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Original Research

Angiotensin II up-regulates monocarboxylate transporters expression in the rat adrenal gland

D. Kirat[1*], S. Shousha[2]

[1] Department of Physiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt

[2] Department of Physiology, Faculty of Veterinary Medicine, Benha University, Moshtohor, Egypt

Abstract: Angiotensin II (Ang II) is a major regulator of aldosterone secretion in the adrenal zona glomerulosa because it up-regulates the expression of a large number of genes involved in aldosterone biosynthesis. The transport of acetate across adrenocortical cells is a crucial step in the de novo synthesis of cholesterol, the steroid precursor of aldosterone. However, whether Ang II can affect this transport remains unknown. The current study aims to investigate the effect of *in vivo* infusion of Ang II on monocarboxylate transporters (MCT1, MCT2, and MCT4) gene expression in the rat adrenal gland. Immunohistochemical analysis and real-time PCR were used to examine the expression of MCTs at the protein and mRNA levels, respectively. The immunohistochemical analysis showed that higher numbers of cells expressed MCT1, MCT2, and MCT4 proteins in the zona glomerulosa and zona fasiculata of the adrenal cortex of Ang II-infused rats. Furthermore, real-time PCR indicated that *in vivo* infusion of Ang II increased the mRNA levels of MCT1, MCT2, and MCT4 in the rat adrenal gland. MCT up-regulation might maximize the intracellular transport of acetate in response to the stimulatory effect of Ang II on aldosterone secretion by the adrenal zona glomerulosa.

Key words: Aldosterone, MCTs, Regulation, Adrenal gland, Angiotensin II.

Introduction

Angiotensin II (Ang II) plays critical roles in numerous physiological processes. Ang II is the key peptide hormone in the renin-angiotensin-aldosterone system that regulates aldosterone levels through its actions on glomerulosa cells of the adrenal gland (1, 2). Aldosterone is principally synthesized in the zona glomerulosa of the adrenal gland by a series of enzymatic reactions that convert cholesterol to aldosterone (1, 2). Biosynthesis of cholesterol, the steroid precursor, involves the conversion of acetate (acetyl CoA) through a series of complex enzymatic steps. Adrenocortical steroidogenesis involves de novo cholesterol biosynthesis, uptake of cholesterol in the blood that is bound to high-density lipoproteins (HDL) and low-density lipoproteins (LDL), cholesterol transfer to mitochondria, and steroid synthesis (3-5).

Ang II regulates adrenal steroid production and gene transcription through several signaling pathways (6). The actions of Ang II on the adrenal glomerulosa to produce aldosterone are often divided into acute (minutes to hours after treatment) and chronic (hours to days after treatment) phases. These actions occur through increased transcription of target genes that are needed for aldosterone biosynthesis (5, 7, 8). After acute stimulation with Ang II, the genes involved in the regulation of cholesterol uptake by adrenocortical cells are up-regulated (6). Ang II has been shown to up-regulate the expression of both scavenger receptor B type I and the LDL receptor (4), and chronic stimulation with Ang II results in the up-regulation of additional genes responsible for cholesterol synthesis and uptake (7). Therefore, chronic exposure to Ang II increases the adrenal cortex's capacity for aldosterone biosynthesis by increasing cholesterol uptake and synthesis, as well as by increasing steroid biosynthetic enzymes.

The availability and transport of acetate across adrenocortical cells is a crucial step for the de novo synthesis of cholesterol and for subsequent aldosterone biosynthesis in adrenal glomerulosa cells. However, whether Ang II can affect this transport remains unknown.

The transport of acetate across the plasma membrane in most cells is largely dependent on certain members of the monocarboxylate transporter (MCT) family. Of the fourteen known MCT isoforms, MCT1, MCT2, and MCT4 have been shown to play a central role in the proton-linked transport of short-chain fatty acids (acetate, propionate, and butyrate) across different cell types (9-13). In the rat adrenal gland, we have identified seven MCT proteins, specifically, MCT1, MCT2, MCT3, MCT4, MCT5, MCT7, and MCT8, and have speculated that the first four members (MCT1-MCT4) are involved in the regulation of steroid hormone biosynthesis in the rat adrenal cortex (14). Therefore, the present study was carried out in an attempt to investigate the effect of *in vivo* infusion of Ang II on MCT1, MCT2, and MCT4 gene expression in the rat adrenal gland and whether increases in aldosterone secretion can up-regulate the expression of MCT1, MCT2, and MCT4 in the rat adrenal zona glomerulosa.

Materials and Methods

Animals and sample preparation

Male Sprague-Dawley rats that ranged from 242 to

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*** Corresponding author:** Doaa Kirat, Department of Physiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt. E-mail address: doaakirat@hotmail.com

295 g in body weight were randomly selected to receive Ang II infusion (n=5) or 0.9% NaCl (vehicle control; n=5). Rats were killed under sodium pentobarbital (35 mg/kg) anesthesia 7 days after the infusion of Ang II or vehicle. Blood was collected from the caudal vena cava just before sacrificing, transferred to chilled tubes containing EDTA, centrifuged at 3,000 rpm for 15 min at 4°C, and then stored at −20°C until assayed for plasma aldosterone.

After the blood was flushed out, whole adrenal glands were quickly removed and placed in ice-cold phosphate-buffered saline (PBS). Rat adrenal samples were frozen at -80 °C until they were either homogenized for RNA extraction or processed for histological and immunohistochemical analyses by fixing them in 4% paraformaldehyde for 24 h, dehydrating them through a series of graded concentrations of ethanol and xylene, and embedding them in paraffin. The experimental protocol was approved by the Laboratory Animal Control Guidelines of Rakuno Gakuen University, which conforms to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health in the USA (NIH Publication No. 86-23, revised 1996).

Angiotensin II infusion

Val5-angiotensin II (Sigma) was infused into anesthetized rats at 0.7 mg/kg/day by subcutaneously implanted osmotic mini pumps (Alzet mini-osmotic pump Model 2001, Durect Corporation, Cupertino, U.S.A.) for 7 days as described previously (15). Control rats underwent an identical surgical procedure using implanted pumps that contained the sterilized vehicle (0.9% NaCl).

Aldosterone analysis

The plasma aldosterone concentrations of rats in treated and control groups were analyzed by radioimmunoassay (RIA) using the SPAC-S aldosterone kit **(**TFB, Inc., Tokyo, Japan) according to the manufacturer instructions.

Antibodies

Polyclonal primary antibodies for MCT1 (chicken anti-rat; AB1286), MCT2 (chicken anti-rat; AB1287), or MCT4 (rabbit anti-rat; AB3314P) were purchased from Chemicon (Temecula, CA, USA). Biotinylated goat antibody to chicken immunoglobulin (IgG) (BA-9010, Vector laboratories, Inc. CA, USA) or biotinylated chicken anti-rabbit IgG (GTX26828, GeneTex, Inc., San Antonio, TX, USA) were used as secondary antibodies.

Immunohistochemical and histological analyses

The immunohistochemical localization of MCT1, MCT2, and MCT4 was performed on paraformaldehyde-fixed paraffin-embedded sections (4 μm thick) of adrenal glands from both control and angiotensin II-treated rats using avidin biotin-peroxidase (Vectastain Elite ABC kit), as previously described (14). Briefly, sections were incubated in 3% (v/v) H_2O_2 in methanol at room temperature for 10 min to quench the endogenous peroxidase activity. Afterwards, non-specific binding sites were blocked for 30 min at 37 ˚C with Block Ace (Snow Brand Co., Ltd., Tokyo, Japan). Subsequently, sections were incubated overnight with MCT1 antibody (1:300), MCT2 antibody (1:30) or MCT4 antibody (1:20) at 4°C. After washing with PBS, an appropriate biotinylated secondary antibody (goat anti-chicken IgG or chicken anti-rabbit IgG) was applied at 1:200 for 30 min. The sections were then washed with PBS and treated with ABC reagent for 30 min. To detect immunoreactivity, sections were treated with 0.5% 3,3′-diaminobenzidine tetrachloride in PBS containing 0.01% H_2O_2. The sections were then counterstained with Mayer's hematoxylin, dehydrated, and cover slipped. Negative control slides were included alongside each staining. For these controls, slides were probed with MCT antibodies that had been pre-absorbed overnight at 4°C with their corresponding peptides (10 μg/ml). Adrenal tissues were histologically examined in hematoxylin and eosin (H&E)-stained sections.

RNA isolation and real-time PCR

Messenger RNA from rat adrenal glands was isolated using a MagNA Pure LC mRNA isolation Kit II (Roche Diagnostics GmbH, Germany), and cDNA was generated by reverse transcription using a Transcriptor first strand cDNA synthesis kit (Roche Diagnostics GmbH, Germany) following the manufacturer's instructions.

Real-time PCR was performed on a LightCycler instrument (Roche Diagnostics) using a LightCycler TaqMan master kit with Universal ProbeLibrary probes (Roche, Mannheim, Germany). PCR primers and probes (Roche Universal ProbeLibrary; Roche) were designed using the Probe Finder software from Roche Applied Science available online at the Universal ProbeLibrary Assay Design Center. For rat MCT1, primer pair (sense: 5′-gaccattgtggaatgttgtcc-3′; antisense: 5′-agcccagtatgtgtatttgtagtctc -3′) and probe no. 79 were used. For rat MCT2, primer pair (sense: 5′- gtgttgcccagttcttttcg -3′; antisense: 5′-tcccactggctatgtacaggt -3′) and probe no. 20 were used. For MCT4, primer pair (sense: 5′- ctgtggctgtgctcatcg -3′; antisense: 5′- tgaacacatacttgtaaactttcgttg -3′) and probe no. 120 were used. Expression of β-actin was used for sample validation and normalization of MCT expression. For rat β-actin, primer pair (sense: 5′-ctaaggccaaccgtgaaaag -3′; antisense: 5′-tacatggctggggtgttga -3′) and probe no. 115 were used. Mean values from two independent experiments performed in duplicate were calculated.

Statistical analyses

The data are expressed as the means ± SE. The values were compared with a one-way analysis of variance (ANOVA) for multiple comparisons or a Student's t test when two groups were compared. A value of $P < 0.05$ or less was considered statistically significant. All data analyses were performed using the VassarStats web site for statistical computation (http://faculty.vassar.edu/lowry/VassarStats.html).

Results

Effects of angiotensin II on the body weight and relative adrenal gland weight of rats

The *in vivo* infusion of Ang II caused significant body weight loss in the rats. As displayed in Figure (1), Ang II–infused rats showed a gradual decrease in their weight for a week after infusion ($P < 0.01$), while the

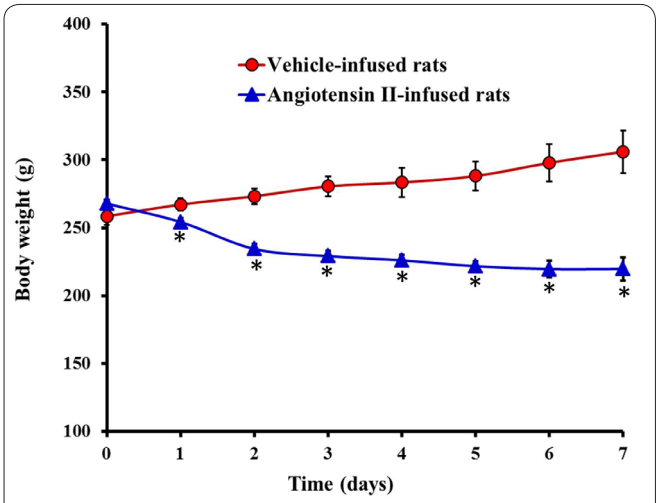

Figure 1. Effect of *in vivo* infusion of angiotensin II on rat body weight. Body weight was determined in vehicle-infused and Ang II–infused rats at indicated times. Shown is the mean ± SE of measurements from five animals per condition per time point. Ang II–infused rats showed a significant gradual decrease in their weight for a week after infusion as indicated by *P<0.01.

vehicle-infused rats continued to gain weight ($P < 0.01$).

The body weight of Ang II–infused rats was significantly lower ($P < 0.05$) than that of control rats. By the 7th day of infusion, the body weight of Ang II–infused rats was reduced 28.2% relative to control rats. Additionally, the relative adrenal weight (whole adrenal weight/body weight; mg/100 g) showed a significant increase ($P < 0.01$) in Ang II-infused rats compared with vehicle-infused rats (29.6±0.72 vs. 18.3±0.65) by the end of infusion period. The increase in the relative adrenal weight of Ang II-infused rats was due to the increase in the weight of the adrenal glands.

Histological examination of the rat adrenal glands indicated that the zona glomerulosa and zona fasciculata of Ang II-infused rats were wider in comparison with those of control rats (data not shown).

The effect of angiotensin II on aldosterone levels in rat blood

As expected, aldosterone levels were greatly increased in Ang II-infused rats relative to controls (Fig. 2). The plasma aldosterone level showed an 8.2-fold increase ($P < 0.01$) in the Ang II-treated group in comparison with the vehicle-infused group (2853±683 pg/ml vs. 346±39 pg/ml), indicating that the dosage of Ang II was effective at increasing endogenous aldosterone secretion.

The effect of angiotensin II on the expression of monocarboxylate transporter proteins in the rat adrenal gland

To examine the effect of *in vivo* infusion of Ang II on the protein expression of proton-linked MCTs (MCT1, MCT2, and MCT4), an immunohistochemical approach was performed on adrenal sections from Ang II-infused and vehicle-infused rats using polyclonal antibodies against rat MCT1, MCT2, and MCT4.

MCT1 and MCT2 proteins were barely detectable in the zona glomerulosa of the adrenal cortex of vehicle-infused rats (Figs. 3A, B and 4A, B).

In contrast, the cells of the zona glomerulosa of Ang II-infused rats exhibited strong positive immunoreactivities for MCT1 and MCT2 proteins (Figs. 3D, E and 4D, E). Additionally, there were more immunoreactive cells with robust staining for the MCT4 protein in the

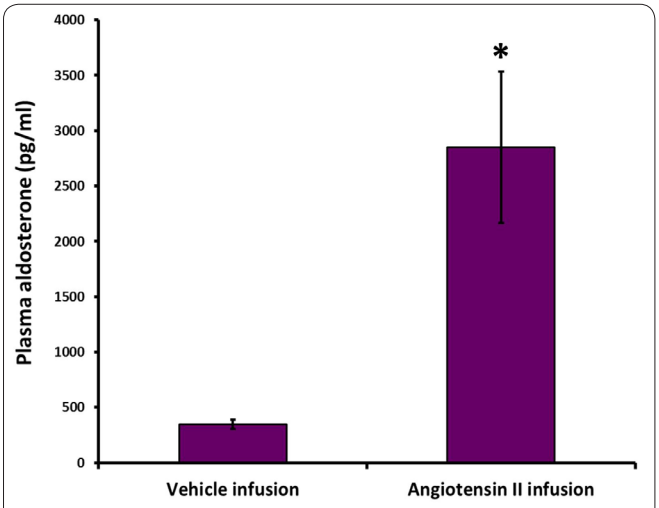

Figure 2. Effect of Ang II on plasma levels of aldosterone. Aldosterone levels in plasma from vehicle- and Ang II–infused animals were determined by radioimmunoassay as described in Methods. Shown is the mean ± SE of duplicate determinations from five rats per condition. The plasma aldosterone levels were significantly increased in Ang II-infused rats relative to controls as indicated by *P<0.01.

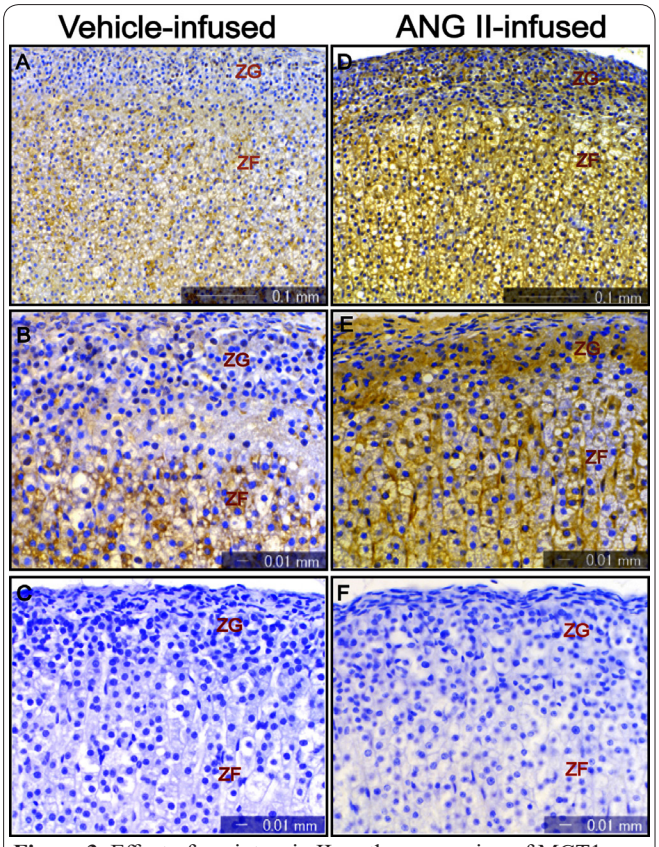

Figure 3. Effect of angiotensin II on the expression of MCT1 protein in the rat adrenal gland as detected by immunohistochemical analysis. Ang II-infused rats showed strong immunoreactive positivities for MCT1 in the adrenal zona glomerulosa (D and E) than that in the zona glomerulosa of control rats (A and B). Notice the absence of MCT1 staining in the zona glomerulosa of controls (A and B) versus their appearance in the corresponding zona in Ang II-infused rats (D and E). Negative controls showed no specific staining for MCT1 (C and F). ZG, Zona glomerulosa; ZF, Zona fasciculate; ZR, Zona reticularis; M, Medulla. Magnification: x20 (A, D); x40 (B-F).

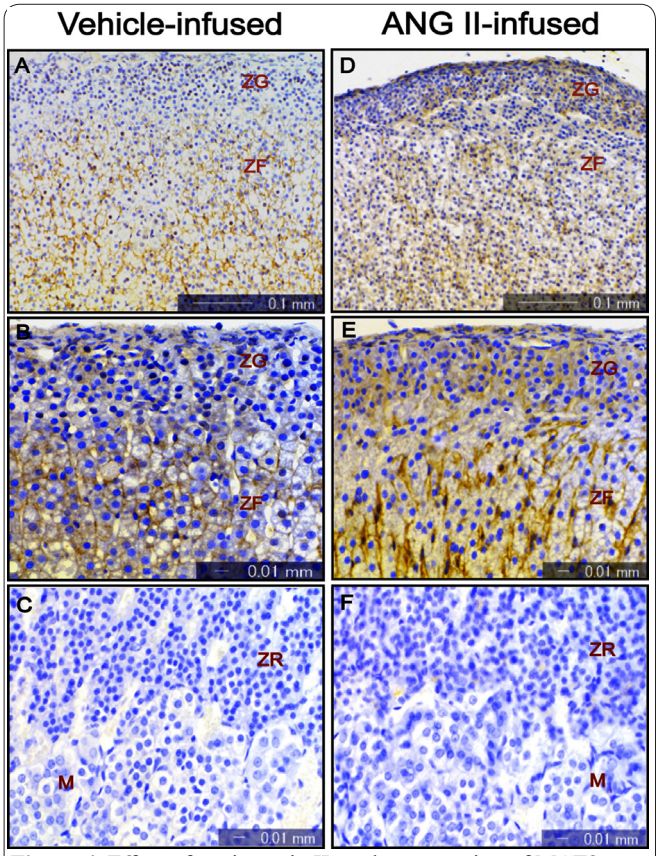

Figure 4. Effect of angiotensin II on the expression of MCT2 protein in the rat adrenal gland as detected by immunohistochemical analysis. *In vivo* infusion of Ang II showed strong immunoreactive positivities for MCT2 in the rat adrenal zona glomerulosa (D and E) than that in the zona glomerulosa of control rats (A and B). Notice the absence of MCT2 staining in the zona glomerulosa of controls (A and B) versus their appearance in the corresponding zona in Ang II-infused rats (D and E). Negative controls showed no specific staining for MCT2 (C and F). ZG, Zona glomerulosa; ZF, Zona fasciculate; ZR, Zona reticularis; M, Medulla. Magnification: x20 (A, D); x40 (B-F).

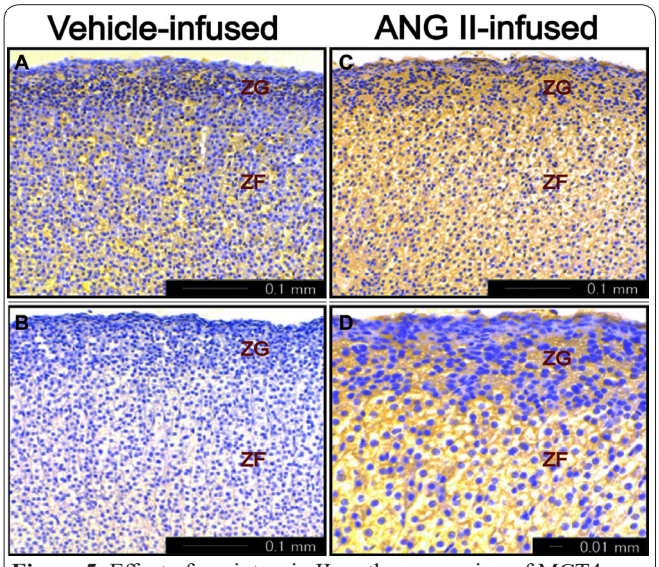

Figure 5. Effect of angiotensin II on the expression of MCT4 protein in the rat adrenal gland as detected by immunohistochemical analysis. *In vivo* infusion of Ang II showed greater immunoreactive intensity for MCT4 in the rat adrenal zona glomerulosa (C and D) than that in the zona glomerulosa of control rats (A). Negative controls showed no specific staining for MCT4 (B). ZG, Zona glomerulosa; ZF, Zona fasciculate; ZR, Zona reticularis; M, Medulla. Medulla. Magnification: x20 (A-C); x40 (D).

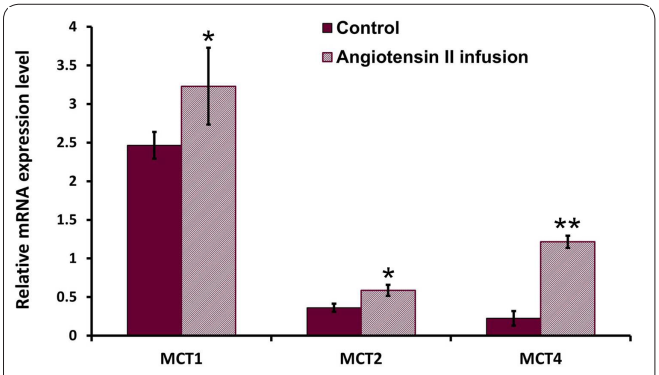

Figure 6. Real-time PCR analysis for transcript levels of MCT1, MCT2, and MCT4 mRNAs rats infused with angiotensin II in comparison to controls. Data were normalized by the amount of β-actin mRNA and are means ± SD from triplicate data.

zona glomerulosa of Ang II-infused rats (Fig. 5C, D) compared with the control group (Fig. 5A).

Furthermore, the immunohistochemical analysis exhibited higher protein expression for MCT1, MCT2, and MCT4 in the cells of the zona fasciculata in Ang II-infused rats (Figs. 3D, E and 4D, E) in comparison with the control rats (Figs. 3A, B, 4A, B, and 5A)

On the other hand, no differences in the patterns of MCT immunoreactivities in the zona reticularis or the medullary cells could be observed between Ang II-treated and control rats (Data not shown). The negative control slides exhibited no specific staining for MCT1 (Fig. 3C, F), MCT2 (Fig. 4C, F), or MCT4 (Fig. 5B).

The effects of angiotensin II on the mRNA levels of monocarboxylate transporters MCT1, MCT2, and MCT4 in the rat adrenal gland

To gain insight into the effects of Ang II on the mRNA levels of MCT1, MCT2, and MCT4, a real-time PCR analysis was performed. Indeed, adrenal glands from Ang II-infused rats exhibited significantly higher levels of MCT1 ($P < 0.05$), MCT2 ($P < 0.05$), and MCT4 ($P < 0.01$) mRNA expression when compared to adrenal glands from the control group (Fig. 6).

Discussion

The novel finding in the present study is the up-regulatory effect of Ang II on MCT expression in the rat adrenal gland. Our results demonstrated that *in vivo* infusion of Ang II for 7 days increases the expression of the MCT1, MCT2, and MCT4 proteins in the rat adrenal zona glomerulosa and fasciculata, as assessed by immunohistochemical analysis (Figs. 3, 4, and 5), and increases their mRNA levels in the rat adrenal gland, as determined by the real-time PCR (Fig. 6). Additionally, our data verified the effects of Ang II on rat body weight, adrenal weight, morphology of the zona glomerulosa and fasciculata, and aldosterone levels (Figs. 1 and 2).

Angiotensin II exerts *in vivo* trophic effects on the adrenal gland and the morphology of both the zona glomerulosa and fasciculata (16-18). Our study demonstrates a marked effect of Ang II on weight loss in rats (Fig. 1). There are several reports that have investigated the effect of systemically infused Ang II on body weight and food intake (19-21). Brink *et al* (1996) (19) reported a 74% decrease in body weight due to the anorexige-

nic effect of Ang II. A subsequent report suggested that decreased body weight that was not attributable to anorexia might be due to decreased muscle protein accumulation as a by-product of increased catabolism (20).

With regard to adrenal gland morphology, our study revealed that both the zona glomerulosa and zona fasiculata were considerably enlarged in rats in response to Ang II infusion. The role of Ang II on zona glomerulosa function is well documented (22). The increased size of the adrenal zona glomerulosa in rats under the effect of Ang II infusion is consistent with observations reported in previous studies (23, 24). *In vivo*, infusion of Ang II via osmotic mini pumps induces proliferation (BrdU positive cell nuclei) in the zona glomerulosa cells of male Wistar rats (24). Similarly, the in situ perfusion of the rat adrenal gland with Ang II stimulates cell growth and increases thymidine incorporation and cell proliferation of glomerulosa cells (25). Moreover, Nussdorfer *et al* (1981) (18) demonstrated that chronic angiotensin treatment induces an increase in the volume of rat zona fasciculata cells and that the renin-angiotensin system is involved in the stimulation of the growth and steroidogenic capacity of the rat zona fasciculata.

Angiotensin II is the major regulator of aldosterone secretion (2, 17). In agreement with previous studies (1, 15, 16, 26), infusion of Ang II in the present study produced a significant elevation in rat blood aldosterone levels (Fig. 2).

It has long been known that adrenocortical cells respond, in vitro and *in vivo*, to Ang II treatment by rapidly inducing the expression of a large number of genes (5, 8). One of the chronic actions of Ang II is to increase the capacity for aldosterone biosynthesis in the adrenal cortex. Kirat *et al* (2009) (14) speculated that MCT1-MCT4 might be involved with the regulation of the steroid hormone biosynthesis in the rat adrenal cortex. The current study suggests that the MCT genes (MCT1, MCT2, and MCT4) are direct targets of Ang II.

Cholesterol is a common precursor of all steroid hormones in mammals. In this regard, Liang *et al* (2007) (7) provided the first demonstration that Ang II increased cholesterol biosynthesis through the up-regulation of several genes, including acetoacetyl coenzyme A thiolase, HMG coenzyme A synthase 1, Isopentenyl-diphosphate-isomerase, lanosterol synthase, Sterol 4 C-methyl oxidase, and emopamil binding protein in human adrenocortical cells. The availability of adequate cholesterol substrate is a critical requirement for optimal aldosterone hormone synthesis (27). Cholesterol can be provided either by de novo biosynthesis or from the uptake of circulating HDL and LDL (2, 27). It is well known that hormonal stimulation of steroid-producing cells results in prompt mobilization of cholesterol esters from intracellular lipid droplets and increased cholesterol uptake from plasma proteins (2, 3, 28, 29). Additionally, the rate of hormone secretion depends on the de novo synthesis of cell-specific steroids (2).

The biosynthesis of aldosterone involves the conversion of acetate to cholesterol. Subsequently, the increase in steroid hormone secretion under the effect of a hormone regulator requires the transport of more acetate for the synthesis of cholesterol. Thus, in the zona glomerulosa cells of the rat adrenal cortex, MCT isoforms (MCT1, MCT2, and MCT4) may be one of the main sources of acetate when cholesterol synthesis is needed to meet the increased demand of aldosterone biosynthesis.

The present study revealed, for the first time, the up-regulating effects of *in vivo* infusion of Ang II on the expression of MCT family members (MCT1, MCT2, and MCT4) in the zona glomerulosa and fasiculata of the rat adrenal cortex. Ang II might represent an important pathway for increasing the capacity of adrenal cells of the zona glomerulosa and fasiculata to transport acetate for the biosynthesis of aldosterone and corticosterone, respectively, in response to Ang II stimulation.

This study, for the first time, showed that Ang II increases the mRNA and protein expression levels of MCT1, MCT2, and MCT4 in the adrenal glands of rats. This up-regulation might maximize the intracellular availability of acetate to support the stimulatory effect of Ang II on aldosterone and corticosterone secretion by adrenal zona glomerulosa and fasiculata cells, respectively. Thus, this study suggests that MCT1, MCT2, and MCT4 have a physiological role in the regulation of aldosterone and corticosterone biosynthesis in rats.

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