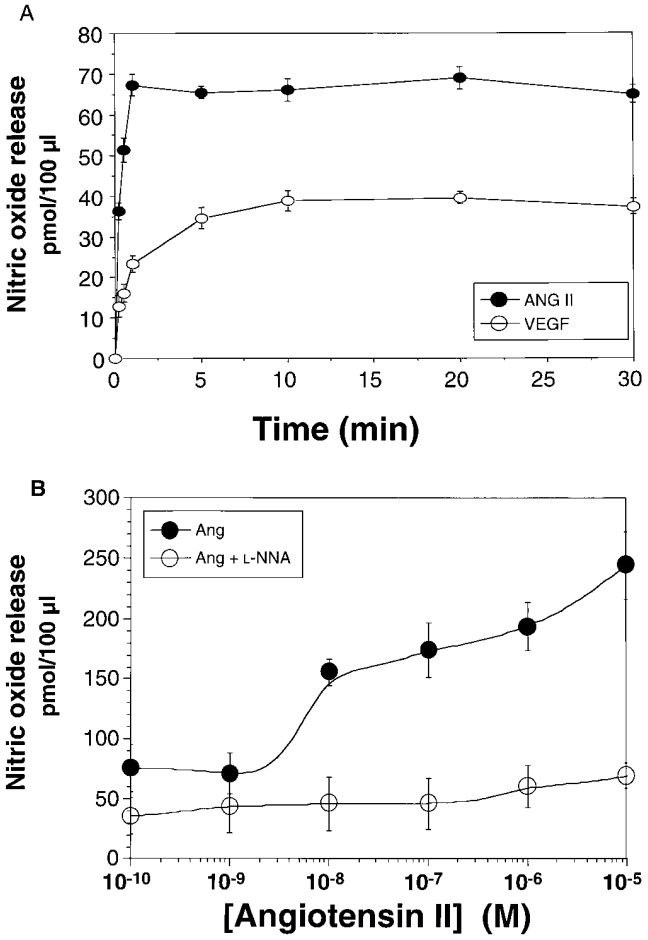

Figure 9. Kinetic and dose dependency of ANG II–evoked nitric oxide release. (*A*) Cells were incubated with 10 nM ANG II (*ANG II*, *filled circle*) or 10 ng/ml vascular endothelial growth factor (*VEGF*, *open circle*) for defined time points. (*B*) Cells were incubated with increasing concentration of ANG II for 30 min in the presence (*open circle*) or in the absence (*filled circle*) of 0.1 mM L-NNA and the supernatants were removed and assayed for NO using a Sievers NOA chemiluminescence analyzer. The appropriate controls (medium alone and medium plus cells) were subtracted from experimental results. Data represents mean±SEM of a single experiment, typical of three separate experiments.

and PTHrP. This study is the first to show that the high levels of AT_1 receptor mRNA and protein are localized in the cytotrophoblast and syncytiotrophoblast of first trimester placental villi, and that AT_1 receptor immunoprotein expression is present in endothelial cells of the villous capillary network and the smooth muscle of placental blood vessels higher up the vascular tree.

One of the important functions of the human placenta is to produce peptide hormones during pregnancy. The differentiated syncytiotrophoblast has endocrine properties including secretion of human chorionic gonadotrophin and human placental lactogen (38). The high levels of AT_1 receptor mRNA together with localization of its AT_1 receptor protein in syncytiotrophoblast, suggest that ANG II may be involved in secretory functions as well as vasoregulation. This view is supported by a recent report that announced that ANG II stimulates estradiol-17β secretion from placental explants via AT_1 receptor activation (39). Formation of estriol, the end product of estradiol-17β oxidation, reflects feto-placental function (40). The reduced levels of placental AT_1 receptors in severe IUGR compared with normal pregnancy may account for reduced estriol reported in this disorder. Abnormal Doppler waveforms in the umbilical artery suggest that increased downstream impedance is associated with depleted numbers of arteries and arterioles in the tertiary stem villi in IUGR (41). The reduced levels of AT_1 receptors is likely to be as a consequence of chronic activation of the placental renin–ANG system in IUGR, however, the possibility that it may simply be a reflection of reduced villous tree elaboration and poor villous morphology can not be excluded.

Syncytiotrophoblast surrounding the placental villi express the potent angiogenic factors, such as basic fibroblast growth factor (FGF-2) and VEGF (for review see reference 42). Administration of ANG II to chick embryo chorioallantois membrane induced pronounced neovascularization via an "unknown AT receptor" subtype (43) and ANG II stimulates expression of FGF-2 in bovine luteal cells (44). Using quantitative receptor autoradiography and competition with selective AT receptor antagonists, a high affinity ANG II recognition site was shown to be pharmacologically distinct from the currently known AT receptor subtypes since it was insensitive to AT_1 or AT_2 receptor-selective ligands at relevant concentrations. It is possible that the angiogenic properties of ANG II may relate to the production of these factors via the non-AT_1/non-AT_2 binding site. However, the presence of this non-AT_1/non-AT_2 binding site was not supported by the binding studies on placental membrane homogenates. There was a small difference in the percentage of the radiolabeled ANG II binding

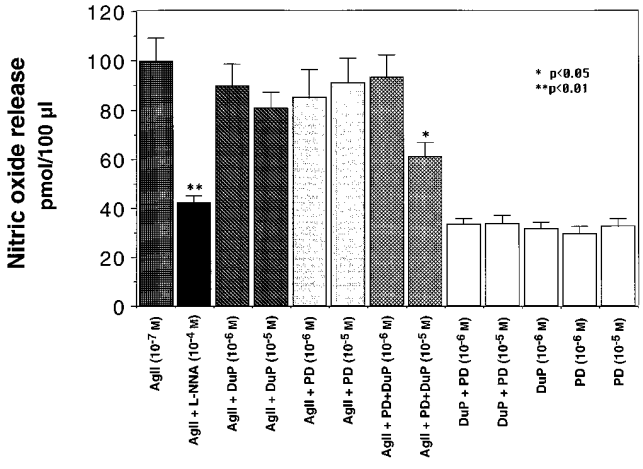

Figure 10. Effect of subtype-selective ANG receptor antagonists on ANG II–evoked nitric oxide release. Cells were incubated for 30 min with 100 nM ANG II alone (*square-hatched bars*), with ANG II plus 100 µM L-NNA (*black bar*), with 1 and 10 µM DuP753 (*striped bars*), with 1 and 10 µM PD123177 (*gray bars*), and with the two antagonists together at 1 µM (*cross-hatched bars*). Open columns show controls in the absence of ANG II. The supernatants were removed and assayed for nitric oxide release. The appropriate controls (medium alone and medium plus cells) were subtracted from experimental results. Data represents mean±SEM of three separate experiments. *$P < 0.01$ compared to ANG II–stimulated NO release.

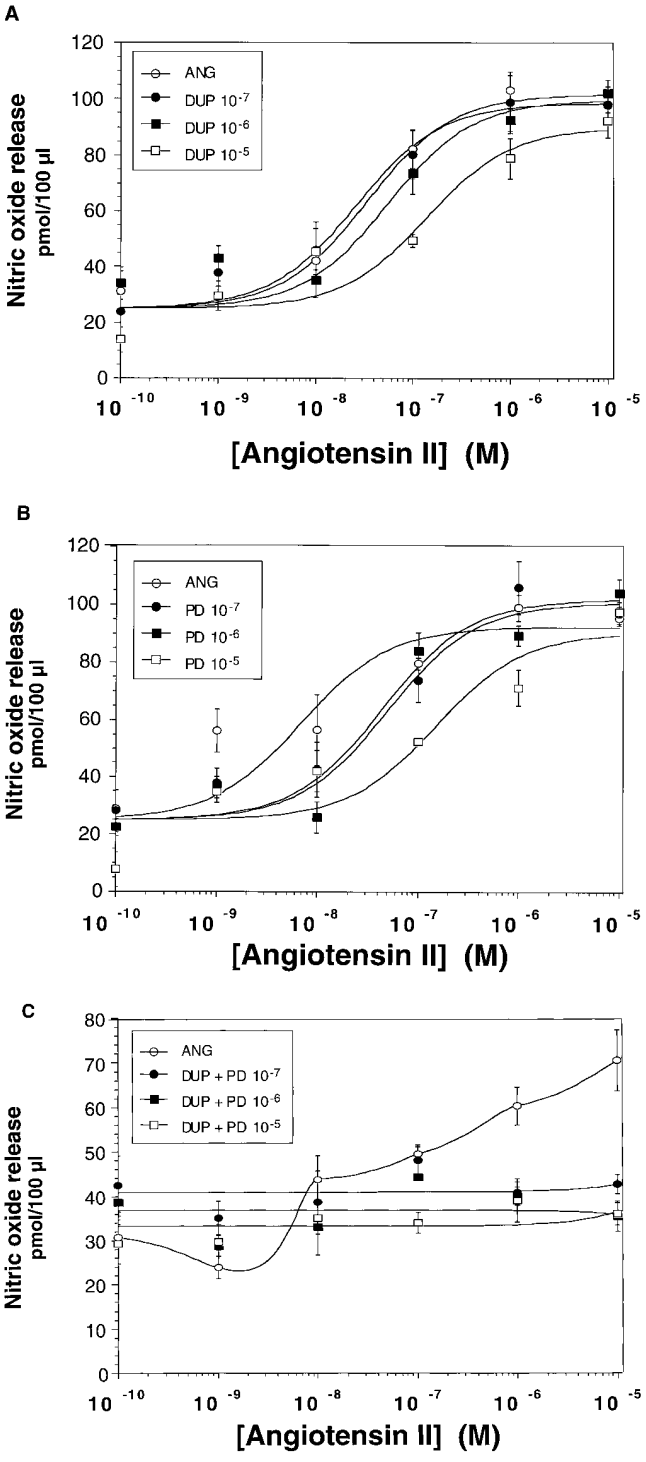

Figure 11. Competition curves using subtype-selective AT receptor antagonists on ANG II–evoked nitric oxide release. Cells were incubated for 30 min with increasing concentrations of ANG II alone (*AgII*, *open circles*) in all experiments. Nitric oxide release (*A*) in the presence of losartan alone at 100 nM (*filled circle*), 1 μM (*filled square*), and 10 μM (*open square*); (*B*) in the presence of PD123177 alone at 100 nM (*filled circle*), 1 μM (*filled square*), and 10 μM (*open square*); and (*C*) in the presence of losartan plus PD123177 at 100 nM (*filled circle*), 1 μM (*filled square*), and 10 μM (*open square*). The supernatants were removed and assayed for NO release. The appropriate controls (medium alone and medium plus cells) were subtracted from experimental results. Data represents mean±SEM of three separate experiments.

that was sensitive to the AT_2 receptor ligand PD123177 between the two preparations (i.e., section or cell membranes of placenta). This difference is more likely to arise from the different procedures used to demonstrate the sites. For instance, tissue sections are likely to include cytosolic binding sites whereas these will have been lost during the preparation of the cell membranes for the homogenate studies. Thus, it is possible that the non-AT_1/non-AT_2 binding site is within the cytosolic compartment of placental tissue. Receptor autoradiographic studies have identified similar high affinity ANG II recognition sites in human endometrium (12) and bovine placentae (Schauser, K., personal communication). Although neither losartan nor PD123177 alone were able to significantly inhibit ANG II–evoked NO release, when cells were stimulated with ANG II in the presence of 10 μM losartan and 10 μM PD123177 in combination, ANG II–evoked NO release was significantly inhibited. This suggests that ANG II could stimulate NO release via either AT_1 or AT_2 receptors as blockage of both receptors are required to suppress the ANG II–evoked NO response. Our work is supported by a recent demonstration of functional interaction between AT_1 and AT_2 receptors in regulating electrophysiological responses of catecholaminergic neurons (18). Both AT_1 and AT_2 receptors were able to cause increases and decreases in neuronal delayed rectified K+ current in response to ANG (100 nM; 18). It is also interesting to note that the AT_2 receptor was shown to mediate renal production of NO in conscious rats (45).

Studies on isolated perfused human placental cotyledon show that NO attenuates, and addition of NOS inhibitors potentiates, the actions of vasoconstrictors in the feto-plancal circulation indicating that NO has a role in maintaining placental blood flow (26, 27, 28). However, the addition of NO to the intervillous space, rather than to the feto-placental circulation it-

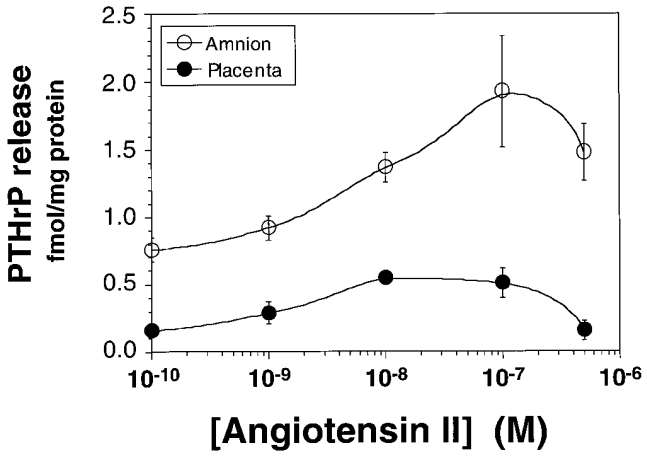

Figure 12. Concentration-dependent ANG II–evoked PTHrP release in amnion and placental explants. Amnion (*open circle*) and placental (*filled circle*) explants were incubated in serum-free medium for 2 h at 37°C in 95% air and 5% CO_2. The explants were then exposed to increasing concentration of ANG II for 20 h. The medium was removed and assayed for PTHrP in triplicate for each sample by a two-site IRMA with antibodies specific for residues 17–27 and 52–61 of PTHrP. The experiments were performed in triplicates for each dose and results are expressed as means (±SEM) of triplicate determination of one typical experiment.

self, has no effect on perfusion pressure (46), suggesting that trophoblast-derived NO may not have a significant influence on the tone of feto-placental blood flow. In severe IUGR, the placental mass is small relative to the fetus (41). Although high levels of NO are cytotoxic, lower levels of NO may inhibit growth of vascular smooth muscle (47) without toxic effects. As ANG II stimulates smooth muscle cell proliferation, its ability to stimulate trophoblast-NO may be to control the proliferation of these cells. ANG II stimulates mRNA expression of PTHrP, and PTHrP inhibits the stimulatory effects of ANG II on DNA synthesis in rat aortic smooth muscle cells (48). This suggests that PTHrP may also modulate the vasoactive and growth-promoting effects of ANG II. The release of PTHrP from placental explants, following stimulation with ANG II further supports our premise that one of the role of ANG II in the placenta may be to regulate placental growth by second messengers, such as NO and PTHrP.

During the first trimester of normal pregnancy there is a significant increase in plasma renin concentration (49), the enzyme that converts angiotensinogen to ANG I, the precursor of ANG II. Interference with the renin–ANG system through the use of ACE inhibitors appears to be associated with poor pregnancy outcome and IUGR (50). The findings presented in this study suggest that ANG II is likely to play a physiological role in pregnancy and that the reduction in placental AT_1 receptors in IUGR may, in part, account for poor placental function in this disorder. This suggests, for the first time, the existence of a cross-talk between AT_1 or AT_2 receptors in trophoblast and that ANG II–stimulated NO and PTHrP may modulate both growth and vascular tone in the placenta.

Acknowledgments

We are grateful to Dr. Wendy Ratcliff for the PTHrP assay. We would like to thank Dr. G.F. Steinfels (DuPont Merck Pharmaceuticals) for the gifts of losartan and PD123177, Mr. S. Horwitt (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for the gift of anti-AT_{1A} antibody and Dr. D.A. Kniss (Ohio State University) for the gift of trophoblast cells. We express our appreciation to Mrs. G. Powell for her technical assistance with the placental explant studies and to the staff at the Birmingham Women's Hospital for their assistance in the collection of tissues.

This work was supported by grants from the British Heart Foundation, Medical Research Council, and Wellcome Trust. M. Shams and X.F. Li were funded by Well Being project grant A2/93 and A2/94.

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