Garlic (*Allium sativum*) modulates the expression of angiotensin II AT$_2$ receptor in adrenal and renal tissues of streptozotocin-induced diabetic rats

Mohamed H. Mansour[*]**, Khaled Al-Qattan, Martha Thomson, Muslim Ali**

Department of Biological Sciences, Faculty of Science, Kuwait University, Kuwait City, Kuwait.
Email: [*]mohamed.shaker@ku.edu.kw

ABSTRACT

The loss of balance between the antagonistic activities of angiotensin II AT$_1$/AT$_2$ receptors has been implicated as a major mediator in the development of hypertension and progressive nephropathy in experimental diabetes. The present study was designed to investigate the potential of garlic to modulate the level of expression of the AT$_2$ receptor in the adrenal and renal tissues of diabetic rats. Three groups of rats were studied after 8 weeks following diabetes induction: normal, streptozotocin-induced diabetic (control diabetic), and garlic-treated diabetic rats. A polyclonal antibody of proven specificity to the AT$_2$ receptor, as verified by Western blotting and employed in immunohistochemical assays, indicated that compared to normal rats, the highest adrenocortical AT$_2$ receptor expression was significantly shifted from the zona glomerulosa to the zona fasciculate/ reticularis, and was significantly reduced in adrenomedullary chromaffin cells of control diabetic rats. In the kidney, STZ treatments were associated with a significant decrease in AT$_2$ receptor expression throughout glomeruli and all cortical and medullary tubular segments. Compared to control diabetic rats, the labeling of the AT$_2$ receptor in the garlic-treated diabetic group was restored among adrenocortical zona glomerulosa cells and adrenomedullary chromaffin cells and significantly reduced in the zona fasiculata, and was also restored in glomeruli and throughout renal cortical and medullary tubular segments, to levels comparable to those observed in normal rats. The capacity of garlic to modulate diabetes-induced AT$_2$ receptor down-regulation may be implicated in restoring the recuperative processes mediated by AT$_2$ receptors, which interfere with the development of hypertension and nephropathy.

Keywords: AT$_2$ Receptor; Garlic; Streptozotocin-Induced Diabetes; Immunohistochemistry

1. INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) is a major regulator of blood pressure and sodium and water homeostasis [1]. The octapeptide angiotensin II (Ang II) is the primary mediator of the RAAS effects. Ang II induces its effects by binding to two major receptor types, AT$_1$ and AT$_2$ [2]. The AT$_1$ receptors are ubiquitously expressed in adult tissues and mediate Ang II-induced vasoconstriction, sodium reabsorption, aldosterone secretion, and cell growth and proliferation [3-5]. The AT$_2$ receptors are expressed abundantly during fetal development, decline after birth and become minimally represented, in comparison with AT$_1$ receptors, in various adult tissues [6]. In these tissues, AT$_2$ receptors target Ang II-induced vasodilatation, apoptosis, antiproliferation, natriuresis and fluid/sodium homeostasis [7-9] and, therefore, can be considered as functional antagonists to the AT$_1$ receptors [10]. Compelling evidence suggest that the alteration in the expression and function of either Ang II receptor type may be the site of regulation that affects the initiation and progression of tissue remodeling in pathophysiological conditions [11-12].

As manifested in diabetic patients and animal models, the hyperglycemia-induced AT$_1$ receptor up-regulation in the adrenal, kidney and other tissues mediates the increase of Ang II local effects in stimulating aldosterone production and over-activating renal sodium transporters causing sodium retention and hypertension [12-14]. Within the kidney, the up-regulated AT$_1$ receptor signaling increases vascular resistance, glomerular capillary pressure and mechanical stretch-induced glomerular injury, and stimulates production of reactive oxygen species and extracellular matrix in the mesangium and tubulo-interstitium [2,14-16], which collectively perpetrates the development of diabetic nephropathy. Interestingly, early diabetes exerts an opposite influence on AT$_2$ receptor and significantly decreases its expression in glomeruli and tubular segments of the kidney [17]. The

down-regulation of intrarenal AT_2 receptor expression implies the subsequent decrease in AT_2-mediated mechanisms that may oppose the detrimental effects of AT_1 receptor signaling, including inhibition of cell growth [18,19] as well as NO/cGMP-dependent vasodilatation and inhibition of renal sodium transporters [20-22]. This could result in an amplification of AT_1-mediated effects on vasoconstriction, glomerulosclerosis or cell hypertrophy, and thus contribute further to progressive injury in diabetic nephropathy [17,23].

Current views suggest that pharmacologically active blockers of the RAAS may be beneficial in the management of diabetic complications including hypertension and nephropathy [24,25]. Angiotensin receptor blockers and angiotensin-converting enzyme inhibitors are used to treat hypertension and to improve renal function in diabetes [26]. Based on the suggestion that a balance between AT_1- and AT_2-receptor-mediated cell-signaling events may be a determinant of progression rate in diabetic nephropathy [17], a debate of preferential use of angiotensin receptor blockers over angiotensin-converting enzyme inhibitors have been initiated. The basis of such preference lies in that angiotensin-converting enzyme inhibitors lower Ang II production, leading to reduction in the functions of AT_1 and AT_2 receptors, whereas angiotensin receptor blockers selectively block AT_1 receptors and leave the AT_2 receptors unopposed to function [26]. Apparently, restoration of AT_2 receptor activity, in early diabetes, may add more therapeutic efficacy in protecting against the development of renal morphologic and functional changes seen during the progression of hypertension and nephropathy.

Currently, the reliance on natural products is gaining popularity in combating various physiological threats, including diabetic complications, associated with the dysregulation of the RAAS [27]. In experimental diabetes, garlic, used either as a whole raw extract or as isolated organosulphur constituents, is one of the best studied herbal remedies with documented anti-diabetic activities [28-32]. These activities include lowering serum glucose and cholesterol levels [30,31,33], inhibiting Ang II and promoting vasodilatation [29], preventing adrenal hypertrophy [34], attenuating oxidative stress [35], inhibiting the formation of advanced glycation endproducts [36], ameliorating hypertension and delaying the progression of diabetic nephropathy [31,32,35,36]. Nonetheless, the potential of garlic in targeting the level of expression of Ang II receptors is, as yet, not fully explored. In a recent study, we reported on the efficacy of an aqueous extract of raw garlic in modulating the up-regulated expression of Ang II AT_1 receptors in the adrenal and renal tissues in early diabetes [37]. In the present study, immunohistochemical evidence is presented for the capacity of garlic treatments to modulate the down-regulated expression of Ang II AT_2 receptors in adrenal and renal tissues of streptozotocin-induced diabetic rats.

2. MATERIALS AND METHODS

2.1. Antibodies and Reagents

Except where noted, all chemicals were reagent grade and purchased from Sigma Chemical Company (St. Louis, MO, USA). 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, peroxidase-conjugated molecular weight standards and nitrocellulose membranes (0.45 μ) were obtained from BioRad (Richmond, CA, USA). An eight amino acid peptide, corresponding to amino acids 10 - 17 (Thr-Ser-Arg-Asn-Ile-Thr-Ser-Ser) of the first extracellular domain, as deduced from the published AT_2 receptor cDNA sequence [38], was synthesized manually on the base-labile linker 4-(hydroxymethyl)-benzoyloxymethyl and supplied by The Protein/DNA Technology Center (The Rockefeller University, NY, USA). The octapeptide was conjugated to bovine serum albumin (BSA) and coupled to CNBr-activated Sepharose 4B as described previously [39]. An aqueous garlic extract (500 mg/ml) was prepared from locally purchased, peeled garlic cloves by homogenization in cold, sterile 0.9% NaCl for 12 min followed by filtration and centrifugation at 200 g for 10 min to remove insolubles as previously described [30].

2.2. Animals

Adult male Sprague-Dawley (SD) rats (England) weighing 150 - 200 gm were bred and raised at the animal house of the Department of Biological Sciences, Kuwait University, and used in the present investigation. Rats were given standard laboratory chow (170 mMol Na^+/kg) and water ad libitum and kept under standard conditions (23°C ± 2°C, 12 h light, 12 h darkness). Diabetic rats, induced by injecting streptozotocin (STZ, 60 mg/kg) intraperitoneally into overnight fasting rats, were determined to be diabetic if they had elevated plasma glucose concentrations ≥ 300 mg/dL five days post-injection, as described previously [40]. STZ-induced diabetic rats were divided into two groups (n = 8): group 1, the control diabetic group, received daily intra-peritoneal injections with saline, and group 2, the garlic-treated group, received daily intraperitoneal injections with 500 mg/kg of the garlic extract. All experiments were carried out at 8 weeks after diabetes induction. All animal procedures

were performed according to the guidelines for animal experimentation of Kuwait University, Faculty of Science.

2.3. Solubilization of Cell-Membranes and Endoglycosidase Treatments

Left adrenal glands and kidneys, excised from anesthetized normal rats, were individually homogenized, solubilized and extracted in 10 mM Tris/HCl, pH 8.0 containing 2 mM phenylmethylsulfonyl fluoride and 2% deoxycholate 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 at 100.000 g for 1 h, and their protein content determined [41] using bovine serum albumin (BSA) in the same buffer as a standard. Cell-membrane lysates of both organs (120 µg protein) were separately precipitated with 20% trichloroacetic acid and ice-cold acetone for 1 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 20 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.

2.4. Western Blotting

Aliquots of solubilized cell-membrane lysates (120 µg protein) collected from adrenal and/or kidney tissues of normal SD rats, and either kept untreated or treated with Endo-F, were individually precipitated, reconstituted in sample buffer and resolved with 10% resolving gels, under non-reducing or reducing conditions, by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [42]. Resolved protein samples were electrophoretically transblotted to 0.45 µ 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 the anti-AT$_2$ receptor antibody (diluted to 1:500 in PBS-Tween buffer, pH 7.2) by incuba-

tion for 2 h at room temperature and then overnight at 4°C with constant agitation. Control blots were prepared by substituting the specific antibody with an equal volume of the anti-AT$_2$ antibody preabsorbed with an 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 metal-enhanced 3,3-diaminobenzidine (DAB) tablets reconstituted in distilled water. Membranes were allowed to air-dry, photographed and relative molecular weights estimated using peroxidase-conjugated Bio Rad broad range molecular weight standards, which were analyzed under identical conditions in parallel to the protein samples.

2.5. Preparation of Tissue Sections

Left adrenal glands and kidneys of anesthetized normal, control diabetic and/or garlic-treated diabetic rats were individually excised and placed in vials containing 3 ml of Bouin's fixative for 24 h - 48 h at room temperature. The tissues were carried through a routine paraffin embedding technique, which included dehydration through a series of ethanol concentrations 50%, 70%, 90% and 100%, clearing in toluene, embedding in paraffin wax, and finally sectioning of the paraffin blocks into 3 µm - 4 µm thick sections 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.

2.6. Labeling of Tissue Sections with Anti-AT$_2$ Antibody

Tissue sections were examined for AT$_2$ receptor distribution by an indirect immunohistochemical labeling technique. Tissue sections were de-waxed 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 µl of the anti-AT$_2$ receptor antibody (diluted to 1:100 with PBS, pH, 7.2). After several washes with 200 µl PBS, pH 7.2, the sections were incubated for 45 min with 300 µl 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 µl of fast DAB reconstituted in water. All sections were counter stained with 100 µl haematoxylin (Gill #1) for 1 min and examined by light microscopy for positive labeling of cells expressing AT$_2$ receptors. Control sections were identically stained by replacing the specific antibody with anti-AT$_2$

antibody preabsorbed with an AT_2 octapeptide/ BSA complex-coated CNBr-activated Sepharose 4B beads. Microphotographs were taken using Olympus AH-3 automated microscope (Tokyo, Japan), equipped with an Olympus Vanox camera. Slides were quantitatively examined by using the Image-Pro Plus 5.1 software program (Media Cybernetics, Silver Spring, MD). Each slide was analyzed for AT_2 receptor labeling in the adrenal cortex and medulla, and in the renal cortex and outer and inner medulla, with three separate fields viewed in each region and four independent samples for each group. Data were expressed as mean values ±SE and statistically analyzed using SPSS, Version 17. Groups were compared with one-way ANOVA and $p < 0.05$ was considered to be significant.
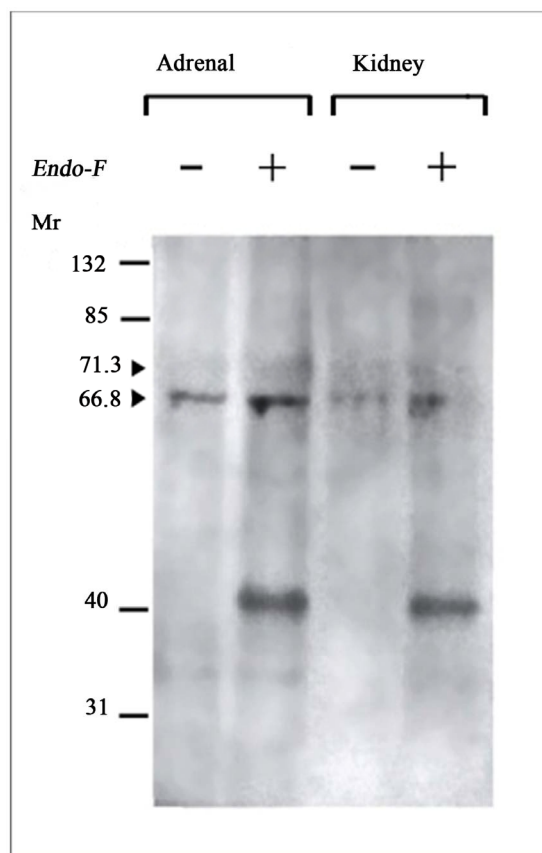
3. RESULTS

3.1. Characterization of the AT_2 Receptor Expressed in Adrenal and Renal Tissues

A polyclonal anti-AT_2 receptor antibody was utilized (at a dilution of 1:500) in probing whole adrenal gland and kidney solubilized proteins (120 μg) of normal rats, which were either untreated or treated with Endo-F and resolved by SDS-PAGE. As judged by Western blotting conducted in the absence of Endo-F treatments, the reactivity of the polyclonal anti-AT_2 receptor antibody was selectively targeted towards 71.3 kDa and 66.8 kDa components in both adrenal gland and kidney lysates (**Figure 1**), analyzed under either reducing or non-reducing conditions.

Following mild treatments with 20 mU of Endo-F and analyses by Western blotting, a major 41 kDa band appeared in addition to the original 71.3 kDa and 66.8 kDa components in the lysates of both organs. It is noteworthy that none of these components were observed in Western blots of either organ analyzed under identical conditions but probed by anti-AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex-coated CNBr-activated Sepharose 4B beads.

3.2. AT_2 Receptor Expression in the Adrenal

The expression and zonal distribution of the AT_2 receptor was immunohistochemically investigated and compared in the adrenal gland of normal, control diabetic and garlic-treated diabetic rats by using a standard indirect labeling technique of de-waxed tissue sections. These sections were treated with aliquots of the polyclonal anti-AT_2 receptor antibody and counter-stained with hematoxylin. Control sections, stained with anti-AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex-coated CNBr⁻ activated Sepharose 4B beads, were investigated in parallel to allow comparisons of selective tissue labeling. Among adrenal cortical zones in normal


Figure 1. Western blot of SDS-PAGE analysis of the AT_2 receptor expressed in adrenal and renal tissues of SD rats. Whole adrenal gland and kidney solubilized proteins (120 μg) of normal rats were either untreated (–) or treated (+) with Endo-F, resolved by SDS-PAGE, blotted and probed by a polyclonal anti-AT_2 receptor antibody. Positions of Bio Rad molecular weight (Mr $\times 10^{-3}$) standards and the estimated molecular weights of the untreated and Endo-F-treated AT_2 receptor are indicated.

rats, light microscopy revealed that moderate labeling with the anti-AT_2 antibody was selectively evident among virtually all cells in the zona glomerulosa, as well as few scattered cells within the zona fasiculata and the zona reticularis, which were otherwise uniformly marked by their entire lack of labeling (**Figure 2(a)** and **Figure 3(a)**). In striking contrast, no immunohistochemical staining for the AT_2 receptor was detectable in the zona glomerulosa, but was selectively notable among most cells constituting the deep zona fasiculata as well as the zona reticularis in control diabetic rats (**Figure 2(b)** and **Figure 3(b)**). In garlic-treated diabetic rats, AT_2 receptor labeling with moderate intensity was restored among cells of the zona glomerulosa, and was notable in the zona reticularis and the outer region of the zona fasiculata, but particularly faded among most of the deep region of the zona fasiculata (**Figure 2(c)** and **Figure 3(c)**).

Similarly, distinct labeling patterns of the AT_2 receptor were observed in the adrenal medulla of the three rat groups. As shown in **Figure 3**, labeling with high to moderate intensity was confined to few chromaffin cells in both normal (**Figure 3(a)**) and garlic-treated diabetic (**Figure 3(c)**) rats, but involved more frequent chromaffin cells in normal rats, and was almost absent in all chromaffin cells of control diabetic rats (**Figure 3(b)**). None of these specific cortical and medullary labeling patterns were observed with adrenal sections treated
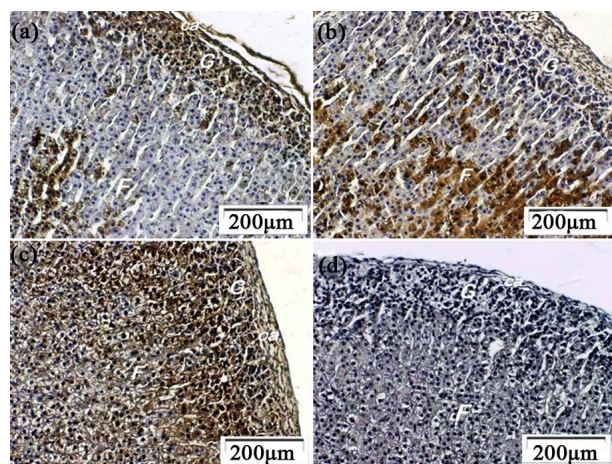
Figure 2. AT_2 receptor expression in the adrenal cortex of normal (A), control diabetic (B) and garlic-treated diabetic (C) rats. (ca) capsule, (G) zona glomerulosa, (F) zona fasciculata. No specific labeling was observed in adrenal tissue sections labeled with the anti-AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex (D). Bar = 200 μm, ×200.
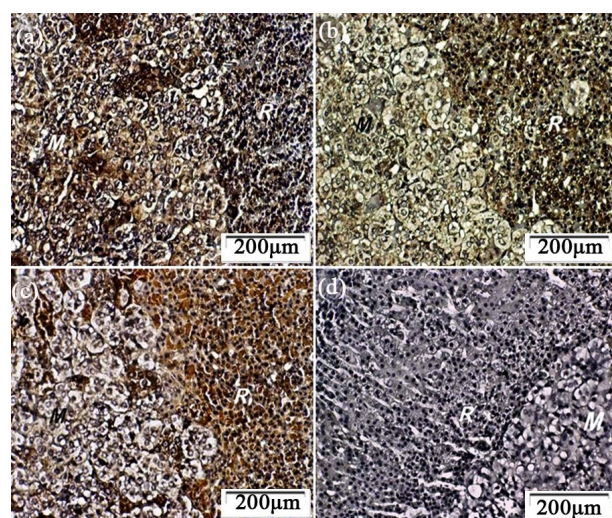
Figure 3. AT_2 receptor expression in the adrenal cortico-medullary region of normal (A), control diabetic (B) and garlic-treated diabetic (C) rats, (R) zona reticularis, (M) medulla. No specific labeling was observed in adrenal tissue sections labeled with the anti-AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex (D). Bar = 200 μm, ×200.

with anti-AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex-coated CNBr⁻ activated Sepharose 4B beads (**Figure 2(d)** and **Figure 3(d)**). As depicted in **Table 1**, quantification of AT_2 receptor staining by computer-based image analysis revealed a significant increase in the expression of AT_2 receptors in the adrenal cortex of control diabetic rats compared with the normal group. The adrenal cortex of garlic-treated diabetic rats was associated with a significant decrease of AT_2 receptor immunostaining compared with control diabetic rats, and comparable to the level observed in the normal group. On the other hand, a significant reduction in AT_2 receptor labeling was observed in the adrenal medulla of control diabetic rats compared with the normal and garlic-treated diabetic rats.

3.3. AT_2 Receptor Expression in the Kidney

In immunohistochemical labeling experiments of kidney sections of the three rat groups, light microscopy revealed that variable patterns of AT_2 receptor labeling were associated with the cortex, the inner stripe of the outer medulla as well as the inner medulla. As depicted in **Figure 4(a)**, cortical nephron segments of normal rats exhibited AT_2 receptor labeling of marked intensity in glomerular endothelial cells and the entire epithelial lining of the distal convoluted tubules, but was uniformly diffused in the epithelial lining of the proximal convoluted tubules. In control diabetic rats, AT_2 receptor labeling was markedly reduced in glomerular endothelial cells, appeared as faded patches at the basolateral side of some of the epithelial cells lining the proximal convoluted tubules, and was evidently lacking among the epithelial lining of the distal convoluted tubules (**Figure 4(b)**). On the other hand, except for the persistent lack of AT_2 receptor labeling in the epithelial lining of the distal convoluted tubules, specific labeling with marked intensity was observed in glomerular endothelial cells and the
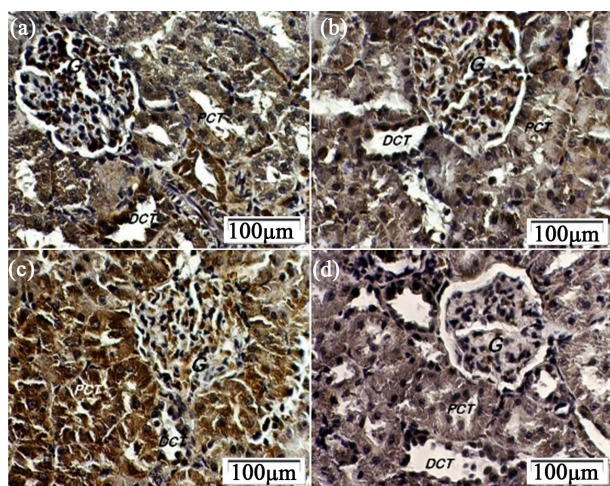
Table 1. AT_2 receptor quantification.

	Normal rats	Control diabetic rats	Garlic-treated diabetic rats
Adrenal			
cortex	2.671 ± 752	$5.890 \pm 635^*$	3.114 ± 110
medulla	7.221 ± 369	$1.265 \pm 223^*$	5.967 ± 776
Kidney			
cortex	5.221 ± 245	$1.523 \pm 447^*$	$12.204 \pm 545^†$
outer medulla	12.567 ± 487	$3.054 \pm 325^*$	13.206 ± 715
Inner medulla	6.321 ± 287	$2.112 \pm 101^*$	5.414 ± 614

Values are means ± SE ($n = 4$). Each value represents the number of pixels that exceeded an arbitrary staining threshold per 300×300-pixel area. $^*P < 0.05$ vs. Garlic-treated diabetic rats and normal rats. $^†P < 0.05$ vs. normal rats.

ABC

entire epithelial lining of the proximal convoluted tubules in garlic-treated diabetic rats (**Figure 4(c)**). As in the cortical regions, distinct labeling patterns of the AT_2 receptor were also observed in the inner stripe of the outer medulla of the three rat groups. In normal rats (**Figure 5(a)**), binding of the anti-AT_2 receptor antibody was evident in the basolateral side of collecting ducts and ascending Henle's loop segments and, to a lesser extent, the basolateral side of collecting tubules, without any obvious involvement of interstitial cells outlining the vasa recta bundles or the epithelial elements of descending Henle's loop segments. Compared to normal rats, AT_2 receptor labeling in control diabetic rats was of significantly reduced intensity in the epithelia of collecting ducts and tubules and ascending Henle's loop segments, and was completely lacking in interstitial cells outlining the vasa recta bundles and the epithelia of descending Henle's loop segments (**Figure 5(b)**). As shown in **Figure 5(c)**, AT_2 receptor labeling was accentuated in the cytoplasm of the entire epithelial lining of collecting ducts and ascending Henle's loop segments and was apparently detectable in interstitial cells outlining the vasa recta bundles, but was absent in collecting tubules and descending Henle's loop epithelia in garlic-treated diabetic rats. In the inner medulla of control and garlic-treated diabetic rats, AT_2 receptor immunostaining was localized to the apical membranes and cytoplasm of inner medullary collecting duct cells (**Figures 6(a)** and **(c)**), whereas in diabetic rats the labeling of these segments was markedly decreased (**Figure 6(b)**).

None of these specific cortical and medullary labeling patterns were observed with renal sections treated with anti-AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex-coated $CNBr^-$ activated Sepharose 4B beads (**Figure 4(d)**, **Figure 5(d)** and **Figure 6(d)**). Quantification of AT_2 receptor staining by computer-based image analysis revealed a significant decrease in AT_2 receptor labeling in the renal cortical and medullary regions of the control diabetic group compared with normal rats. Medullary regions of garlic-treated diabetic
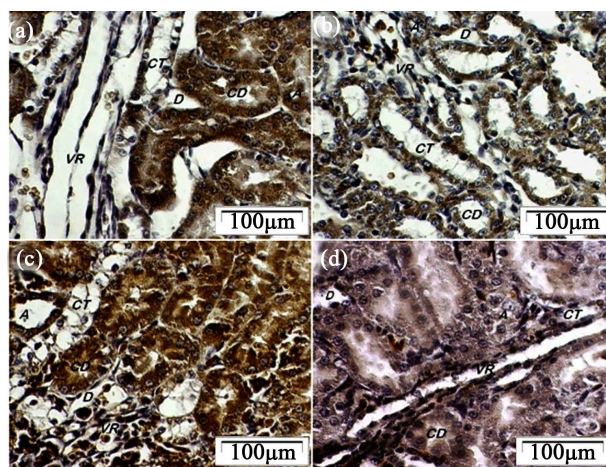
Figure 5. AT_2 receptor expression in the outer medulla of the kidney of normal (A), control diabetic (B) and garlic-treated diabetic (C) rats. (A) ascending limb of Henle's loop, (CD) collecting duct, (CT) collecting tubule, (D) descending limb of Henle's loop, (VR) vasa recta bundle. No specific labeling was observed in kidney tissue sections labeled with the anti-AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex (D). Bar = 100 μm, ×100.
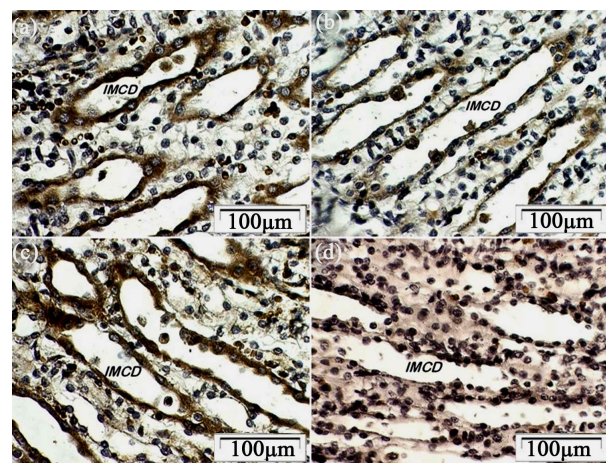
Figure 4. AT_2 receptor expression in the renal cortex of normal (A), control diabetic (B) and garlic-treated diabetic (C) rats. (DCT) distal convoluted tubule, (G) glomerulus, (PCT) proximal convoluted tubule. No specific labeling was observed in kidney tissue sections labeled with the anti-AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex (D). Bar = 100 μm, ×100.

Figure 6. AT_2 receptor expression in the inner medulla of the kidney of normal (A), control diabetic (B) and garlic-treated diabetic (C) rats. (IMCT) inner medullary collecting duct. No specific labeling was observed in kidney tissue sections labeled with the anti-AT_2 antibody preabsorbed with an AT_2 octapeptide/BSA complex (D). Bar = 100 μm, ×100.

rats were marked by levels of AT_2 receptor labeling that were significantly higher than the control diabetic group and comparable to those observed in normal rats. In the cortical region, AT_2 receptor labeling of garlic-treated diabetic rats was significantly higher than the normal and control diabetic groups (**Table 1**).

4. DISCUSSION

Garlic has a wide range of therapeutic applications. In experimental diabetes, sulfur-containing and non-sulfur constituents of garlic work synergistically to exhibit antithrombotic, antioxidant, hypocholesterolaemic, hypoglycaemic as well as hypotensive potentials, which collectively ameliorate the development and progression of diabetic complications, including nephropathy and hypertension [28-36]. Nonetheless, neither the precise mechanisms that underlie the anti-diabetic potentials of garlic nor the nature of the key receptor system(s) targeted, are fully understood. In hypertensive diabetic rats, altered gene and protein expression of the Ang II AT_1 and AT_2 receptor types and the loss of balance between their antagonistic activities, in renal and other tissues, have been directly implicated in the development of early changes associated with diabetic nephropathy [2,4, 5,12,14,17,21]. In a recent report, the remarkable capacity of garlic treatments to modulate the upregulated expression of AT_1 receptors, associated with hypersecretion of aldosterone and the impairment of renal vascular and tubular functions in early diabetes, has been demonstrated [37]. The present study was designed to investigate the level of expression of the AT_2 receptor in the adrenal and renal tissues of normal, control diabetic and garlic-treated diabetic rats.

In the present study, AT_2 receptor expression in adrenal and renal tissues was investigated using a polyclonal anti-AT_2 antibody of proven specificity to an epitope mapping within the N-terminal extracellular domain of the AT_2 polypeptide [43]. In Western blots, this antibody was selectively targeted towards 71.3 kDa and 66.8 kDa components in both adrenal gland and kidney lysates, which were in direct agreement with the estimated molecular weight of glycosylated AT_2 receptors previously detected in rat and other mammalian tissues [17,44,45]. Also consistent with the established presence of five highly-conserved N-linked glycosylation sites along the AT_2 receptor sequence [45], mild enzymatic deglycosylation treatments resulted in the detection of a lower molecular weight component of 41 KDa in both organs, which corresponded exactly to the predicted mass of the deglycosylated protein back-bone deduced from the cDNA sequence of the AT_2 receptor [38,46]. In normal rats, the binding of the anti-AT_2 antibody was immunohistochemically demonstrable in the zona glomerulosa of the adrenal cortex and scattered chromaffin cells of the

adrenal medulla and in glomeruli and proximal and distal convoluted tubules in the renal cortex, collecting tubules and ducts and ascending Henle's loop segments in the inner stripe of the outer renal medulla, as well as inner renal medullary collecting ducts. These selective labeling patterns, observed in adrenal and renal tissues, were all in accordance with AT_2 receptor distribution in both organs as previously determined by immunohistochemical, in vitro autoradiographic localization and in situ hybridization techniques [6,17,47,48]. None of the glycosylated or deglycosylated forms of the receptor observed in Western blots or the selective immunohistochemical labeling patterns were detectable when the antibody was preabsorbed with an AT_2 octapeptide, corresponding to amino acids 10 - 17 of the first extracellular domain of the receptor polypeptide, thus establishing the specificity of the polyclonal antibody in selectively binding the AT_2 receptor in adrenal and renal tissues.

Confirming the reported loss of balance in the levels of AT_1 and AT_2 receptor expression in early diabetes [2, 4,5,12,14,17,21], STZ treatments in the present study were paralleled by quantitative and qualitative alterations in the pattern of AT_2 receptor expression in both the adrenal gland and the kidney. Compared to normal rats, the highest adrenocortical AT_2 receptor expression was significantly shifted from the zona glomerulosa to the zona fasciculate/reticularis, and was significantly reduced in adrenomedullary chromaffin cells of STZ-treated, control diabetic rats. In this rat group, the reduction of AT_2 receptor expression in the zona glomerulosa was in direct contrast to the increase in AT_1 receptor expression observed in this zone in an earlier report [37] and may imply a decrease in AT_2-mediated inhibition of cell growth [18,19] and the amplification of AT_1-mediated effects on cell hypertrophy and aldosterone synthesis and release [2-5], leading to electrolyte imbalance and the development of hypertension. The significant reduction of AT_2 receptor expression in adrenomedullary chromaffin cells may be also implicated in a possible loss of the synergistic AT_1/AT_2 receptor cross-talk suggested to regulate basal and stress-induced tyrosine hydroxylase transcription rates [49], and thereby leading to dysregulation of adrenomedullary catecholamine secretions. Interestingly, the up-regulatory shift in AT_2 receptor expression in the zona fasiculata/reticularis, which was also observed earlier for AT_1 receptor expression [37], has been consistently observed with either receptor type under various abnormal physiological and pathophysiological conditions including dietary sodium restriction, sodium loading, stimulated levels of aldosterone or Ang II, and water deprivation [47,50-52]. Although the exact functional significance of AT_1/AT_2 receptor perturbations in these zones is still to be elucidated, their altered expression may represent a signifi-

cant adaptive factor in adrenal tissue remodeling under various pathological conditions, including early diabetes. In the kidney, the present study also revealed that STZ treatments were associated with a significant decrease in AT_2 receptor expression throughout all nephronal segments including glomeruli and proximal and distal convoluted tubules in the cortex, collecting tubules and ducts and ascending Henle's loop segments in the inner stripe of the outer medulla, as well as inner medullary collecting ducts. The observed decrease in total intrarenal expression of AT_2 receptors is in direct accordance with previous observations in the same model of experimental diabetes [17], and contrasts the documented effects of STZ treatments in upregulating intrarenal expression of AT_1 receptors [1,2,4,11,12,24,26,37]. Thus, in early diabetes, the expected decline in AT_2-mediated mechanisms may potentiate the pathological augmentation of intrarenal AT_1-mediated activities promoting sodium retention and hypertension, glomerulosclerosis and proteinuria, tubulo-interstitial cell hypertrophy and hyperplasia associated with stimulation of extracellular matrix production, which are the hallmark of diabetic nephropathy [2,12-16].

A major observation of the present investigation was the significant modulation of the AT_2 receptor expressed in adrenal and renal tissues of garlic-treated diabetic rats. Compared to the control diabetic rats, AT_2 receptor expression was restored among adrenocortical zona glomerulosa cells and adrenomedullary chromaffin cells and significantly reduced in the zona fasiculata of garlic-treated diabetic rats. In the renal tissues of this rat group, AT_2 receptor expression was also significantly restored in glomeruli and throughout renal cortical and medullary tubular segments, to levels comparable to those observed in normal rats. Along with the reported efficacy of garlic treatments in reducing diabetes-induced AT_1 receptor up-regulation [37], the capacity of garlic in modulating diabetes-induced AT_2 receptor down-regulation may imply not only reversing the detrimental consequences of excessive AT_1 receptor signaling, which is pivotal in the dysregulation of adrenal secretions and alterations in renal hemodynamic and tubular functions, but also restoring the recuperative processes mediated by AT_2 receptors in both organs. This notion may be supported by the documented AT_2-mediated activities in inhibiting aldosterone hypersecretion and regulating catecholamine levels in the adrenal gland [1,7-9,49], inhibiting the sodium pump [53] and Na^+-, K^+-ATPase activity [20-22] in renal proximal tubules thereby promoting natriuresis/diuresis and hypotension, and inhibiting vasoconstriction and cell hypertrophy [7-9,18,19] thereby interfering with excessive renal glomerular and tubular remodeling. In this regard, our observations may lend support to the documented efficacy of garlic treat-

ments in ameliorating diabetic complications in STZ-treated rats [28-36] and further suggest that garlic may alleviate diabetic-induced hypertension and nephropathy by restoring the diabetic-induced loss of balance in AT_1/AT_2 receptor expression in key target organs. Future studies on the significance of constituent garlic metabolites, acting individually or in concert, may clarify the mechanisms that underlie its beneficial capacity in modulating the expression of selective receptor molecules implicated in diabetes-associated disorders.

5. ACKNOWLEDGEMENTS

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