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Angiotensin-(1–7) through AT_2 receptors mediates tyrosine hydroxylase degradation via the ubiquitin–proteasome pathway

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Abstract

Hypothalamic norepinephrine (NE) release regulates arterial pressure by altering sympathetic nervous system activity. Because angiotensin (Ang) (1–7) decreases hypothalamic NE release and this effect may be correlated with a diminished NE synthesis, we hypothesize that Ang-(1–7) down-regulates tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamines biosynthesis. We investigated the effect of Ang-(1–7) on centrally TH activity and expression. TH activity was evaluated by the release of tritiated water from ^{3}H-L-tyrosine. TH expression and phosphorylation were determined by western blot. Hypothalami from normotensive or spontaneously hypertensive rats pre-incubated with Ang-(1–7) showed a significant decrease in TH specific activity. Ang-(1–7) caused a decrease in TH phosphorylation at Ser19 and Ser40 residues. The heptapeptide induced a decrease in TH expression that was blocked by an AT_2 receptor antagonist and not by an AT_1 or Mas receptor antagonist, suggesting the involvement of AT_2 receptors. The proteasome inhibitor MG132 blocked the Ang-(1–7)-mediated TH reduction. In addition, Ang-(1–7) increased the amount of TH–ubiquitin complexes, indicating that the Ang-(1–7)-mediated TH degradation involves ubiquitin conjugation prior to proteasome degradation. We conclude that Ang-(1–7) down-regulates TH activity and expression centrally leading to a decrease in the central NE system activity.

Keywords: angiotensin-(1–7), AT_2 receptors, neuronal cultures, spontaneously hypertensive rats, tyrosine hydroxylase, ubiquitin–proteasome pathway.

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The renin–angiotensin system (RAS) is one of the major systems in the regulation of cardiovascular function and fluid homeostasis. A complete RAS exists in the brain and comprises all necessary precursors and enzymes required for formation and metabolism of its components (Veersingham and Raizada 2003; Saavedra 2005). Within the brain, angiotensin (Ang) II contributes to cardiovascular regulation via its action at various hypothalamic and medullary areas resulting in elevated blood pressure, augmented drinking behavior, attenuation of the baroreflex, enhancement of sympathetic outflow, and augmented vasopressin release (Veersingham and Raizada 2003; Saavedra 2005).

Angiotensin II is certainly not the only biologically active peptide of the RAS as it has been determined that other peptides contribute to or actually oppose the pressor and proliferative actions of Ang-II (Ferrario *et al.* 1997; Trask and Ferrario 2007; Santos *et al.* 2008). Among them, Ang-(1–7) is an endogenous counter-regulator of Ang-II because it produces vasodilation, natriuresis, and diuresis, facilitates

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Abbreviations used: Ang, angiotensin; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum; NE, norepinephrine; Neu, neurofilament protein; RAS, renin–angiotensin system; SHR, spontaneously hypertensive rats; TH, tyrosine hydroxylase; Ub, ubiquitin; WKY, Wistar–Kyoto.

the baroreflex control of blood pressure and inhibits angiogenesis and cell growth (Ferrario *et al.* 1997; Trask and Ferrario 2007; Santos *et al.* 2008). At the central level, Ang-(1–7) acts at sites involved in the control of the cardiovascular function, thus contributing to blood pressure regulation (Ferrario *et al.* 1997; Trask and Ferrario 2007; Santos *et al.* 2008). In fact, intrahypothalamic injections of Ang-(1–7) in spontaneously hypertensive rats (SHR) blocked the pressor response elicited by Ang-II (Höcht *et al.* 2006). This action may result from its activity on neurotransmitter release; i.e. the peptide not only diminishes hypothalamic norepinephrine (NE) release (Gironacci *et al.* 2004a) but also blocks the stimulatory effect of Ang-II on this mechanism (Gironacci *et al.* 2004b); in addition, Ang-(1–7) modulates the release of glutamate and taurine at the caudal ventrolateral medulla which in turn contribute to its hypotensive effect (Kubo *et al.* 1993). The G protein-coupled receptor Mas has been shown to bind Ang-(1–7) and is involved in many of its biological actions (Santos *et al.* 2003, 2008). In fact, a dense Mas receptor immunoreactivity was reported in neurons of cardiovascular-related areas of the medulla and forebrain (Becker *et al.* 2007).

Sympathetic nervous system overactivity contributes to the development and maintenance of human essential hypertension (Wyss and Carlson 1999). One of the physiological mechanism by which Ang-II exerts its pressor effect involves the modulation of sympathetic activity and the regulation of catecholamine metabolism in the brain nuclei typically associated with the control of blood pressure (Veersingham and Raizada 2003; de Wardener 2001). In contrast to Ang-II, Ang-(1–7) reduces NE release in hypothalami isolated from SHR (Gironacci *et al.* 2004a) and in this way, it may decrease sympathetic activity contributing to blood pressure regulation. As the diminished NE release caused by Ang-(1–7) may be correlated with a diminished NE synthesis, we hypothesize that Ang-(1–7) might down-regulate L-tyrosine hydroxylase (EC 1.14.16.2) (TH), the enzyme that catalyzes the first and rate-limiting step in catecholamines biosynthesis (Fujisawa and Okuno 2005). Thus, our aim was to investigate the effect of Ang-(1–7) on centrally TH activity and expression in normortensive and SHR rats.

Materials and methods

Materials

Horse serum (HS) and Dulbecco's modified Eagle's medium (D-MEM) were from Invitrogen (Carlsbad, CA, USA). MG132 was from Calbiochem (San Diego, CA, USA). Dnase I and cytosine arabinose were purchased from Sigma Chemical Co. (St Louis, MO, USA). Losartan were purchased from Dupont (Boston, MA, USA). PD123319 was a gift from Dr Jack Hodges at Parke Davis (Detroit, MI, USA). Ang-(1–7) and [D-Ala7]-Ang-(1–7) were synthesized in our laboratory by the Merrifield solid-phase procedure, as previously described (Gironacci *et al.* 2004b). The crude peptide was purified and characterized as a single component by HPLC. It showed the correct amino acid composition and sequence. Peptide purity (> 97%) was confirmed by matrix assisted laser desorption mass spectrometry. All other chemicals were analytical grade reagents of the highest purity available.

Animals

Male Wistar–Kyoto (WKY) or SHR were used in our study. The study was approved by the Animals Studies Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Tyrosine hydroxylase enzymatic assay

Tyrosine hydroxylase activity was determined according to the method described by Bobrovskaya *et al.* (2004) which measures the conversion of L-[3,5-^{3}H]-tyrosine to L-[5-^{3}H]-DOPA, a process that releases a single [^{3}H]-H_2O molecule per L-[3,5-^{3}H]-tyrosine molecule that is hydroxylated by TH activity.

Isolated hypothalami from 3-month-old male WKY or SHR were pre-incubated in Krebs solution in the absence or presence of 0.1 or 1 μmol/L Ang-(1–7) during 15 min. Lower concentrations of Ang-(1–7) were not tested because our previous works about the effect of Ang-(1–7) on NE release were performed with 0.1 μmol/L Ang-(1–7) (Gironacci *et al.* 2000, 2004a,b). Hypothalami were homogenized in water and 400 μg protein present in the homogenate was incubated for 10 min at 25°C in a reaction mixture, pH 7.2, containing 60 mmol/L K_3PO_4, 2 mmol/L β-mercaptoethanol, 25 μmol/L L-tyrosine, 36 μg/mL catalase, 2 mmol/L tetrahydrobiopterin, and 10 μCi/mL L-[3,5-^{3}H]-tyrosine (specific activity 40 Ci/mmol; NEN Life Science Products, Inc., Boston, MA, USA) in a final volume of 100 μL. The reaction was stopped by the addition of 600 μL of 7.5% charcoal suspended in 1 mol/L HCl. L-[3,5-^{3}H]-Tyrosine, [^{3}H] L-DOPA, and intermediate metabolites bound to charcoal, but not [^{3}H]-H_2O that was formed from L-[3,5-^{3}H]-tyrosine by TH activity. The samples were mixed thoroughly and centrifuged at 10 000 *g* for 15 min. The supernatant was counted in a liquid scintillation counter. Proteins were quantified by the Bradford protein assay (Bradford 1976). Results were expressed as enzymatic specific activity that is nmol product/mg protein/h.

Cell culture

Neuronal cultures were prepared using a slight modification of the method described by Yu *et al.* (1996). Hypothalamus–brainstem areas of 1-day-old WKY or SHR rats were dissociated with 18 U/mL papain (Worthington, Lakewood, NJ, USA) at 37°C for 30 min. The hypothalamic block contained the paraventricular, supraoptic, anterior, lateral, posterior, dorsomedial, and ventromedial nuclei. The brainstem block contained medulla oblongata and pons. After being centrifuged 5 min at 600 *g*, the resultant pellet was triturated in D-MEM containing Dnase I (0.01 mg/mL) and centrifuged at 800 *g* for 5 min. Cells were resuspended in D-MEM containing 10% HS (D-MEM/HS) and plated in poly-L-lysine-pre-coated tissue culture dishes. After 24 h, the medium was replaced with D-MEM/HS containing 10 μmol/L cytosine arabinose for 48 h to avoid

growing of non-neuronal cells. After that, neurons were allowed to complete differentiation in D-MEM/HS. The corresponding experimental treatment of the cells was carried out in a 24 h serum-starved condition.

Immunocytochemistry

Neuronal cultures were characterized by immunocytochemistry using a monoclonal mouse antibody directed against neurofilament protein (Invitrogen) or brain microtubule-associated proteins 2 (Sigma Chemical Co.) to detect neurons, and a polyclonal anti-TH rabbit antibody or monoclonal anti-TH mouse antibody (Chemicon, San Francisco, CA, USA) to detect catecholaminergic neurons. Ang-(1–7) immunostaining was also investigated in neuronal cultures using a polyclonal Ang-(1–7) antibody obtained in our laboratory. Anti-Ang-(1–7) antibody was raised against the C-terminal of Ang-(1–7), so it may recognize also Ang-(3–7) and Ang-(2–7). Among others, Angs formed in the brain (Karamyan and Speth 2007), sequences like Ang-II, Ang-(2–8), Ang-(3–8), Ang-(4–8), Ang-(2–10), Ang-(1–10) or Ang-(1–5), may not be recognized by this antibody as its C-terminal is different. Radioimmunoassay was performed to test the specificity of anti-Ang-(1–7) antibody (dilution 1/5000). Data in Fig. 1 show that the antibody raised against Ang-(1–7) did not recognize Ang-I, Ang-II, or Ang-III (affinity 1000-fold lower than for Ang-(1–7).

Neuronal cultures were fixed in 4% *p*-formaldehyde at 25°C. Non-specific staining was blocked by incubation with 1.5% goat serum in phosphate-buffered saline containing 0.2% Triton during 30 min. Cells were incubated with rabbit anti-TH antibody (1 : 500) plus mouse anti-neurofilament protein antibody (1 : 100) or with rabbit anti-Ang-(1–7) antibody (1 : 900) plus mouse anti-microtubule-associated proteins 2 antibody (1 : 500) in phosphate-buffered saline containing 0.2% Triton overnight at 4°C. Cells were incubated with anti-rabbit IgG FITC conjugate (Sigma Chemical Co.) and anti-mouse IgG AlexaFluor 594 (Invitrogen) (1 : 100) for 3 h at 25°C. Microscopic observation was performed by epifluorescence using an Olympus (Westmont, IL, USA) BX50 microscope. For negative controls, cells were treated with normal goat serum in the absence of the primary antibody or with anti-Ang-(1–7) antibody pre-incubated with 10 μmol/L Ang-(1–7), and no stain was visible (data not shown).

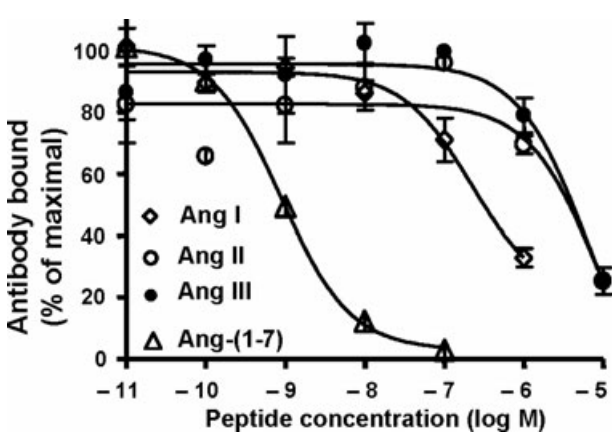

Fig. 1 Characterization of anti-Ang-(1–7) antibody. Radioimmunoassay with the anti-Ang-(1–7) antibody (dilution 1/5000) in the presence of increasing concentrations of Ang-I, Ang-II, Ang-III, or Ang-(1–7) was performed to test the specificity of anti-Ang-(1–7) antibody.

Western blot

After treatments, cells were homogenized in ice-cold buffer, pH 7.4, containing 24 mmol/L HEPES, 1 mmol/L EDTA, 2 mmol/L tetrasodium pyrophosphate, 70 mmol/L sodium fluoride, 1 mmol/L β-glycerophosphate, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 2 μg/mL leupeptin. Homogenates were centrifuged at 20 000 *g* for 30 min at 4°C. Equal amount of proteins were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membrane. Non-specific binding sites on the membrane were blocked by incubation with 5% milk in Tris-buffered saline solution containing 0.1% Tween 20. Membranes were subsequently probed with either mouse anti-TH (1 : 500) (Chemicon) to measure TH expression or with rabbit anti-TH phospho-Ser19 (1 : 300) (Chemicon) or anti-TH phospho-Ser40 (1 : 300) (Chemicon) to measure TH phosphorylation, followed by incubation with goat anti-mouse or goat anti-rabbit IgGs coupled to horseradish peroxidase (Amersham Biosciences, Piscataway, NY, USA). Immunoreactive bands were visualized by chemiluminescence detection (ECL Plus reagent; Amersham Biosciences) and quantified by densitometry. Protein loading in gels was evaluated by reblotting membranes with anti-β-actin antibody.

For immunoprecipitation, 500 μg protein present in cells homogenates were incubated with anti-ubiquitin (Ub) antibody (1 μg/mL) (Santa Cruz Biotechnology Laboratories, Santa Cruz, CA, USA) overnight at 4°C. After incubation, immunocomplexes were purified with protein G–Sepharose and analyzed by immunoblotting using anti-TH antibody as described above.

Statistical analysis

The data are presented as mean ± SEM. Statistical significance was assessed by one- or two-way ANOVA followed by Bonferroni post-tests (GraphPad Prism 4; GraphPad Software, Inc., San Diego, CA, USA). Values of $p < 0.05$ were considered significant.

Results

Figure 2 shows TH specific activity in hypothalami isolated from WKY and SHR rats. Basal TH enzymatic activity was significantly higher in SHR than in WKY rats. When hypothalami from SHR were pre-incubated with 0.1 μmol/L or 1 μmol/L Ang-(1–7) during 15 min, a significant decrease in TH specific activity was observed (Fig. 2). Therefore, we further investigated the effect of Ang-(1–7) on TH phosphorylation and expression in hypothalamic neuronal cultures. Figure 3(a–c) shows that neurofilament-positive cells contain TH, demonstrating that they are mainly catecholaminergic neurons. Ang-(1–7) immunostaining was also found in TH immunoreactive neurons (Fig. 3f), suggesting the endogenous presence of the peptide in these catecholaminergic neurons.

Tyrosine hydroxylase activity can be regulated by protein phosphorylation at serine residues by a variety of protein

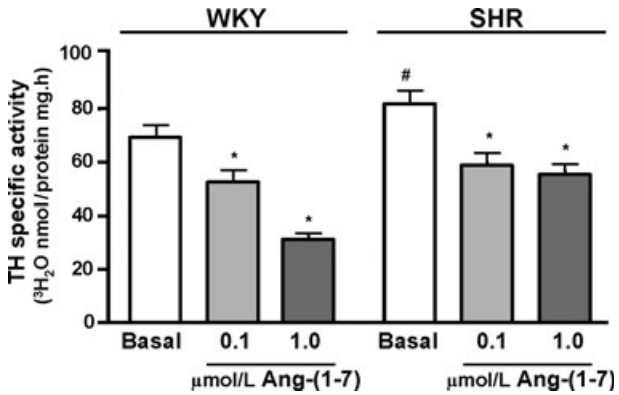

Fig. 2 Effect of Ang-(1–7) on TH specific activity in hypothalami isolated from WKY and SHR. Hypothalami were pre-incubated with 0.1 or 1 μmol/L Ang-(1–7) during 15 min and TH specific activity determined as described in Materials and methods. Basal TH specific activity in hypothalami from WKY was 69 ± 4 nmol/mg protein/h and from SHR was 82 ± 5 nmol/mg protein/h. Values are mean ± SEM (n = 10); $^*p < 0.05$ when compared with the respective control and $^\#p < 0.05$ when compared with WKY control.

kinases (Dunkley *et al.* 2004). Phosphorylation of TH at Ser19 has no effect on TH activity, but phosphorylation at Ser19 alters the conformation of TH to allow increased accessibility of Ser40 to kinases leading to an increase in the enzyme activity (Dunkley *et al.* 2004; Lehmann *et al.* 2006). As observed in Fig. 4, basal phosphorylation of both TH at Ser19 and Ser40 was greater in neuronal cultures from SHR compared with WKY, which is consistent with the increased TH activity seen in SHR (Fig. 2). When neuronal cultures from WKY or SHR were incubated for 15 min with 0.1 μmol/L Ang-(1–7) a decrease in TH phosphorylation was observed in both strains (Fig. 4). Membranes reblotted with anti-TH showed that TH expression was not modified with respect to the control in any of the conditions assayed, suggesting that changes in TH phosphorylation are not because of modifications in TH expression (Fig. 4).

Basal endogenous TH levels were 1.7-fold greater in neuronal cultures from SHR than in those from WKY (Fig. 5a). Treatment with 0.1 μmol/L Ang-(1–7) during 30 min decreased TH expression in neuronal cultures from both strains (Fig. 5b). No effect on TH expression was observed with longer time periods of treatment, which might be because of Ang-(1–7) degradation. To test this hypothesis, TH content was examined at 60 min when cells were stimulated twice with Ang-(1–7) at time 0 and 30 min. Under this experimental condition, a decrease in TH content was observed (data not shown), demonstrating in this way that Ang-(1–7) degradation is responsible for the recovery in TH levels after 60 min. In addition, Ang-(1–7) metabolism in neuronal cultures was investigated by incubating the cells for increasing time periods with 0.1 μmol/L Ang-(1–7) and identifying peptides by HPLC coupled to electrospray ionization-ion trap mass spectrometry. We observed that Ang-(1–7) levels decreased while metabolites of lower molecular weight increased after 60-min incubation. Only traces of the peptide were detectable after 3 h-incubation (data not shown).

Concentration–response assays were performed by incubation of neuronal cultures with Ang-(1–7) during 30 min; 0.1 μmol/L Ang-(1–7) caused a decrease of 27 ± 8% TH expression in WKY and of 29 ± 4% in SHR while a higher concentration (1 μmol/L) had no effect (Fig. 5c). A lower concentration (0.01 μmol/L) of Ang-(1–7) decreased TH expression although this diminution did not reach statistical significance in SHR neuronal cultures (Fig. 5c).

We investigated then the mechanism by which Ang-(1–7) down-regulated TH expression. We carried out our study in

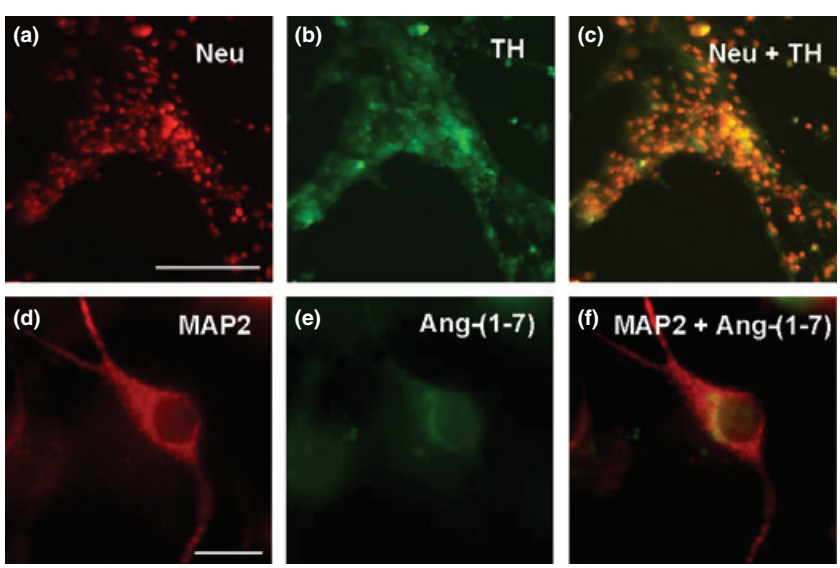

Fig. 3 Immunoreactivities against the neurofilament specific neuronal protein (Neu), a neuronal marker, and TH, a catecholaminergic marker, in hypothalamic-brainstem neuronal culture are shown in (a and b), respectively. (c) Represents the combination of red and green demonstrating Neu and TH colocalization. Magnification, 40× (a, b, and c) and scale bar, 50 μm. (d) Demonstrates immunoreactivities against brain microtubule-associated proteins (MAP2), a neuronal marker, (e) Ang-(1–7) immunoreactivity, and (f) represents colocalization of MAP2 and Ang-(1–7). Magnification, 100x (d, e, and f) and scale bar, 10 μm.

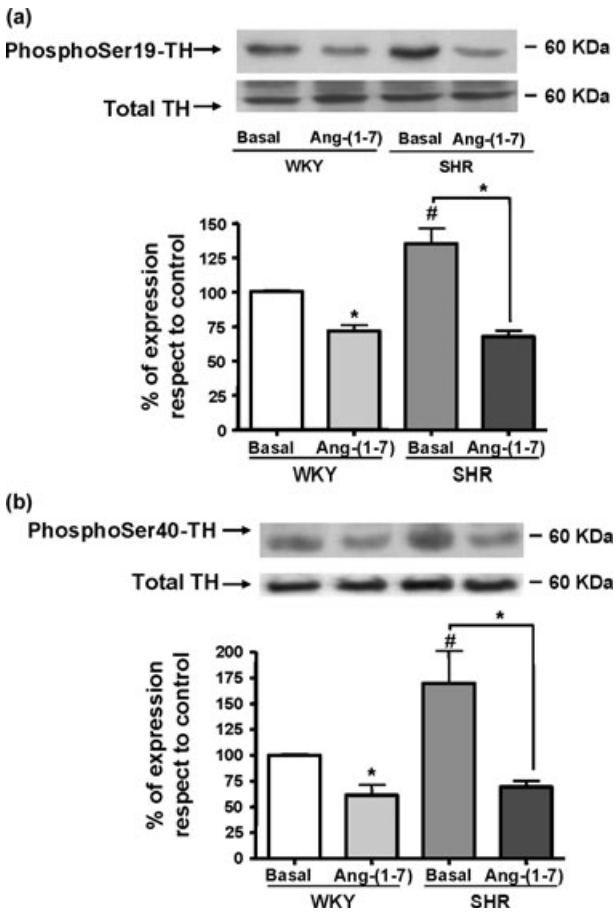

Fig. 4 Effect of Ang-(1–7) on TH phosphorylation at Ser19 (a) and Ser40 (b) in WKY and SHR hypothalami-brainstem neuronal cultures. Cultures were treated with Krebs buffer in the presence or absence of 0.1 μmol/L Ang-(1–7) for 15 min. TH-phospho-Ser19 or TH-phospho-Ser40 were measured by western blot as described in Materials and methods and normalized to TH content in the same sample. TH abundance was measured by reblotting the same membranes with anti-TH and no changes were observed. Results are expressed as the percentage of the response detected in control WKY cells, taking them as 100% i.e. those incubated with Krebs buffer alone. Each bar represents the mean ± SEM of three determinations from four separate cell culture preparations; $^{*}p < 0.05$ when compared with the respective basal strain and $^{\#}p < 0.05$ when compared with WKY control.

SHR because it has been shown that Ang-(1–7) acts as a counter-regulator of Ang-II actions, especially in situations of Ang-II overactivity as in hypertension (Ferrario *et al.* 1997). To study the Ang-receptor subtypes coupled to the inhibitory activity of Ang-(1–7) on TH expression, the effects of selective antagonists for Mas, AT_1-, and AT_2-receptor subtypes were assessed; 1 μmol/L PD123319, an AT_2 receptor antagonist, blocked the effect of 0.1 μmol/L Ang-(1–7), suggesting the involvement of AT_2 receptors. The action of Ang-(1–7) on TH expression was modified by neither 1 μmol/L [D-Ala7] Ang-(1–7), the Mas receptor antagonist, nor 1 μmol/L losartan, an AT_1 receptor antagonist (Fig. 6). Higher concentrations of the Mas antagonist were not assayed because the affinity constant for Mas receptors is in the nmol/L range (Santos *et al.* 2003), so in our conditions Mas receptors should be saturated with 1 μmol/L [D-Ala7] Ang-(1–7). The antagonists *per se* did not modify TH expression.

The decrease in TH levels caused by Ang-(1–7) might be because of increased degradation of the protein. As the Ub–proteasome system is the major pathway for intracellular protein degradation (Kornitzer and Ciechanover 2000), we examined the involvement of the Ub-proteasomal pathway in the Ang-(1–7)-induced down-regulation of TH expression. Neuronal culture cells were pre-treated for 15 min with the proteasome inhibitor MG132 (1 μmol/L dissolved in dimethylsulfoxide) followed by incubation with Krebs solution or 0.1 μmol/L Ang-(1–7) for 30 min. The decrease in TH expression induced by Ang-(1–7) was blocked by MG132 pre-treatment, suggesting a proteasome-dependent TH degradation (Fig. 7a).

Before degradation by the proteasome, most of substrates are labeled by conjugation with multi-Ub chains (Glickman and Ciechanover 2002). To investigate whether Ub conjugation to TH was required prior to Ang-(1–7)-induced TH proteosomal degradation, neuronal cultures were incubated with Krebs solution or 0.1 μmol/L Ang-(1–7) for 30 min in the presence of the proteasome inhibitor to prevent degradation of ubiquitinated TH, and subjected to immunoprecipitation with anti-Ub antibody following by immunoblotting with anti-TH antibody. Figure 7b shows a series of bands that correspond to TH with a multiple number of attached polyubiquitin chains. The intensity of these bands was increased by 1.8-fold in lysates prepared from Ang-(1–7)-treated cells, suggesting that Ang-(1–7) enhances TH ubiquitination in neuronal cultures from SHR.

Discussion

Our study shows that Ang-(1–7) diminishes centrally TH activity and expression, and this effect involves AT_2 receptors and the proteasome–Ub pathway stimulation.

Increased catecholaminergic neuromodulation has been reported in SHR, characterized by increased NE transporter, α_{2A}-receptors, and TH genes expression (Reja *et al.* 2002a,b). TH mRNA and enzymatic activity were higher in neuronal cultures from newborn SHR in comparison to WKY (Yu *et al.* 1996). In fact, a positive correlation between systolic arterial blood pressure and TH gene expression exists in the brainstem of SHR (Reja *et al.* 2002a). Intracerebroventricular administration of a TH antisense gene to SHR decreases systolic blood pressure with reduced TH protein, TH activity, and catecholamine synthesis (Kumai *et al.* 2001). Accordingly, our present results show that the activity and expression of TH is higher in SHR compared

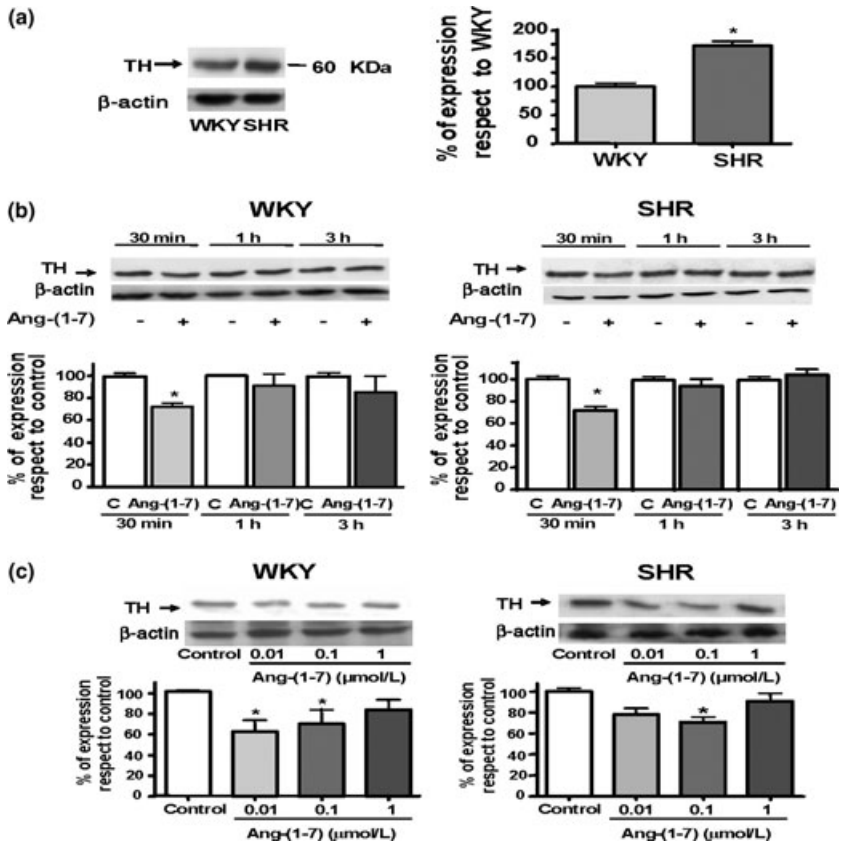

Fig. 5 (a) Basal expression of TH in both WKY and SHR hypothalami-brainstem neuronal cultures. TH expression was measured by western blot as described in Materials and methods and is expressed as a percentage of the response detected in WKY cells. Each bar represents the mean ± SEM of three determinations from four separate cell culture preparations; *$p < 0.05$ when compared with WKY. (b) Time-dependent effect of Ang-(1–7) on TH expression in hypothalami-brainstem neuronal cultures from WKY (left panel) and SHR (right panel). Neuronal cultures were incubated in the absence (C) or presence of 0.1 μmol/L Ang-(1–7) for the indicated time periods. TH expression was measured by western blot as described in Materials and methods and is expressed as the percentage of the response detected in control basal cells, taking them as 100% i.e. those incubated with Krebs buffer alone. Each bar represents the mean ± SEM of three determinations from four separate cell culture preparations; *$p < 0.05$ when compared with the corresponding control. (c) Concentration–response effect of Ang-(1–7) on TH expression in hypothalami-brainstem neuronal cultures from WKY (left panel) and SHR (right panel). Neuronal cultures were incubated for 30 min with varying concentrations of Ang-(1–7) (0.01–1 μmol/L). TH expression was measured by western blot as described in Materials and methods and is expressed as a percentage of the response detected in control basal cells, i.e. those incubated with Krebs buffer alone. Each bar represents the mean ± SEM of three determinations from four separate cell culture preparations; *$p < 0.05$ when compared with control.

with WKY. Altogether these results suggest that TH plays a key role in hypertension development. In fact, Rao *et al.* (2007) have reported that common variation in the TH proximal promoter contributes to inheritable alteration in multiple autonomic traits, biochemical, and physiological, and the ultimate disease trait of hypertension, suggesting that treatments targeting the adrenergic pathway might be beneficial in hypertension if administered to subjects at specific genetic risk.

Angiotensin-II stimulates TH activity and its mRNA levels in neuronal cultures from the hypothalamus–brainstem of SHR through AT_1 receptors activation (Yu *et al.* 1996). Similar results were observed when Ang-II was infused intracerebroventricularly into hypothalami and brainstems of adult SHR (Yu *et al.* 1996). In contrast, our present results demonstrate that Ang-(1–7) decreased TH activity in hypothalami from WKY and SHR, which reflects a reduced catecholamine biosynthesis that may be correlated with the diminished NE release caused by the peptide (Gironacci *et al.* 2000, 2004a). In this way, Ang-(1–7) opposes the stimulatory effect of Ang-II on noradrenergic neurotransmission, supporting the hypothesis that Ang-(1–7) elicits a counter-regulatory role on Ang-II actions.

The enzymatic activity of TH is positively regulated by its phosphorylation. TH is phosphorylated at Ser8, Ser19, Ser31, Ser40, Ser153, and Ser404 by a variety of protein

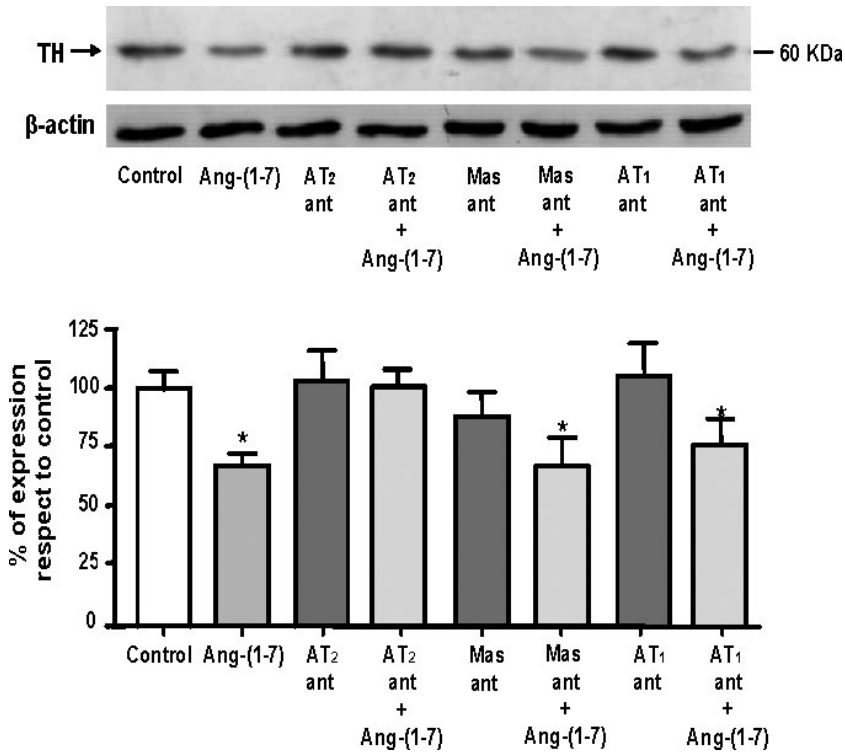

Fig. 6 Effect of AT_2-, AT_1-, and Mas receptor antagonists on the inhibitory response of Ang-(1–7) on TH expression in hypothalami-brainstem neuronal cultures from SHR. Cells were pre-incubated for 15 min with the AT_2 receptor antagonist PD123319 (AT_2 ant) (1 μmol/L), or the AT_1 receptor antagonist losartan (AT_1 ant) (1 μmol/L), or the Mas receptor antagonist [D-Ala7]-Ang-(1–7) (Mas ant) (1 μmol/L) or Krebs buffer alone and then incubated for a further 30 min with 0.1 μmol/L Ang-(1–7). TH expression was measured by western blot as described in Materials and methods and is expressed as a percentage of the response detected in control basal cells, i.e. those incubated with Krebs buffer alone. Each bar represents the mean ± SEM of three determinations from four separate cell culture preparations; $^*p < 0.05$ when compared with control.

kinases (Bobrovskaya *et al.* 2004; Dunkley *et al.* 2004; Fujisawa and Okuno 2005). Among them, phosphorylation of Ser40 causes the most prominent activation of TH (Bobrovskaya *et al.* 2004; Dunkley *et al.* 2004). Phosphorylation of TH at Ser19 or Ser8 has no effect on TH activity, but phosphorylation at Ser19 alters the conformation of TH to allow increased accessibility of Ser40 to kinases leading to an increase in the enzyme activity (Dunkley *et al.* 2004; Lehmann *et al.* 2006). Funakoshi *et al.* (1991) reported that activation of TH by Ser phosphorylation is maximal at 5 min, lasting up to 20 min. Our results showed that after 15 min of stimulation, Ang-(1–7) decreases TH phosphorylation at Ser19 and 40 in neuronal cultures from hypothalamic-brainstem of WKY and SHR, in contrast to the reported enhanced-TH activity and phosphorylation of TH at Ser19, 31, and 40 caused by Ang-II in rat mediobasal hypothalamic cultures (Ma *et al.* 2004). As a result, by altering TH phosphorylation, the peptide decreases TH activity, resulting in a decreased neurotransmitter synthesis. Thus, the reduced TH activity caused by Ang-(1–7) might be because of a decrease in TH phosphorylation and not to a decrease in TH content at this early time point (Fig. 4), because the amount of TH was not modified after treatment with Ang-(1–7) for 15 min.

In contrast, exposure of neuronal cultures to Ang-(1–7) for 30 min induced TH down-regulation (present results). The lack of response of Ang-(1–7) on TH expression for longer time periods might be because of its rapid degradation because of its peptidergic nature. In accord, TH content was reduced after 60 min when Ang-(1–7) was added twice, at 0 and 30 min, demonstrating that Ang-(1–7) degradation is responsible for the recovery of TH levels after 60 min stimulation with Ang-(1–7) (data not shown). In fact, we observed that Ang-(1–7) levels decreased while metabolites of lower molecular weight increased after 60-min incubation, and only traces of the peptide were detectable after 3 h-incubation.

The fact that a higher concentration of Ang-(1–7) (1 μmol/L) did not modify either TH enzymatic activity or endogenous TH protein levels might be because of desensitization of Ang receptors (Sasamura *et al.* 1994). It may also happen that higher concentrations of Ang-(1–7) may bind to AT_1 receptors leading to AT_1 receptors activation, and in this way, antagonize Ang-(1–7) inhibitory effects on TH. In fact,

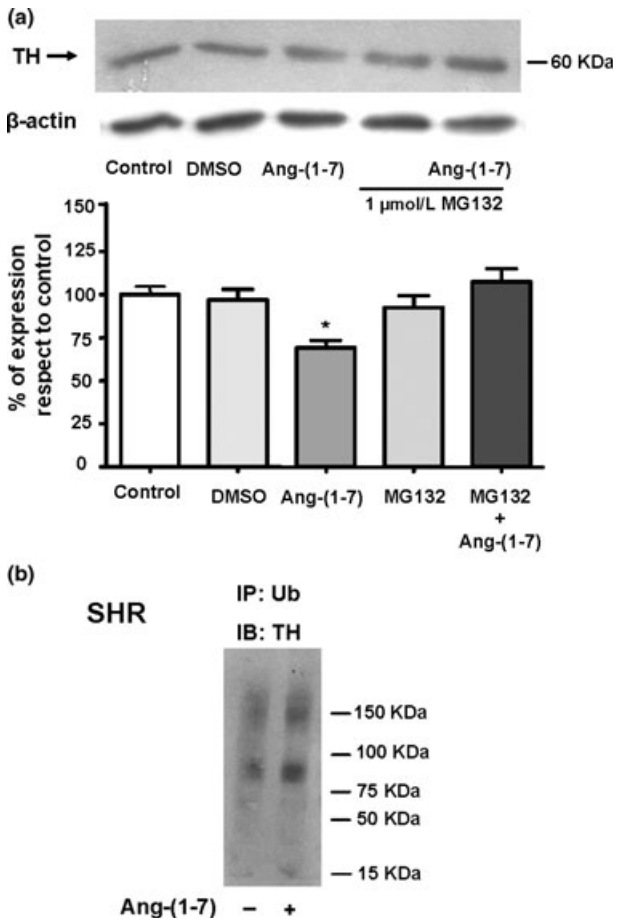

Fig. 7 (a) Effect of proteasome inhibition on Ang-(1–7)-induced down-regulation of TH in hypothalami-brainstem neuronal cultures from SHR. Cells were pre-incubated for 15 min with the proteasome inhibitor 1 μmol/L MG132 or Krebs alone and then incubated for another 30 min in the absence or presence of 0.1 μmol/L Ang-(1–7). TH expression was measured by western blot as described in Materials and methods and is expressed as a percentage of the response detected in control basal cells, i.e. those incubated with Krebs buffer alone. Each bar represents the mean ± SEM of three determinations from three separate cell culture preparations; $*p < 0.05$ when compared with control. (b) TH ubiquitination: neuronal cultures from SHR were treated with 0.1 μmol/L Ang-(1–7) or Krebs alone for 30 min in the presence of the proteasome inhibitor to prevent degradation of ubiquitinated TH (Ub-TH). After immunoprecipitation with anti-Ub antibody, western blot was performed using anti-TH antibody as described in Materials and methods. A representative blot from one of four separate independent experiments is shown.

Ang-(1–7) recognizes AT_1 receptors, although with a lower affinity than that displayed by Ang-II (Diz and Ferrario 1996; Gironacci *et al.* 1999). Supporting our hypothesis, it was shown that AT_1 receptors antagonize the central hypotensive effect caused by high concentrations of Ang-(1–7) (Höcht *et al.* 2008). In accord, Ang-(1–7) evoked a depressor response in SHR via AT_2 receptors activation during AT_1 receptor blockade (Walters *et al.* 2005).

Although Mas receptors have been shown to bind Ang-(1–7) and to be involved in many of its biological actions (Santos *et al.* 2003, 2008), our present results demonstrate that the Ang-(1–7)-decreased TH expression is independent of Mas receptors activation. In contrast, the decreased TH expression evoked by Ang-(1–7) was mediated by AT_2 receptor activation (present results), which are expressed in neuronal cultures from 1-day-old SHR hypothalamus and brainstem (Sumners *et al.* 1993). Various central effects of Ang-(1–7) are impaired by AT_2 antagonists; i.e. the inhibited NE release in rat hypothalamus from normotensive and SHR rats (Gironacci *et al.* 2000, 2004a), the Ang-(1–7)-induced neuronal excitation in the paraventricular nucleus (Felix *et al.* 1991), the prostaglandin release in astrocytes (Jaiswal *et al.* 1991), or the phosphoinositide turnover enhancement in neonatal rat brain (Pereyra-Alfonso *et al.* 2007) were blocked by AT_2 antagonists, reflecting an interaction of Ang-(1–7) with these receptors.

While the classical actions of Ang-II are mediated through AT_1 receptor activation, stimulation of AT_2 receptors may offset or oppose the AT_1 mediated actions of Ang-II on cell growth, blood pressure, and fluid intake (Horiuchi *et al.* 1999). Both AT_1 and AT_2 receptors appear to have antagonistic roles on noradrenergic neurotransmission. For instance, in cultured porcine chromaffin cells AT_1 increases catecholamine synthesis whereas stimulation of AT_2 decreases it (Takekoshi *et al.* 2002); the Ang-II-facilitated NE release is mediated by AT_1 receptors (Timmermans *et al.* 1993), whereas Ang-(1–7) inhibitory action on NE release is coupled to AT_2 receptors (Gironacci *et al.* 2004b). In accord, the Ang-II-stimulated TH activity and expression at the central level was blocked by an AT_1 antagonist while an AT_2 antagonist was without effect, demonstrating that the effect of Ang-II is mediated by AT_1 and not by AT_2 receptors (Yu *et al.* 1996), whereas the Ang-(1–7)-mediated reduction in TH expression in catecholaminergic neurons is coupled to AT_2 receptors (present results). In addition, AT_2 receptors stimulation in the rostral ventrolateral medulla exhibited an inhibitory effect on sympathetic outflow while AT_1 receptors displayed the opposite effect (Gao *et al.* 2008).

It has been reported that AT_2 receptors bind directly to AT_1 receptors and thereby antagonize receptor function. This heterodimerization may modulate Ang-II responses (AbdAlla *et al.* 2001). It is reasonable to hypothesize that Ang-(1–7), acting through AT_2 receptors, may induce AT_1–AT_2 heterodimerization leading to inhibition of AT_1-mediated sympathoexcitatory effects of Ang-II. Further experiments have to be performed to elucidate this hypothesis.

The Ub–proteasome system is a multicatalytic protease complex found in almost all living cells and is responsible for selective protein degradation in the cytoplasm (Glickman and Ciechanover 2002). Degradation of a protein via this system involves covalent conjugation of Ub to the target protein, followed by its recognition by the 26S proteasome and

degradation of the polyubiquitinated protein into small peptides (Glickman and Ciechanover 2002). Proteasome complexes have been detected in neurons from rat brains and neuronal cultures (Adamo *et al.* 1994). It has previously been reported that Ang-II decreases insulin receptor substrate-1 protein levels, a protein that under insulin receptor stimulation coordinates downstream signaling events, in vascular smooth muscle cells via proteasome-dependent degradation (Taniyama *et al.* 2005). This Ang-II-mediated effect impairs the vasodilator and glucose transport properties of insulin and contributes to insulin resistance (Taniyama *et al.* 2005). Our present results demonstrate that the Ang-(1–7)-induced down-regulation of TH expression in neuronal cultures from SHR is mediated by the Ub–proteasome pathway stimulation. Thus, Ang-(1–7) stimulates both Ub conjugation to TH and its subsequent proteasome recognition, which in turn increases TH degradation, leading to a decreased cellular content of TH.

In conclusion, we have demonstrated that Ang-(1–7) acts as a negative modulator of central TH in normotensive and hypertensive rats at two levels: activity and expression of the enzyme. Ang-(1–7) diminishes TH enzymatic activity by reducing its phosphorylation. Ang-(1–7) also down-regulates TH expression by increasing its degradation through AT_2 receptor activation by a proteasome-dependent pathway. This is another example of an AT_2-mediated effect that opposes an AT_1-mediated effect, reducing in this way TH endogenous content. Furthermore, it is the first time that the Ub–proteasome stimulation is reported for Ang-(1–7), demonstrating a novel mechanism of action to down-regulate a protein involved in hypertension development (Rao *et al.* 2007). These findings together with the fact that Ang-(1–7) inhibits NE release from SHR hypothalami (Gironacci *et al.* 2004a) and that it is endogenously present in catecholaminergic neurons (present results) lead us to suggest that the peptide elicits a negative neuromodulatory role on the sympathetic nervous system at the central level, thus contributing to blood pressure regulation.

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