Differential responses to salt supplementation in adult male and female rat adrenal glands following intrauterine growth restriction

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Abstract

In low sodium-induced intrauterine growth restricted (IUGR) rat, foetal adrenal steroidogenesis as well as the adult renin–angiotensin–aldosterone system (RAAS) is altered. The aim of the present study was to determine the expression of cytochrome P450 aldosterone synthase (P450aldo) and of angiotensin II receptor subtypes 1 (AT₁R) and 2 (AT₂R) in adult adrenal glands and whether this expression could be influenced by IUGR and by high-salt intake in a sex-specific manner. After 6 weeks of 0.9% NaCl supplementation, plasma renin activity, P450aldo expression and serum aldosterone levels were decreased in all groups. In males, IUGR induced an increase in AT₁R, AT₂R, and P450aldo levels, without changes in morphological appearance of the zona glomerulosa (ZG). By contrast, in females, IUGR had no effect on the expression of

AT₁R, but increased AT₂R mRNA while decreasing protein expression of AT₂R and P450aldo. In males, salt intake in IUGR rats reduced both AT₁R mRNA and protein, while for AT₂R, mRNA levels decreased whereas protein expression increased. In females, salt intake reduced ZG size in IUGR but had no effect on AT₁R or AT₂R expression in either group. These results indicate that, in response to IUGR and subsequently to salt intake, P450aldo, AT₁R, and AT₂R levels are differentially expressed in males and females. However, despite these adrenal changes, adult IUGR rats display adequate physiological and adrenal responses to high-salt intake, via RAAS inhibition, thus suggesting that extra-adrenal factors likely compensate for ZG alterations induced by IUGR.

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Introduction

The renin–angiotensin system (RAS) via angiotensin II (Ang II) plays a key role in the regulation of blood pressure and hydro–mineral balance through the control of vascular tone and in renal sodium reabsorption either directly or through stimulation of adrenal aldosterone production (Schiffrin & Touyz 2004, Hunyady & Catt 2006, Otis & Gallo-Payet 2007, Otis *et al.* 2007). Ang II binds two major receptors, the Ang II type-1 receptor (AT₁R) and type-2 receptor (AT₂R). Binding of Ang II to the widely expressed AT₁R plays a predominant role in the capacity of the adrenal zona glomerulosa (ZG) to produce aldosterone (Capponi 2004, Schiffrin & Touyz 2004, Hunyady & Catt 2006) via cytochrome P450 aldosterone synthase (P450aldo; Bassett *et al.* 2004, Capponi 2004). In contrast to several adult tissues, AT₂R is relatively highly expressed in the adult adrenal glands (ZG and medulla; Breault *et al.* 1996, Frei *et al.* 2001, Macova *et al.* 2008). In various pathological situations, it is generally assumed that Ang II stimulation of the AT₂R counteracts the effects of AT₁R (de Gasparo *et al.* 2000, Schiffrin & Touyz 2004).

Interestingly, the RAS has been identified both as a target for the programming of adult diseases (McMillen & Robinson

2005) and as an important contributor for the detrimental cardiovascular consequences of salt intake (Meneton *et al.* 2005). In our laboratory, an animal model of impaired foetal growth was developed by administering a low-sodium diet to rats during the last week of gestation (Roy-Clavel *et al.* 1999), inducing an important activation of the maternal renin–angiotensin–aldosterone system (RAAS) in comparison to normal pregnant rats (Bedard *et al.* 2005). We recently showed that AT₁R and P450aldo mRNA expression was enhanced in intrauterine growth restriction (IUGR) foetal adrenal glands and was associated with an increase in serum aldosterone levels (Bibeau *et al.* 2010). Since Ang II does not cross the placenta (Clark *et al.* 1990), these changes are not dependent on maternal RAAS over-activation observed in low-sodium restricted dams. As young adults (12-week-old), offspring of this IUGR model have been shown to manifest a modest increase in systolic blood pressure, but a disrupted RAAS as well as renal dysfunction, evidenced by reduced glomerular filtration which was not related to a lower number of glomeruli (Battista *et al.* 2002). These modifications were less pronounced in IUGR males, suggesting differing effects between adult males and females following these adverse intrauterine conditions. Such observations have also been

reported in IUGR induced by a low-salt diet prior to and throughout pregnancy (Lopes *et al.* 2008).

Since we previously showed that IUGR offspring of dams on a low-sodium diet exhibit altered foetal adrenal steroidogenesis (Battista *et al.* 2002, 2005, Bibeau *et al.* 2010), we hypothesized that the regulation of aldosterone secretion by adrenal glands is likely influenced by IUGR. The aims of the present study were therefore to characterize the consequence of IUGR on adult male and female adrenal glands (histology, AT₁R and AT₂R expression, and steroidogenesis) and, secondly, to determine whether adrenal glands of these IUGR offsprings are vulnerable to the influence of 0.9% NaCl supplementation.

Materials and Methods

Animals, experimental design, tissue preparation and physiological measurements

This study was approved by our institutional Animal Care Committee, which is accredited by the Canadian Council on Animal Care. Female Sprague–Dawley rats (Charles River Canada, St-Constant, Québec, Canada) weighing 225–250 g were mated with a known fertile male. Day 1 of pregnancy was determined by the presence of spermatozoa in morning vaginal smears. All animals were housed under controlled lighting (0600–1800 h) and temperature (21 ± 3 °C). The dams were randomly assigned to one of two diets for the last 7 days of gestation (term = day 23). One group ($n = 13$ dams) were fed a normal diet containing 0.2% sodium (basal diet 5755; PMI Feed, Inc., Ren's Feed and Supplies, Oakville, Ontario, Canada) and tap water. They gave birth to the control offspring group. The second group ($n = 14$ dams) received a 0.03% sodium diet (low-sodium diet 5881; PMI Feed, Inc.) and demineralized water. Their offspring suffered from IUGR (Battista *et al.* 2002). After parturition, all dams received a regular diet (0.23% sodium, Teklab global 18% protein rodent diet 2018, Harlan Teklab, Montréal, Québec, Canada) and tap water. Litters were not culled because in a previous report (Roy-Clavel *et al.* 1999) we showed that litter size was unaffected by the maternal low-sodium diet (IUGR: 13 ± 1 pups versus control: 14 ± 1 pups). The control and IUGR animals were weaned at 4 weeks of age and separated into male and female sub-groups. At 6 weeks of age, males and females were randomly separated into two additional groups (0.9% NaCl supplementation and non-supplemented), according to whether or not they received the sodium supplement (0.9% NaCl in tap water), until killed at 12 weeks.

At the end of week 12, body weights were recorded for male and female (random undetermined oestrous cycle stage) animals which were then decapitated (0800–1000 h). Trunk blood was collected for hormone and electrolyte analysis. Adrenal glands were removed, weighed, and processed. Under manual compression, the adrenal cortex containing only the ZG was gently separated from the medulla to which

the zona fasciculata/reticularis (ZFR) remained attached. For each litter, adrenal zonae glomerulosa or fasciculata/reticularis from two rats of the same sex were pooled (corresponding to $n = 1$). They were immediately snap-frozen in liquid nitrogen and stored at -80 °C. For histological analyses, five adrenal glands per group were immediately immersed in 4% paraformaldehyde and subsequently embedded in paraffin. Sections (5 µm thick) were cut and stained with haematoxylin and eosin for histological examination.

Adrenal histology

Before inclusion in paraffin, each ovoid gland was trimmed down to about one-third of its larger portion. Then, in the following serial 5 µm sections, the section containing the largest diameter of medulla was chosen to measure the width of ZG. Slides were examined with a Nikon Eclipse 2000 inverted microscope (Nikon, Mississauga, Ontario, Canada) equipped for epi-illumination. Images were acquired with a Hamamatsu ORCA-ER digital camera using 10× or 20× objectives. The width of the ZG was measured on two slides representing the largest diameter of the gland, using the calliper integrated to the MetaMorph Imaging System v4.5 software package (Universal Imaging Corp., Westchester, PA, USA). Mean width was calculated using a parameter analysis module, with three separate measurements performed for each slide of the gland, and five glands in each group.

Blood sample collection, hormone measurements and analysis

After decapitation, a first blood sample (0.5 ml) was collected into a vacutainer tube (Becton Dickinson, Franklin Lakes, NJ, USA) containing 15% dipotassium EDTA solution to quantify plasma renin activity (PRA). A second sample was drawn into a plain vacutainer tube to determine serum steroid concentration. A final blood sample was collected in a lithium heparin vacutainer tube for measurement of plasma electrolytes. After centrifugation of all blood samples at 1550 *g* for 20 min at 4 °C, the serum or plasma was stored at -80 °C.

PRA was quantified indirectly as described elsewhere (Roy-Clavel *et al.* 1999). Briefly, Ang I, generated per 2 h incubation period, was measured by RIA with an antibody purchased from Peninsula Laboratories (San Carlos, CA, USA; Gutkowska *et al.* 1977). Aldosterone and corticosterone were analyzed directly from serum with commercial RIA kits (aldosterone: Intermedico, Montréal, Québec, Canada; corticosterone: Mediacorp, Montréal, Québec, Canada). Plasma sodium and potassium were measured with specific electrodes.

RNA isolation and reverse transcriptase-PCR

Total RNA from six adrenal zonae glomerulosa or fasciculata/reticularis in each group was extracted by TRIzol Reagent (Invitrogen Canada, Inc.). Final RNA pellets were dissolved in an appropriate volume of diethylpyrocarbonated water and stored at -80 °C. RNA concentration was

established by absorbance measurement at 260 nm, with sample integrity ascertained by the 260/280 nm ratio. Quality was verified by ethidium bromide fluorescence.

PCR primers specific for the genes of interest were designed with PRIMER3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), based on sequence data from the National Center for Biotechnology Information (Table 1). For AT₁R, since AT_{1b} expression is the predominant form in the rodent adrenal gland (Gasc *et al.* 1994), the AT_{1b} sequence primers were selected for our studies.

Single-stranded cDNA was created by reverse transcriptase (RT; SuperScript II RNase H-Reverse Transcriptase, Invitrogen) and PCR (Taq DNA Polymerase, Invitrogen) was performed according to the procedure detailed by the manufacturer. The annealing step was carried out for 1 min at temperatures established for each gene in adrenal ZG or ZFR (Table 1). The extension step was undertaken at 72 °C for 1 min. PCR results were collected during the exponential phase, since the number of cycles needed for amplification was determined for each gene in each tissue (Table 1). To ensure that genomic DNA was not amplified, RT was performed without enzyme and PCR was conducted as previously described. PCR products were assessed by electrophoresis on 1% agarose gels and visualized by ethidium bromide fluorescence. Amplification products were quantified by Alpha Imager software (Alpha Innotech Corporation, San Leandro, CA, USA). The procedure was conducted on triplicate samples and the results reported on glyceraldehyde-3-phosphate dehydrogenase as internal control.

AT₁R and AT₂R and P450aldo, P45011β protein expression

Frozen adrenal zonae glomerulosa or zonae fasciculata/reticularis (*n*=6/group) were homogenized in lysis buffer containing 50 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, and 1 × complete EDTA-free protease inhibitor cocktail tablet (Roche) and centrifuged at 12 000 *g* for 30 min at 4 °C. Supernatants were collected and subsequently resuspended in Laemmli buffer. For comparative purposes, proteins (25 µg for AT₁R, 10 µg for AT₂R, 50 µg for P450aldo, and 20 µg for P45011β) were separated by SDS-PAGE (12% for AT₁R and AT₂R and 10% for P450aldo and P45011β) and then transferred electrophoretically onto nitrocellulose membranes (Fisher Scientific, Nepean, Ontario, Canada). Nonspecific sites on the membranes were blocked for 1 h at room temperature in 5% skim milk PBS (1.4 M NaCl, 2.7 mM KCl, 100 mM Na₂HPO₄, 17.6 mM KH₂PO₄, pH 7.4)/0.1% Tween-20. Overnight incubation at 4 °C was conducted with rabbit anti-AT₁R (1/100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or AT₂R (1/500; Santa Cruz Biotechnology, Inc.), mouse anti-P450aldo (1/500; Chemicon, Temecula, CA, USA), or P45011β (1/1000, Chemicon). Membranes were then washed and incubated with HRP-conjugated anti-mouse or anti-rabbit IgG (1/5000; Amersham) for 45 min at room temperature. Equal protein

Table 1 PCR primers designed (by alpha DNA) for genes of interest

Gene name (gene symbol)—gene number	Forward primer (FP) (5'–3')	Reverse primer (RP) (5'–3')	Product size (bp)	Tissue	Optimal conditions	
					Temperature (°C)	Cycle (number)
Angiotensin receptor type 1 _b (AT _{1b} R) – NM_031009	FP: GCTAAGCAGCTCACTCACTAC	RP: AACTCTTGACCTCCCATCTC	363	ZG	56	30
Angiotensin receptor type 2 (AT ₂ R) – NM_012494	FP: GAAGGACAACCTTCAGTTTTC	RP: CAAGGGGAACACTACATAAGATGC	497	ZG	56	30
Cytochrome P450 11β-hydroxylase (P45011β) – XM_343262	FP: TCATATCCGAGATGGTAGCA	RP: GCTCAGGTCTTTGGGAACAC	399	ZFR	52	22
Cytochrome P450 aldosterone synthase (P450aldo) – NM_012538	FP: GGATGTCCAGCAAGTCTC	RP: ATTAGTCTGCCACAATGC	324	ZG	59	29
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) – NM_017008	FP: GGTGATGCTGGTGCTGAGTA	RP: GGATGCAGGGGATGATGTTCT	369	ZG	58	22
				ZFR	58	20

Table 2 Effects of 0.9% NaCl supplementation on body weight, adrenal gland weight and relative adrenal gland weight of 12-week-old control and intrauterine growth restriction (IUGR) male and female rats. Results are expressed as means ± s.e.m. (n=20–25 rats/group)

	Control		IUGR	
	Non-supplemented	0.9% NaCl	Non-supplemented	0.9% NaCl
Males				
Body weight (g)	444 ± 8	445 ± 8	426 ± 7	430 ± 5
Adrenal gland weight (mg)	57 ± 2	56 ± 1	56 ± 2	54 ± 1
Relative adrenal gland weight (% × 10 ⁻³)	12.8 ± 0.3	12.9 ± 0.3	12.9 ± 0.3	12.6 ± 0.3
Females				
Body weight (g)	259 ± 5	266 ± 6	245 ± 5	261 ± 5
Adrenal gland weight (mg)	63 ± 2	67 ± 2	60 ± 1	62 ± 2
Relative adrenal gland weight (% × 10 ⁻³)	24.3 ± 0.8	25.3 ± 0.5	24.8 ± 0.3	23.5 ± 0.5

loading was determined on the same membrane with mouse anti-β-actin primary antibody (1/100 000; Novus Biologicals, Inc., Littleton, CO, USA) and with HRP-conjugated sheep anti-mouse IgG or donkey anti-rabbit (1/5000; Amersham). Immunoreactive bands were visualized with the ECL western blotting analysis system (GE HealthCare Biosciences, Inc., Montréal, Québec, Canada) and quantified by Alpha Imager software (Alpha Innotech Corporation). Results are expressed as densitometric units relative to β-actin.

Statistical analyses

Data are expressed as means ± s.e.m. Results were tested for normality using a Shapiro–Wilk test. If normality could not be assumed, the data were normalized using a log₁₀ transformation. The (normalized) data were tested for the effects and interactions of sex, IUGR, and 0.9% NaCl supplementation by the General Linear Model Univariate Analysis procedure (SPSS Statistics 16; IBM Company, Chicago, IL, USA). Since significant interactions were

Figure 1 Effects of IUGR and 0.9% NaCl supplementation on adrenal zona glomerulosa width. Adrenal glands were immediately immersed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm thick) were cut and stained with haematoxylin–eosin for general histological examination. (A) Representative illustration of adrenal gland cortex. Scale bar, 30 μm. ZG, zona glomerulosa and ZFR, zona fasciculata-reticularis. (B) Results are expressed as means ± s.e.m. (n=5/group). Salt-supplemented groups significantly different from their non-supplemented counterparts are indicated by **P*<0.05.

found for sex in several parameters, all the studied parameters were analyzed separately for sex. Two-factor ANOVA was thus performed on the effects of IUGR and 0.9% NaCl supplementation, followed by the Newman–Keuls multiple range test. Values were considered to be significantly different if $P < 0.05$. When significant interactions between IUGR and 0.9% NaCl supplementation were found, an unpaired Student's *t*-test was conducted to compare salt-supplemented groups to their non-supplemented counterpart as well as to compare both non-supplemented groups (IUGR versus control). Owing to multiple comparisons, the minimum level of statistical significance was then assumed to be $P < 0.05$.

Results

Effects of IUGR and 0.9% NaCl supplementation on body weight and adrenal glands

Body weight at 12 weeks of age, as well as weight and relative weight of adrenal glands were unchanged between groups of males and females (Table 2).

Histological examination of the adrenal glands indicated that, in males, neither IUGR nor 0.9% NaCl supplementation altered the width of the ZG (Fig. 1, a–d). In females, two-factor ANOVA analysis revealed that NaCl supplementation induced an important reduction in ZG width in IUGR animals (Fig. 1, h versus g, $P < 0.05$, Student's *t*-test, Fig. 1B, right panel).

Effects of IUGR and 0.9% NaCl supplementation on electrolytes and circulating hormonal levels

Plasma sodium and potassium levels in males or in females were not affected by IUGR (data not shown). Similarly, NaCl supplementation did not modify plasma sodium concentrations in either male or female rats. However, plasma potassium levels in females but not in males were decreased by NaCl intake ($P < 0.05$, two-factor ANOVA).

In males, PRA was unaltered by IUGR (Fig. 2A, left panel) but was decreased in NaCl supplementation-treated rats, both in control and IUGR groups ($P < 0.05$, Newman–Keuls test). In females (Fig. 2A, right panel), PRA was similarly reduced in NaCl-supplemented controls and in non-supplemented IUGR ($P < 0.05$, Newman–Keuls test). The presence of both conditions further reduced PRA.

Serum aldosterone levels were reduced by IUGR but only significantly in males (Fig. 2B). NaCl supplementation induced the expected decrease in aldosterone both in control and in IUGR rats, including both males and females. In contrast, serum corticosterone was not affected by IUGR or by NaCl supplementation in either males or females (Fig. 2C).

Effects of IUGR and 0.9% NaCl supplementation on AT_1R and AT_2R expression in adrenal ZG

In males (Fig. 3A, left panel), IUGR increased AT_1R mRNA levels compared with control groups ($P < 0.05$,

Newman–Keuls test). However, NaCl supplementation decreased the AT_1R mRNA levels in both control and IUGR male groups ($P < 0.05$, Newman–Keuls test). At the protein level, AT_1R protein expression was increased in non-supplemented IUGR males compared with their controls ($P < 0.05$, Student's *t*-test; Fig. 3B, left panel). Moreover, NaCl intake had an opposite effect in control and IUGR groups. Indeed, a small increase was observed in control animals ($P = 0.06$, Student's *t*-test), conversely to a decrease in IUGR animals ($P < 0.05$, Student's *t*-test). In females, both IUGR and NaCl supplementation had no effect on either AT_1R mRNA or protein expression (Fig. 3A and B, right panel).

In males, AT_2R mRNA expression was increased by IUGR and decreased by NaCl supplementation (Fig. 4A, left panel; $P < 0.05$, Newman–Keuls test), similarly to that observed for AT_1R mRNA expression. Surprisingly, protein

Figure 2 Effects of IUGR and 0.9% NaCl supplementation on (A) plasma renin activity, (B) serum aldosterone, and (C) serum corticosterone in 12-week-old control and IUGR animals. Results are expressed as means $\pm$ S.E.M. ($n = 17$ –26 rats per group). Salt-supplemented groups significantly different from their non-supplemented counterparts are indicated by * $P < 0.05$, while non-supplemented groups significantly different from each other are indicated by † $P < 0.05$.

Figure 3 Effects of 0.9% NaCl supplementation on the expression of AT₁R mRNA (A) and protein (B) in adrenal zona glomerulosa from 12-week-old control and IUGR rats. Representative gels for PCR amplification products and immunoblots are shown. For purposes of conciseness, two samples/group are illustrated. Data from densitometric analysis are expressed in arbitrary units. Results are expressed as means $\pm$ S.E.M. ($n=6$ /group). Salt-supplemented groups significantly different from their non-supplemented counterparts are indicated by * $P<0.05$, while non-supplemented groups significantly different from each other are indicated by [†] $P<0.05$.

expression was not influenced by IUGR, but was enhanced by NaCl intake ($P<0.05$, two-factor ANOVA; Fig. 4B, left panel). In females, IUGR increased AT₂R mRNA expression (Fig. 4A, right panel; $P<0.05$, Newman–Keuls test) and decreased protein expression (Fig. 4B, right panel; $P<0.05$, two-factor ANOVA). NaCl supplementation did not show any effect on expression of AT₂R mRNA nor protein.

Effects of IUGR and NaCl supplementation on steroidogenesis in adrenal ZG

In males, as observed for AT₁R and AT₂R, P450aldo mRNA expression was increased by IUGR and decreased by NaCl supplementation (Fig. 5A, left panel; $P<0.05$, two-factor ANOVA). A similar pattern was also observed for protein expression (Fig. 5B, left panel; $P<0.05$, two-factor ANOVA). In females, IUGR had no effect on P450aldo mRNA expression, which was decreased by NaCl supplementation. (Fig. 5A, right panel; $P<0.05$, Newman–Keuls test). Protein expression was decreased by IUGR and NaCl supplementation (Fig. 5B; $P<0.05$, two-factor ANOVA).

In males, P45011 β mRNA expression was not affected by IUGR or by 0.9% NaCl supplementation (Fig. 6A, left panel), although protein level was increased by NaCl supplementation ($P<0.05$, two-factor ANOVA, Fig. 6B left panel). In females, P45011 β mRNA expression was increased only in IUGR NaCl-supplemented animals (Fig. 6A, right panel; $P<0.05$, two-factor ANOVA). Protein level was not affected by IUGR or by NaCl supplementation (Fig. 6B, right panel).

Discussion

The present study was undertaken to assess the consequences of IUGR on the expression of Ang II receptors and on adrenal steroidogenesis enzymes in the adult rat submitted to excess sodium ingestion, a condition often observed in the human adult population. We show herein that, in adult males, mRNA expression of Ang II receptors, both AT₁R and AT₂R, as well as of P450aldo was increased by IUGR, while sodium supplementation consistently decreased this expression. Female rats hardly manifested any changes in the

Figure 4 Effects of 0.9% NaCl supplementation on the expression of AT₂R mRNA (A) and protein (B) in adrenal zona glomerulosa from 12-week-old control and IUGR rats. Representative gels for PCR amplification products and immunoblots are shown. For purposes of conciseness, two samples/group are illustrated. Data from densitometric analysis are expressed in arbitrary units. Results are expressed as means $\pm$ S.E.M. ($n=6$ /group). Salt-supplemented groups significantly different from their non-supplemented counterparts are indicated by * $P<0.05$, while non-supplemented groups significantly different from each other are indicated by [†] $P<0.05$.

Figure 5 Effects of 0.9% NaCl supplementation on the expression of P450aldo mRNA (A) and protein (B) in adrenal zona glomerulosa from 12-week-old control and IUGR rats. Representative gels for PCR amplification products and immunoblots are shown. For purposes of conciseness, two samples/group are illustrated. Data from densitometric analysis are expressed in arbitrary units. Results are expressed as means $\pm$ S.E.M. ($n=6$ /group). Salt-supplemented groups significantly different from their non-supplemented counterparts are indicated by * $P<0.05$, while non-supplemented groups significantly different from each other are indicated by † $P<0.05$.

above mRNA expressions in response to IUGR and/or NaCl supplementation, except for P450aldo, which was decreased by the latter condition. Protein expression of AT₁R, AT₂R, and P450aldo was less consistently altered by the experimental conditions, except for P450aldo which was decreased by NaCl supplementation. These results demonstrate that IUGR has different sex-related impacts on the RAAS and adrenal mineralocorticoid synthesis following NaCl supplementation.

Adrenal changes induced by IUGR

It is quite striking that an adverse foetal environment impacts differentially the adult male and female adrenal glands. With regard to gland morphology, the size of the adrenal ZG was not altered by IUGR. From a regulatory point of view, increased AT₁R expression (mRNA and protein) was associated with increased P450aldo (mRNA and protein) in males, whereas no such relationship was observed in females. In fact, P450aldo protein levels were decreased along with an increase in AT₂R mRNA expression and a decrease in AT₂R

protein levels. This discordance between mRNA and protein expression could be linked to the various regulatory mechanisms involved in translational and post-translational regulation (Li *et al.* 1999, Zhang *et al.* 2004). Contrary to Lopes *et al.* (2008), who also used a low-sodium diet to induce IUGR in rats, PRA was not altered in IUGR males but was reduced in IUGR females. This discrepancy is undoubtedly linked to the timing of the diet (4 weeks before mating and throughout gestation versus end of the 3 weeks of gestation in this study) and by the severity of sodium restriction (0.15 vs 0.03% herein). Nonetheless, the above reports strongly suggest that adverse foetal environment has different impacts on RAAS regulation. Indeed, Bogdarina *et al.* have studied the epigenetic RAS in foetal programming of hypertension. They showed a link between foetal insults (maternal low-protein diet) and epigenetic modification of genes at 1 week of age (Bogdarina *et al.* 2007). The authors also demonstrated that, later in life, changes in expression of certain genes, including AT_{1b}, were likely to be secondary to development

Figure 6 Effects of 0.9% NaCl supplementation on the expression of P45011β mRNA (A) and protein (B) in adrenal zona glomerulosa from 12-week-old control and IUGR rats. Representative gels for PCR amplification products and immunoblots are shown. For purposes of conciseness, two samples/group are illustrated. Data from densitometric analysis are expressed in arbitrary units. Results are expressed as means $\pm$ S.E.M. ($n=6$ /group). Salt-supplemented groups significantly different from their non-supplemented counterparts are indicated by * $P<0.05$.

of hypertension (Bogdarina *et al.* 2007), suggesting that gene expression is differently regulated with age. A role for glucocorticoids in AT_{1b} gene expression was also shown (Bogdarina *et al.* 2010).

In the present study, circulating aldosterone levels were reduced in IUGR versus controls, despite altered adrenal P450aldo expression (increase in males and decrease in females). The discrepancy between circulating aldosterone levels and P450aldo mRNA and protein expression in males is quite surprising. This suggests that, in IUGR males, other factors are implicated in the modulation of P450aldo activity. It could be that the electron transfer rate by the NADPH-linked redox system (adrenodoxin and adrenodoxin reductase; Lisurek & Bernhardt 2004) or the membrane composition of the mitochondria may affect mitochondrial cholesterol trafficking (Seybert 1990, Jefcoate 2002). The amount of precursors may also be responsible for these differences. It is speculated that this regulation is exerted at the level of cytochrome P450 side-chain cleavage, the first enzyme in steroidogenesis (Brochu *et al.* 1991), by a post-translational phosphorylation of the enzyme (Bureik *et al.* 2002). Since past research on the regulation of mitochondrial steroid hydroxylase systems has focused on the transcriptional level and not on post-translational modifications, further studies are needed to identify the specific mechanism(s) involved. The present data confirm, however, that an adverse foetal environment can induce long-term changes in adrenal glands that are influenced by the sex of the affected offspring.

Adrenal changes induced by NaCl supplementation

NaCl supplementation markedly reduced PRA, serum aldosterone, AT₁R expression (protein and mRNA in males), AT₂R mRNA (also in males) and protein expression (females) as well as P450aldo expression (mRNA and protein in both sexes). Such reduction in RAAS activity following high-salt intake is well documented (Sagnella *et al.* 1990, Cholewa & Mattson 2001). Reduction in the size of adrenal ZG in females under NaCl supplementation is in agreement with the observations reported by Hartroft & Hartroft (1955). However, no such observation was found in males, again suggesting sex-related changes of the adrenal ZG in response to NaCl supplementation in IUGR animals. The discrepancy between this previous study (Hartroft & Hartroft 1955) and the present findings in males could be explained by the use of a different strain of rats (Wistar versus Sprague–Dawley). Indeed, response to treatment has been reported to be influenced by rat strains (Garland *et al.* 1989) and even by rat suppliers (Pollock & Rekito 1998). Proliferation of glomerulosa cells has been shown to be controlled by Ang II and mediated by AT₁R-dependent and -independent processes (McEwan *et al.* 1999). The mechanisms implicated in the atrophy of this zone by salt supplementation are not well characterized, although apoptosis could be involved (McEwan *et al.* 1996a).

In control males, NaCl supplementation decreased mRNA levels but increased protein expression for both AT_{1b}R and AT₂R compared with non-supplemented animals. Following NaCl supplementation, decreased Ang II levels would preclude any down-regulation in receptor protein expression, even leading to a certain relative increase in levels compared with non-supplemented control rats. This could also apply for AT₂R expression levels in IUGR males. However, for AT₁R expression, IUGR affected the response to NaCl supplementation (decreased mRNA and protein compared with non-supplemented IUGR), suggesting a different regulatory control in these animals. In control and IUGR females, NaCl supplementation was not found to be a major regulator of AT₁R and AT₂R expression, again indicating differential responses between males and females to salt supplementation. To date, the majority of experiments reported on the regulation of ATR have been performed using low-sodium intake or plasma Ang II infusions (McEwan *et al.* 1996b, 1999, Mulrow 1999). To our knowledge, the few studies available concerning salt supplementation on both mRNA and protein expression of AT₁R and AT₂R are somewhat inconsistent. While lower AT₁R protein expression (Nora *et al.* 2000) and reduced number of AT₁R binding sites (Douglas & Catt 1976) following high-salt intake have been described, other studies meanwhile have shown unaltered AT₁R mRNA expression (Jo *et al.* 1996, Schmid *et al.* 1997). Only one study has reported an absence of effect of salt supplementation on AT₂R protein expression in the rat adrenal gland (Nora *et al.* 2000).

While AT₂R expression is low in several adult tissues, there is a general consensus that AT₂R promotes vasodilatation, growth inhibition, cell differentiation and apoptosis (de Gasparo *et al.* 2000, St-Louis *et al.* 2001, Gendron *et al.* 2003). However, in the adrenal ZG, AT₂R expression is maintained at a high level, even in the adult (Breault *et al.* 1996, Frei *et al.* 2001). Its role is not yet clearly defined, since it does not modify proliferation or protein synthesis in isolated cells (Otis *et al.* 2005). However, several recent data have suggested that AT₂R may ensure a protective role not only in the cardiovascular and renal systems (Carey & Padia 2008, Siragy 2009), but also in metabolic homeostasis, brain disorders and even in cancer (for reviews, see Henriksen (2007), Roberge *et al.* (2007), Horiuchi *et al.* (2010) and Steckelings *et al.* (2010)). The change in protein levels of AT₁R and AT₂R in IUGR males in the present study led to a decrease in AT₁R to AT₂R ratio, which may have contributed to the observed reduction in aldosterone synthesis. In these conditions, Ang II binding to the AT₂R may counteract the stimulation of aldosterone synthesis induced through the AT₁R. Conversely, in control males and in both groups of females, Ang II receptor expression does not explain the observed decrease in P450aldo and aldosterone levels. In fact, a long list of compensatory factors may be involved, since it is already known that aldosterone secretion is under multifactorial regulation, even if Ang II is considered as its most important modulator

(Ehrhart-Bornstein *et al.* 1998). As also shown by Ye *et al.* (2003), P45011 β mRNA did not respond herein to salt supplementation in control males and females. However, an increase was noted in IUGR females, suggesting again that the sex of these IUGR animals influences the response to salt intake.

In summary, the present study shows that adult male and female rats submitted to IUGR exhibit differential responses regarding the expression of both AT_{1b} and AT₂ receptors of Ang II, as well as aldosterone synthesis, in controls and under NaCl supplementation. Moreover, salt supplementation affected mRNA as well as protein expression for both receptor subtypes in males, as opposed to females where supplementation was not found to be a major regulator, again indicating sex-related differences. However, our results suggest that impaired adrenal steroidogenesis is compensated by extra- and intra-adrenal factors which enable to cope with NaCl supplementation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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