Renal molecular mechanisms underlying altered Na$^+$ handling and genesis of hypertension during adulthood in prenatally undernourished rats

Leucio D. Vieira-Filho[1,2], Edjair V. Cabral[1,2], Juliane S. Farias[1], Paulo A. Silva[2,3], Humberto Muzi-Filho[2,3], Adalberto Vieyra[2,3] and Ana D. O. Paixão[1,2]*

[1]*Department of Physiology and Pharmacology, Federal University of Pernambuco, Professor Moraes Rego Avenue, Cidade Universitária, Recife 50670-901, Brazil*
[2]*National Institute of Science and Technology for Structural Biology and Bioimaging, Rio de Janeiro, Brazil*
[3]*Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*

Abstract

In the present study, we investigated the development of hypertension in prenatally undernourished adult rats, including the mechanisms that culminate in dysfunctions of molecular signalling in the kidney. Dams were fed a low-protein multideficient diet throughout gestation with or without α-tocopherol during lactation. The time course of hypertension development followed in male offspring was correlated with alterations in proximal tubule Na$^+$-ATPase activity, expression of angiotensin II (Ang II) receptors, and activity of protein kinases C and A. After the establishment of hypertension, Ang II levels, cyclo-oxygenase 2 (COX-2) and NADPH oxidase subunit expression, lipid peroxidation and macrophage infiltration were examined in renal tissue. Lipid peroxidation in undernourished rats, which was very intense at 60 d, decreased at 90 d and returned to control values by 150 d. During the prehypertensive phase, prenatally undernourished rats exhibited elevated renal Na$^+$-ATPase activity, type 2 Ang II receptor down-regulation and altered protein kinase A:protein kinase C ratio. Stable late hypertension coexisted with highly elevated levels of Ang II-positive cells in the cortical tubulointerstitium, enhanced increase in the expression of p47^{phox} (NADPH oxidase regulatory subunit), marked down-regulation of COX-2 expression, expanded plasma volume and decreased creatinine clearance. These alterations were reduced when the dams were given α-tocopherol during lactation. The offspring of well-nourished dams treated with α-tocopherol exhibited most of the alterations encountered in the offspring of undernourished dams not treated with α-tocopherol. Thus, alterations in proximal tubule Na$^+$ transport, subcellular signalling pathways and reactive oxygen species handling in renal tissue underpin the development of hypertension.

Key words: Undernutrition-induced hypertension: Ouabain-resistant renal Na$^+$-ATPase: Tissue angiotensin II signalling pathways: α-Tocopherol

Maternal undernutrition during the perinatal period can imprint cardiovascular, renal and metabolic dysfunctions in later life of the offspring[1]. The intra-uterine programming hypothesis[2] states that early changes induced by a poor intra-uterine environment (e.g. nutritional) permanently affect tissue structure and function[3]. These might be beneficial changes for the fetus in the short term, leading to increased viability in adverse intra-uterine conditions, but later increase the risk of morbidity. Changes in renal function induced by intra-uterine undernutrition have been reported to be correlated with the development of hypertension during adulthood[4]. These changes include reduction in the number of nephrons[4,5], increased Na$^+$ reabsorption[6,7], activation of the systemic and local renin–angiotensin system (RAS)[8,9] and elevated tissue oxidative stress[10,11]. We have demonstrated that intra-uterine programming induced by undernutrition retards nephrogenesis along with decreased levels of angiotensin II (Ang II) in renal cortical cells[12] and that α-tocopherol reprogrammes the Ang II-linked regulatory network linked to the renal ouabain-resistant Na$^+$-ATPase affected by placental undernutrition[11].

Renal proximal tubule Na$^+$ reabsorption plays a central role in the long-term regulation of blood pressure in rats and humans[13,14]. The recently cloned and purified

ouabain-resistant Na^+-ATPase[15] is considered to be responsible for the fine-tuning of proximal tubule Na^+ reabsorption in this segment and therefore is critical for the regulation of arterial pressure[16,17]. This pump is regulated by many hormones and auto/paracrine hormones, including Ang II[18]. As this peptide seems to be responsible for the regulation of more than 50 % of proximal Na^+ transport[19], Ang II-modulated ouabain-resistant Na^+-ATPase is clearly relevant to alterations in arterial pressure induced during intra-uterine programming. We found that intra-uterine undernutrition programmes a loss of response of this ATPase to physiological concentrations of Ang II in proximal tubule membranes isolated from 90-d-old offspring of dams that were undernourished during gestation[11]. Treatment with α-tocopherol during lactation has also been found to lead to the recovery of a normal response to Ang II[11]. In relation to its response to α-tocopherol, this pump is also affected by reactive oxygen species (ROS)[16], with Ang II and cyclo-oxygenase 2 (COX-2) in most tissues and organs (including the kidney) playing a central role in their generation and handling[20–25]. The rationale for measuring COX-2 expression is thus based on the key role that COX-2-mediated PG-derived products play in the regulation of glomerular filtration rate and renal fluid handling[26,27]. COX-2 also participates in the pathogenesis of renal oxidative stress in conjunction with Ang II with parallel up-regulation of NADPH oxidase expression[23,28–31].

The introduction of molecular alterations from an early age in prenatally undernourished pups and the mechanisms that culminate in renal injury when hypertension is established during adulthood have not been studied. The correlation of possible modifications in molecular signalling with tension levels during the expected phases of prehypertension, hypertension development and permanently established hypertension remain unclear.

In the present study, we investigated the time course of the development of hypertension in rats programmed by placental undernutrition and explored the development of key markers of renal function during prehypertension and during hypertension development and establishment. We also addressed specific aspects related to the modifications of proximal tubule Na^+-ATPase activity and its renal RAS regulatory components during the silent phase that could potentially precede hypertension development. To elucidate the alterations in key renal processes that potentially link RAS to ROS when maternal undernutrition-induced hypertension is established in the adult offspring, we assessed the tissue levels of Ang II and expression of COX-2 and NADPH oxidase and the possible reprogramming of the modifications following the administration of maternal α-tocopherol during lactation.

Experimental methods and materials

Ethical considerations

Experimental procedures were approved by the Committee for Experimental and Animal Ethics at the Federal University of Pernambuco (no. 23076.012671/2008-09) and carried out in accordance with its guidelines related to the Uniform Requirements for Manuscripts Submitted to Biomedical Journals.

Materials

Ang II, α-tocopherol, bovine serum albumin, furosemide, HEPES, EDTA, histone 4B, phenylmethanesulfonyl fluoride, trypsin inhibitor (type IIS), methyl green and 5,5′-dithio-bis-2-nitrobenzoic acid were purchased from Sigma-Aldrich. The creatinine colorimetric assay kit was obtained from Labtest. Calphostin C and the protein kinase A (PKA) inhibitor (PKAi$_{(5-24)}$ peptide) were obtained from Calbiochem/Merck KGaA. Rabbit polyclonal antibodies against Ang II receptors (angiotensin II receptor 1 (AT_1R) and angiotensin II receptor 2 (AT_2R)), COX-2 and p47^{phox} (regulatory subunit of NADPH oxidase) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibodies against gp91^{phox} (catalytic subunit of NADPH oxidase) and ED1 (surface macrophage antigen) were also purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody against Ang II was obtained from Peninsula Laboratories. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies and the ECL™ Western blot detection reagents were purchased from GE Healthcare Life Biosciences. Anti-rabbit and anti-mouse biotinylated antibodies and the avidin–biotin–peroxidase complex were obtained from Vector Laboratories. Radioactive orthophosphate ($^{32}P_i$) was obtained from the Brazilian Institute of Energy and Nuclear Research. [γ-^{32}P]ATP was prepared according to the method reported by Maia et al.[32]. All other reagents of highest purity were obtained from different suppliers.

Animal groups

Female Wistar rats (aged 90–120 d and weighing 200–250 g) were housed in individual cages at 21°C under a 12 h light–12 h dark cycle, with free access to water and standard chow, and randomly assigned to the experimental groups. Standard chow was given to the control (well-nourished) group of dams (n 14) throughout mating and pregnancy, whereas the undernourished group (n 14) was fed a low-protein multideficient diet (see the Diets subsection). The total food intake of undernourished dams fed this diet was consistently approximately 20 % lesser than the intake of well-nourished females fed the control diet[11,33]. Thus, despite the energy content of both diets being approximately the same, the total energy intake of the undernourished dams was low. Dams (n 7 from the C and U groups) were given daily 1 ml/kg body weight of maize oil by oral administration, the α-tocopherol vehicle, from parturition to weaning, whereas the other dams (n 7 and n 6 from the CT and UT groups, respectively) were given α-tocopherol (350 mg/kg body weight). The dose chosen had previously been shown to restore nephrogenesis[12] and Ang II signalling in the proximal tubules of prenatally undernourished rats aged 90 d[11]. The dose was supraphysiological; the control diet provided approximately 10 mg/100 g body mass daily, calculated from the maternal daily food intake per 100 body mass during

lactation and the α-tocopherol content of the chow. Besides its protective effect in nephrogenesis[12], this dose was chosen aiming to provide a dietary amount of vitamin sufficient to produce antioxidant effects in rats, i.e. to mimic a situation in which ROS are quenched[34]. The vitamin was diluted in maize oil, which provided approximately 0·01 mg of total tocopherols/100 g body mass (in 0·1 ml/100 g body mass) per d, a negligible amount when compared with the daily dose of 10 and 350 mg α-tocopherol/100 g body mass given to the C/U and CT/UT groups, respectively.

Litters from both groups of dams were culled to eight pups at birth. Only male offspring were subsequently studied, but some females were kept until weaning (25 d after birth) to ensure that there were eight animals per litter. At 25 d (weaning), the male pups were divided into the following four groups: offspring of control dams maintained on maize oil (C, *n* 34); offspring of undernourished dams maintained on maize oil (U, *n* 30); offspring of control dams treated with α-tocopherol (CT, *n* 43); offspring of undernourished dams treated with α-tocopherol (UT, *n* 28). After weaning, males in these four groups were randomly designated to different experimental protocols and were killed at 30, 60 and 150 d of age. Some of the offspring were killed by decapitation at 30 d (C, *n* 11; U, *n* 9; CT, *n* 13; and UT, *n* 8) and 60 d (C, *n* 12; U, *n* 13; CT, *n* 16; and UT, *n* 13). Proximal tubule membrane preparations obtained from their kidneys were used to measure the activities of Na$^+$-ATPase, protein kinase C (PKC) and PKA and the expression of AT$_1$R and AT$_2$R.

Other animals aged 30, 60 and 150 d (C, *n* 11; U, *n* 8; CT, *n* 14; and UT, *n* 7) were placed in metabolic cages to measure water balance, food intake, urinary creatinine clearance and proteinuria. After collecting urine for 24 h, a blood sample was immediately collected from the tail vein for creatinine clearance measurement. Before being put into metabolic cages, the animals were allowed to acclimatise for 6 h on three consecutive days. Urinary and serum creatinine levels were quantified using a colorimetric assay kit (Labtest), and urinary protein levels were determined by the Folin phenol method[35], using bovine serum albumin as a standard.

Systolic arterial pressures (SAP) were recorded at different intervals (see the Measurement of blood pressure subsection and Fig. 1) from 60 to 150 d of age. Finally, at 150 d, animals in the four experimental groups were anaesthetised with Na$^+$ pentobarbital (60 mg/kg body weight, intraperitoneally) to determine plasma volume and killed by exsanguination. Their kidneys were sectioned for immunolocalisation of Ang II and ED1 (surface macrophage antigen), and two different classes of whole-kidney homogenates were prepared: one for the measurement of lipid peroxidation and reduced glutathione levels and the other for the immunodetection of COX-2 and the gp91^{phox} catalytic subunit and p47^{phox} regulatory subunit of NADPH oxidase.

Diets

Malnutrition was induced with a low-protein multideficient diet[36] mimicking those used in impoverished regions with high rates of undernutrition worldwide. We aimed to induce
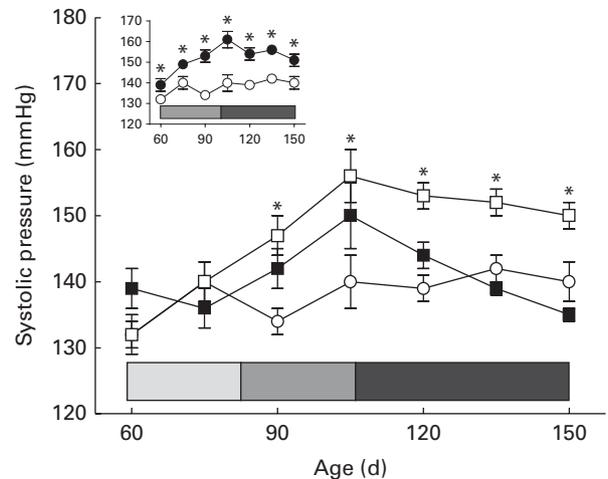

Fig. 1. Development of prenatal undernutrition-induced hypertension in the offspring and influence of α-tocopherol administration to the dams during lactation. Systolic arterial pressure (SAP) measurements in conscious rats were made with tail-cuff plethysmography. The groups of rats were as follows: offspring of control dams (C, *n* 11); offspring of dams that were undernourished during gestation (U, *n* 8); offspring of control dams treated with α-tocopherol during lactation (CT, *n* 14); offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation (UT, *n* 7). Main panel: Evolution of arterial pressure in the C (○), U (□) and UT (■) groups. Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different from those of the C group (*P*<0·05; two-way ANOVA followed by the Student–Newman–Keuls test). The horizontal bar delimits the phases of prehypertension (□), hypertension development (□) and established hypertension (■) in the U group. Inset: Evolution of arterial pressure in the C (○) and CT (●) groups. Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different from those of the C group (*P*<0·05; two-way ANOVA followed by Bonferroni test). The horizontal bar delimits the hypertension-development (□) and established-hypertension (■) phases in the CT group. The phase of established hypertension in the U and CT groups was defined from the beginning of a plateau (at 105 d) of SAP values that were statistically higher than those recorded in the C group.

deficiency to investigate its impact on Na$^+$-ATPase activity, on its related Ang II-linked regulatory pathways and on associated processes that, together with Ang II, affect redox balance and intrarenal blood circulation with possible establishment of hypertension. This diet was initially prepared according to data obtained from the food consumption surveys of 918 families (5939 persons) in three physiographic zones of Pernambuco State (Brazil), which mimics basic food patterns in many underdeveloped and developing countries worldwide[36]. Similar basic food patterns have been found in more than thirty countries in Near and Middle East, Central and South America[36]. The ingredients of the diet (% w/w) were beans (*Phaseolus vulgaris*, brown beans, 18·3), manioc flour (64·8), jerked meat (3·7) and sweet potato (12·8). The average amounts of major nutrients (% w/w) measured in three different chow samples chosen at random during the present study were as follows: protein, 9; carbohydrate, 80; lipid, 2. Na$^+$ content ranged between 5·6 and 6·5 mmol/100 g. The total energy of the diet was 1548 kJ/100 g dry weight. The nutrient content of the control diet was as follows (% w/w): protein, 23; carbohydrate, 44; lipid, 5. Na$^+$ content averaged 5·2 mmol/100 g. The total energy of the control diet was 1,477 kJ/100 g dry weight.

Adequacy in terms of energy was comparable to the control diet (approximately 1500 kJ/100 g), but only a small fraction was from fats (30 kJ/100 g against 340 kJ/100 g in the control diet). Besides the accentuated global protein restriction (8 g% against 23 g%), the diet consumed by the populations initially investigated was also deficient in protein ($>90\%$ was provided by beans and roots and $<10\%$ by meat) and contained high amounts of carbohydrates from the manioc flour and sweet potato commonly used by these populations. For these reasons, this diet is referred to as a low-protein multideficient diet.

Measurement of blood pressure

The development of SAP in conscious rats was assessed at different ages using tail-cuff plethysmography (IITC Life Science B60-7/16''; Life Science Instruments). Before acquiring SAP data, the animals were trained in a heated container at 32°C for three consecutive days to adapt them to the experimental procedures. SAP was measured between 09.00 and 15.00 hours. To avoid circadian variations, one rat was selected at random from each group for the first set of pressure measurements. As there were four groups, this takes about 70–75 min. Another rat from each group was selected at random over 70–75 min, etc. until the pressure in all rats was measured. The four rounds usually finished at about 15.00 hours. This ensured that each group had one rat tested in the same interval of 70–75 min as all the others. Based on the manufacturer's guideline, the same tail-cuff size was used throughout the investigation, and data obtained for each day were an average of three to five consecutive measurements.

Measurement of plasma volume

Plasma volume was measured in offspring rats at 150 d using the Evans Blue dye, as described previously[37].

Estimation of lipid peroxidation and reduced glutathione levels in kidneys

Lipid peroxidation in the membranes of kidneys was estimated by measuring the levels of thiobarbituric acid-reactive substances (TBARS)[38] in the second class of whole homogenates. Following sampling for plasma volume measurement at 150 d, the rats were killed and the renal hilar region was sliced for the immunolocalisation of Ang II- and ED1-positive cells, and the upper parts of the left kidneys were homogenised in 150 mM-KCl (1 g tissue: 5 ml solution). About half of each homogenate was immediately used for the measurement of TBARS levels[12], with butanol being used as the extraction solvent. A standard curve was obtained using 1,1,3,3-tetraethoxypropane.

The levels of the reduced form of glutathione were quantified by measuring the levels of non-protein sulfhydryl groups in the homogenates as per the method of Sedlak & Lindsay[39], with slight modifications. The other half of each homogenate was mixed with an equal volume of 10 % (w/v) TCA to precipitate proteins, which were centrifuged at $1000\,\mathbf{g}$ for 10 min. Sequentially, 1 ml of the supernatant was mixed with 1·5 ml of a reaction medium containing 100 mM-Tris (pH 8·9) and 0·4 mM-5,5′-dithio-bis-2-nitrobenzoic acid. The samples were incubated at room temperature for 5 min, and the absorbance was measured at 412 nm. The results were compared with a standard curve using α-cysteine and corrected for the protein content of the initial homogenates.

Preparation of whole-kidney homogenates for immunodetection of cyclo-oxygenase 2 and NADPH oxidase in rats at 150 d

After collecting the hilar transverse slices for immunolocalisation, the remaining renal tissue was minced on ice in 250 mM-sucrose, 10 mM-HEPES–Tris (pH 7·4), 2 mM-EDTA, 0·15 mg/l trypsin inhibitor (type IIS) and 1 mM-phenylmethanesulfonyl fluoride (1 g tissue: 4 ml solution). The suspension was homogenised at 1200 rpm for 1 min using a Potter Elvehjem homogeniser with a Teflon pestle. Aliquots were stored at -20°C and thawed only once before assay. The total protein content of each homogenate was measured by the Folin phenol reagent method[35].

Isolation of proximal tubule cell membranes

Purified plasma membrane preparations and controls for residual contamination of intracellular membranes were obtained as described previously[11,33,40], using male offspring at 30 and 60 d. Only the outer cortex (cortex corticis) was used, as $>90\%$ of the cell population in this region corresponds to proximal tubules[41]. The protein content of the preparations was measured as described above[35].

Measurement of ouabain-resistant Na^+-ATPase activity

Ouabain-resistant furosemide-sensitive Na^+-ATPase activity in the renal membranes was quantified by the difference between the inorganic phosphates released from ATP in the absence and presence of 2 mM-furosemide, as described elsewhere[11], except that unlabelled ATP was used. Enzyme activity was measured in the absence (basal activity) and presence of 10^{-12} M-Ang II. The stimulatory effect of Ang II on the ouabain-resistant Na^+-ATPase is mediated by an $AT_1R \rightarrow$ phospholipase Cβ (PLCβ) $\rightarrow$ PKC signalling pathway, all components of which are present in purified plasma membranes from proximal tubule cells[18,42]. The preservation of these components in the isolated membranes allows investigation of the response of the pump to Ang II *in vitro* after different treatments or conditions *in vivo*[11,43].

Determination of protein kinase C and protein kinase A activities

Protein kinase activities were measured by the incorporation of the γ-phosphoryl group of [γ-^{32}P]ATP (approximately 1 MBq/nmol) into histone 4B in the presence and absence of

the specific PKC and PKA inhibitors[11,44]. Radioactivity incorporated into the histone was quantified by liquid scintillation.

SDS-PAGE and Western blotting

Ang II receptors and COX-2 and NADPH oxidase subunits were immunodetected in the isolated renal membranes (AT_1R and AT_2R) or whole-kidney homogenates (COX-2 and NADPH oxidase subunits) using their specific antibodies after separation of the proteins by SDS-PAGE (10%). Proteins were transferred onto nitrocellulose membranes at 350 mA. Non-specific binding prevention and washing procedures followed were as those reported by Vieira-Filho *et al.*[11].

For immunoblotting of AT_1R and AT_2R, protein samples were separated by SDS-PAGE, successively probed with the corresponding primary antibodies (1:500 dilution) and secondary antibodies (1:2500 dilution), and visualised using a chemiluminescence kit (ECL™ Western blotting system)[45]. The antibody dilutions used for immunoblotting of COX-2 were 1:500 (primary) and 1:2500 (secondary), whereas those used for immunoblotting of the NADPH oxidase subunits were 1:200 and 1:1000, respectively; more details are given in the legends of Figs. 3, 6 and 7. Densitometric analyses were carried out using Scion Image for Windows Alpha 4.0.3.2 (Scion Corporation), available at www.scionCorporation.com at the time the study was carried out. Protein expression was normalised to protein load using Ponceau Red staining, giving the same accuracy as β-actin[43].

Immunohistochemistry

Hilar transverse slices of kidneys collected at 150 d were used for the immunolocalisation of Ang II- and ED1-positive cells, as described by Cabral *et al.*[46]; more details are given in the legends of Figs. 5 and 9.

Statistical analysis

Data are presented as means with their standard errors. Differences between the experimental groups were assessed as described in the figure legends after confirming normal distribution using the Kolmogorov–Smirnov test. Depending on the experimental design, the hypothesis to be tested, the parameter to be investigated and the distribution of the data, we used two-way ANOVA followed by Bonferroni, one-way ANOVA followed by Student–Newman–Keuls, unpaired Student's *t* or one-sample *t* test, as indicated in the corresponding table and figure legends. Differences were considered significant at $P < 0.05$.

The determination of Na^+-ATPase, PKC and PKA activities was carried out in triplicate; for blood pressure, recordings were obtained at three to five consecutive time points. Standard deviations of each set of experiments were divided by the mean of the set and multiplied by 100 to calculate the intra-assay CV. Finally, we calculated the mean CV percentage to check intra-assay reproducibility, which gave a value $< 10\%$ for all parameters in the four experimental groups.

Results

Development of hypertension in the offspring of prenatally undernourished dams: beneficial and harmful effects of α-tocopherol administered during lactation

The offspring of undernourished dams had lower birth weights than the control group (5·0 (SEM 0·1) (*n* 58) *v.* 6·4 (SEM 0·1) g (*n* 77); $P < 0.001$), but all groups had similar arterial pressure until 60 d of age (Fig. 1). Nevertheless, after a period of prehypertension during young age, when their tension levels were similar to those of the control group (Fig. 1, main panel, light grey portion of the horizontal bar), the offspring of undernourished dams entered a phase characterised by hypertension development until a stage of well-established and persistently elevated SAP levels (at 90 d). The offspring of undernourished dams that received α-tocopherol throughout lactation had normal pressure, which remained so throughout adulthood (as far as investigated). Although α-tocopherol normalised blood pressure in the prenatally malnourished group, this was not the case when the vitamin was administered to well-nourished dams (Fig. 1, inset). Their offspring entered an earlier phase in which systolic pressure increased (compare the length of the light grey horizontal bar), reaching a stable state of hypertension throughout adulthood.

Main general and renal parameters during the prehypertensive, hypertension-development and established-hypertension phases

At 30 d, the four offspring groups (C, U, CT and UT) had normal body weights. Their food and water intake, haematocrit and major renal parameters (urinary flow, proteinuria and creatinine clearance) were similar and within the normal range (Supplementary Table S1, available online). The profiles of rats at 60 d remained similar in all four groups, with the usual growth-dependent variations in body weight, food and water intake, and urinary output (Supplementary Table S2, available online). This normal picture persisted till 90 d of age (Supplementary Table S3, available online); i.e. it was preserved during the hypertension-development period in the U and CT groups (Fig. 1).

Table 1 demonstrates that neither the renal mass nor the renal mass:body mass ratio was modified in the adult offspring of undernourished dams. The preserved renal mass, however, corresponds to kidneys with an abnormal structure already detected at 25–30 d of age[5,12,47], which certainly constitutes the basis for the abnormal functions found at 150 d (Table 1). The normal renal picture in the prehypertensive and hypertension-development phases changed critically during the established-hypertension phase in the U group (Table 1). At 150 d, this group had significantly decreased creatinine clearance, accompanied by an expanded plasma volume. These alterations were, respectively, totally and partially suppressed in the offspring of undernourished dams that received α-tocopherol (UT). The CT group that exhibited identical changes in creatinine clearance and plasma volume compared with the U group also had an increased

Table 1. General parameters of adult offspring in the established-hypertension phase (150 d of age)

(Mean values with their standard errors)

	C (n 7–11)		U (n 6–8)		CT (n 10–13)		UT (n 6–7)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
BW (g)	354·6[a]	8·8	370·5[a]	6·2	374·6[a]	6·8	356·0[a]	4·4
Kidney weight (g)	1·14[a]	0·03	1·12[a]	0·03	1·29[b]	0·03	1·11[a]	0·03
Kidney weight/BW (100 ×)	0·32[a]	0·01	0·30[a]	0·01	0·34[b]	0·01	0·31[a]	0·01
Food intake (g/100 g BW per 24 h)	6·9[a]	0·5	7·2[a]	0·4	6·3[a]	0·5	7·5[a]	0·2
Haematocrit	0·49[a]	0·01	0·49[a]	0·01	0·48[a]	0·01	0·47[a]	0·02
Urinary flow (ml/100 g BW per 24 h)	3·9[a]	0·3	3·8[a]	0·2	3·9[a]	0·2	4·7[a]	0·4
Water intake (ml/100 g BW per 24 h)	10·4[a]	0·5	10·1[a]	0·4	10·5[a]	0·8	12·9[a]	0·5
Water balance (ml/100 g BW per 24 h)	6·5[a]	0·5	6·3[a]	0·4	6·6[a]	0·7	8·1[a]	0·4
Proteinuria (mg/100 g BW per 24 h)	24·5[a]	1·0	25·3[a]	1·4	27·1[a]	1·8	22·3[a]	1·7
Creatinine clearance (ml/100 g BW per min)	0·49[a]	0·03	0·37[b]	0·02	0·37[b]	0·02	0·41[a,b]	0·04
Plasma volume (ml/100 g BW)	4·11[a]	0·15	4·82[b,c]	0·16	4·78[c]	0·17	4·35[a,b]	0·10

C, offspring of control dams; U, offspring of dams that were undernourished during gestation; CT, offspring of control dams treated with α-tocopherol during lactation; UT, offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation; BW, body weight.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$; two-way ANOVA followed by Bonferroni test).

kidney weight at the end of the established-hypertensive phase (Table 1 and Fig. 1).

Alterations in the proximal tubule ouabain-resistant Na⁺-ATPase activity and in components of the angiotensin II-linked signalling pathways during the prehypertensive phase

The main remarkable features regarding the effect of prenatal undernutrition on the proximal tubule Na⁺-ATPase activity of offspring during the prehypertensive phase (before 60 d; Fig. 2(A) and (B)) were up-regulation of basal (unstimulated) activity, loss of normal sensitivity towards Ang II and recovery of the control profile after the administration of α-tocopherol to the dams during lactation. No differences were found between the C and CT groups.

During this phase, which was silent in terms of SAP, changes in AT₂R expression in proximal tubule membranes were detected only in the U and CT groups in 60-d-old rats (Fig. 3). The changes were mirror images: decreased in the U group but increased to an equivalent degree in the CT group (Fig. 3(D)). Maternal α-tocopherol totally restored the AT₂R levels in the prenatally undernourished rats (UT).

With respect to the two protein kinase activities functionally linked to the renal Na⁺-ATPase activity via Ang II receptors[18,48], the effect of undernutrition and the response to α-tocopherol varied with time and differed in some respects depending on which kinase was being considered. The UT and CT groups exhibited similar up-regulation of PKC activity due to the influence of undernutrition (Fig. 4(A)). At the end of the prehypertensive phase, there was a huge increase in PKC activity (Fig. 4(B)). PKA activity was strongly decreased in the U group at 30 d, and the levels returned to control values in the UT group (Fig. 4(C)). At 60 d, the activity of this kinase was up-regulated to the same level in both the placentally undernourished U group (without α-tocopherol treatment) and the well-nourished group of offspring of dams that received the vitamin (CT). Conversely, α-tocopherol almost completely restored the control activity in prenatally undernourished rats (Fig. 4(D)).

Renal alterations during the established-hypertension phase induced by prenatal undernutrition or by α-tocopherol treatment alone in well-nourished dams during lactation

The Ang II-positive tubulointerstitial cell counts doubled in the U and CT groups (Fig. 5(A) and (C)), with no change in glomeruli (Fig. 5(B) and (D)), during the established-hypertension phase. This is further evidence that the same specific effect on the local RAS had been achieved under two different conditions: undernutrition during fetal life (U) and administration of α-tocopherol alone to well-nourished dams (CT). The number of tubulointerstitial Ang II-positive cells returned to normal values in the UT group (Fig. 5(C)).

The renal expression of COX-2 (Fig. 6) during the same period of SAP evolution also demonstrated that the two different treatments had the same effect – as shown in Fig. 5 – but as the mirror image of that in Ang II-positive cells. COX-2 levels were significantly decreased in the U and CT groups.
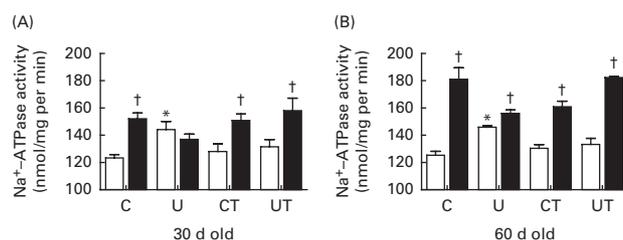

Fig. 2. Activity of ouabain-resistant furosemide-sensitive Na⁺-ATPase in renal proximal tubules of the prenatally undernourished group during the prehypertensive phase: effect of maternal α-tocopherol during lactation. The groups of rats were as follows: offspring of control dams (C, n 11); offspring of dams that were undernourished during gestation (U, n 8); offspring of control dams treated with α-tocopherol during lactation (CT, n 14); offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation (UT, n 7). □, Unstimulated (basal) Na⁺-ATPase activity. ■, Na⁺-ATPase activity in the presence of 10^{-12} M-angiotensin II (Ang II). Rats at 30 d (A) and 60 d (B) of age. Values are means of five to eight triplicate assays using different membrane preparations, with their standard errors represented by vertical bars. * Mean value was significantly different from that of the corresponding age-matched C group ($P < 0.05$; two-way ANOVA followed by Bonferroni test). † Mean value was significantly different from the corresponding non-stimulated (no Ang II) mean value ($P < 0.05$; unpaired Student's t test).
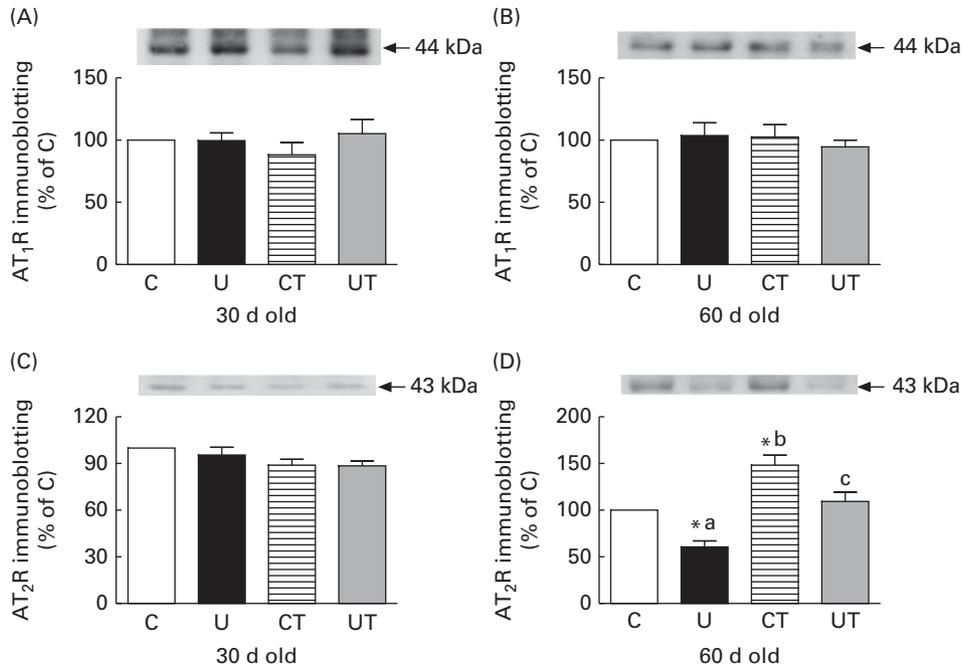
Fig. 3. Expression of ((A) and (B)) type 1 and ((C) and (D)) type 2 angiotensin II receptors (AT₁R and AT₂R) in renal proximal tubule cell membranes of rats aged 30 or 60 d. The groups of rats were as follows: offspring of control dams (C, *n* 11); offspring of dams that were undernourished during gestation (U, *n* 8); offspring of control dams treated with α-tocopherol during lactation (CT, *n* 14); offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation (UT, *n* 7). The upper part of each panel shows a representative immunodetection in a single gel. Each lower graph corresponds to densitometric data presented as means of three to five determinations made using different membrane preparations, with their standard errors represented by vertical bars. * Mean value was significantly different from that of the C group ($P<0.05$; one-sample *t* test). a,b,c Mean values with unlike letters were significantly different ($P<0.05$; one-way ANOVA followed by the Student–Newman–Keuls test).
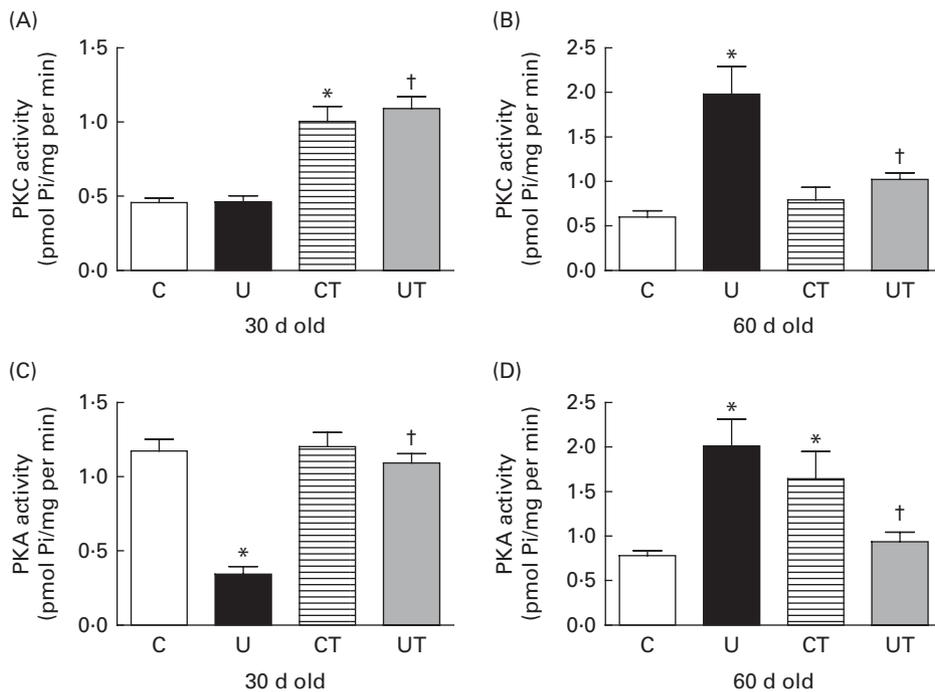

Fig. 4. Activities of ((A) and (B)) protein kinase C (PKC) and ((C) and (D)) protein kinase A (PKA) in the renal proximal tubule cell membranes of rats aged 30 or 60 d. The groups of rats were as follows: offspring of control dams (C, *n* 11); offspring of dams that were undernourished during gestation (U, *n* 8); offspring of control dams treated with α-tocopherol during lactation (CT, *n* 14); offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation (UT, *n* 7). Values are means of three to four determinations made using different membrane preparations, with their standard errors represented by vertical bars. * Mean value was significantly different from that of the C group ($P<0.05$; two-way ANOVA followed by Bonferroni test). † Mean value was significantly different from that of the U group ($P<0.05$; two-way ANOVA followed by Bonferroni test). Pi, inorganic phosphate.
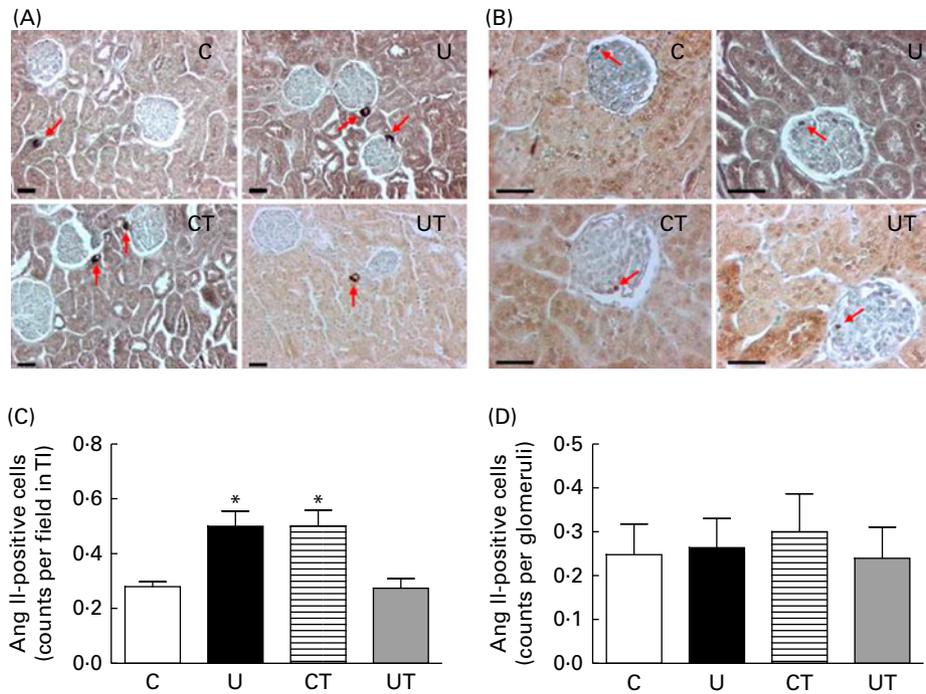
Fig. 5. Immunolocalisation of angiotensin II (Ang II)-positive cells in renal tubulointerstitium (TI) (A) and glomerulus (B) during the established-hypertension phase in prenatally undernourished rats. Rats aged 150 d were used for immunohistochemistry. The groups of rats were as follows: offspring of control dams (C, n 11); off-spring of dams that were undernourished during gestation (U, n 8); offspring of control dams treated with α-tocopherol during lactation (CT, n 14); offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation (UT, n 7). (A) Representative fields (magnification 200 ×) showing Ang II-positive cells in the TI (red arrows) of the C, U, CT and UT groups. (B) Representative fields (magnification 400 ×) showing Ang II-positive cells in glomeruli (red arrows). The images were obtained from a hilar transverse cortical section (6 μm thick). (C) and (D) Graphs corresponding to cell count data in TI and glomeruli, respectively, presented as means, with their standard errors represented by vertical bars and obtained as follows. The numbers of rats in the C, U, CT and UT groups were 5, 5, 4 and 5, respectively. In (C), one hilar transverse section from each rat provided thirty fields of cortical TI, where the number of Ang II-positive cells was counted using light microscopy in a grid of 300 000 μm². In (D), each rat is represented by fifty individual glomeruli. * Mean value was significantly different from that of the C group ($P < 0.05$; two-way ANOVA followed by Bonferroni test). (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

The expression of the catalytic (gp91^{phox}), but not the regulatory (p47^{phox}), subunits of NADPH oxidase was significantly up-regulated (70 %) in the U group (Fig. 7). Neither lipid peroxidation (Fig. 8(A)) nor changes in renal reduced form of glutathione levels (Fig. 8(B)) accompanied the dysregulation of NADPH oxidase. The number of ED1-positive tubulointerstitial cells was similar to that of p47^{phox} in U rats (Fig. 9(A) and (C)), with no differences in the glomeruli among the four groups (Fig. 9(B) and (D)).

Discussion

The silent prehypertensive phase (Fig. 1, main panel) in young rats programmed by intra-uterine undernutrition has been shown to be accompanied by a hidden up-regulation of proximal tubule ouabain-resistant Na$^+$-ATPase activity and alterations in its local RAS regulatory components (Figs. 2–4). It follows that the increased activity of the pump responsible for the fine-tuning of Na$^+$ reabsorption[16,17] leads to a gradual, initially subtle, increase in body Na$^+$ content[49], which culminates in an expanded plasma volume (Table 1) and the onset of permanent hypertension in later adulthood. The same earlier programmed molecular alterations could also underpin an abnormal interplay between Ang II, COX-2 and ROS signalling in renal tissue, which in turn might contribute to a sustained elevation of arterial pressure. This view is

supported by the observations that, during adulthood, the prenatally undernourished group exhibited more Ang II-positive cells in the cortical tubulointerstitium after hypertension had been established, down-regulation of COX-2 expression,
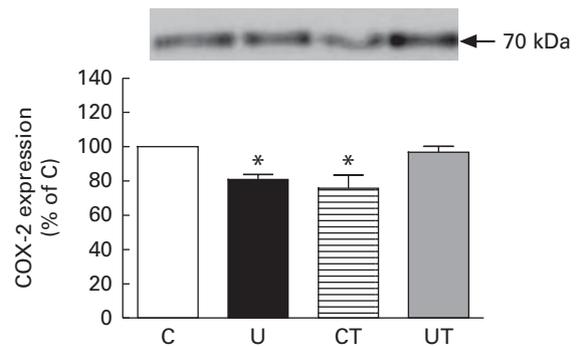

Fig. 6. Immunodetection of cyclo-oxygenase 2 (COX-2) in whole-kidney homogenates obtained from 150-d-old rats. The groups of rats were as follows: offspring of control dams (C, n 11); offspring of dams that were undernourished during gestation (U, n 8); offspring of control dams treated with α-tocopherol during lactation (CT, n 14); offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation (UT, n 7). The upper part of the figure shows representative Western blots for COX-2. The graph bars show densitometric representations (means with their standard errors represented by vertical bars) of five to ten immunodetections in different homogenates. * Mean value was significantly different from that of the C group ($P < 0.05$; one-sample t test).
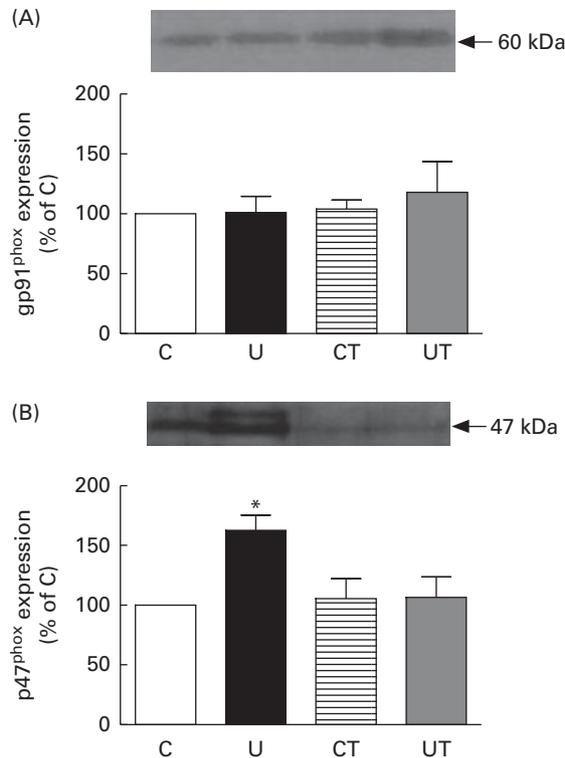
Fig. 7. Expression of the gp91[phox] catalytic (A) and p47[phox] regulatory (B) subunits of renal NADPH oxidase during the established-hypertension phase in prenatally undernourished rats. The groups of rats were as follows: offspring of control dams (C, *n* 11); offspring of dams that were undernourished during gestation (U, *n* 8); offspring of control dams treated with α-tocopherol during lactation (CT, *n* 14); offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation (UT, *n* 7). The upper part of each panel presents representative Western blots for each NADPH subunit. The graph bars show densitometric representations (means with their standard errors represented by vertical bars) of three to four Western blots obtained from different homogenates. *Mean value was significantly different from that of the C group ($P < 0.05$; one-sample *t* test).

overexpression of the p47[phox] regulatory subunit of NADPH oxidase, and enhanced infiltration of macrophages (Figs. 5–7 and 9). Overstimulated local Ang II and decreased COX-2 expression in the adult offspring of undernourished dams could affect the renal microvasculature as well as Na$^+$ reabsorption mediated by the ouabain-resistant Na$^+$-ATPase, contributing further to their permanent hypertensive state at 90 d (Fig. 1, main panel). It is generally held that the Ang II- and COX-2-mediated pathways are central players in ROS signalling, as well as in renal homeostasis[22–24,50]. Renal tubulointerstitial inflammation (Fig. 9(A)) probably results from high local Ang II and oxidative stress levels, as in models of salt challenge[24,50,51] and offspring undernutrition induced by low-maternal protein diet during the final period of gestation[52].

ROS signalling is implicated in the earlier processes that culminate in the onset and maintenance of elevated arterial pressure in prenatally undernourished adult rats. This is evident from the total reprogramming of both hypertension (Fig. 1, main panel) and Na$^+$-ATPase activity modifications (Fig. 2(A) and (B)) by maternal α-tocopherol during lactation (Fig. 9(A)).

The very high levels of ROS at 30 d[12] may be responsible for the lack of response of Na$^+$-ATPase to Ang II in the U group (Fig. 2(A)) when there were no changes in the abundance of AT$_1$R and AT$_2$R (Fig. 3(A) and (C)). However, other possibilities, such as changes (i) in the phosphorylation status of these receptors and in the kinases associated with the Ang II signalling pathways[53–55] and (ii) in the ratio of glycosylated: non-glycosylated AT$_1$R[56], should be considered. Increased levels of ROS resulting from a possible Ang II-induced[44] up-regulation of p47[phox] expression (Fig. 7) can augment Na$^+$-ATPase activity by scavenging NO[16], a mechanism that might in turn have implications for a renal microvasculature affected by increased local Ang II (Fig. 5) and decreased COX-2 expression (Fig. 6) levels. It is interesting that maternal α-tocopherol also completely reprogrammed undernutrition-induced late modifications in tubulointerstitial Ang II, COX-2 expression, p47[phox] up-regulation and macrophage infiltration in the kidney (Figs. 5–7 and 9). The last observations shed more light on the ROS-mediated mechanisms that underlie the genesis and sustainability of hypertension in the programmed adult offspring[57]. A constellation of co-stressors that include humoral factors, the nervous system and the
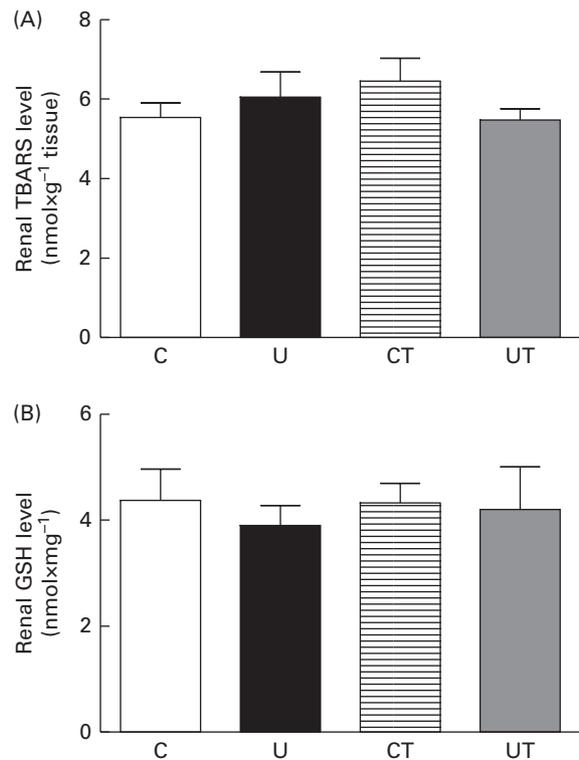

Fig. 8. Levels of thiobarbituric acid-reactive substances (TBARS) (A) and reduced glutathione (GSH) (B) in renal homogenates during the established-hypertension phase (at 150 d) in prenatally undernourished rats. The groups of rats were as follows: offspring of control dams (C, *n* 11); offspring of dams that were undernourished during gestation (U, *n* 8); offspring of control dams treated with α-tocopherol during lactation (CT, *n* 14); offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation (UT, *n* 7). Values are means of seven to fourteen (TBARS) and six to twelve (GSH) duplicate determinations made using kidneys from different animals, with their standard errors represented by vertical bars.
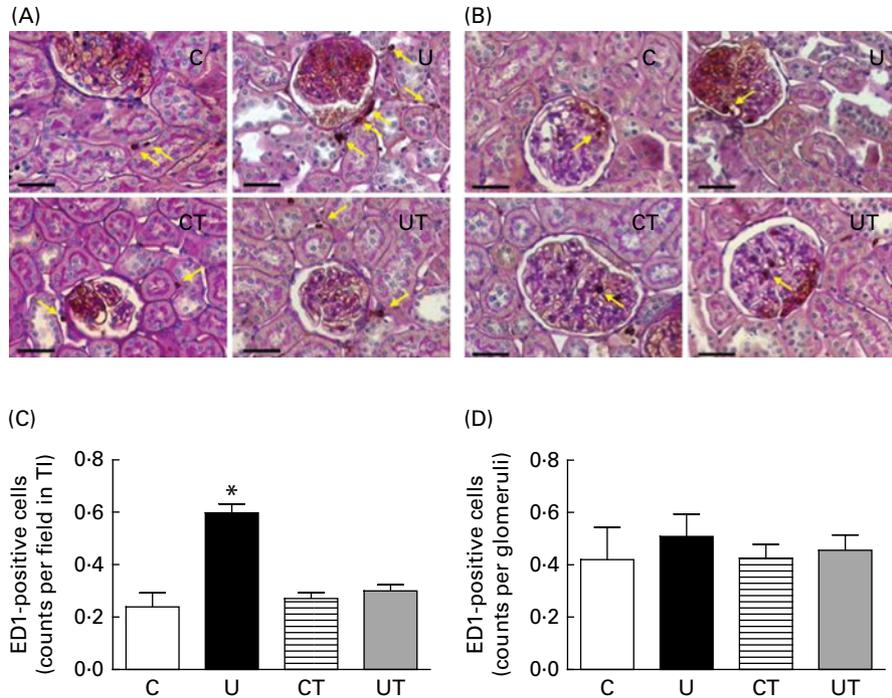
Fig. 9. Infiltration of macrophages in renal tissue during the established-hypertension phase in prenatally undernourished rats. The groups of rats were as follows: offspring of control dams (C, *n* 11); offspring of dams that were undernourished during gestation (U, *n* 8); offspring of control dams treated with α-tocopherol during lactation (CT, *n* 14); offspring of dams that were undernourished during gestation and treated with α-tocopherol during lactation (UT, *n* 7). (A) Representative fields (magnification 400 ×) showing ED1-positive cells in tubulointerstitium (TI; yellow arrows). (B) Representative fields (magnification 400 ×) showing ED1-positive cells in glomeruli (yellow arrows). The images were obtained from a hilar transverse cortical section (6 μm thick). (C) and (D) Cell counts in TI and glomeruli, respectively, presented as means with their standard errors represented by vertical bars and obtained as follows. The numbers of rats in the C, U, CT and UT groups were 5, 5, 4 and 5, respectively. In (C), one hilar transverse section from each rat provided sixty fields of cortical TI, where the number of ED1-positive cells was counted using light microscopy in a grid of 75 000 μm². In (D), each rat is represented by sixty individual glomeruli. * Mean value was significantly different from that of the C group ($P < 0.05$; two-way ANOVA followed by Bonferroni test). (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

immune system could collaborate in the genesis of hypertension via Ang II and ROS, as has been reviewed recently[58]. It is puzzling that despite the up-regulation of p47phox expression, renal TBARS and reduced form of glutathione remained unmodified in the programmed adult offspring (Fig. 8). Maternal undernutrition leads to increased oxidative stress in younger rats, which is gradually attenuated with time[11,59]. At 60 d of age, the offspring of undernourished dams had renal TBARS levels 80 % higher than those encountered in control rats[59]. This difference became smaller at 90 d (20 %), as the result of the significant decrease found in the U group with the maintenance of TBARS levels in the control group[11], and finally disappeared at 150 d (Fig. 8(A)). Thus, the imprinted up-regulation of p47phox expression can be considered a relic of a history of elevated ROS levels and altered ROS signalling. Consideration should be given, however, to the possibility that the attenuation of renal TBARS in the U group is helped by an improved balance between oxidant and antioxidant processes as the result of an augmented H_2O_2-induced abundance of mitochondrial and extra-mitochondrial antioxidant enzymes[60,61], although other studies have reported the opposite[62].

The effect of placental undernutrition on rats at 60 d that leads to a huge decrease in AT₂R expression, and the complete abrogation of this effect by α-tocopherol during lactation (Fig. 3(D)), points to this window of growth as a relevant moment – and this type of Ang II receptor as a specific target – for the influence of ROS on the programming of hypertension, the onset of which begins 3 weeks later (Fig. 1, main panel). AT₂R decreases Na⁺ reabsorption[63], and down-regulation of the expression of this receptor in proximal tubule cells could contribute to the subtle and progressive increase in body Na⁺ content during the prehypertensive phase, as has been proposed above. The fact that AT₁R expression remained unmodified throughout the prehypertensive period (Fig. 3(A) and (B)) supports the idea that programming selectively affects renal AT₂R, possibly through a specific effect on the intracellular signalling proteins to which it is coupled, which seem to participate in the spatio-temporal modulation of AT₂R functions[64]; later modifications[11] might result from compensatory or contra-regulatory effects during the development of hypertension. When intra-uterine undernourished rats were subjected to a salt-loading challenge between weaning and 60 d of age, no further changes occurred in blood arterial pressure and plasma volume, despite an additional increase in TBARS levels[59]. This is evidence that normal renal Na⁺ handling coexists with silent modifications in local RAS and initially elevated ROS levels.

The 30–60 d period seems to be a special window for the effect of prenatal undernutrition on the Ang II-linked and kinase-mediated effects in renal tissue during the prehypertensive phase. These kinases have opposite affects on

Na$^+$-ATPase activity: PKC participates in its stimulation by Ang II[18] and PKA in counterbalancing the effects of Ang II via smaller peptides[48]. The marked decrease in PKA activity in 30-d-old offspring with normal PKC activity in the undernourished group (and therefore a decrease in the PKA/PKC balance) (Fig. 4(A) and (C)) could underpin one of the mechanisms responsible for the abnormal proximal tubule Na$^+$-ATPase activity, the proposed subtle increase in local and systemic Na$^+$ content, and the later development of hypertension. As the activities of both kinases were similarly up-regulated at 60 d points to an oscillating dynamic of the processes that probably contribute to the small, though significant, recovery of the normal response of Na$^+$-ATPase to Ang II (Fig. 2(B)). In summary, an undernutrition-provoked imbalance between stimulatory and inhibitory Ang II-related signalling components in renal tubules during the prehypertensive phase could foster the up-regulation of ouabain-resistant Na$^+$-ATPase activity and proximal tubule Na$^+$ reabsorption.

In the context of undernutrition-induced alterations in the receptors present in the membranes of proximal tubule cells, the above-postulated oscillating dynamic of different processes could involve the activation/deactivation of D1-like receptors. We know little about the influence of dopamine on the ouabain-resistant renal Na$^+$-ATPase, which is in contrast with all that is known with respect to its influence on the (Na$^+$ + K$^+$)-ATPase present in proximal tubules[65]. However, a possible effect mediated through altered signals involving D1-like receptors emerges from the observations that PKA activity was initially low (Fig. 4(C)), as also from the loss of response to Ang II. This is what would be expected in the case of activation of these receptors followed by their deactivation promoted by the higher local oxidative stress, as found in renal cells in culture[66]. Late increase in PKC activity could be a result – at least in part – of this deactivation, as well as the recovery of the response to Ang II. In F344 rats, diminished D$_1$R and exaggerated AT$_1$R functions have been implicated in the development of hypertension[67]. Crosstalk between AT$_1$R and D$_1$R[63] supports the hypothesis of D1-like receptor participation in the ensemble of altered mechanisms that we have described.

In young adult rats and in intact cells, PKC-dependent activation of the ouabain-resistant Na$^+$-ATPase could be further amplified as the result of an increase in the apical Na$^+$ entry, as found in other nephron segments of rats subjected to placental protein restriction[7,68]. Fetal PKC activity could be inhibited during placental life if hypoxia accompanied undernutrition. However, it was clearly restored in the pups after birth, as indicated by the normal levels at 30 d of age in the U group, and it progressed to a clear up-regulation (Fig. 2(B)) when SAP began increasing (Fig. 1).

Our novel and unexpected observation was that the influence of α-tocopherol – and therefore the global effects of ROS – on the genesis of hypertension and renal dysfunction in undernourished offspring is more complex than previously supposed. The central issue is that α-tocopherol, when administered to well-nourished dams during lactation, mimics the effects of prenatal undernutrition in the development of SAP with age (Fig. 1, inset), also with earlier hypertension development. However, the alterations in RAS signalling (Figs. 3 and 4) differ from those in the undernourished group without α-tocopherol. We can only hypothesise that the different selective effects of the drug on AT$_2$R (Fig. 3(D)), accompanied by alternating and opposing modifications of both protein kinases (Fig. 4), possibly in conjunction with other kinases[63,69], account at least in part for the earlier hypertension development by a mechanism that is independent of Na$^+$-ATPase (Fig. 2). During the established-hypertension phase, the comparable increase in renal Ang II-positive cell number and decrease in COX-2 expression in the U and CT groups could explain similar tension values (Fig. 1, main panel and inset, respectively), together with the similarly expanded intravascular compartment and decreased creatinine clearance. The harmful side effects of α-tocopherol alone warrant further studies (VS Ribeiro, EV Cabral, LD Vieira-Filho, A Vieyra and ADO Paixão, unpublished results). However, they seem to be in line with the proposal that an oxidant/antioxidant balance in renal tissue[70], with some ROS being beneficial[71], is crucial for ion and fluid homeostasis as well as arterial blood pressure regulation[72].

In conclusion, key alterations in renal signalling pathways and ROS handling in the offspring of undernourished dams have been demonstrated, which could help in the elucidation of the mechanism(s) related to the onset of hypertension that remains stable throughout adulthood.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114513004236

References

1. Fowden AL, Giussani DA & Forhead AJ (2006) Intrauterine programming of physiological systems: causes and consequences. *Physiology (Bethesda)* **21**, 29–37.

2. Barker DJ, Gluckman PD, Godfrey KM, et al. (1993) Fetal nutrition and cardiovascular disease in adult life. Lancet 341, 938–941.

3. Zandi-Nejad K, Luyckx VA & Brenner BM (2006) Adult hypertension and kidney disease: the role of fetal programming. Hypertension 47, 502–508.

4. Langley-Evans SC, Welham SJ & Jackson AA (1999) Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. Life Sci 64, 965–974.

5. Paixão AD, Maciel CR, Teles MB, et al. (2001) Regional Brazilian diet-induced low birth weight is correlated with changes in renal hemodynamics and glomerular morphometry in adult age. Biol Neonate 80, 239–246.

6. Bertram C, Trowern AR, Copin N, et al. (2001) The maternal diet during pregnancy programs altered expression of the glucocorticoid receptor and type 2 11β-hydroxysteroid dehydrogenase: potential molecular mechanisms underlying the programming of hypertension in utero. Endocrinology 142, 2841–2853.

7. Manning J, Beutler K, Knepper MA, et al. (2002) Upregulation of renal BSC1 and TSC in prenatally programmed hypertension. Am J Physiol Renal Physiol 283, F202–F206.

8. Langley-Evans SC & Jackson AA (1995) Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets. Comp Biochem Physiol A Physiol 110, 223–228.

9. Sahajpal V & Ashton N (2003) Renal function and angiotensin AT1 receptor expression in young rats following intrauterine exposure to a maternal low-protein diet. Clin Sci (Lond) 104, 607–614.

10. Evans RG, Majid DS & Eppel GA (2005) Mechanisms mediating pressure natriuresis: what we know and what we need to find out. Clin Exp Pharmacol Physiol 32, 400–409.

11. Vieira-Filho LD, Lara LS, Silva PA, et al. (2011) Placental malnutrition changes the regulatory network of renal Na-ATPase in adult rat progeny: reprogramming by maternal α-tocopherol during lactation. Arch Biochem Biophys 505, 91–97.

12. Vieira-Filho LD, Cabral EV, Santos FTJ, et al. (2011) Alpha-tocopherol prevents intrauterine undernutrition-induced oligonephronia in rats. Pediatr Nephrol 26, 2019–2029.

13. Roos JC, Kirchner KA, Abernethy JD, et al. (1984) Differential effect of salt loading on sodium and lithium excretion in Dahl salt-resistant and -sensitive rats. Hypertension 6, 420–424.

14. Burnier M, Biollaz J, Magnin JL, et al. (1994) Renal sodium handling in patients with untreated hypertension and white coat hypertension. Hypertension 23, 496–502.

15. Rocafull MA, Thomas LE & del Castillo JR (2012) The second sodium pump: from the function to the gene. Pflügers Arch 463, 755–777.

16. Bełtowski J, Borkowska E, Wójcicka G, et al. (2007) Regulation of renal ouabain-resistant Na$^+$-ATPase by leptin, nitric oxide, reactive oxygen species, and cyclic nucleotides: implications for obesity-associated hypertension. Clin Exp Hypertens 29, 189–207.

17. Queiroz-Madeira EP, Lara LS, Wengert M, et al. (2010) Na$^+$-ATPase in spontaneous hypertensive rats: possible AT1 receptor target in the development of hypertension. Biochim Biophys Acta 1798, 360–366.

18. Rangel LB, Lopes AG, Lara LS, et al. (2005) PI–PLCβ is involved in the modulation of the proximal tubule Na$^+$-ATPase by angiotensin II. Regul Pept 127, 177–182.

19. Wang X, Armando I, Upadhyay K, et al. (2009) The regulation of proximal tubular salt transport in hypertension: an update. Curr Opin Nephrol Hypertens 18, 412–420.

20. Wolf G (2000) Free radical production and angiotensin. Curr Hypertens Rep 2, 167–173.

21. Sachse A & Wolf G (2007) Angiotensin II-induced reactive oxygen species and the kidney. J Am Soc Nephrol 18, 2439–2446.

22. Silva LA, Vieira-Filho LD, Barreto IS, et al. (2011) Prenatal undernutrition changes renovascular responses of nimesulide in rat kidneys. Basic Clin Pharmacol Toxicol 108, 115–121.

23. Green T, Gonzalez AA, Mitchell KD, et al. (2012) The complex interplay between cyclooxygenase-2 and angiotensin II in regulating kidney function. Curr Opin Nephrol Hypertens 21, 7–14.

24. Lara LS, McCormack M, Semprum-Prieto LC, et al. (2012) AT1 receptor-mediated augmentation of angiotensinogen, oxidative stress, and inflammation in ANG II-salt hypertension. Am J Physiol Renal Physiol 302, F85–F94.

25. Brand S, Amann K & Schupp N (2013) Angiotensin II-induced hypertension dose-dependently leads to oxidative stress and DNA damage in mouse kidneys and hearts. J Hypertens 31, 333–344.

26. Ichihara A, Imig JD, Inscho EW, et al. (1998) Cyclooxygenase-2 participates in tubular flow-dependent afferent arteriolar tone: interaction with neuronal NOS. Am J Physiol 275, F605–F612.

27. Flores D, Liu Y, Liu W, et al. (2012) Flow-induced prostaglandin E$_2$ release regulates Na and K transport in the collecting duct. Am J Physiol Renal Physiol 303, F632–F638.

28. Kömhoff M, Grone HJ, Klein T, et al. (1997) Localization of cyclooxygenase-1 and -2 in adult and fetal human kidney: implication for renal function. Am J Physiol 272, F460–F468.

29. Harris RC & Breyer MD (2001) Physiological regulation of cyclooxygenase-2 in the kidney. Am J Physiol Renal Physiol 281, F1–F11.

30. Vaziri ND, Bai Y, Ni Z, et al. (2007) Intra-renal angiotensin II/AT1 receptor, oxidative stress, inflammation, and progressive injury in renal mass reduction. J Pharmacol Exp Ther 323, 85–93.

31. Tian XY, Wong WT, Leung FP, et al. (2012) Oxidative stress-dependent cyclooxygenase-2-derived prostaglandin F$_{2\alpha}$ impairs endothelial function in renovascular hypertensive rats. Antioxid Redox Signal 16, 363–373.

32. Maia JCC, Gomes SL & Juliani MH (1983) Preparation of (gamma-^{32}P)- and (alpha-^{32}P)-nucleoside triphosphates with high specific activity. In Genes and Antigens of Parasites, a Laboratory Manual, 2nd ed., pp. 146–167 [CM Morel, editor]. Rio de Janeiro: Editora Fundação Oswaldo Cruz.

33. Vieira-Filho LD, Lara LS, Silva PA, et al. (2009) Placental oxidative stress in malnourished rats and changes in kidney proximal tubule sodium ATPases in offspring. Clin Exp Pharmacol Physiol 36, 1157–1163.

34. Franco MCP, Akamine EH, de Oliveira MA, et al. (2003) Vitamins C and E improve endothelial dysfunction in intrauterine-undernourished rats by decreasing vascular superoxide anion concentration. J Cardiovasc Pharmacol 42, 211–217.

35. Lowry OH, Rosebrough NJ, Farr AL, et al. (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193, 265–275.

36. Teodósio NR, Lago ES, Romani SA, et al. (1990) A regional basic diet from northeast Brazil as a dietary model of experimental malnutrition. Arch Latinoam Nutr 40, 533–547.

37. Cardoso HD, Cabral EV, Vieira-Filho LD, et al. (2009) Fetal development and renal function in adult rats prenatally subjected to sodium overload. Pediatr Nephrol 10, 1959–1965.

38. Buege JA & Aust SD (1978) Microsomal lipid peroxidation. *Methods Enzymol* **52**, 302–310.

39. Sedlak J & Lindsay RH (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* **25**, 192–205.

40. Vieyra A, Nachbin L, de Dios-Abad E, *et al.* (1986) Comparison between calcium transport and adenosine triphosphatase activity in membrane vesicles derived from rabbit kidney proximal tubules. *J Biol Chem* **261**, 4247–4255.

41. Proverbio F & Del Castillo JR (1981) Na^+-stimulated ATPase activities in kidney basal–lateral plasma membranes. *Biochim Biophys Acta* **646**, 99–108.

42. Lara LS, Correa JS, Lavelle AB, *et al.* (2008) The angiotensin receptor type 1–G_q protein-phosphatidyl inositol phospholipase C β–protein kinase C pathway is involved in activation of proximal tubule Na^+-ATPase activity by angiotensin(1–7) in pig kidneys. *Exp Physiol* **93**, 639–647.

43. Luzardo R, Silva PA, Einicker-Lamas M, *et al.* (2011) Metabolic programming during lactation stimulates renal Na^+ transport in the adult offspring due to an early impact on local angiotensin II pathways. *PLoS One* **6**, e21232.

44. Cabral LM, Wengert M, da Ressurreição AA, *et al.* (2007) Ceramide is a potent activator of plasma membrane Ca^{2+}-ATPase from kidney-promixal tubule cells with protein kinase A as an intermediate. *J Biol Chem* **282**, 24599–24606.

45. Axelband F, Dias J, Miranda F, *et al.* (2012) Angiotensin-(3–4) counteracts the angiotensin II inhibitory action on renal Ca^{2+}-ATPase through a cAMP/PKA pathway. *Regul Pept* **177**, 27–34.

46. Cabral EV, Vieira-Filho LD, Silva PA, *et al.* (2012) Perinatal Na^+ overload programs raised renal proximal Na^+ transport and enalapril-sensitive alterations of Ang II signaling pathways during adulthood. *PLoS One* **7**, e43791.

47. Lucas SR, Costa Silva VL, Miraglia SM, *et al.* (1997) Functional and morphometric evaluation of offspring kidney after intrauterine undernutrition. *Pediatr Nephrol* **11**, 719–723.

48. Lara LS, Vives D, Correa JS, *et al.* (2010) PKA-mediated effect of MAS receptor in counteracting angiotensin II-stimulated renal Na^+-ATPase. *Arch Biochem Biophys* **496**, 117–122.

49. Meneton P, Jeunemaitre X, de Wardener HE, *et al.* (2005) Links between dietary salt intake, renal salt handling, blood pressure, and cardiovascular diseases. *Physiol Rev* **85**, 679–715.

50. Tian N, Moore RS, Braddy S, *et al.* (2007) Interactions between oxidative stress and inflammation in salt-sensitive hypertension. *Am J Physiol Heart Circ Physiol* **293**, H3388–H3395.

51. Rodríguez-Iturbe B, Franco M, Tapia E, *et al.* (2012) Renal inflammation, autoimmunity and salt-sensitive hypertension. *Clin Exp Pharmacol Physiol* **39**, 96–103.

52. Stewart T, Jung FF, Manning J, *et al.* (2005) Kidney immune cell infiltration and oxidative stress contribute to prenatally programmed hypertension. *Kidney Int* **68**, 2180–2188.

53. Yang H, Lu D & Raizada MK (1997) Angiotensin II-induced phosphorylation of the AT_1 receptor from rat brain neurons. *Hypertension* **30**, 351–357.

54. García-Caballero A, Olivares-Reyes JA, Catt KJ, *et al.* (2001) Angiotensin AT_1 receptor phosphorylation and desensitization in a hepatic cell line. Roles of protein kinase C and phosphoinositide 3-kinase. *Mol Pharmacol* **59**, 576–585.

55. Li XC & Zhuo JL (2011) Phosphoproteomic analysis of AT_1 receptor-mediated signaling responses in proximal tubules of angiotensin II-induced hypertensive rats. *Kidney Int* **80**, 620–632.

56. Pedrosa R, Villar VA, Pascua AM, *et al.* (2008) H_2O_2 stimulation of the Cl^-/HCO_3^- exchanger by angiotensin II and angiotensin II type 1 receptor distribution in membrane microdomains. *Hypertension* **51**, 1332–1338.

57. Chabrashvili T, Kitiyakara C, Blau J, *et al.* (2003) Effects of ANG II type 1 and 2 receptors on oxidative stress, renal NADPH oxidase, and SOD expression. *Am J Physiol Regul Integr Comp Physiol* **285**, R117–R124.

58. Montezano AC & Touyz RM (2012) Oxidative stress, Noxs, and hypertension: experimental evidence and clinical controversies. *Ann Med* **44**, Suppl. 1, S2–S16.

59. Magalhães JC, da Silveira AB, Mota DL, *et al.* (2006) Renal function in juvenile rats subjected to prenatal malnutrition and chronic salt overload. *Exp Physiol* **91**, 611–619.

60. Judge S, Jang YM, Smith A, *et al.* (2005) Age-associated increases in oxidative stress and antioxidant enzyme activities in cardiac interfibrillar mitochondria: implications for the mitochondrial theory of aging. *FASEB J* **19**, 419–421.

61. Lambertucci RH, Levada-Pires AC, Rossoni LV, *et al.* (2007) Effects of aerobic exercise training on antioxidant enzyme activities and mRNA levels in soleus muscle from young and aged rats. *Mech Ageing Dev* **128**, 267–275.

62. Gomes P, Simão S, Silva E, *et al.* (2009) Aging increases oxidative stress and renal expression of oxidant and antioxidant enzymes that are associated with an increased trend in systolic blood pressure. *Oxid Med Cell Longev* **2**, 138–145.

63. Gildea JJ, Wang X, Shah N, *et al.* (2012) Dopamine and angiotensin type 2 receptors cooperatively inhibit sodium transport in human renal proximal tubule cells. *Hypertension* **60**, 396–403.

64. Rodrigues-Ferreira S & Nahmias C (2010) An ATIPical family of angiotensin II AT2 receptor-interacting proteins. *Trends Endocrinol Metab* **21**, 684–690.

65. Aperia A, Holtbäck U, Syrén ML, *et al.* (1994) Activation/deactivation of renal Na^+, K^+-ATPase: a final common pathway for regulation of natriuresis. *FASEB J* **8**, 436–439.

66. Asghar M, Chugh G & Lokhandwala MF (2009) Inflammation compromises renal dopamine D1 receptor function in rats. *Am J Physiol Renal Physiol* **297**, F1543–F1549.

67. Chugh G, Lokhandwala MF & Asghar M (2012) Altered functioning of both renal dopamine D_1 and angiotensin II type 1 receptors causes hypertension in old rats. *Hypertension* **59**, 1029–1036.

68. Alwasel SH & Ashton N (2012) Segmental sodium reabsorption by the renal tubule in prenatally programmed hypertension in the rat. *Pediatr Nephrol* **27**, 285–293.

69. Wruck CJ, Funke-Kaiser H, Pufe T, *et al.* (2005) Regulation of transport of the angiotensin AT2 receptor by a novel membrane-associated Golgi protein. *Arterioscler Thromb Vasc Biol* **25**, 57–64.

70. Huang HS, Ma MC, Chen J, *et al.* (2002) Changes in the oxidant–antioxidant balance in the kidney of rats with nephrolithiasis induced by ethylene glycol. *J Urol* **167**, 2584–2593.

71. Schreck C & O'Connor PM (2011) NAD(P)H oxidase and renal epithelial ion transport. *Am J Physiol Regul Integr Comp Physiol* **300**, R1023–R1029.

72. Panico C, Luo Z, Damiano S, *et al.* (2009) Renal proximal tubular reabsorption is reduced in adult spontaneously hypertensive rats: roles of superoxide and Na^+/H^+ exchanger 3. *Hypertension* **54**, 1291–1297.