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Influence of thyroid disorders on the kidney expression and plasma activity of aminopeptidase A

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Objective. Thyroid disorders may affect blood pressure and renal function modifying factors of the plasmatic and kidney renin-angiotensin system such as aminopeptidase A (AP A) that metabolizes angiotensin II to angiotensin III. We investigated the expression of AP A in the kidney, as well as its enzymatic activity in the plasma of euthyroid, hyperthyroid, and hypothyroid adult male rats.

Methods. Hyperthyroidism was induced by daily subcutaneous injections of tetraiodothyronine. Hypothyroid rats were obtained by administration of methimazole in drinking water. Expression of AP A was determined by Western blot analysis. Plasma AP A activity was measured fluorometrically using glutamyl-β-naphthylamide as substrate.

Results. While hyperthyroid rats exhibited lower levels of plasma AP A activity than controls, the kidney of hyperthyroid animals expressed significantly higher AP A than controls and hypothyroid animals.

Conclusions. A discrepancy between the high expression of AP A in kidney of hyperthyroid rats and the low activity of AP A measured in plasma and kidney of hyperthyroid animals was found. The posttranslational influence of environmental biochemical factors may be in part responsible for that divergence.

Key words: hyperthyroidism, hypothyroidism, aminopeptidases, arylamides

Thyroid disorders affect blood pressure and renal function through changes in the components of the local renin-angiotensin system such as aminopeptidase A (AP A or GluAP), which metabolizes angiotensin II (Ang II) to angiotensin III (Ang III), consequently regulating their functions (Segarra et al. 2006). It has been shown that while hypothyroidism increases, hyperthyroidism decreases the kidney AP A activity in cortex and medulla, respectively (Segarra et al. 2006). These changes may account for alterations in renal function during thyroid disorders (Vargas et al. 2006). However, we have recently hypothesized that aminopeptidase activities are influenced in part by the biochemical environment created by the fluctuating levels of factors such as cholesterol and steroids, especially testosterone, in the internal milieu (Martinez et al. 1998; Ramirez et al. 2008; Segarra et al. 2008). Alterations in serum cholesterol (Sauter et al. 1997) and testosterone levels (Bruni et al. 1975) are well documented in hypothyroidism and hyperthyroidism. Therefore, the objective of the present study was to investigate the status of AP A expression in the kidney of euthyroid, hyperthyroid, and hypothyroid adult male rats as well as its enzymatic activity in plasma.

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Materials and Methods

Animals and treatment. To analyze the expression of AP A, nine adult male Wistar rats weighing 180-200 g at the beginning of the experiments were randomly divided into three groups: Controls (euthyroid) (n=3), hypothyroid (n=3) and hyperthyroid (n=3). The animals were kept in a temperature-controlled room (24±1°C) and housed in standard laboratory cages with a 12h/12h light/dark schedule. Standard chow and tap water were provided *ad libitum* during all experimental period. Hyperthyroidism was induced with daily subcutaneous injections of tetraiodothyronine (300 μg/kg/day). Hypothyroid rats were obtained with 0.03% methimazole in drinking water (Segarra et al. 2006). For plasma determinations of AP A, twenty one adult male Wistar rats weighing 180-200 g were divided into three groups (each one n=7): Controls, hyperthyroid, and hypothyroid. After 6 weeks of treatment, the animals were anaesthetized with equithensin (2 ml/kg body weight) and the right kidneys were quickly removed and cooled in dry ice. Blood samples were obtained from the left cardiac ventricle and plasma was isolated after centrifugation at 2000xg during 10 min at 37°C. The experiments were performed according to the European Union guidelines for the ethical care of animals.

Western blot analysis. Expression of AP A was determined by Western blot analysis. Briefly, the kidney was homogenized with a tissue grinder in a solution containing sucrose 250 mM, protease inhibitor cocktail tablets (Roche Diagnostics, Barcelona, Spain), 1 % Triton X100, and Tris-HCl 10 mM at pH 7.6, and centrifuged at 1000xg for 15 min at 4°C. Protein concentrations were determined in the supernatant using a DC Protein Assay kit (Bio-Rad). 30 μg of protein were subjected to SDS-PAGE using 8% gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain, which verified the uniformity of protein load and transfer efficiency across the test samples. After blocking with Tris-based saline buffer containing 5% nonfat milk, the membrane was probed with goat anti-AP A polyclonal antibody (Everest Biotech, Upper Heyford, UK) used at 1 μg/ml in blocking buffer at 4°C overnight. Bound antibodies were detected with a secondary horseradish peroxidase-conjugated rabbit anti-goat antibody (KPL, Gaithersburg, MD, USA). Membrane was retested with rabbit anti-alpha tubulin polyclonal antibody (Labfrontier, Seoul, Korea) at 0.1 μg/ml, as a loading control, and bound antibodies were detected with a secondary HRP-conjugated goat anti-rabbit antibody (Biomedal, Sevilla, Spain). The bands were visualized using the enhanced chemiluminescence system ECL Plus (Amersham, Amersham, UK), and the images were captured using an autoradiography film, scanned and quantified with the program Image J (NIH).

Aminopeptidase A activity was determined in a fluorometric assay using glutamyl-β-naphtylamide as the substrate according to the method of Tobe et al. (1980) modified previously (Villarejo et al. 2014) as follows: 25 ml of plasma was incubated during 120 min at 37°C with 1 ml of the substrate solution (2.72 mg/100 ml glutamyl-β-naphtylamide, 10 mg/100 ml BSA, 10 mg/100 ml DTT and 0.555 g/100 ml $CaCl_2$ in 50 mM HCl-Tris, pH 7.4). To stop the enzymatic reaction, 1 ml of 0.1 M of acetate buffer, pH 4.2 was added to the medium. Because of the enzymatic activity, the ß-naphthylamine was released by hydrolysis from the substrate and then determined by fluorimetry at 412 nm emission wavelength with an excitation wavelength of 345 nm. Quantification of proteins was performed colourimetrically at 595 nm by the method of Bradford (1976), using the dye Coomassie Brilliant Blue G250 and BSA as a standard. Specific AP A activity was expressed as pmol of glutamyl-ß-naphthylamide hydrolyzed per min per mg of protein.

Statistical analysis. Student's t test was used to analyze the possible differences between groups. Differences were considered statistically significant at $p<0.05$ level.

Results

Results are shown in Fig. 1 and Fig. 2. In plasma, hyperthyroid rats demonstrated slight but significantly lower levels of AP A ($p=0.049$) than euthyroid (control) rats. Hyperthyroid animals also showed lower levels of AP A than hypothyroid but without reaching statistical significance ($p=0.053$). No differences were observed between control and hypothyroid animals (Fig. 1).

The kidney of hyperthyroid animals expressed significantly higher AP A protein levels than controls ($p<0.05$ + 67%) and hypothyroid animals ($p<0.01$ + 77%). No differences were observed between control and hypothyroid rats (Fig. 2).

Discussion

It is known that while hyperthyroidism exhibits high levels of plasma renin activity (PRA) and increased car-

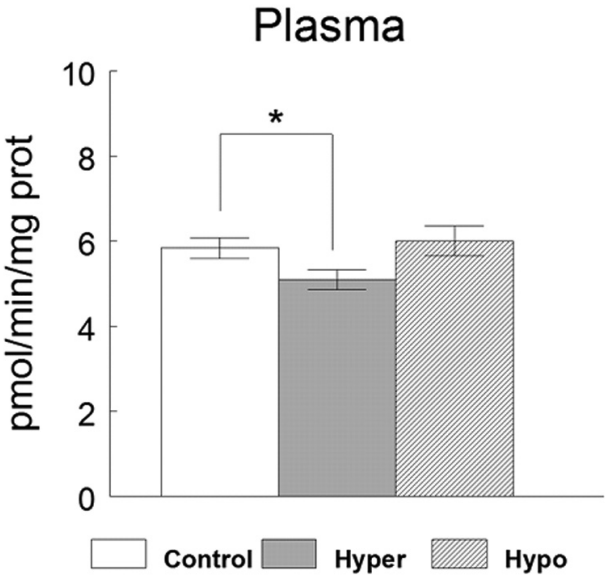

Fig. 1. Values represent mean ± S.E.M. levels of aminopeptidase A activity in plasma of euthyroid (Control), hyperthyroid (Hyper) and hypothyroid (Hypo) adult male rats. Aminopeptidase A activity is expressed as pmol of glutamyl-β-naphthylamide hydrolyzed per min per mg of protein. * $p < 0.05$.

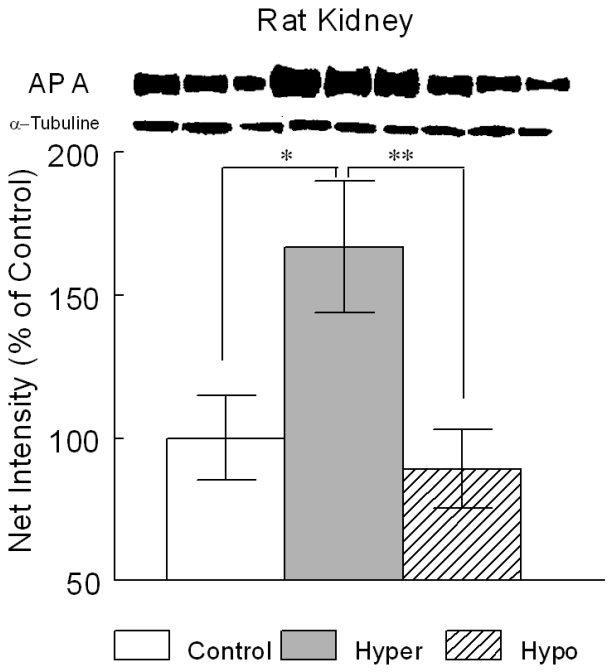

Fig. 2. *Top*: Western blot analysis of the expression of aminopeptidase A in the whole kidney of euthyroid (Control), hyperthyroid (Hyper) and hypothyroid (Hypo) adult male rats. α–tubulin was used as a loading control. *Botton*: Densitometry analysis expressed as percentage (mean ± S.E.M.) of net intensity from control non-treated rats.

diac output with high levels of blood pressure, hypothyroid rats show a decreased cardiac output with low levels of blood pressure compared with euthyroid animals (Vargas et al. 2006). Decreased levels of AP A activity in plasma of hyperthyroid rats suggest a lower metabolism of Ang II and, in consequence, an increased availability and longer hypertensive effect of this peptide. Therefore, the present results obtained in plasma would be in agreement with those observations. In addition, renin activity and Ang II levels are increased in the kidney of hypertensive rats (Vargas et al. 2006) which, also agree with previous results that demonstrated decreased levels of AP A in the kidney of hyperthyroid rats (Segarra et al. 2006). Again, we could suggest that lower levels of AP A activity in the kidney of hyperthyroid rats implies a lower metabolism of Ang II, which therefore exert its action for a longer period.

These previous results are therefore in marked contrast with the present ones in which the expression of AP A is significantly higher in hyperthyroid rats in comparison with control and hypothyroid rats. These results however agree with a previous report showing an increased renal renin and Ang II levels and an enhanced renal renin mRNA expression in hyperthyroid rats (Kobori et al. 1998). Thus, the increased protein expression does not necessarily correlate with an increased protein activity (Lawrence 2001). The present results suggest that although the protein expression is increased, its activity could be inhibited by undetermined factors. Other authors have reported similar discrepancies. *In vitro*, aminopeptidase Y gene expression was less abundant in yeast grown in a minimal nutritional medium than in a rich medium whereas its activity was increased (Herrera-Camacho and Suarez-Rendueles 1996). Similar discrepancy has been observed between angiotensin converting enzyme (ACE) and neutral endopeptidase mRNA levels and the corresponding enzyme activities in olfactory bulb and striatum of alcohol-preferring and alcohol-avoiding rat lines developed through selective out breeding (Winkler et al. 1998). Another study has demonstrated non-proportional changes in PRA and renal renin mRNA levels after administration of an ACE inhibitor (Morishita et al. 1993). PRA was significantly elevated while there was no change in renin mRNA. Similar imbalance with reduced insulin receptor gene expression and increased in insulin receptor tyrosine kinase activity in hippocampus has been also observed in the brain insulin system after streptozotocin ICV injection (Grunblatt et al. 2007). Dopamine receptor antagonist (haloperidol) did not affect aminopeptidase

N mRNA expression but decreased its activity in the rat frontal cortex (Waters et al. 1997). All these results suggest that the discrepancy between mRNA expression and enzymatic activity may be linked to post-transcriptional events. Our data along with the observations reported in the literature support the hypothesis of a key influence of the biochemical environmental on AP A activity (Ramirez et al. 2008). We have previously demonstrated the direct influence *in vitro* of cholesterol and steroids on AP A activity (Martinez et al. 1998). These results have already been widely discussed (Ramirez et al. 2008). Since changes in cholesterol and steroid levels *in vivo* paralleled with changes in AP A activity, we hypothesize a partial influence of these factors on the enzyme activity: cholesterol and testosterone may be amongst the post-translational factors. Thyroidectomy in rats with intact gonads resulted in a significant decrease in serum testosterone in males. Administration of thyroxine to thyroidectomized rats restored normal serum testosterone levels (Bruni et al. 1975). In addition, it has been well established that hypothyroidism is characterized by hypercholesterolaemia and that hyperthyroidism exhibits decreased plasma levels of total cholesterol (Sauter et al. 1997). Therefore, since thyroid disorders modified cholesterol and testosterone levels, these changes may account in part for a post-transcriptional influence that explain the discrepancy observed between AP A expression and activity. Thyroid hormones may also complementarily influence AP A activity. In addition, the present results may suggest a compensatory response of the kidney to the lower enzymatic activity and higher presence of Ang II in hyperthyroid rats.

In conclusion, a discrepancy between the AP A expression in kidney and AP A activity in plasma and kidney was found. This divergence may support our hypothesis of an influence of surrounding biochemical factors, such as cholesterol and steroids, on AP A activity (Ramirez et al. 2008) and highlight the (relative) importance of components of the biochemical environment on the control of some factors of the renin-angiotensin system.

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