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The Marked Disparity between the Sizes of Angiotensin Type 2 Receptors from Different Tissues Is Related to Different Degrees of *N*-Glycosylation

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SUMMARY

We recently described the photoaffinity labeling and partial characterization of the angiotensin type 2 (AT_2) receptor from human myometrium. In the present study, specific receptors for angiotensin II (AII) were also analyzed in a murine fibroblast cell line (R3T3) and a rat pheochromocytoma cell line (PC-12). Dose-displacement experiments with PD 123319 (an AT_2-selective antagonist) completely inhibited ^{125}I-AII binding, whereas L-158,809 (an AT_1-selective antagonist) had no significant effect on ^{125}I-AII binding, thus revealing that these two cell lines express exclusively AT_2 sites. High yields of covalent and selective labeling of AT_2 receptors from human myometrium, R3T3 cells, and PC-12 cells were obtained with the photosensitive analogue ^{125}I-[Sar^1,Val^5,*p*-benzoyl-Phe^8]AII. Gel permeation chromatography of Triton X-100-solubilized AT_2 receptors from the three different sources revealed similar Stokes' radii of about 65 Å. Interestingly, upon electrophoresis under reducing conditions, marked disparities were observed between the apparent molecular masses of AT_2 receptors from the three different sources. As observed previously, AT_2 receptors from human myometrium showed a molecular mass of 68 ± 4.6 kDa. AT_2 receptors from PC-12 cells showed a larger molecular mass of 113 ± 12 kDa, whereas AT_2 receptors from R3T3 cells showed a molecular mass of 91 ± 7.8 kDa. After endoglycosidase digestion with an enzyme that cleaves *N*-linked saccharides, the molecular masses of the denatured AT_2 receptors of human myometrium, R3T3 cells, and PC-12 cells were decreased by 54%, 66%, and 73%, to 31.3 ± 1.6 kDa, 30.9 ± 0.7 kDa, and 30.6 ± 0.8 kDa, respectively. Kinetic studies with AT_2 receptors from human myometrium revealed a complex, multiple-step process of deglycosylation involving at least three different sites of *N*-linked saccharides. These results suggest that the disparity in the sizes of AT_2 receptors from different sources is mostly related to different degrees of *N*-glycosylation. They also imply that the AT_2 receptor contains at least three asparagine-linked sites of glycosylation.

AII is the active component of the renin-angiotensin system and regulates a wide variety of physiological phenomena, including vascular contraction, aldosterone secretion, catecholamine release, glycogenolysis, and renal filtration (1, 2). AII has recently been shown to promote myoproliferation in heart and vascular tissue (3). The recent development of selective ligands has led to the identification of two AII receptor subtypes, designated AT_1 and AT_2 (4). The AT_1 receptor is preferentially recognized by DuP753 (losartan), whereas AT_2 receptors are preferentially recognized by PD 123177 (5–7). DuP753 antagonizes all of the known physiological actions of AII, indicating that these phenomena are related to the activation of AT_1 receptors (8).

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AT_2 receptors are present in a wide variety of tissues, including rat adrenal medulla (7, 9, 10), rat brain (11, 12), rat ovarian granulosa cells (13), rabbit and human uterus (6, 14–16), and mesenchymal tissues of rat fetus (17). Although most of the physiological actions of AII are mediated by the AT_1 receptor subtype, some recent data suggest a physiological role for AT_2 receptors. Cultured neurons from neonatal rat brain contain mostly AT_2 receptors that mediate a reduction in basal cGMP levels (18). This reduction is blocked by PD 123177 or CGP 42112A but not by DuP753. Furthermore, it was reported that particulate guanylate cyclase activity of rat adrenal glomerulosa cells and PC-12W cells was inhibited by AII (19). This effect, which could not be reproduced by two independent groups (20, 21), was shown to depend on the activation of a phosphotyrosine phosphatase that was not blocked by DuP753, suggesting that it was mediated through AT_2 receptors. In human astrocytes, prostaglandin synthesis appears to be partly related to the activation of AT_2 receptors (22).

ABBREVIATIONS: AII, angiotensin II; AII-Bpa, [Sar^1,Val^5,*p*-benzoyl-Phe^8]angiotensin II; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PNGase-F, glycopeptidase-F; AT_1 and AT_2, angiotensin type 1 receptor and angiotensin type 2 receptor.

The biochemical and molecular properties of the AT_2 receptor are still poorly understood. We recently reported the successful covalent labeling of AT_2 receptors in human myometrium using a novel and potent photosensitive analogue, ^{125}I-AII-Bpa (23). In the present study, we investigated the biochemical properties of AT_2 receptors from different sources by using the same photoaffinity labeling approach. We showed that the AT_2 receptor is a highly glycosylated protein and, interestingly, that the saccharide content contributes significantly to the size heterogeneity of AT_2 receptors from different tissues.

Experimental Procedures

Cell culture. A culture of Swiss 3T3 cells that expressed AII binding was found. This culture was designated as R3T3 and the cells were morphologically identical to other strains of Swiss 3T3 cells (24). R3T3 cells were grown under conditions described for Swiss 3T3 cells (25), using Dulbecco's modified essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO). All experiments used cells grown as a monolayer. PC-12 cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 4500 mg/liter glucose and 10% fetal bovine serum (GIBCO), as described (26).

Preparation of human myometrium membranes. The uteri used in this study were from 36–52-year-old women who had given birth to one to three children and were within days 6–18 of their menstrual cycles. In most of the cases, the pathological causes for the surgery were prolapse and menorrhagia. Immediately after the surgery, the uterus was kept on ice in medium containing 20 mM sodium bicarbonate. The myometrium was then sliced and homogenized for 2 min with a Sorvall Omni-mixer set at force 10. The material was rehomogenized for three periods of 10 sec with a Polytron homogenizer set at force 11. The homogenate was subjected to centrifugation at 35,000 × *g* for 20 min. All procedures were performed at 0°. The pellet was resuspended at a final concentration of 2–5 mg of protein/ml in medium containing 25 mM Tris·HCl, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mg/ml BSA, 0.1 mg/ml bacitracin, and 50 μg/ml soybean trypsin inhibitor. Aliquots were stored at −70°. The protein content was determined by the method of Lowry *et al.* (27).

Preparation of R3T3 and PC-12 cell membranes. Crude membranes of R3T3 and PC-12 cells were prepared by scraping the cells into 5 mM Tris buffer, pH 7.4, containing 10 μM leupeptin, 10 μM bestatin, 10 μM pepstatin A, and 100 μM phenylmethylsulfonyl fluoride. Cells were then homogenized, using a Brinkmann Polytron, and centrifuged at 1000 × *g* for 10 min. The supernatant was removed and then recentrifuged at 50,000 × *g* for 30 min. The resulting pellets were resuspended at 1 mg/ml in 10 mM phosphate buffer, pH 7.4, and either used immediately or stored at −70°.

AII binding assay. R3T3 cell membranes (2–3.5 μg of protein) or PC-12 cell membranes (6–10 μg of protein) were incubated in medium containing 25 mM Tris·HCl, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mg/ml BSA, 0.1 mg/ml bacitracin, and 50 μg/ml soybean trypsin inhibitor. ^{125}I-AII and selected concentrations of L-158,809, PD 123319, and AII were added in a final incubation volume of 500 μl. Incubations were performed at 37° for 180 min. Nonspecific binding was determined in the presence of 1 μM AII. Incubations were terminated by vacuum filtration through presoaked glass fiber filters (Whatmann GF/C) and three rapid washes with 3 ml of cold medium. The receptor-bound radioactivity was analyzed by γ counting.

Photoaffinity labeling. Human myometrium membranes, R3T3 cell membranes, or PC-12 cell membranes (2 mg of protein) were incubated with 2 μCi of ^{125}I-AII-Bpa in 1 ml of medium containing 25 mM Tris·HCl, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mg/ml BSA, 0.1 mg/ml bacitracin, and 50 μg/ml soybean trypsin inhibitor. L-158,809 (1 μM) was added to prevent any interaction with AT_1 receptors. After 45 min at room temperature, the membranes were washed by centrifugation (three times) and irradiated under filtered UV light (365 nm) [the mercury vapor lamps (JC-Par-38) were purchased from Westinghouse and the Raymaster black light filters (no. 5873) were purchased from Gates and Co. Inc. (Long Island, NY)] for 30–60 min at 0°. Irradiated membranes were incubated for 60 min at 37°, in denaturing buffer containing 60 mM Tris·HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 125 mM dithiothreitol, and 3% (w/v) bromphenol blue. The material was then aliquoted and stored at −20° until further use.

Endoglycosidase digestion. Endoglycosidase digestion was performed according to protocols of Carson *et al.* (28) and Tarentino *et al.* (29), with slight modifications. Briefly, photolabeled membranes (2 mg of protein) were solubilized for 1 hr at 37° in medium containing 25 mM Tris·HCl, pH 7.3, 1% (w/v) SDS, 0.1 mg/ml bacitracin, and 50 μg/ml soybean trypsin inhibitor. Aliquots of the solubilized material (corresponding to 75–150 μg of protein) were added to deglycosylation buffer containing 250 mM sodium phosphate, pH 8.6, 0.6% (w/v) Nonidet P-40, 0.1 mg/ml bacitracin, and 50 μg/ml soybean trypsin inhibitor. PNGase-F was added to a final volume of 100 μl. Incubations were performed at 37° for 16–24 hr. An equal volume (100 μl) of denaturing buffer (as described for photoaffinity labeling) was added and the incubations were extended for 1 hr at 37°. The resulting material was immediately analyzed by SDS-polyacrylamide gel electrophoresis or was stored at −20°.

SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out as described by Laemmli (30). Denatured proteins (25–50 μg) were subjected to electrophoresis on a 9% polyacrylamide gel and were run at 95 V for approximately 3 hr. Gels were stained with 0.05% (w/v) Coomassie brilliant blue and dried before autoradiography on Kodak X-Omat AR film. Exposures lasted for 3–7 days.

Gel permeation chromatography. Photolabeled membranes were solubilized for 1 hr at 0° in medium containing 25 mM Tris·HCl, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mg/ml bacitracin, 50 μg/ml soybean trypsin inhibitor, and 1% (v/v) Triton X-100. The solubilized material (200 μl, 500 μg of protein) was applied to a 10- × 370-mm Sepharose CL-6B column (SR 10/50; Pharmacia). Elution was performed with the aforementioned buffer (containing 0.2%, v/v, Triton X-100) at a flow rate of 0.17 ml/min, which was maintained with a peristaltic pump. Fractions of 850 μl were collected. The column was calibrated with the standard proteins thyroglobulin (669 kDa), ferritin (440 kDa), and catalase (232 kDa) (Pharmacia).

Materials. BSA, bacitracin, soybean trypsin inhibitor, and PNGase-F were from Sigma Chemical Co. (St. Louis, MO). L-158,809 and PD 123319 were generous gifts from Merck and Parke-Davis Warner-Lambert, respectively. AII-Bpa was synthesized in our laboratories. ^{125}I-AII and ^{125}I-AII-Bpa (specific activity, 985 Ci/mmol) were prepared with Iodogen as described by Fraker and Speck (31). The products were purified to apparent homogeneity by high performance liquid chromatography (reverse phase C-18), and their specific radioactivity was determined by self-displacement in the binding system.

Results

^{125}I-AII binding to R3T3 and PC-12 cell membranes. ^{125}I-AII binding to PC-12 cell membranes was saturable and of high affinity. In competitive binding studies, AII inhibited ^{125}I-AII binding with an IC_{50} of 0.2 nM (Fig. 1A). Scatchard analysis of the binding data was consistent with a single set of high affinity sites (Fig. 1A, *inset*) with a K_d of 0.17 nM and a maximal binding capacity (B_{max}) of 3.6 pmol/mg of protein. The specificity of the AII binding sites was analyzed in competitive binding experiments performed with selective AII analogues. As shown in Fig. 1B, AII and PD 123319 (an AT_2-selective analogue) showed high affinities for the binding site, with IC_{50} values of 0.2 nM and 22 nM, respectively. L-158,809 (an AT_1-selective analogue) was unable to significantly compete for the

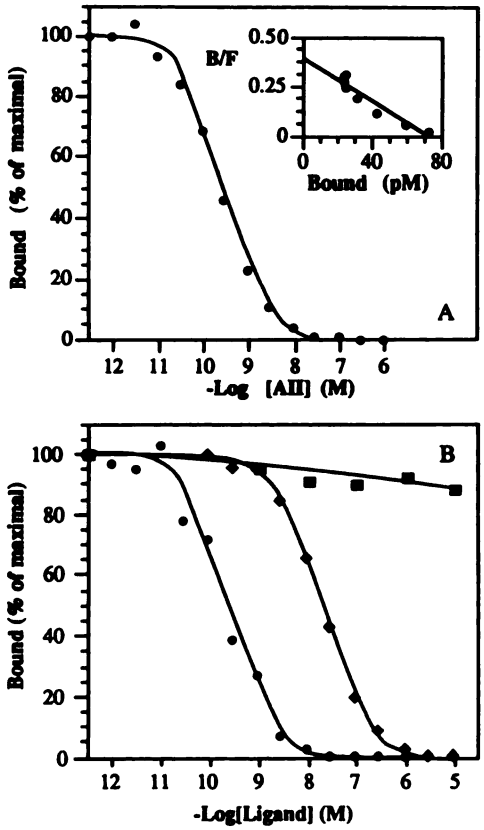

Fig. 1. A, Effect of increasing concentrations of AII on ^{125}I-AII specific binding to PC-12 cell membranes. PC-12 cell membranes (10 μg of protein) were incubated at 37° in medium containing ^{125}I-AII (42,438 cpm) and increasing concentrations of unlabeled AII. After 3 hr, the incubation was stopped by filtration. *Inset*, Scatchard plot of the same binding data. B, Inhibitory effect of increasing concentrations of AII (●), PD 123319 (♦), and L-158,809 (■) on ^{125}I-AII binding to PC-12 cell membranes. Membranes (6 μg of protein) were incubated at 37° in medium containing ^{125}I-AII (44 364 cpm) and increasing concentrations of peptide or nonpeptide analogue. After 3 hr, the incubation was stopped by filtration. Data are expressed as values relative to the total binding observed in the absence of unlabeled ligand and were corrected for nonspecific binding. These experiments, performed in triplicate, are representative of three such observations.

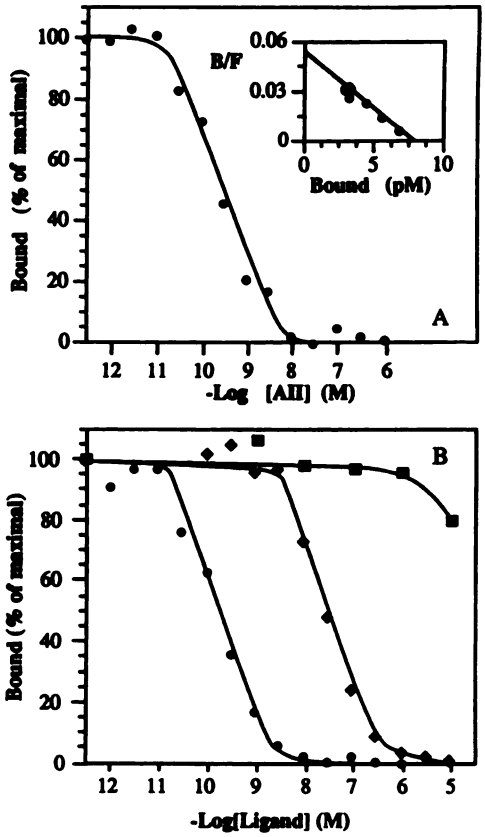

Fig. 2. A, Effect of increasing concentrations of AII on ^{125}I-AII specific binding to R3T3 cell membranes. R3T3 cell membranes (2 μg of protein) were incubated at 37° in medium containing ^{125}I-AII (42,438 cpm) and increasing concentrations of unlabeled AII. After 3 hr, the incubation was stopped by filtration. *Inset*, Scatchard plot of the same binding data. B, Inhibitory effect of increasing concentrations of AII (●), PD 123319 (♦), and L-158,809 (■) on ^{125}I-AII binding to R3T3 cell membranes. Membranes (3.5 μg of protein) were incubated at 37° in medium containing ^{125}I-AII (56,999 cpm) and increasing concentrations of peptide or nonpeptide analogue. After 3 hr, the incubation was stopped by filtration. Data are expressed as values relative to the total binding observed in the absence of unlabeled ligand and were corrected for nonspecific binding. These experiments, performed in triplicate, are representative of three such observations.

binding site. These results reveal that PC-12 cells contain high levels of AT_2 binding sites. Similarly, AII binding to R3T3 cell membranes was saturable and of high affinity, with an apparent K_d of 0.15 nM and B_{max} of 2 pmol/mg of protein (Fig. 2A). Competitive studies showed that AII and PD 123319 completely displaced ^{125}I-AII binding, with IC_{50} values of 0.2 nM and 28 nM, respectively (Fig. 2B). L-158,809 had no significant effect on ^{125}I-AII binding to R3T3 cell membranes. These results confirm that R3T3 cells express exclusively the AT_2 receptor subtype, as reported previously (24).

Gel permeation chromatography. Photolabeled AT_2 receptors from human myometrium were solubilized with Triton X-100 and chromatographed on Sepharose CL-6B. The bulk of radioactivity eluted as a symmetrical peak corresponding to a protein with a Stokes' radius of 66 ± 5 Å (mean ± standard deviation, three experiments) (Fig. 3, *upper*). A minor peak corresponding to the free tracer was also eluted with the total volume of the column. These results are similar to those obtained by Capponi and Catt (32) for nitroazidobenzoyl-AII-labeled AII receptors from dog myometrium. Photolabeled AT_2 receptors from R3T3 cells (Fig. 3, *lower*) and PC-12 cells (Fig. 3, *middle*) also eluted as symmetrical peaks, with Stokes' radii of 61 ± 4 Å and 67 ± 5 Å, respectively (mean ± standard deviation, three experiments). Gel permeation experiments did not reveal any significant differences between the sizes of AT_2 receptors from the three different sources.

SDS-polyacrylamide gel electrophoresis. Photolabeled AT_2 receptors were solubilized in denaturating buffer and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Fig. 4B shows that AT_2 receptors from the three different sources migrated as bands of heterogeneous apparent molecular masses. The broadness of the bands suggested a glycoprotein nature. AT_2 receptor from human myometrium (Fig. 4B, *lane 1*) showed a molecular mass of 68 ± 5 kDa (mean ± standard deviation, 30 experiments), a value identical to that found by Capponi and Catt (32) in dog myometrium. AT_2 receptor from R3T3 cells (Fig. 4B, *lane 2*) showed a much higher molecular mass of 91 ± 7 kDa (mean ± standard deviation, six experiments), a value similar to that reported earlier using disuccinimidyl suberate cross-linking (24). AT_2

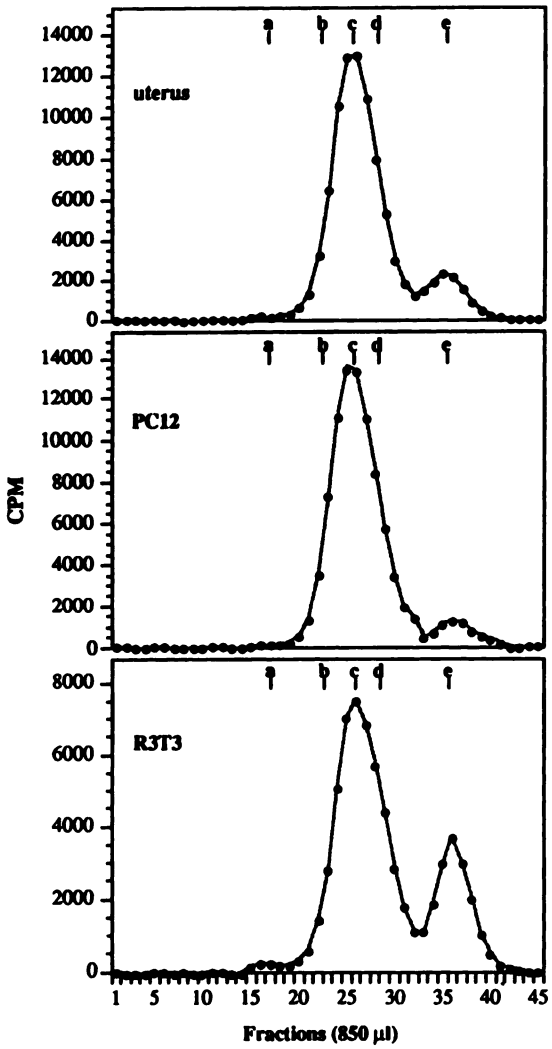

Fig. 3. Gel permeation analysis of ^{125}I-AII-Bpa-AT_2 receptor complexes derived from particulate cell membrane fractions of human myometrium (*upper*), PC-12 cells (*middle*), and R3T3 cells (*lower*). Membranes (0.5–2 mg of protein) were incubated with ^{125}I-AII-Bpa (≈2 µCi) for 45 min at room temperature, in the dark. The medium also contained L-158,809 (1 µM) to prevent any interaction with AT_1 receptors. The membranes were then washed and irradiated for 1 hr. After irradiation, they were solubilized with 1% (v/v) Triton X-100 in medium containing 25 mM Tris·HCl, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mg/ml bacitracin, and 50 µg/ml soybean trypsin inhibitor. After centrifugation at 15,000 × *g* for 5 min, aliquots of the solubilized material were loaded onto a Sepharose CL-6B size exclusion column. The flow rate was adjusted to 0.17 ml/min and 850-µl fractions were collected. Molecular mass standards depicted in the graphs are as follows: *a*, plasmid of 16,000 base pairs (≈10,000 kDa); *b*, thyroglobulin (669 kDa); *c*, ferritin (440 kDa); *d*, catalase (232 kDa); *e*, ^{125}I-AII (1172 Da). Similar results were obtained in three independent experiments.

receptor from PC-12 cells (Fig. 4B, *lane 3*) showed an even higher molecular mass of 113 ± 12 kDa (mean ± standard deviation, four experiments). Fig. 4A shows that, in competitive experiments, photoaffinity labeling of AT_2 receptors from the three different sources was unaffected by high concentrations (1 µM) of L-158,809 (Fig. 4A, *lanes 1*) but was completely abolished by high concentrations (1 µM) of PD 123319 (Fig. 4A, *lanes 2*), indicating selective labeling of the AT_2 receptor subtype.

Deglycosylation studies. To further characterize the nature of the AT_2 receptor, photolabeled membranes were treated

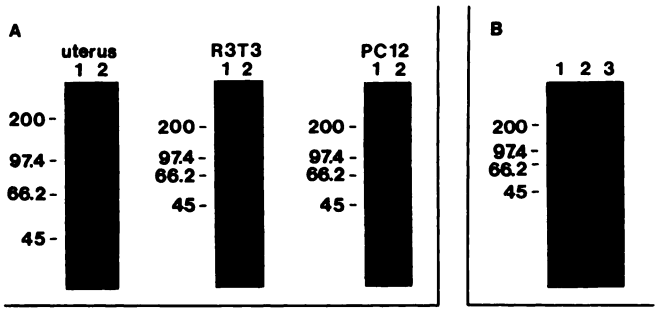

Fig. 4. A, Selective photoaffinity labeling of AT_2 receptors from different sources. Membranes from different sources were incubated with ^{125}I-AII-Bpa (2 µCi) and L-158,809 (1 µM) (*lanes 1*) or L-158,809 (1 µM) plus PD 123319 (1 µM) (*lanes 2*) and were labeled as indicated in the legend to Fig. 3. The material was solubilized in denaturating buffer and subjected to gel electrophoresis on a 9% separating gel, followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of at least four separate experiments. B, Membranes from three different sources, i.e., human myometrium (*lane 1*), R3T3 cells (*lane 2*), and PC-12 cells (*lane 3*), were labeled with ^{125}I-AII-Bpa (2 µCi) as indicated in the legend to Fig. 3. The material was solubilized in denaturating buffer and subjected to gel electrophoresis on the same separating gel, under the conditions described for A.

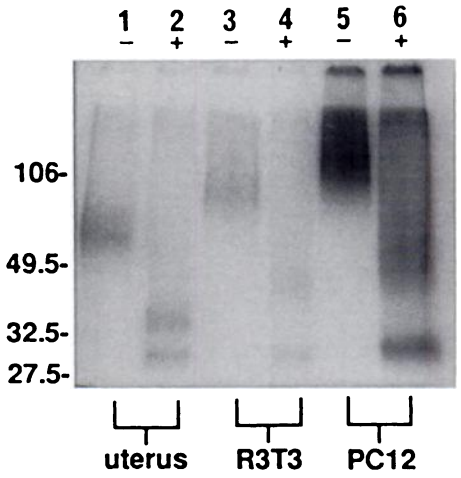

Fig. 5. Deglycosylation of photolabeled AT_2 receptors. Membranes from human myometrium (uterus), R3T3 cells, and PC-12 cells were photolabeled, solubilized, and incubated in the presence (+) or in the absence (–) of PNGase-F (3 units/ml) for 18 hr at 37°, under the conditions described in Experimental Procedures. Aliquots were then denatured and subjected to gel electrophoresis on a 9% separating gel, followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. This experiment is representative of at least three similar observations.

with the endoglycosidase PNGase-F, which cleaves *N*-linked saccharides (29). Fig. 5, *lane 2*, shows that endoglycosidase treatment of AT_2 receptors from human myometrium resulted in two products, of 40 ± 4 kDa and 31 ± 2 kDa (mean ± standard deviation, 25 experiments). As shown previously, untreated receptors migrated as a large band of 68 kDa (Fig. 5, *lane 1*). Endoglycosidase treatment of photolabeled R3T3 cell membranes resulted in two products, of 46 ± 3 kDa and 31 ± 1 kDa (mean ± standard deviation, three experiments) (Fig. 5, *lane 4*). The untreated receptor is shown in Fig. 5, *lane 3*. Endoglycosidase treatment of photolabeled PC-12 cell membrane resulted in two products, of 55 ± 4 kDa and 31 ± 1 kDa (mean ± standard deviation, three experiments) (Fig. 5, *lane 6*). The untreated receptor is shown in Fig. 5, *lane 5*. These

results clearly reveal the glycoprotein nature of the AT_2 receptor. They also suggest a stepwise process of deglycosylation that yields different intermediate products depending on the tissue source. It is interesting to note that the end product of deglycosylation is identical (molecular mass of 31 kDa) in all three tissues.

To further define the stepwise process of deglycosylation, photolabeled AT_2 receptors from human myometrium were subjected to PNGase-F digestion for different periods of time. Fig. 6 clearly shows that deglycosylation occurred in three distinct steps. Fig. 6, *lanes 1–3*, reveals the appearance of a first deglycosylation product within the first few minutes of incubation (5–30 min) with a low concentration of PNGase-F (3 units/ml). This product showed a molecular mass of 58 kDa, which represents a 12% size reduction, compared with the control receptor (molecular mass of 66 kDa) (Fig. 6, *lane 7*). Fig. 6, *lanes 4–6*, reveals the appearance of a second deglycosylation product after prolonged incubation (1–24 hr) with low concentrations of PNGase-F (3 units/ml). This second product showed a molecular mass of 38 kDa, corresponding to a size reduction of 42%, compared with untreated receptor (Fig. 6, *lane 7*). The stepwise process of deglycosylation is well illustrated in Fig. 6 by the disappearance of the first deglycosylation product concomitant with the appearance of the second deglycosylation product. Fig. 6 also reveals the appearance of a third deglycosylation product with a molecular mass of 31 kDa after 24 hr of incubation with PNGase-F (3 units/ml). Prolonged incubations, for up to 60 hr, with high concentrations of PNGase-F (60 units/ml) did not reveal any further deglycosylation (Fig. 7). These results, together with the sharpness of the 31-kDa band, suggest that this band corresponds to a completely deglycosylated protein.

Discussion

We previously reported the use of a new photosensitive analogue of AII (^{125}I-AII-Bpa) for the covalent labeling of AT_2 receptors from human myometrium (23). This photosensitive analogue recognized AT_2 receptors with high affinity and provided a high yield of covalent incorporation upon photolysis. In the present study, we used this analogue for the labeling of AT_2 receptors from different sources.

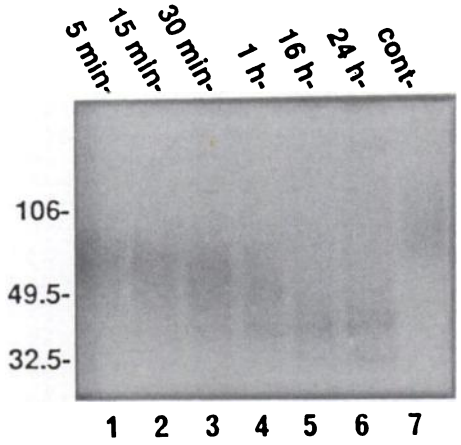

Fig. 6. Time course of deglycosylation of photolabeled AT_2 receptors from human myometrium. Membranes from human myometrium were treated for different periods of time as indicated in the legend to Fig. 5. At the end of the incubation, the proteins were denatured and subjected to gel electrophoresis on a 9% separating gel, followed by autoradiography. *Lane 7* (*cont*), incubation for 24 hr without PNGase-F. Protein standards of the indicated molecular masses (kDa) were run in parallel. This experiment is representative of three similar observations.

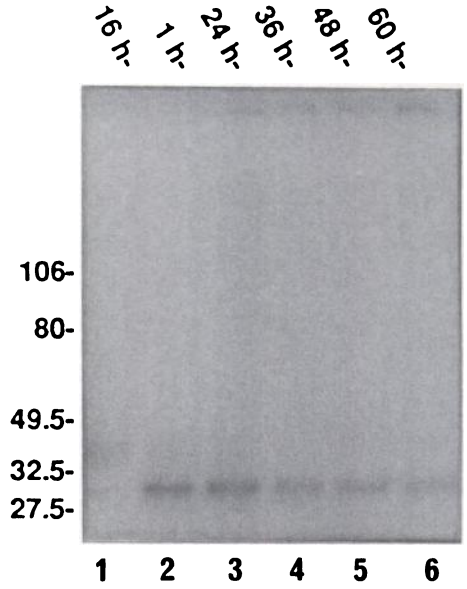

Fig. 7. Complete digestion of photolabeled AT_2 receptors from human myometrium. Membranes from human myometrium were treated, as indicated in the legend to Fig. 5, with 3 units/ml PNGase-F (*lane 1*) or 60 units/ml PNGase-F (*lanes 2–6*) for different periods of time. At the end of the incubation, the proteins were denatured and subjected to gel electrophoresis on a 9% separating gel, followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. This experiment is representative of two similar observations.

As reported previously, the murine fibroblast cell line R3T3 (24) and the rat pheochromocytoma cell line PC-12 express high affinity binding sites for AII. Dose-displacement experiments with the selective nonpeptidic antagonists L-158,809 and PD 123319 confirmed that R3T3 and PC-12 cells express exclusively the AT_2 receptor subtype. This receptor showed high affinity for AII (K_d of approximately 0.2 nM in both membrane preparations) and PD 123319 (IC_{50} of approximately 25 nM in both membrane preparations) but did not show binding affinity for L-158,809, an AT_1-selective analogue of AII. R3T3 cells and PC-12 cells contain large amounts of AT_2 receptors (B_{max} of approximately 4 pmol/mg of protein in PC-12 cells and about 2 pmol/mg of protein in R3T3 cells). It must be mentioned that different batches of R3T3 cells showed important variations in the maximal amount of AT_2 receptor (varying from as little as a few femtomoles to as much as 2 pmol/mg of protein). This has recently been shown to be due to the growth state of the cells (33); however, the variation in density of AT_2 receptors had no effect upon the size of the photolabeled bands. The concentration of AT_2 receptors in PC-12 cell membranes (4 pmol/mg of protein) was significantly higher than that found in another rat pheochromocytoma cell line (PC-12W), where B_{max} values varied from 4 to 326 fmol/mg of protein (20, 21, 34). Although they are likely to be similar, it is unknown whether the AT_2 receptors in PC-12W cells are identical to the AT_2 receptors from PC-12 cells used in this study.

Conflicting reports recently appeared in the literature concerning the size of the AT_2 receptor. With a disuccinimidyl suberate cross-linking approach, the AT_2 receptor of R3T3 cells was found to have a molecular mass of 100 kDa (24). With a

similar approach, AT_2 receptors of rat ovarian granulosa cells were shown to have a molecular mass of 79 kDa (13). With a photoaffinity labeling approach, we recently reported a molecular mass of 68 kDa for AT_2 receptor of human myometrium (23). It is unlikely that these size disparities are due to different approaches or different experimental conditions, because the results of the present study revealing heterogeneous sizes of AT_2 receptors from different sources were obtained with the same photoaffinity labeling approach under identical experimental conditions. Our results suggest, rather, that the size heterogeneity of the AT_2 receptor is related to the tissue where it is expressed. With two different approaches, similar molecular mass values of 91 kDa (this study) and 100 kDa (24) were found for AT_2 receptors of R3T3 cells. Also, under different experimental conditions, molecular mass values of 68 kDa were found in human (Ref. 23 and this study) and dog (32) myometrium. It is interesting to note that the study on the characterization of the AII receptor of dog myometrium (32) was performed in 1980, at a time when no selective analogue was available to discriminate between AT_1 and AT_2 subtypes. Because the properties of this receptor are very similar to those observed by us in human myometrium and because myometrium from different species seems to express predominantly the AT_2 receptor subtype (6, 14–16, 23, 35), this study could retrospectively be considered as the first biochemical characterization of the AT_2 receptor subtype.

AT_2 receptors of human myometrium, R3T3 cells, and PC-12 cells are glycoproteins. The glycoprotein nature of those receptors was suspected from the broadness of the electrophoretic bands and it was directly confirmed by the effect of endoglycosidase digestion. The carbohydrate moieties contributed significantly to the molecular masses of those receptors. Treatments with PNGase-F reduced the estimated sizes of the labeled receptors by >50%, and by up to 73% for the AT_2 receptor of PC-12 cells, revealing a high level of glycosylation. On the basis of the size reduction observed at the different stages of deglycosylation, we could not find any common pattern of deglycosylation for the different receptors from the three different sources, suggesting that each receptor contains different carbohydrate moieties. A common characteristic of those receptors is their *N*-glycosylation, as revealed by the effect of PNGase-F, which is known to cleave *N*-linked saccharides. The stepwise deglycosylation process implies (at least for the AT_2 receptor of human myometrium) the presence of three asparagine-linked sites of glycosylation. A second common characteristic of AT_2 receptors from the three different sources involves the similar sizes of their final deglycosylation products. The sharpness of the band and its stability after prolonged periods of incubation in the presence of a high concentration of endoglycosidase suggest that the 31-kDa protein is completely deglycosylated. The analysis of a larger range of AII target tissues expressing the AT_2 receptor will be necessary to clarify whether the AT_2 receptor shows a continuous size distribution in the various target tissues or a range of discrete sizes that would correlate with the presence of specific degrees of glycosylation at one or more sites on the extracellular domain of the molecule.

After the submission of our manuscript, the AT_2 receptor from rat fetus and rat pheochromocytoma cells was cloned and sequenced by two independent teams (36, 37). This receptor is a 363-amino acid protein with a deduced molecular mass of 41 kDa. This value is slightly larger than that observed after complete deglycosylation of the AT_2 receptor. A simple explanation for this discrepancy would be proteolytic cleavage occurring during the deglycosylation experiments. However, the consistent observation of a 31-kDa protein, the great stability of this protein even after prolonged incubations at 37°, and the absence of any lower molecular mass proteins argue against the possibility of proteolytic degradation of the AT_2 receptor under our experimental conditions. Rather, we believe that the discrepancy is merely due to a limitation of the electrophoresis technique, which does not always provide the actual molecular masses of proteins. A similar phenomenon was observed in the case of the AT_1 receptor when Carson *et al.* (28) determined a molecular mass of 34 kDa for the deglycosylated AT_1 receptor of bovine adrenal cortex, whereas Sasaki *et al.* (38) and Murphy *et al.* (39) revealed a molecular mass of 41 kDa after cloning and sequencing the protein.

The recent sequencing of the rat AT_2 receptor also revealed the presence of five potential *N*-glycosylation sites, located exclusively in the amino-terminal hydrophilic domain of the AT_2 receptor. On the basis of the different steps of deglycosylation observed during our study, we suggest the presence of at least three sites of *N*-glycosylation on the AT_2 receptor of human myometrium. If AT_2 receptors of human myometrium and rat fetus are identical proteins, this would suggest that only three of the five putative sites of glycosylation are effectively glycosylated. Our results, however, cannot exclude the possibility that a single step of deglycosylation may correspond to the simultaneous removal of two or more sugars from different sites of the protein.

The basis for the size disparities between AT_2 receptors from human myometrium, R3T3 cells, and PC-12 cells seems to be related to their different degrees of glycosylation, but the functional significance of these disparities is not yet clear. Actually, no definite role has been assigned to the AT_2 receptor (8). It is possible that there is more than one gene for the AT_2 receptor and that these are differentially expressed in the various AII target tissues. Two genes, coding for the AT_1 type 1A and type 1B receptors, have already been identified in rodents (40–43). Another possibility is that different post-translational modifications of a single gene product occur within individual target tissues. Further studies are needed to determine the role of carbohydrates in establishing the pharmacological properties (affinity and activity) of the AT_2 receptor. Carbohydrates could also contribute to the stability of the receptor, its intracellular trafficking, and its membrane targeting. The cloning and expression of the AT_2 receptor will certainly furnish interesting information about these questions.

In conclusion, we have characterized the biochemical properties of AT_2 receptors from three different tissues. We have shown that AT_2 receptors are highly glycosylated proteins. We also showed that the size of glycosylated AT_2 receptors from different tissues is very heterogeneous and that the basis for this heterogeneity is the varying degree of glycosylation. It will be interesting, once a physiological action is assigned to the AT_2 receptor, to investigate the functional significance of glycosylation.

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