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Full Length Research Paper

Immunohistochemical Analysis of Angiotensin II AT1 and AT2 Receptor Isoforms in Sprague-Dawley Rat and Meriones crassus Adrenal Glands

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Employing specific polyclonal anti-AT_1 and anti-AT_2 antibodies, AT_1 and AT_2 receptor expression was immunohistochemically demonstrable within adrenal tissues in Sprague-Dawley rats and the desert rodent *Meriones crassus*. Among adrenal cortical zones in rats, AT_1 receptor labeling was evident in zona glomerulosa and zona reticularis. In contrast, AT_1 receptor labeling was confined to the zona glomerulosa and the deep zona fasiculata in *Meriones crassus*. AT_1 receptor labeling was, however, equally observed among ganglion and chromaffin cells constituting the adrenal medulla of both animal models. AT_2 receptor labeling was faint in all adrenal regions in rats. However, intensity was high in the deep zona fasiculata, and medullary chromaffin and ganglion cells in *Meriones crassus.* Two-dimensional Western blotting, in the presence or absence of endoglycosidase-F, revealed that structurally distinct spectra of AT_1 and AT_2 receptor isoforms are expressed in the adrenal tissues of each animal model. These spectra were constituted by molecular isoforms with distinct patterns of charge microheterogeneity unique to each receptor type in each animal model. In both species, heterogeneity of AT_1 and AT_2 receptor isoforms may be attributed in part to differential post-translational glycosylation mechanisms of the receptor polypeptide backbones, which may be critical in differentially fine-tuning adrenal functions in lab-reared and desert rodents.

Key words: Angiotensin II receptors, adrenal gland, desert rodents, N –linked glycosylation, immunohistochemistry.

INTRODUCTION

The renin-angiotensin system is one of the most potent systems that regulate blood pressure, electrolyte balance, and extracellular fluid volume (Ichihara et al., 2004). The effects of the principal component of this system, angiotensin II (Ang II), are triggered by its interaction with specific receptors in a variety of tissues (Belloni et al., 1998; Johren et al., 2003; Bird and Pattison, 2004). Major advances in understanding the physiological consequences of Ang II-Ang II receptor interactions have been attributed to the development of selective non-peptide Ang II receptor antagonists, which established the occurrence of at least two major types of Ang II receptors referred to as AT_1 and AT_2 (Chiu et al., 1994). At a more fundamental level, the structural heterogeneity of these receptor types has been confirmed by cDNA sequence data (Koike et al., 1995), which revealed only 30% homology in the coding regions of both types. Two subtypes of the rodent AT_1 receptor (AT_{1A} and AT_{1B}) have been identified and sequenced and these have about 96% sequence identity in the coding region (Inagami et al., 1994). The deduced sequence of the rat AT_1 receptor shows a protein of 359 amino acids with a molecular weight of 40 kDa, seven putative transmembrane domains, and three potential N-glycosy-lation sites (Murphy et al., 1991). The AT_2 receptor is also a seven transmembrane domain receptor, represented by a 363

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amino acid polypeptide with a molecular mass of 41 kDa and five potential N- glycosylation sites located in in the extracellular N-terminal domain (Mukoyama et al., 1993). In a variety of tissues, AT_1 receptors are coupled to ion channels and phosphoinositide breakdown and appear to mediate most of the known physiological effects of Ang II (Capponi, 1996). On the other hand, the coupling of AT_2 receptors has been implicated in modulating guanylate cyclase activity and T-type calcium channels in selective cell types but, as yet, is not fully explored (Dinh et al., 2001; Porrello et al., 2009).

In the adrenal gland, both Ang II receptor types have been localized in different species by a variety of detection methods (Allen et al., 2000). Based on immunohistochemistry (Wang et al., 1998; Giles et al., 1999), *in vitro* autoradiography (Zhuo et al., 1996; Lehoux et al., 1997) and Northern blot and radioligand binding studies (Gasc et al., 1994; Wang et al., 1998), all mammals studied express both AT_1 and AT_2 receptors in the cortex and medulla, but with marked species-dependent differences observed in the distribution and proportions of both receptors. In the cortex, AT_1 receptors predominate in the zona glomerulosa of all species including rodents, monkeys and humans (Zhuo et al., 1996). Ang II receptors occur in very low or undetectable concentrations in the zona fasciculata and reticularis in most mammals examined except in the human, canine, and bovine adrenals, where a moderate to high density of AT_1 and AT_2 receptors coexists (Ouali et al., 1993; Allen et al., 2000; Bird and Pattison, 2004). In the medulla, moderate levels of AT_1 receptors occur in catecholamine-releasing chromaffin cells of most species examined (Israel et al., 1995; Dinh et al., 2001). In contrast, high levels of AT_2 receptors occur in the medulla of most species, although the density is much lower in humans (Zhuo et al., 1996). The distribution of AT_1 receptors in zona glomerulosa cells of the cortex and chromaffin cells of the medulla in various mammalian adrenal glands is consistent with the known regulatory effects of Ang II on the biosynthesis and release of aldosterone and catecholamines from the adrenal glands (Allen et al., 2000; Jezova et al., 2003). The direct involvement of AT_1 receptor in aldosterone secretion has been particularly strengthened by the observed up-regulation of mRNA and protein levels of this receptor type under conditions of low sodium diet, renovascular hypertension or water deprivation (Giacchetti et al., 1996; Chatelain et al,. 2003). The physiological role of AT_2 receptors in the adrenal gland is, on the other hand, largely unknown, but accumulating evidence suggest that their major role may involve the functional antagonism of the mineralocorticoid-releasing and growth-promoting effects of AT_1 receptors in cortical zona glomerulosa cells (AbdAlla et al., 2001), the stimulating effect in the secretion of endogenous ouabain from cortical cells (Laredo et al., 1997) as well as the synergistic effect with AT_1 receptors in regulating catecholamine synthesis and release by adrenomedullary cells (Mazzocchi et al., 1998; Jezova et al., 2003).

Animals living in arid environments (e.g. the desert rodent *Meriones crassus*) must deal with major problems of a high ambient temperature, a rapid evaporative water loss, and the scarcity of water. In these animal models, Ang II is expected to play a central widespread role in the regulatory mechanisms for electrolytes and blood volume control. Although the physiological significance of Ang II has been the focus of numerous studies in various animal models, the cellular and molecular bases of Ang II effects on the tissues of desert animals in general, as well as the nature of the receptors involved in particular, are still to be resolved (Al-Qattan et al., 2006). The present study is thus designed to explore, in a comparative context, the tissue distribution pattern and structural characteristics of Ang II AT_1 and AT_2 receptor isoforms expressed in the adrenal gland of the lab-reared Sprague-Dawely (SD) rats and the desert rodent *Meriones crassus* (*M. crassus*).

Our investigation is aimed at providing a correlation between the distinctive tissue localization and structural characteristics of the Ang II receptor family, and the differential Ang II- Ang II receptor interactions, putatively operating in lab-reared and desert rodents.

MATERIALS AND METHODS

Animals and reagents

Adult male SD rats (England) weighing 100 g raised at the animal house of the Department of Biological Sciences, Kuwait University were used. Adult males of the desert gerbil *M. crassus*, Sundevall's jird, weighing 100 g were captured from Kabid area (40 km west of Kuwait City) and used within few days of captivity. Adult female New Zealand rabbits (weighing 2 to 3 kg) were used for immunization. All animals were given standard laboratory chow (170 mmol Na^+/kg) and water *ad libitum* and kept under standard conditions (23 ± 2°C, 12 h light, 12 h darkness). Animals were treated in strict accordance with the recommendations of the declaration of Helsinki and the guidelines for animal experimentation of Kuwait University, Faculty of Science.

Except where noted, all chemicals were reagent grade and purchased from Sigma Chemical Company (St. Louis, MO, USA). Polyclonal anti-AT_1 receptor antibody (sc-1173, rabbit IgG specific to an epitope mapping within the N-terminal extracellular domain of the human AT_1 polypeptide), polyclonal anti-AT_2 antibody (sc-7421, rabbit IgG specific to an epitope mapping within the N-terminal extracellular domain of the human AT_2 polypeptide) and peroxidase–conjugated goat anti-rabbit IgG antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Ca, USA). Gel electrophoresis reagents, ampholines, peroxidase– conjugated molecular weight and pI standards, and nitrocellulose membranes (0.45 ∝) were obtained from BioRad (Richmond, CA, USA). Eight amino acid peptides, corresponding to amino acids 14 to 21 (Ile-Gln-Asp-Asp-Cys-Pro-Lys-Ala) of the first extracellular domain as deduced from the published AT_1 receptor cDNA sequence (Murphy et al., 1991), and corresponding to amino acids

10 to 17 (Thr-Ser-Arg-Asn-Ile-Thr-Ser-Ser) of the first extracellular domain as deduced from the published AT_2 receptor cDNA sequence (Feng et al., 2005), were synthesized manually on the base-labile linker 4-(hydroxymethyl)- benzoyloxymethyl and supplied by The Protein/DNA Technology Center (The Rockefeller University, NY, USA). The octapeptides were conjugated to bovine serum albumin (BSA) and coupled to CNBr-activated Sepharose 4B as described previously (Al-Qattan et al., 2006).

Preparation of adrenal gland sections

The left adrenal gland of anesthetized SD rats and *M. crassus* was excised and placed in 3 ml of Bouin's fixative for 24 to 48 h at room temperature. The tissues were processed for routine paraffin embedding, which included dehydration through a series of ethanol concentrations 50, 70, 90 and 100%, clearing in toluene, embedding in paraffin wax, and finally 3 to 4 ∝m sections were cut on a rotary microtome. The sections were picked up on clean slides after spreading them in a water bath at 40°C. The slides were air-dried to be used for subsequent staining.

Labeling of adrenal gland sections with anti-AT_1 and anti-AT_2 receptor antibodies

Tissue sections were examined for AT_1 and AT_2 receptor distribution by an indirect immunohistochemical labeling technique. Adrenal gland sections were dewaxed in xylene, hydrated with a series of 90, 75 and 60% ethanol and washed with PBS, pH 7.2. Sections were quenched by the addition of 10% normal goat serum and 0.3% hydrogen peroxide in PBS, pH 7.2 for 1 h and then individually labeled for 45 min in a humidified chamber with 300 ∝l of either the anti-AT_1 or anti-AT_2 receptor antibody (1:100 dilution, each). After several washes with 200 ∝l PBS, pH 7.2, the sections were incubated for 45 min with 300 ∝l of peroxidase-conjugated goat anti-rabbit IgG antibody (diluted to 1:200 in PBS, pH 7.2), followed by a 10 min treatment with 400 ∝l of 3,3-diaminobenzidine tablets (fast DAB) reconstituted in water. All sections were counter stained with 100 ∝l haematoxylin (Gill #1) for 1 min and examined by light microscopy for positive labeling of cells expressing AT_1 or AT_2 receptors. Control sections were identically stained by replacing the specific antibodies with either the anti-AT_1 or the anti-AT_2 antibody, pre-absorbed with their respective AT_1 or AT_2 octapeptide/BSA complex-coated CNBr-activated Sepharose 4B beads. Other hydrated sections were stained with haematoxylin for 3 to 4 min and eosin for 2 to 3 min and examined in parallel for comparative purposes by light microscopy. Photomicrographs were taken using Olympus AH-3 automated microscope (Tokyo, Japan), equipped with an Olympus Vanox camera.

Solubilization of adrenal cell-membranes in deoxycholate

Adrenal glands collected from SD rats and *M. crassus* were individually homogenized, solubilized and extracted in 10 mM Tris/HCl, pH 8.0 containing 2 mM phenylmethylsulfonyl fluoride and 2% deoxycholate (Al-Qattan et al., 2006) by an automatic homogenizer followed by sonic disruption and stirring at room temperature for 2 h and three cycles of freezing at -20°C and thawing at room temperature. Solubilized cell membrane lysates were recovered in supernatants following centrifugation of reaction mixtures at 100,000 *g* for 1 h, and their protein content determined by the method of Lowry et al. (1951) using BSA in the same buffer as a standard.

Endoglycosidase treatments

Adrenal cell-membrane lysates of SD rats and *M. crassus* (120 µg protein) were separately precipitated with 20% trichloroacetic acid and ice-cold acetone for I h at -20°C, washed for 1 h with acetone at -20°C and reconstituted in 50 µl of 100 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% Nonidet P-40 containing 200 mU of Endo-F (Endo-ß-N-acetylglucosaminidase F, from *Flavobacterium meningosepticum*, 600 U/mg, Sigma Chem. Comp. , St. Louis, MO). Samples were incubated for 18 h at 37°C, precipitated with equal volume of 20% trichloroacetic acid, washed with cold acetone and dried under nitrogen gas before analysis by polyacrylamide gel electrophoresis. Control samples were similarly treated but in the absence of Endo-F.

Two-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis

Aliquots of solubilized cell-membrane lysates (120 ∝g protein) collected from adrenal tissues of SD rats and *M. crassus*, and either untreated or treated with Endo-F, were individually resolved by two-dimensional (2-D) sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were reconstituted for 4 h at 37°C in first dimension sample buffer (9.5 M urea, 2% NP-40, 0.4% ampholines "pH 3 to 10" and 1.6% ampholines "pH 5 to 7") and analyzed essentially as described by O'Farrell (1975). First dimension isoelectric focusing tube gels were focused at 750 V (constant voltage) for 3.5 h, equilibrated for 15 min in equilibration buffer (62.5 mM Tris/HCl, pH 6.8, 2.3% SDS and 10% glycerol) and resolved in the second dimension with 12% slab SDS-PAGE using a Bio Rad Mini-Protein II 2-D cell according to Laemmli (1970). Slab gels were equilibrated in electrophoretic transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3 containing 20% methanol) for 15 min for subsequent analyses by Western blotting.

Western blotting

Protein samples in adrenal cell-membrane lysates resolved by 2-D SDS-PAGE were electrophoretically transblotted to 0.45 ∝ nitrocellulose membranes at 100 V (constant voltage) for 1.5 h at 4°C in electrophoretic transfer buffer, pH 8.3 using a Bio Rad Mini Trans-Blot electrophoretic transfer cell. Nitrocellulose membranes were washed 3 times with 200 mM PBS, pH 7.2 containing 0.05% Tween 40 (PBS – Tween buffer), each for 15 min with constant agitation and nonspecific binding sites blocked by incubation for 1 h in blocking buffer (3% BSA in PBS - Tween buffer, pH 7.2). Membranes were then washed thrice in PBS – Tween buffer, pH 7.2 and subsequently probed by either anti-AT_1 or anti-AT_2 antibody (each diluted to 1:500 in PBS – Tween buffer, pH 7.2, respectively) by incubation for 2 h at room temperature and then overnight at 4°C with constant agitation. Control blots were prepared by substituting the specific antibodies with either the anti-AT_1 or the anti-AT_2 antibody, pre-absorbed with their respective AT_1 or AT_2 octapeptide/BSA complex-coated CNBr-activated Sepharose 4B beads. Following the incubation time, membranes were washed thrice in PBS – Tween buffer, pH 7.2, treated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit IgG antibody (diluted to 1:1000 in PBS – Tween buffer, pH 7.2) and the reactions visualized by treatments with DAB tablets reconstituted in distilled water. Membranes were allowed to air-dry, photographed and relative molecular weights and pIs estimated using peroxidase-conjugated Bio Rad broad range molecular weight and pI standards, which were analyzed under identical conditions in parallel to the protein samples.

RESULTS

Localization of the AT_1 and AT_2 receptors in adrenal tissues of SD rat and *M. crassus*

The expression and zonal distribution of the AT_1 and AT_2 receptors was immunohistochemically investigated and compared in both SD rats and *M. crassus*.

Of the different adrenal areas, light microscopy revealed

that both AT_1 and AT_2 receptor expression was primarily associated with cortical zones, and to a relatively much lesser extent in the medulla, in both animal models. Nonetheless, a uniformed labeling of the two receptor types was only evident within the deep region of the cortical zona fasiculata and the medulla of *M. crassus* (Figure 1). Among adrenal cortical zones in SD rat, intense labeling with the anti-AT_1 receptor antibody was selectively evident in both the zona glomerulosa and the zona reticularis and was also observed among scattered cells within the zona fasiculata, which were otherwise uniformly marked by their less pronounced labeling pattern (Figures 2A and C). Labeling with the anti- AT_2 antibody was selectively prominent in confined areas outlining the capillary sinusoids separating the cell cords of the zona fasiculata, but was notably of faded intensity among all cellular constituents of the three cortical zones (Figures 3A and C). In contrast, labeling of *M. crassus* adrenal with either anti-AT_1 or anti-AT_2 antibodies resulted in a similar pattern, in which apparent labeling was observed within the deep region of the zona fasiculata (Figures 1B and D). Within this area, the labeling appeared to be more intense compared to the less intense labeling observed within the zona glomerulosa, and the much faded staining of the outer region of the zona fasiculata or the zona reticularis, using either antibodies (Figures 2B and D; 3B and D).

Similarly, distinct labeling patterns of the SD rat adrenal medulla were observed with the anti-AT_1 and the anti-AT_2 antibodies. As shown in Figure 2E, labeling of high intensity with the anti-AT_1 was selectively confined to ganglion cells as well as the cell-surface of few chromaffin cells, but was of faded intensity in both cell types with the anti-AT_2 antibody (Figure 3E). Conversely, intense to moderate labeling was equally observed with both antibodies among ganglion cells and the cytoplasm of several, but not all, chromaffin cells constituting the adrenal medulla of *M. crassus* (Figures 2F and 3F). It is noteworthy that none of the specific cortical and medullary labeling patterns were observed with adrenal sections, treated with aliquots of the anti-AT_1 antibody pre-absorbed with an AT_1 octapeptide/BSA complex (Figures 2G and H) or with anti-AT_2 antibody pre-absorbed with an AT_2 octapeptide/BSA complex (Figures 3G and H), of either animal models.

Structural characterization of the AT_1 receptor expressed in SD rat and *M. crassus* adrenal tissues

The anti-AT_1 antibody was utilized (at a dilution 1:500) in probing whole adrenal solubilized proteins (120 µg), which were either untreated or treated with Endo-F and resolved by 2-D Western blotting. As judged by 2-D Western blotts conducted in the absence of Endo-F treatments, the reactivity of the anti-AT_1 antibody was selectively targeted towards a major 74.1 kDa component, in addition to another minor 69.2 kDa component, in both SD rat (Figure 4A) and *M. crassus* (Figure 4B) adrenal lysates. However, each of these components was constituted by a number of equal-sized molecular isoforms that express distinct patterns of charge microheterogeneity in each animal model. In SD rat, the 74.1 kDa component was constituted by two major acidic charge variants with pIs of 6.2 and 6.3 and three less-prominent isoforms with pIs of 5.9, 6.1 and 6.6 (Figure 4A), whereas its counterpart in *M. crassus* resolved into a molecular cluster exhibiting both size and charge microheterogeneity (Figure 4B). This cluster included six 74.1 KDa isoforms with distinct pIs of 5.0, 5.1, 5.2, 5.3, 5.5 and 5.7 in addition to three minor 76.0 kDa charge variants with pIs of 5.7, 6.1 and 6.2. Similarly, the 69.2 kDa component was focused as a single fuzzy spot with a pI of 6.3 in SD rat (Figure 4A), whereas its counterpart in *M. crassus* resolved into a cluster of size and charge variants of 69.2 to 72.3 kDa in the pI range of 5.4 to 5.6 (Figure 4B).

The putative association of the 74.1 and 69.2 kDa components with oligosaccharides was investigated by testing their susceptibility to Endo-F treatments and analyses 2-D Western blotting. Treatments of the SD rat and *M. crassus* adrenal lysates with 200 mU of Endo-F reduced the extensive molecular weight and acidic charge microheterogeneity expressed by the untreated 74.1 kDa and 69.2 kDa components into homogeneous spots, with an apparent shift in molecular weight as well as charge towards relatively more basic pIs, in both animal models (Figures 4C and D). Given the known specificity of Endo-F in cleaving linkages in the core of complex- and high-mannose-type N-linked glycans (Elder and Alexander, 1982), the shift towards a lower molecular and basic pI was consistent with the removal of complex-type glycan units carrying variable acidic moieties. In SD rat, the deglycosylated targets of the anti-AT_1 antibody focused as four 40 to 44 kDa components, each with an identical pI of 7.1 to 7.2 (Figure 4C). Interestingly, the deglycosylated targets of the anti-AT_1 antibody exhibited in *M. crassus* a much simpler pattern and focused as a major 41 kDa and a minor 42 kDa components, each with an identical pI of 7.0 (Figure 4D). It is noteworthy that none of the glycosylated or deglycosylated components were observed in Western blots, of either animal models, analyzed under identical conditions but probed by aliquots of the anti-AT_1 antibody, pre-absorbed with AT_1 octapeptide/BSA complex-coated CNBr-activated sepharose 4B beads.

Structural characterization of the AT_2 receptor expressed in SD rat and *M. crassus* adrenal tissues

The anti-AT_2 antibody was utilized (at a dilution 1:500) in probing whole adrenal solubilized proteins (120 µg), which were either untreated or treated with Endo-F and

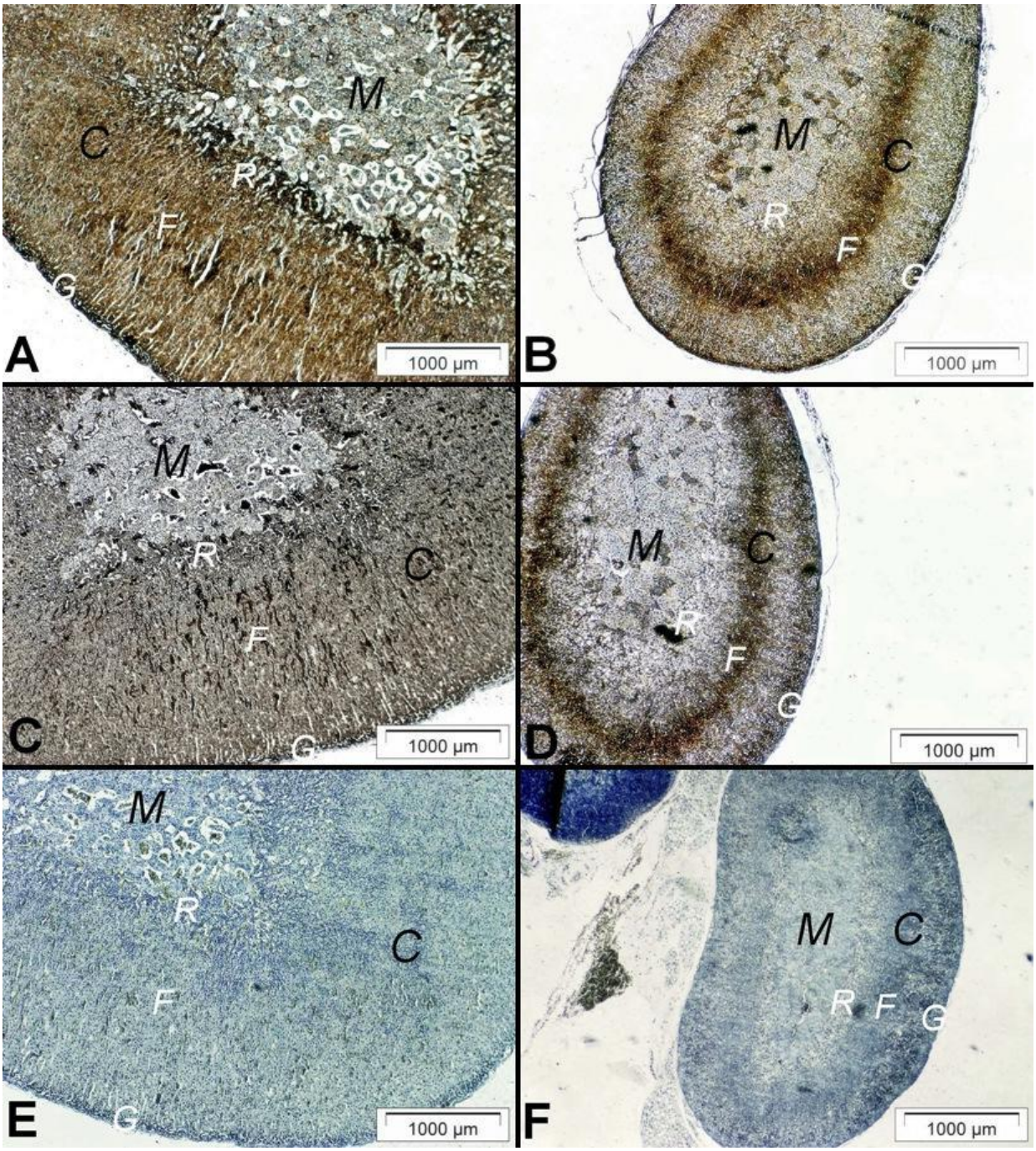

Figure 1. Photomicrograph of SD rat (A, C, E) and *M. crassus* (B, D, F) adrenal glands. Immunohistochemical distribution patterns of AT_1 (A, B) and AT_2 (C, D) receptors. (C) cortex, (G) zona glomerulosa, (F) zona fasciculata, (R) zona reticularis, (M) medulla. No specific labeling with either the anti-AT_1 or the anti-AT_2 antibodies pre-absorbed with their respective AT_1 or AT_2 octapeptide/BSA complex-coated CNBr-activated Sepharose 4B beads (E, F). X40, Bar = 1000 µm.

resolved by 2-D Western blotting. Figure 5 shows that, in the absence of Endo-F, the reactivity of the polyclonal anti-AT_2 receptor antibody were selectively targeted towards a 71.3 kDa component, in both SD rat and *M. crassus*. This component was constituted by five equal-sized acidic isoforms exhibiting charge microheterogeneity in the pI range of 5.2 to 5.6 (Figure 5A), whereas its counterpart in *M. crassus* resolved into a molecular cluster exhibiting both size and charge microheterogeneity (Figure 5B). This cluster included five 70.2 kDa acidic isoforms in the pI range of 5.0 to 5.4, in addition to 71.3, 72.1 and 77.4 kDa components, each being focused as a fuzzy

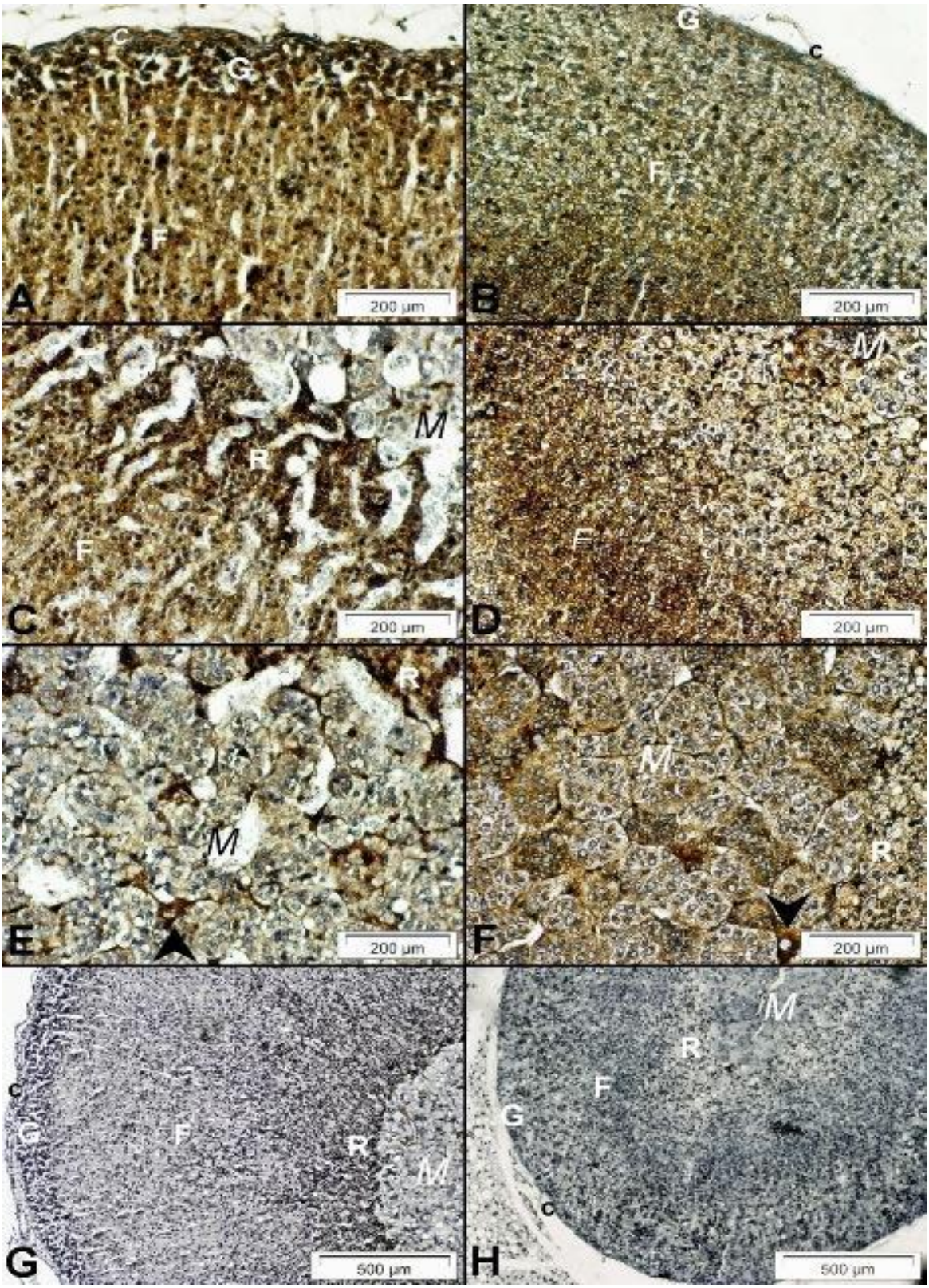

Figure 2. Immunohistochemical localization of the AT_1 receptor in the adrenal tissues of SD rat (A, C, E; X200, G; X100) and *M. crassus* (B, D, F; X200, H; X100). (*c*) capsule, (C) cortex, (G) zona glomerulosa, (F) zona fasciculata, (R) zona reticularis, (M) medulla, (arrowhead) ganglion cells. No specific labeling was observed in the adrenal of SD rat (G) or *M. crassus* (H) labeled with the anti-AT_1 antibody pre-absorbed with an AT_1 octapeptide/BSA complex. A-F: Bar = 200 µm, G and H: Bar = 500 µm.

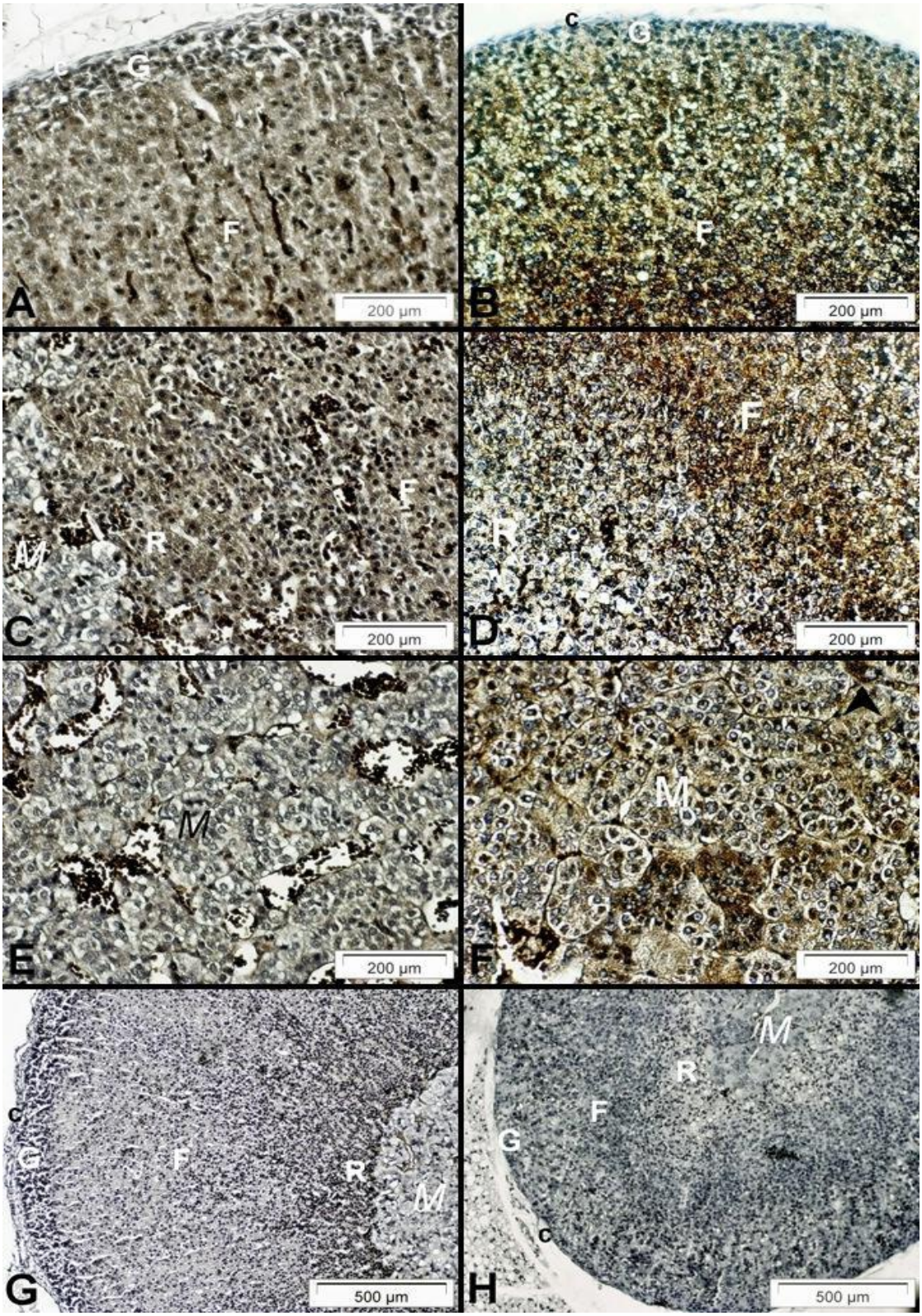

Figure 3. Immunohistochemical localization of the AT_2 receptor in the adrenal tissues of SD rat (A, C, E; X200, G; X100) and *M. crassus* (B, D, F; X200, H; X100). (*c*) capsule, (C) cortex, (G) zona glomerulosa, (F) zona fasciculata, (R) zona reticularis, (M) medulla, (arrowhead) ganglion cells. Note the nonspecific labeling of erythrocytes located within cortical and medullary sinusoids in SD rat adrenal (C, E). No specific labeling was observed in the adrenal of SD rat (E) or *M. crassus* (F) labeled with the anti- AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex. A-F: Bar = 200 µm, G and H: Bar = 500 µm.

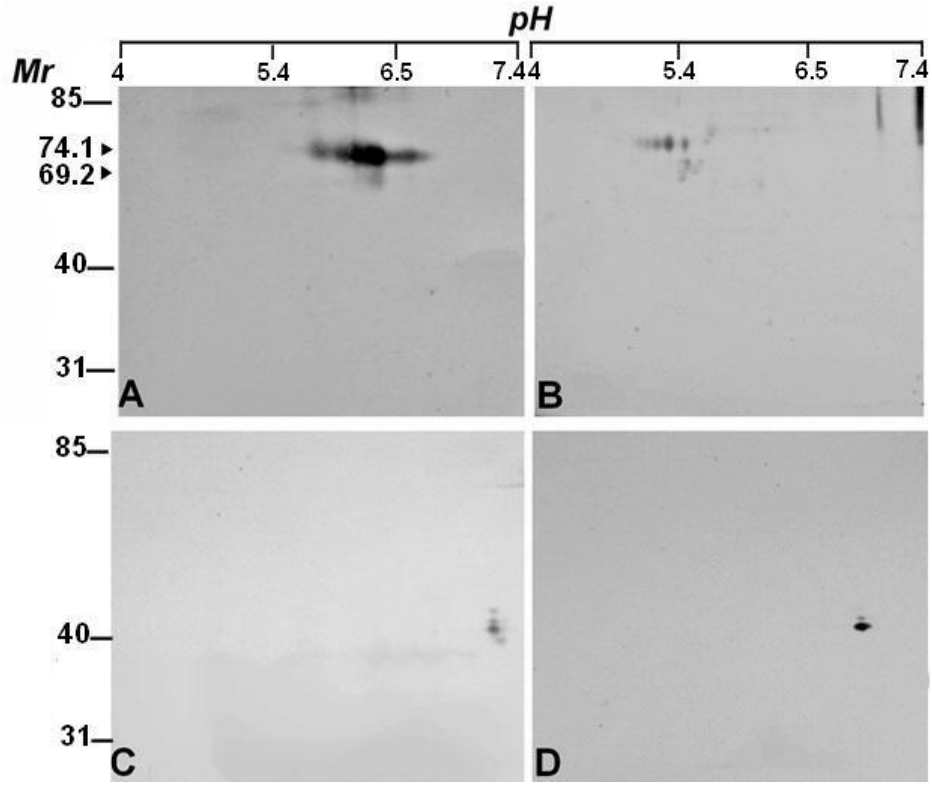

Figure 4. Western blots of two-dimensional SDS-PAGE analysis of AT_1 receptor isoforms expressed in SD rat (A and C) and *M. crassus* (B and D) adrenal glands. (A and B) untreated, (C and D) Endo-F-treated. Positions of Bio Rad 2-D SDS-PAGE isoelectric points and molecular weight (Mr X 10^{-3}) standards are indicated. Also shown are estimated molecular weights of the glycosylated AT_1 receptor isoforms.

spot with a pI of about 5.4. In addition, a 66.8 kDa component, which focused as multiple spots within the pI ranges of 5.2 to 5.6, and a 58.2 kDa component, which also focused as multiple spots within the pI ranges of 5.0 to 5.5, were observed in SD rat and *M. crassus*, respectively. Following treatments with Endo-F, the microheterogeneous pattern exhibited by the 71.3 kDa, in addition to either the 66.8 kDa or the 58.2 kDa components, disappeared and was replaced by two 42 kDa charge variants with pIs of 6.7 and 7.0 in SD rat (Figure 5C) and two 41 kDa spots with pIs of 6.8 and 7.2 in *M. crassus* (Figure 5D), which apparently represent the completely deglycosylated AT_2 receptor forms in both animal models. In both SD rat and *M. crassus*, none of the glycosylated or deglycosylated components were observed in Western blots analyzed under identical conditions but probed by the anti-AT_2 antibody pre-absorbed with AT_2 octapeptide/BSA complex-coated CNBr- activated Sepharose 4B beads.

DISCUSSION

Employing specific polyclonal anti-AT_1 and anti-AT_2 antibodies, selective zonal expression patterns of the AT_1 and AT_2receptor types were immunohistochemically evident in the adrenal gland of both SD rats and *M. crassus*. These specific labeling patterns were not detectable utilizing either antibodies, pre-absorbed with their respective synthetic AT_1 and/or AT_2 receptor octapeptides, thus confirming the specificity of both antibodies in binding their respective Ang II receptor type. Within the adreno-cortical region of both animal models, the relatively more intense AT_1 receptor labeling among zona glomerulosa cells was paralleled by the faded labeling of the AT_2 receptor type. This imbalanced expression of both receptor types within this zone is in direct agreement with previous reports on different mammalian species, including humans (Zhuo et al., 1996), employing immunohistochemical (Giles et al., 1999) or radioligand binding and autoradiography (Lehoux et al., 1997) to detect the expressed polypeptide, or *in situ* hybridization and PCR techniques detecting intra-cytoplasmic mRNA (Gasc et al., 1994; Wang et al., 1998) of both receptors. In all incidences, irrespective of the species tested or variations in detection methods, the zona glomerulosa seemed to be the common structural denominator for high density AT_1 receptor expression,

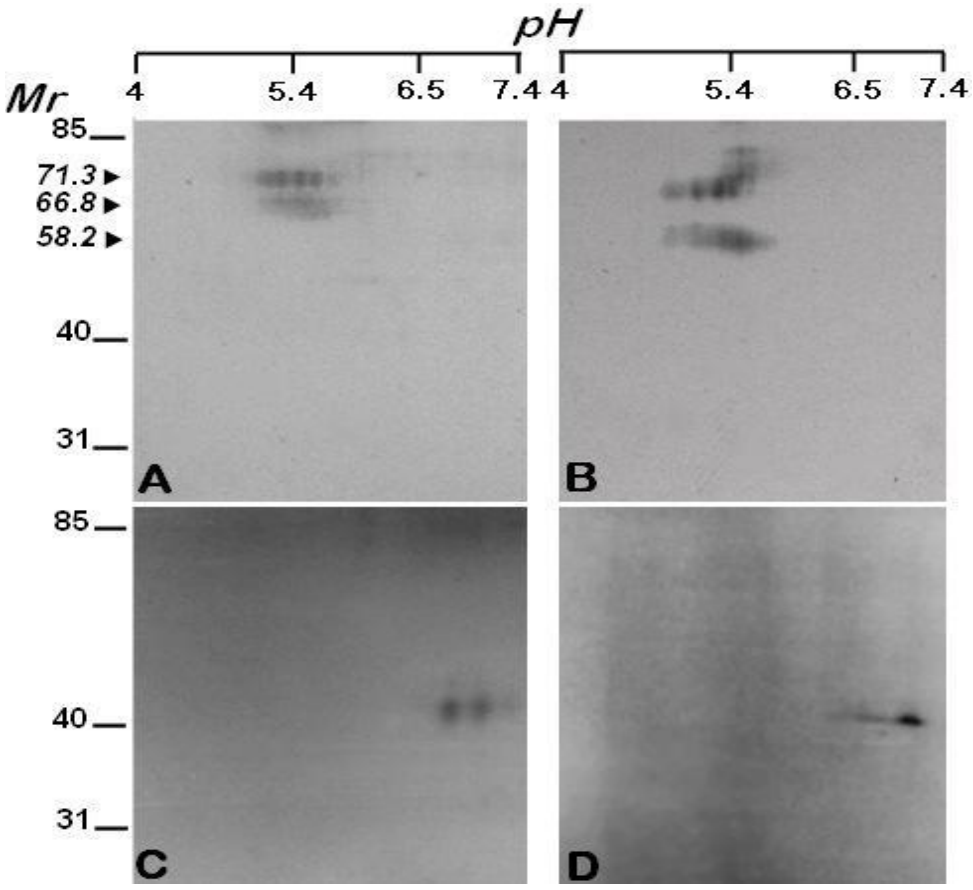

Figure 5. Western blots of two-dimensional SDS-PAGE analysis of AT_2 receptor isoforms expressed in SD rat (A and C) and *M. crassus* (B and D) adrenal glands. (A and B) untreated, (C and D) Endo-F-treated. Positions of Bio Rad 2-D SDS-PAGE isoelectric points and molecular weight (Mr X 10^{-3}) standards are indicated. Also shown are estimated molecular weights of the glycosylated AT_2 receptor isoforms.

and may thus be confirmed as the primary adrenal site for the regulatory role of Ang II in the synthesis and release of aldosterone (Gupta et al., 1995; Kakiki et al., 1997; Belloni et al., 1998) in all mammals, including desert rodents.

As suggested earlier, the putative significance of the AT_2 receptor, expressed with low density in the zona glomerulosa, in either counterbalancing the AT_1–mediated aldosterone-releasing and/or mitogenic func-tions (Bottari et al., 1992; Nakajima et al., 1995; Tanabe et al., 1998; AbdAlla et al., 2001), or in targeting the Ang II-mediated secretion of endogenous ouabain (Laredo et al., 1997; Shah et al., 1998), may also not be excluded.

As revealed in the present investigation, a clear inter-species difference in AT_1 and AT_2 receptor expression was observed among cells within the zona fasiculata/ zona reticularis. In these deep cortical zones, the low to moderate expression of the two receptor types in SD rats was contrasted by their intense co-expression in the deep region of the zona fasiculata in *M. crassus*. As reported in previous studies (Zhuo et al., 1996; Allen et al., 2000; Dinh et al., 2001), the observed low expression of both receptors in the zona fasiculata/zona reticularis of SD rats is typical of rodents, as well as most mammals studied, where Ang II signaling through either receptor types is apparently not directly implicated in regulating corticosterone synthesis or release (Lehoux et al., 1997; Tanabe et al., 1999; Aguilar et al., 2004). However, apparently these cortical zones are targets of inter-species discrepancies, since as observed in *M. crassus*, and contrary to most mammals, cells of both zones express high density of both AT_1 and AT_2 receptors also in canine, bovine as well as human adrenal glands (Ouali et al., 1993; Zhuo et al., 1996; Allen et al., 2000). Interestingly, the level of expression of both receptor types, within these zones, seems to be selectively prone to various modulatory signals in lab-reared rats stimulated by different physiological conditions. As revealed by immune-flourescence, autoradiography and *in situ* hybridization, an up-regulatory shift in both AT_1 and AT_2 receptor expression within the zona fasiculata/zona reticularis was observed in rats kept under dietary sodium restriction

(Lehoux et al., 1997; Wagner et al., 1998). However, within these cortical zones, this up-regulatory shift was selective for the AT_1 receptor, with no alterations in the level of expression of the AT_2 receptor, in rats subjected to sodium loading (Sun et al., 1996), stimulated levels of endogenous Ang II (Wagner et al., 1998) or water deprivation (Chatelain et al., 2003). On the other hand, in aldosterone-treated rats, the highest adrenal AT_1 receptor expression was shifted from the zona glomerulosa to the zona fasciculata, and was paralleled by a significant reduction in the level of expression of the AT_2 receptor in all regions of the gland (Wang et al., 1998). Collectively, within deep adrenal cortical zones, the exact functional significance of this species-dependent or experimentally-induced variability in levels of the expressed Ang II receptor type, is still to be elucidated. Nonetheless, given the suggested implication of an Ang II-dependent phospholipase D-mediated cortisol secretion in bovine zona fasiculata cells (Rabano et al., 2004), its is tempting to speculate that the observed high level of AT_1 and AT_2 receptors, co-expressed in the zona fasiculata/zona reticularis in *M. crassus*, may represent a significant adaptive variant in selectively shaping Ang II-mediated adrenal functions in rodents coping with a stressful arid environment.

In accordance with previous reports (Israel et al., 1995; Zhuo et al., 1996; Dinh et al., 2001), the expression of both AT_1 and AT_2 receptors was demonstrable among selective chromaffin and ganglion cell bodies in the adrenal medulla of both SD rat and *M. crassus*. However, in SD rat, the labeling of both receptor types was confined to a relatively few number of chroma-ffin/ganglion cells, and was marked by a high intensity labeling of the AT_1 receptor compared to the faded labeling of the AT_2 receptor type. In *M. crassus*, labeling of both receptors was equally of a moderate-high intensity and involved more frequent, but not all, chromaffin/ganglion cells. Although the predominant biological effects of Ang II within the adrenal medulla is believed to be mediated primarily via the AT_1 receptor type (Wong et al., 1990; Armando et al., 2001), early autoradiographic (Israel et al., 1995; Allen et al., 2000) and immunoflourescence (Jezova et al., 2003) studies have indicated that the AT_1 receptor represents only 5 to 10% of the total number of Ang II receptors and that, with the exception of humans (Zhuo et al., 1996), AT_2 receptors predominate in the medulla of all mammals, including rats.

Compared to these reports, the discrepancy in the ratio of the expressed AT_1/AT_2 receptors in the medulla, as observed in SD rat in the present immunohistochemical investigation, may be attributed to variations in the sensitivity and specificity of the detection methods and/or the utilized antibodies. Nonetheless, both receptor types have been suggested to act synergistically, yet through independent pathways, in regulating tyrosine hydroxylase transcription, and subsequently controlling basal and stress-induced adrenomedullary catecholamine synthesis and release (Jesova et al., 2003; Armando et al., 2004). Indeed, the distinct pattern of AT_1/AT_2 receptor expression observed in *M. crassus*, as well as humans (Cavadasa et al., 2003), still suggest that this ratio may be governed by species-dependent mechanisms, which may prove significant in balancing the proposed AT_1/AT_2 receptor cross-talk to regulate secretagogue activities of the adrenal medulla under different physiological/environmental conditions.

A novel observation of the present investigation was stemmed from the structural assessment of the expressed AT_1 and AT_2 receptors by 2-D Western blotting. Employing the anti-AT_1 receptor antibody in this assay, in the presence and absence of Endo-F, revealed that the reactivity of the antibody was selectively targeted towards a major 74.1 kDa fully-glycosylated component, in addition to another minor 69.2 kDa partially-glycosylated component, in both SD rat and *M. crassus*. Under similar conditions, the reactivity of the polyclonal N-19 anti-AT_2 receptor antibody was selectively targeted towards a fully-glycosylated 71.3 kDa component, in addition to another partially-glycosylated 66.8 kDa component in SD rat or a 58.2 kDa component in *M. crassus*. However, each of these components was apparently constituted by a number of molecular isoforms that express distinct patterns of charge and/or size micro heterogeneity unique to each receptor type and/or animal model. The estimated range of molecular weight for the constituent AT_1 and AT_2 receptor isoforms in both species was comparable to the predominant 60 to 78 KDa Ang II receptors previously detected in the adrenal gland (Belloni et al., 1998), as well as other tissues in different species (Marsigliante et al., 1996; Servant et al., 1996; Giles et al., 1999; Al-Qattan et al., 2006), thus confirming the specificity of the antibodies used in this study in binding their respective Ang II receptor type. Given that the anti-AT_1 receptor antibody used is not subtype-specific, the expected co-detection of the structurally homologous AT_{1A} and AT_{1B} receptor subtypes (Sandberg et al., 1992; Inagami et al., 1994) may account, at least in part, for the micro heterogeneous pattern observed in SD rat AT_1 receptors. The putative occurrence of a subtype heterogeneity for the AT_2 receptor as reported in early reports (Reagan et al., 1996) has been recently ruled-out (Feng et al., 2005), and thus may not contribute to the micro heterogeneous pattern observed in SD rat AT_2 receptors. Nonetheless, the reported inter-species conservation of sequences within N-terminal extra-cellular domains of either AT_1 or AT_2 receptor types, as revealed in various mammalian AT_1 and AT_2 receptor cDNAs (Inagami et al., 1994; Feng and Douglas, 2000), may provide the basis for the detection of cross-reacting homologues to AT_1 and AT_2 polypeptides in *M. crassus*.

As revealed by Endo-F treatments, and given its known specificity in cleaving linkages in the core of N-linked glycans (Elder and Alexander, 1982), it seems plausible that the differential addition of high-mannose type or complex type N-linked glycan units, of different sizes and compositions, is a major source of the intra- and

inter-species structural variability of the expressed AT_1 and AT_2 receptor isoforms. This assumption is supported by the observed collapsed micro heterogeneity, as well as the obvious shift towards lower molecular weight, following the enzymatic deglycosylation of the mature, fully-expressed AT_1 and AT_2 receptors in both animal models. Also supportive of this assumption is the documented presence of three (Murphy et al., 1991) and five (Servant et al., 1996), highly-conserved N-linked glycosylation sites along the AT_1 and AT_2 receptor sequences, respectively. Interestingly, the deglycosylated AT_1 receptor forms in SD rat were represented by a pattern of multiple components in the molecular weight range of 40 to 44, which are likely representing allelic variants corresponding to the structurally-related subtypes known to constitute the AT_1 receptor family (Dinh et al., 2001). In contrast, AT_1 subtype heterogeneity was apparently limited in *M. crassus*, since the deglycosylated receptor pattern was represented by only a major 41 kDa and a minor 42 kDa components. In both animal models, the deglycosylated AT_2 receptor pattern was represented by two 41 to 42 kDa components, which may reflect the lack of subtype heterogeneity of the AT_2 receptor gene, as previously suggested by Feng et al. (2005). It is noteworthy that the predominant molecular weight range of 41 to 42 kDa estimated for the deglycosylated receptors was in direct agreement with the predicted 41 to 42 kDa of the protein back-bones of cDNA sequences of both AT_1 and AT_2 receptor types, in various mammals (Murphy et al., 1991; Mukoyama et al., 1993; Feng et al., 2005).

The observed expression of structurally-distinct AT_1 and AT_2 receptor isoforms, dictated genetically as well as by differential degrees of glycosylation, clearly supports early biochemical and pharmacological studies indicating the extensive heterogeneity of Ang II receptors in different tissues and/or species (Chiu et al., 1994; Zhuo et al., 1996; Dinh et al., 2001). Apparently, differential tissue-specific and/or species-specific post-translational regulatory mechanisms would be decisive in the expression of differentially glycosylated AT_1 and AT_2 receptor isoforms within the adrenal tissue of SD rat, and may account for the structurally unique spectra of AT_1 and AT_2 receptor isoforms observed in *M. crassus*. The potential for the differential glycosylation state to alter the optimal expression of selective isoforms of either receptor types (Servant et al., 1996; Jayadev et al., 1999) may have important implications in regulating the various adaptive physiological responses mediated by Ang II-receptor interactions in key tissues. In particular, the variable pattern of expression of distinct AT_1 and AT_2 receptor isoforms in the adrenal may add another level of complexity to mechanisms controlling the synthesis and release of aldosterone, cortisol and catecholamines in different species. In this regard, the present observations are indicative of a significant role of AT_1 and AT_2 receptor isoform spectra in differentially fine-tuning adrenal functions in water/electrolyte homeostasis and stress responses in lab-reared and desert rodents.

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