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Inhibition of Angiotensin II receptors during pregnancy induces malformations in developing rat kidney

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ABSTRACT

Evidence suggests that Angiotensin II plays an important role in the complex process of renal organogenesis. Rat kidney organogenesis starts between E13–14 and lasts up to 2 weeks after birth. The present study demonstrates histologic modifications and changes in receptor localisation in animals born from mothers treated with Angiotensin II, Losartan or PD123319 (1.0 mg/kg/day) during late pregnancy. Angiotensin II-treated animals exhibited very well developed tubules in the renal medulla in coincidence with higher AT_1 binding. Control animals exhibited angiotensin AT_2 binding in the outer stripe of the outer medulla, while in the Angiotensin II-treated animals binding was observed to the inner stripe. In Angiotensin II-treated 1-week-old animals, the nephrogenic zone contained fewer immature structures, and more developed collecting tubules than control animals. Treatment with Losartan resulted in severe renal abnormalities. For newborn and 1-week-old animals, glomeruli exhibited altered shape and enlarged Bowman spaces, in concordance with a loss of [^{125}I]Angiotensin II binding in the cortex. Blockade with PD123319 led to an enlarged nephrogenic zone with increased number of immature glomeruli, and less glomeruli in the juxtamedullary area. Autoradiography showed a considerable loss of AT_1 binding in the kidney cortex of PD123319-treated animals at both ages. The present results show for the first time histomorphological and receptor localisation alterations following treatment with low doses of Losartan and PD123319 during pregnancy. These observations confirm previous assumptions that in the developing kidney Angiotensin II exerts stimulatory effects through AT_1 receptors that might be counterbalanced by angiotensin AT_2 receptors.

1. Introduction

Nephrogenesis is the result of a complex interplay among proliferation, cell-to-cell communication, inductive events and cellular movements. Kidney development begins at E13–E14 (Saxen, 1987, 1999), as the result of the interaction between the ureteric bud epithelium and adjacent metanephric mesenchyma. The mesenchyma condenses around the tip of the branching ureter and differentiates to a variety of cell lineages. Several growth factors, cytokines and extracellular matrix proteins are considered responsible for this biological phenomenon (Wolf, 1999; Burrow, 2000).

All components of the renin–angiotensin system show early expression during kidney development. Angiotensin II, the active peptide of the renin–angiotensin system recognises two receptor subtypes, AT_1 and AT_2, differentially blocked by either Losartan or PD123319 ((1-(4-dimethylamino)-3-methylphenyl)-methyl-5-diphenil-acetyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5*c*] pyridine-6-carboxylic acid), respectively (De Gasparo et al., 2000). New evidence suggests that Angiotensin II has important effects on structural development and functional differentiation of the kidney in perinatal life (Lasaitiene et al., 2006). Angiotensin II AT_2 receptors upregulates the paired homeobox 2 (Pax 2) mediating mesenchymal to epithelial transformation as well as apoptosis (Zhang et al., 2004).

Angiotensin II AT_1 and AT_2 receptors are developmentally regulated, being angiotensin AT_2 receptors more abundant at early stages of development, while AT_1 receptors are expressed later (Ciuffo et al., 1993; Tufro-McReddie and Gomez, 1993; Aguilera et al., 1994; Norwood et al., 1997; de Gasparo et al., 2000; Wolf, 2002; Garcia-Villalba et al., 2003). The high expression of angiotensin AT_2 receptors during fetal and early postnatal life implies an important role in cellular differentiation, organ development, the regulation of growth, cell proliferation and apoptosis (De Gasparo et al., 2000). In the rat kidney, the density of angiotensin AT_2 receptors, with specific localisation, rapidly declines after birth (Ciuffo et al., 1993; Aguilera et al., 1994; Kakuchi et al., 1995; Norwood et al., 1997). Autoradiography as well as *in situ* hybridisation showed an localisation of angiotensin AT_2 receptors or its mRNA in the nephrogenic zone containing immature glomeruli (Ciuffo et al., 1993; Shanmugan

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et al., 1995; Norwood et al., 1997), while more developed glomeruli express AT_1 receptors. In a recent paper, we showed that angiotensin AT_2 receptors in rat fetal kidneys induced protein tyr-dephosphorylation, a process associated with growth control (Alvarez et al., 2003).

The importance of the renin–angiotensin system in the normal kidney development has been demonstrated by a number of studies using gene targeting or pharmacological interruption of the renin–angiotensin system (Lasaitiene et al., 2006). In recent years, several mouse strains that carry null mutated Angiotensin II receptor genes (*Agtr1a*, *Agtr1b*, *Agtr2*, *Agt*) have been developed (Hein et al., 1995; Ichiki et al., 1995; Ito et al., 1995; Niimura et al., 1995; Nagata et al., 1996; Oliverio et al., 1998; Tsuchida et al., 1998; Nishimura et al., 1999; Mangrum et al., 2002). None of the murine Angiotensin II single receptor mutants display the severe abnormal phenotypes disclosed by the Angiotensinogen mutant (*Agt* −/−) (Nagata et al., 1996) and the double mutant *Atgr1a* and *Atgr1b* null mice (Tsuchida et al., 1998).

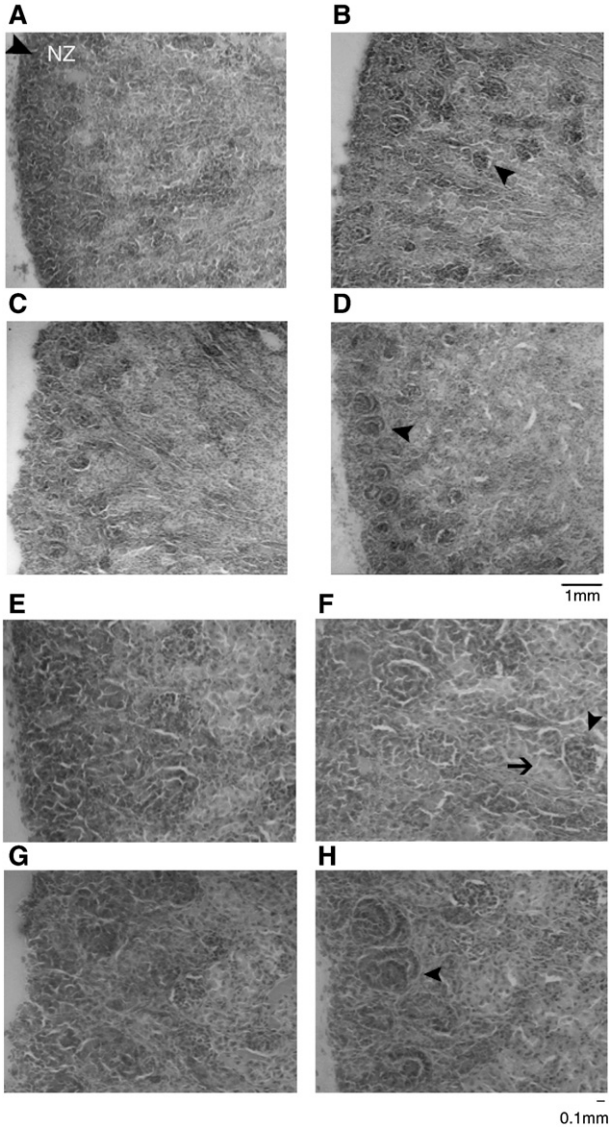

Fig. 1. Histology (H&E) of the cortical and sub-cortical area of newborn rat kidneys. Representative histology from control (A) and treated animals: Losartan (B), Angiotensin II (C) and PD123319 (D) (n=24 for each treatment). H&E magnification 40×. E–H: Enlarged view (100×) of the same sections (A–D) showing a detail of the nephrogenic zone (NZ) with S-shaped and comma-shaped bodies. Arrowhead in B points to the same glomeruli as in F. Arrow points to convoluted tubules in F. Arrowhead in D points to the same glomeruli as in H.

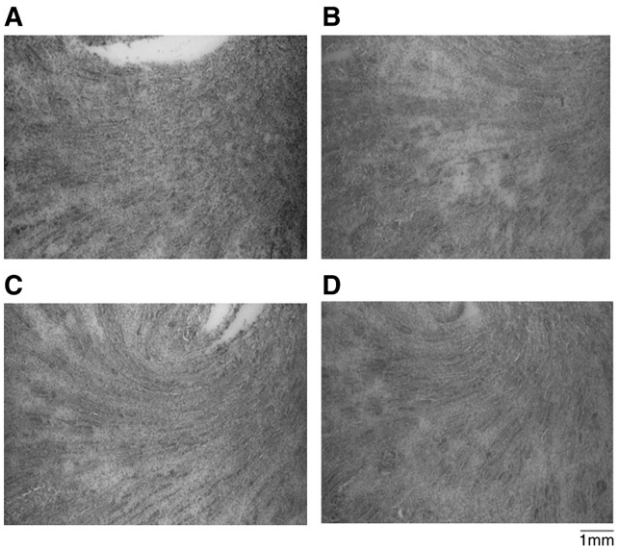

Fig. 2. Histology (H&E) of newborn rat kidneys medulla from control (A) and treated animals: Losartan (B), Angiotensin II (C) and PD123319 (D). Magnification 40×.

Treatments to neonatal rats with inhibitors of the angiotensin-converting enzyme (ACE) or Losartan perturb medullary tubulogenesis (Tufro-McReddie et al., 1995; Friberg et al., 1997; Mc Causland et al., 1997; Chen et al., 2004). Similarly, blockade of AT_1 receptors by Losartan induces alteration in the expression of the major histocompatibility complex (MHC) II in the loop of Henle (Lasaitiene et al., 2004). A main functional finding in adult rats subjected to neonatal inhibition of the renin–angiotensin system is impairment of the urine concentrating ability, which is causally related to medullar atrophy and tubulointerstitial abnormalities (Friberg et al., 1997; Guron et al., 1997; Guron and Friberg, 2000). In this regard, Lasaitiene et al. (2006) reviewed the general characteristics of mammalian kidney development and the mechanisms by which Angiotensin II mediates tubulogenesis and nephrovascular development, studied by means of pharmacological blockade in the rat. In a model of renal damage following renal ablation (Vazquez et al., 2005), Angiotensin II induced an increase of angiotensin AT_2 receptor expression, an effect reverted by the selective antagonist PD123319. On the other hand, in a model of cardiac hypertrophy induced by *in vivo* treatment with Angiotensin II, PD123319 regulated the gene expression of different growth factors in the adult rat heart (Lakó-Futó et al., 2003).

Alwan et al. (2005) recently reviewed the teratogenic effects after maternal treatment with large doses of AT_1 receptor antagonist of the 'sartan' family. Akil et al. (2005) observed that treatment during rat pregnancy with Losartan induces an arrest in nephrovascular maturation. A case report of renal malformation was observed in human (Daikha-Dahmane et al., 2006).

Gribouval et al. (2005) reported mutations in genes of the renin–angiotensin system in humans that are associated with autosomal recessive renal tubular dysgenesis. By applying gene targeting methods, different knock-out mice have been generated (Ito et al., 1995; Niimura et al., 1995; Nagata et al., 1996; Nishimura et al., 1999; Mangrun et al., 2002). Inactivation of the AT_{1A} gene results in animals with a lower blood pressure and renal malfunctions, as well as renal architectural malformations (Oliverio et al., 1998). Mice lacking both AT_{1A} and AT_{1B} receptors exhibited reduced growth and abnormal kidney structure (Tsuchida et al., 1998). Saavedra et al. (2001) reported increased AT_1 receptor expression in kidney glomeruli of mice lacking angiotensin AT_2 receptors.

An inter-regulation between both Angiotensin II receptor subtypes has been postulated, suggesting that angiotensin AT_2 receptors may antagonise the effects of AT_1, particularly on growth effects (Ciuffo et al.,

1993; Tufro-McReddie and Gomez, 1993; Tufro-McReddie et al., 1995; Wolf, 2002; Lakó-Futó et al., 2003; Vázquez et al., 2005). To add up information on the impact of inhibition of the renin–angiotensin system during fetal kidney development, the aims of the present study were to examine: 1) histological changes of the newborn rat kidney following treatment during late pregnancy with Angiotensin II or its antagonists; 2) changes in receptor localisation of treated animals.

2. Materials and methods

2.1. Experimental design

Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

Pregnant Wistar rats weighing 250 g were kept in a dark–light cycle (12:12 h), maintained at 22±1 °C and fed with standard rodent food and water *ad libitum*. On the 13th day of pregnancy, miniosmotic pumps (Alzet model 2001; Palo Alto, CA) were implanted subcutaneously between the scapulae bones under light ether anaesthesia. Alzet miniosmotic pumps were filled with sterile saline vehicle (control animals), Angiotensin II (Sigma Chemical Co, St. Louis, MO), PD123319 (RBI-Sigma), or Losartan (provided by DuPont-Merck Pharmaceutical Co, Wilmington, DE). The doses used for the different treatments, 1.0 mg/kg/day, corresponded to 41 μg/kg/h. For Angiotensin II this dose was comparable to the one used by Lakó-Futó et al., (2003). On the other hand, doses used for PD123319 and Losartan were considerably lower than the ones used by other authors, usually 30 mg/kg/day and 10 mg/kg/day, respectively (Lakó-Futó et al., 2003; Chen et al., 2004; Lasaitiene et al., 2004).

Pregnant rats were divided into four experimental groups: group 1 (Control) rats ($n=4$) were subjected to vehicle administration; group 2 rats ($n=4$) received the AT_1 antagonist Losartan; group 3 rats ($n=4$) were subjected to Angiotensin II; and group 4 rats ($n=4$) received the AT_2 antagonist PD123319. From each dam, 5–6 pups/treatment group were sacrificed at two stages, newborn or 1-week-old, for each different experimental condition. After birth, neonatal rats were sacrificed by decapitation, kidneys were immediately removed, snap frozen in isopentane at −30 °C and stored at −80 °C.

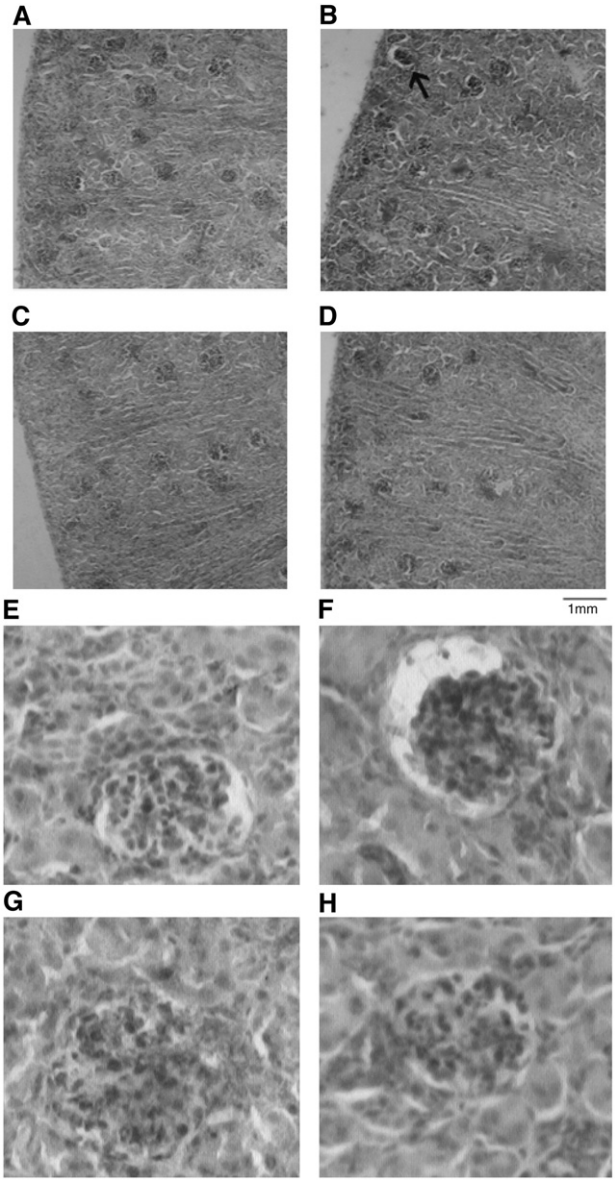

Fig. 3. Histology of sagittal sections of kidneys from 1-week-old control and treated animals. Representative histology from control (A) and treated animals, Losartan (B), Angiotensin II (C), and PD123319 (D) ($n=24$ for each treatment). A–D: Histology of the cortical and sub-cortical area, H&E, magnification 40×. E–H: Enlarged view (400×) of glomeruli from the juxtamedullary area from the same sections. F shows an ischemic glomeruli from Losartan-treated kidney.

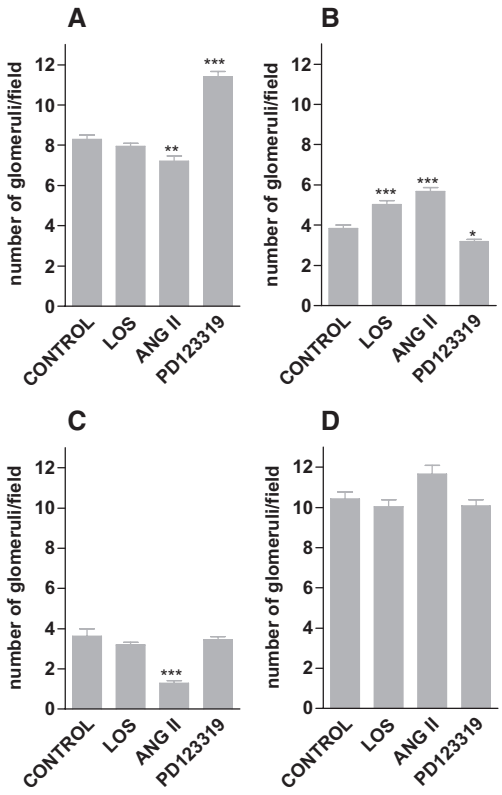

Fig. 4. Relative number of glomeruli in kidney from control and treated animals. Glomeruli/field were counted in the nephrogenic zone and renal cortex from control and prenatally treated animals with Angiotensin II (Ang II), Losartan (Los) or PD123319, at different stages of development (newborn and 1-week-old). ***: $p<0.001$, **$p<0.01$, *$p<0.05$ vs control. A. Glomeruli number in the nephrogenic zone from newborn animals. B. Glomeruli number in the renal cortex from newborn, excluding nephrogenic zone. C. Glomeruli number in the nephrogenic zone from 1-week-old animals. D. Glomeruli number in the renal cortex from 1-week-old animals. Values are means ±SEM from six to eight animals from each treatment.

2.2. Histological analysis

Parallel sections to those used for autoradiography were stained with haematoxylin and eosin (H&E) for comparative determination of morphology and architectural structures. The histological evaluation

and quantitative analyses were performed on H&E stained sections from sagittal sections of the kidneys including cortex, medulla and renal pelvis structures. Kidneys with evidence of traumatic artefacts were not considered. The light microscopic examination (microscope Nikkon Eclipse 50i) was based on the standard morphological criteria. The number of glomeruli per field was counted in 10 fields per kidney and 5–6 kidneys were analysed for each treatment group at 100× magnification. Glomeruli were counted at the nephrogenic zone and the rest of the cortex (excluding nephrogenic zone). Similarly, glomeruli with enlarged Bowman's space were counted.

2.3. Autoradiography of Angiotensin II receptors

Binding by autoradiography was performed as described previously (Ciuffo et al., 1993). Sagittal sections, 16 μm thick, were cut in a cryostat at −20 °C (Microm, Zeiss Inc), thaw-mounted on gelatine-coated glass slides, and dried overnight in a desiccator at 4 °C before incubation. Briefly, sections were preincubated during 15 min at 22 °C in 10 mM sodium phosphate buffer pH 7.4, containing 120 mM NaCl, 5 mM disodium EDTA, 0.005% bacitracin (Sigma), and 0.2% proteinase free bovine serum albumin (Sigma). Sections were incubated *in vitro* with 0.2 nM [^{125}I]Angiotensin II (DuPont-NEN, sp. act. 2200 Ci/mmol), a concentration below the Kd value, for 120 min in fresh buffer containing the ligand. Non-specific binding was determined with an excess of Angiotensin II (10^{-6} M). After incubation, slides were rinsed four consecutive times, 1 min each, in fresh ice-cold 50 mM Tris buffer, pH 7.6, followed by 30 s in ice-cold water, and then the sections were allowed to dry under a steam of cool air.

For Angiotensin II receptor subtype identification, consecutive sections were incubated with 0.2 nM [^{125}I]Angiotensin II, in the presence of 10^{-6} M of AT_1 antagonist Losartan, to define AT_2 receptors, or 10^{-6} M PD123319, to define AT_1 subtype. The dried labelled sections were apposed against Kodak BioMax MR film in X-ray cassettes. Films were developed with D19 Kodak developer for 4 min at 4 °C, after 2 or 10 days exposition.

Quantification: Autoradiographic images were quantified by densitometry with Scion software for Windows. Images were captured from films with low-exposure time (2 days). For statistical comparisons, values from all groups were obtained within the same film. Results were expressed in optical density units from a 256 grey scale.

2.4. Statistics analysis

Mean and standard error were calculated for every data set. Differences between groups were evaluated using one-way Analysis of Variance (ANOVA) followed by Tukey–Kramer Multiple Comparisons Test. A probability of less than 0.05 was assumed to be significant.

3. Results

Four different treatments were performed: Control, Angiotensin II, Losartan and PD123319 by administrating to pregnant rats, either vehicle or solutions at a concentration of 1.0 mg/kg/day. We found no statistically significant differences among groups when compared either kidney or body weight. Kidney weight (mg) for newborn pups: 29.03±0.13 (Control, n=6), 29.13±0.16 (Losartan, n=6), 29.15±0.15 (Angiotensin II, n=6), 29.05±0.12 (PD123319, n=6). Kidney weight (mg) for 1-week-old animals: 63.53±0.82 (Control, n=6), 63.38±0.74 (Losartan, n=6), 63.40±0.78 (Angiotensin II, n=6), 63.35±0.69 (PD123319, n=6).

3.1. Histological analysis

3.1.1. Newborn animals

From every treatment group, sagittal sections of kidneys consecutive to those used for autoradiography were stained with H&E and examined under light microscopy. For newborn animals, the typical architecture of developing kidney was observed in animals treated with saline (Fig. 1A). In this group (control), a gradient of developing

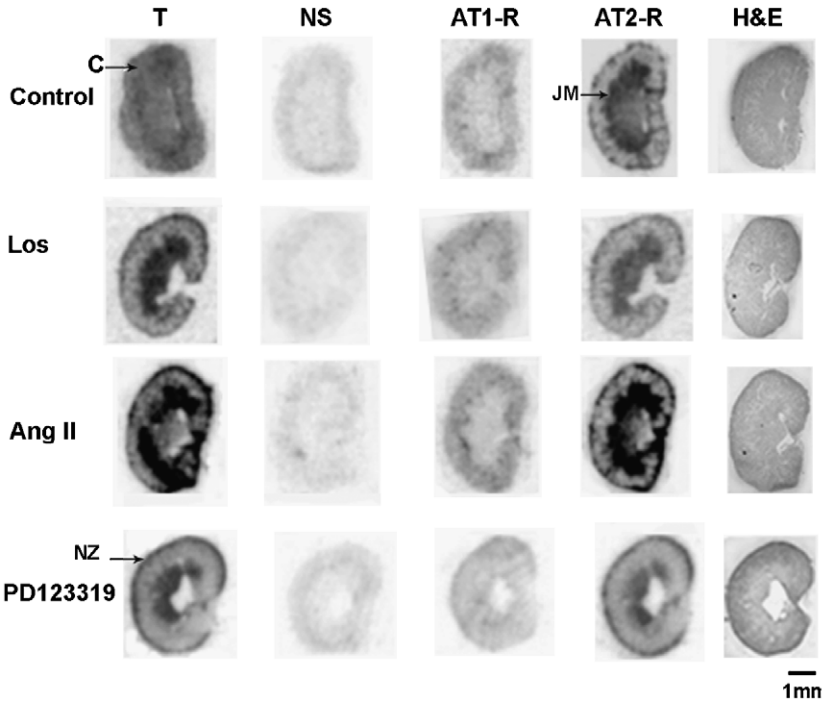

Fig. 5. Autoradiographic localisation of Angiotensin II receptors in rat kidneys from newborn rat born from treated mothers. Parasagittal consecutive sections of kidneys were incubated with [^{125}I]Angiotensin II in the absence (Total binding, T) or in the presence of Angiotensin II (NS, 10^{-6} M). Receptor subtype was identified with an excess of Losartan (AT_2 receptors, 10^{-6} M, AT_2-R) or PD123319 (AT_1 receptors, 10^{-6} M, AT_1-R) in the incubation media. Different rows correspond to Control animals, Losartan (Los), Angiotensin II (Ang II) or PD123319 treatments during late pregnancy. Arrows point to different structures. NZ: nephrogenic zone, C: cortex, JM: juxtamedullary area. H&E: sections stained with haematoxylin and eosin. Representative data from four independent treatments of the pregnant mothers, 5–6 animals for each treatment group.

glomeruli was present, with an important nephrogenic zone and a very well defined capsule.

Treatment with Losartan led to a disorganised nephrogenic zone, where the comma-shaped and S-shaped bodies were present at different levels of the cortex, not limited to the nephrogenic zone, in contrast with the control group. Fig. 1B shows a representative image of animals born from mothers treated with Losartan. In the deep cortex, some of the glomeruli exhibited an enlarged Bowman's space as compared to control animals (Fig. 1B, arrowhead).

Animals treated with Angiotensin II exhibited a nephrogenic zone thinner and with less S-shaped bodies (Fig. 1C) than control animals, and the capsule was disorganised. Animals treated with the AT_2 antagonist, PD123319, showed a thicker nephrogenic zone containing a high number of immature structures such as, comma-shaped and S-shaped bodies (Fig. 1D). Fig. 1(E–H) shows an enlarged view of the cortex (magnification 100×). Convoluted tubules were considerably more developed in animals treated with either Losartan or PD123319, than in control or Angiotensin II-treated animals (Fig. 1F, arrow).

At this developmental stage, as expected, medullary structures were not as well developed as in control animals, while the medulla appeared more developed in Angiotensin II-treated animals (Fig. 2A and C). Animals treated with either Losartan or PD123319 exhibited a poor development of tubular structures (Fig. 2B and D) as comparing to controls.

3.1.2. 1-week-old animals

Control animals sacrificed on day 8 after birth still evidenced a developmental stage on their kidney architecture, as described (Ciuffo et al., 1993). Control animals (Fig. 3A) exhibited the expected typical structures. Glomeruli at different stages of development were present in the cortex area (Fig. 3A), the most mature in the juxtamedullary area.

Kidneys of 1-week-old rats born from mothers treated during pregnancy with Losartan exhibited glomeruli with altered structures (Fig. 3B and F) and a lower number of mature glomeruli were observed in the deep cortex (Fig. 3B). Fig. 3F shows a representative image of glomeruli with enlarged Bowman's space, observed in Losartan-treated animals. This observation is in agreement with previous ones from Akil et al. (2005) in fetal kidney (E18). As in newborn, an excessive development of convoluted tubules was present in the cortex (data not shown).

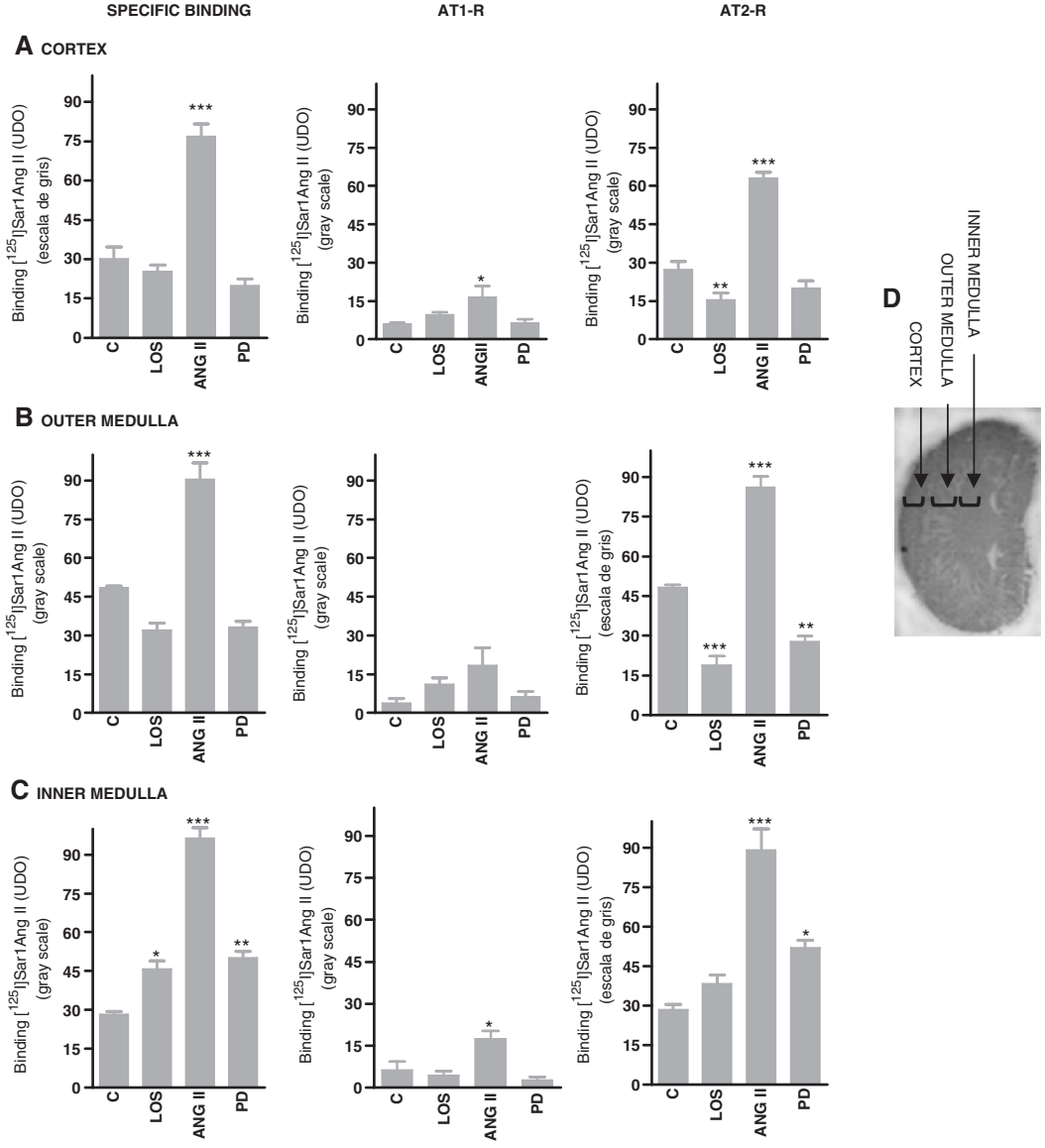

Fig. 6. Quantification of the [^{125}I]Angiotensin II binding (autoradiography) to different structures of rat kidney from newborn rats born from treated mothers. Bars are mean ± SEM of specific binding, AT_1 subtype (AT_1-R) and AT_2 subtype (AT_2-R). A: Binding to the cortex for the different treatment groups. B: Binding to the outer medulla for the different treatment groups. C: Binding to the inner medulla for the different treatment groups. D: H&E where the quantified areas are indicated. ***: $p<0.001$, **$p<0.01$, *$p<0.05$ vs control.

Examination of rat kidney sections from 1-week-old animals born of pregnant rats exposed to Angiotensin II showed a significant decrease of the nephrogenic zone with scarce number of developing glomeruli. The number of glomeruli in the cortex was lower than in control animals (Fig. 3A and C) although normal appearing glomeruli were present in the deep cortex (Fig. 3G). Medullary rays were well developed, corresponding to the kidney's structure of adult animals rather than to 1-week-old. These structures were absent in control animals.

Histological examination of 1-week-old kidneys from animals treated with the AT_2 antagonist PD123319 showed a poorly organised kidney (Fig. 3D). A lower number of developed glomeruli was observed. Besides, glomeruli present in the cortex appear less developed than those present in control animals. In these animals, convoluted tubules look poorly developed or practically absent, as well the same trend was observed for the medullary rays of the collecting tubules. The medulla appeared unorganised, and no structure could be observed in this area.

Fig. 3E–H shows images of glomeruli observed in 1-week-old animals at higher magnification where some of the above mentioned structures are shown.

3.2. Glomeruli quantification

The number of glomeruli per field was quantified as described under Materials and methods. For newborn animals the number of glomeruli in the nephrogenic zone was lower in animals born from mothers treated with Angiotensin II ($p<0.01$) and higher in those born from mothers treated with PD123319 ($p<0.001$) (Fig. 4A). When the cortex area (excluding nephrogenic zone) was considered, the number of glomeruli was higher in Losartan-treated and Angiotensin II-treated groups ($p<0.001$), and lower in PD123319-treated group ($p<0.05$) as compared to control animals (Fig. 4B).

In 1-week-old animals, the number of glomeruli/field in the nephrogenic zone was considerably lower than in newborn animals, as maturation proceeded. Pups born from mothers treated with Angiotensin II, exhibited a lower number of glomeruli at the nephrogenic zone ($p<0.001$) (Fig. 4C). In the cortex (excluding nephrogenic zone), the number of glomeruli was comparable for all the treatments (Fig. 4D). At the stage of 1-week-old, we observed that treatment with Losartan during late pregnancy produced a 30% of glomeruli with enlarged Bowman's space, shown in Fig. 3F.

3.3. Autoradiographic localisation of Angiotensin II receptors in rat kidneys

Figs. 5 and 7 show representative images of binding by autoradiography for all treatment groups at the two developmental stages, newborn (Fig. 5) and 1-week-old (Fig. 7). The different treatments to the pregnant rats are ordered by rows: Control, Losartan (Los), Angiotensin II (Ang II) and PD123319. Total binding, non-specific binding, subtypes AT_1 and AT_2 and H&E, are depicted by the different columns. Non-specific binding was low in all cases. Figs. 6 and 8 show the quantified data from autoradiography.

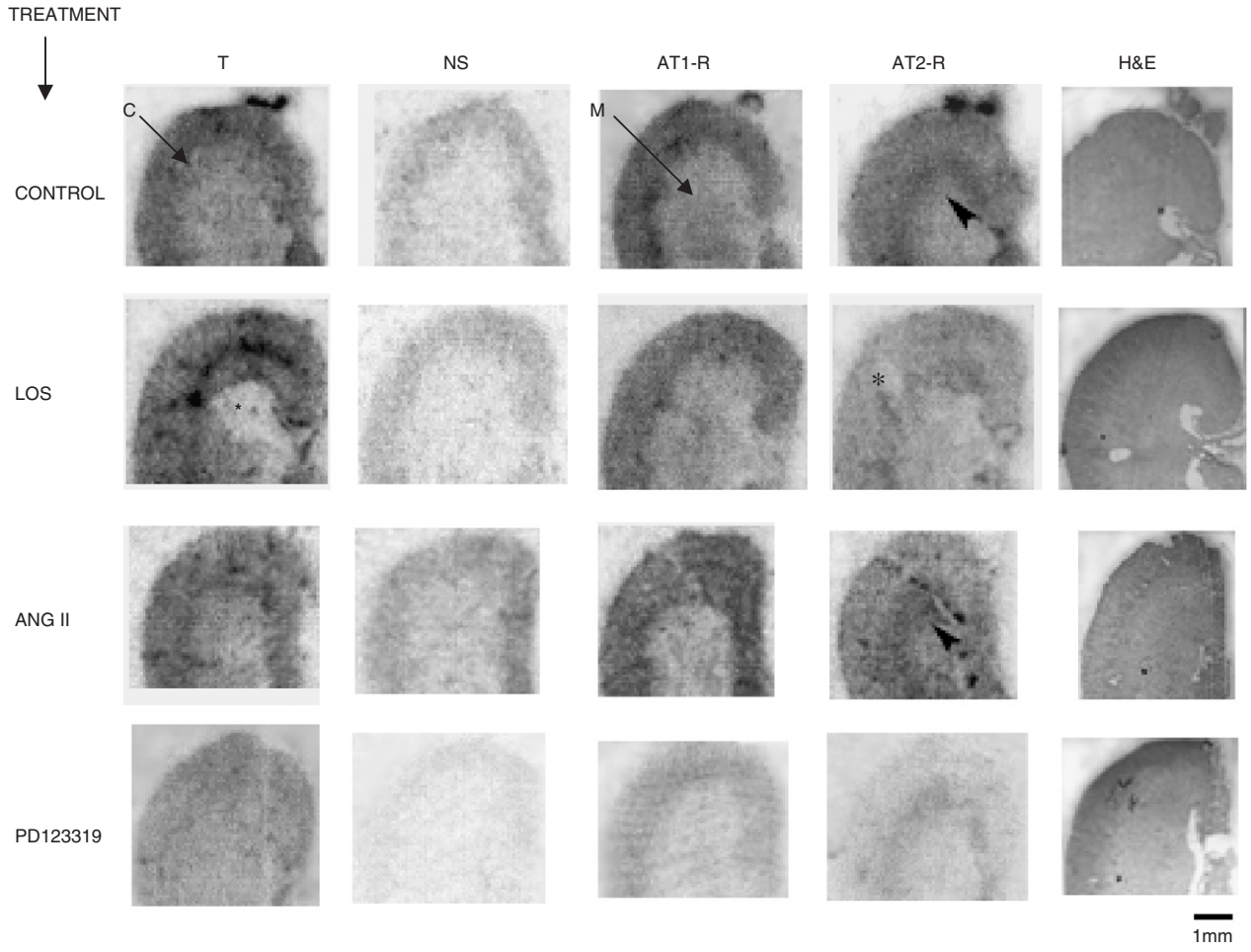

Fig. 7. Autoradiographic localisation of Angiotensin II receptors in rat kidneys from 1-week-old rats born from treated mothers. Parasagittal consecutive sections of kidneys were incubated with [^{125}I]Angiotensin II in the absence (Total binding, T) or in the presence of Angiotensin II (NS, 10^{-6} M). Receptor subtype was identified with an excess of Losartan (AT_2 receptors, 10^{-6} M, AT_2-R) or PD123319 (AT_1 receptors, 10^{-6} M, AT_1-R) in the incubation media. Different rows correspond to Control animals, Losartan (Los), Angiotensin II (Ang II) or PD123319 treatments during late pregnancy. Arrows point to different structures. C: cortex, M: medulla. * points binding to the convoluted tubules. Arrowhead points to the ISOM. H&E: sections stained with haematoxylin and eosin. Representative data from four independent treatments to the pregnant mothers (20–24 animals from each treatment).

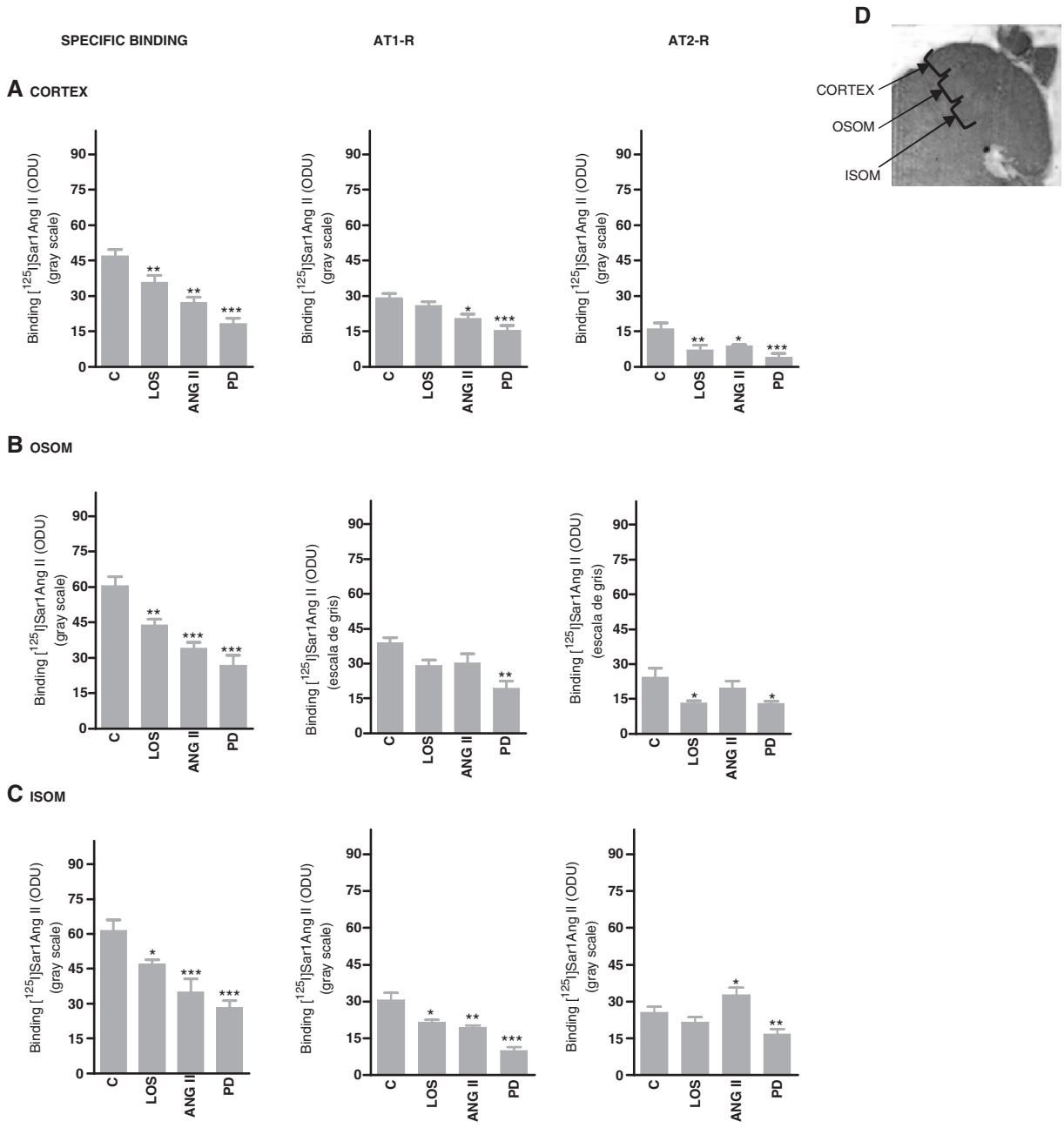

Fig. 8. Quantification of the [^{125}I]Angiotensin II binding (autoradiography) in different structures of rat kidney from 1-week-old rats born from treated mothers. Bars are mean ± SEM of specific binding, AT_1 subtype (AT_1-R) and AT_2 subtype (AT_2-R). A: Binding to the cortex for the different treatment groups. B: Binding in the outer stripe of the outer medulla (OSOM) for the different treatment groups. C: Binding to the inner stripe of the outer medulla (ISOM) for the different treatment groups. D: H&E indicates where the areas were quantified. ***: $p<0.001$, **$p<0.01$, *$p<0.05$ vs control.

3.3.1. Newborn animals

Fig. 5 shows [^{125}I]Angiotensin II binding to newborn rat kidneys from the different treatment groups. Kidneys from control animals (Fig. 5, upper row) exhibited binding to the nephrogenic zone, cortex and medulla. At this developmental stage, most of the binding corresponds to angiotensin AT_2 receptors, mainly localised to the nephrogenic zone and to the outer stripe of the outer medulla (OSOM). Lower AT_1 receptor binding was observed, mainly localised to the cortex (Fig. 5).

Animals treated with Losartan (Fig. 5, second row) exhibited binding localised at the nephrogenic zone and the medulla. AT_2 binding, localised to the nephrogenic area and to the outer medulla (Fig. 5), was significantly lower in this group (Fig. 6). Binding localisation to the medulla (inner medulla) differed from that observed in control animals, where binding was spread over the whole medulla. As mentioned above, the nephrogenic zone has a poor development as comparing with control animals and, consequently, lower AT_2 binding (Fig. 6). Papillary atrophy was observed in animals treated with Losartan, as well as in those treated with PD123319 (Fig. 5, H&E), in agreement with previous observations (Chen et al., 2004).

The animals born from mothers treated with Angiotensin II at newborn stage, showed in general a higher intensity of binding than control animals, present in all areas (Fig. 5, third row). Total specific binding, as well as AT_1 and AT_2 binding were significantly increased in the cortex and the medulla (Fig. 6). The strongest binding was observed in the nephrogenic zone and the medulla, predominantly of the AT_2 subtype. The cortex exhibited higher AT_1 binding than control animals (Fig. 6). Binding was spread out all over the medulla, as it is

observed in control animals and differed from the one shown by Losartan-treated animals.

In PD123319 treated animals, binding was present in the nephrogenic zone and the inner medulla (Fig. 5). There were almost no differences between total binding and AT_2 binding, while in control or Angiotensin II-treated animals a clear binding attributed to AT_1 receptors appeared in the cortex (Fig. 6). The nephrogenic zone is enlarged and very well defined in these animals, as shown at the histological level, and exhibits high AT_2 binding. In the OSOM, binding was lower than in control animals (Fig. 6). Although AT_1 binding is present in the cortex, it is diffuse and not associated with structures. When compared to control, the absence of binding in this area of PD123319 treated animals, suggests the absence of structures, either glomeruli or the origin of collecting tubules (Figs. 1 and 4). This observation agrees with the histological analysis, since a lack of mature glomeruli was observed (Figs. 1D and 5).

3.3.2. 1-week-old animals

In control animals, both receptor subtypes, AT_1 and AT_2 were observed, localised in both areas, cortex and medulla (Figs. 7 and 8). AT_1 binding was present in the cortex, including the juxtamedullary area, while AT_2 binding was observed in the cortex and in the OSOM (Fig. 7, first row, arrowhead). AT_1 binding was associated to glomerular structures in the cortex.

Animals treated with Losartan exhibited lower binding ($p<0.01$) than control animals (Figs. 7 and 8). AT_1 binding localised to the ISOM was lower than in control animals ($p<0.5$). AT_2 binding was spread and diffuse over different areas but the OSOM was lower than in control animals, as pointed out for newborn (Fig. 8). In Losartan-treated animals, low binding was observed in correspondence with poorly developed tubular structures (Fig. 7, *).

For kidneys from Angiotensin II-treated animals total binding was significantly ($p<0.01$) lower than for control animals to the different structures (Figs. 7 and 8). Localisation of AT_1 binding was comparable to that of control animals, present in the cortex and juxtamedullary area (Fig. 7). Comparison with control animals evidenced a change in the binding pattern to the medulla (Fig. 7, arrowhead). While in control animals, AT_2 binding was present in the OSOM, in Angiotensin II-treated animals it was localised to the ISOM. AT_2 binding localised to the ISOM (Fig. 7 third row, arrowhead) was higher than in control group (Fig. 8). In the renal cortex, AT_2 binding was diffuse and lower than in control animals (Fig. 8).

In kidneys of animals born from mothers treated with PD123319, the total binding was significantly ($p<0.001$) lower than in control or Angiotensin II-treated animals (Figs. 7 and 8). This observation correlates with the lower number and poor development of glomeruli (Fig. 4). AT_1 and AT_2 binding to the different kidney areas were significantly reduced in these animals (Fig. 8). Also, binding in the juxtamedullary area was absent in agreement with the lack of medullary structures observed in histological preparations (Fig. 7).

4. Discussion

Previous studies on pharmacological blockade of Angiotensin II receptors have been reported. Important morphological changes in kidney development have been reported following Losartan treatment, mainly performed postnatally (Mc Causland et al., 1997; Chen et al., 2004; Lasaitiene et al., 2004). Akil et al. (2005) reported the effect on renal development of blockade during late pregnancy with high doses (10 mg/kg/day) of Losartan. Alwan et al. (2005) recently reviewed the teratogenic effects after maternal treatment with large doses of AT_1 receptor antagonists of the 'sartan' family on both animal studies and human cases. Most of these studies pointed out the induction of renal malformations following Losartan administration either during pregnancy or postnatally. The present results show for the first time histomorphological and receptor localisation alterations following treatment during pregnancy with low doses of Losartan. The presence of glomeruli exhibiting enlarged Bowman's space was a constant in most of these reports, also observed in a case report in human (Daikha-Dahmane et al., 2006). Alteration of the medulla development was reported for knock-out animals, the *Agt−/−* and the double *Agtr1a−/−;Agtr1b−/−* nullizygote (Nagata et al., 1996; Oliverio et al., 1998; Tsuchida et al., 1998).

Previous studies of pharmacological blockade of Angiotensin II AT_2 receptors with PD123319 were performed in newborn animals and no alterations were reported (Tufro-McReddie et al., 1995; Mc Causland et al., 1997). In the present work we observed that blockade of Angiotensin II AT_2 receptors during pregnancy induced an arrest in glomerular maturation and tubular growth, as evidenced by the increased number of immature glomeruli under the capsule in newborn animals. We observed an increased binding of the AT_2 subtype in the nephrogenic zone in concordance with the histological observation and the number of glomeruli present in this area. Animals at 1-week-old exhibited reduced binding in the cortex, suggesting that treatment during the period of organogenesis, seriously disrupts the pattern of renal development, which is not recovered a week after birth. *Agtr2−/−* null mice display congenital anomalies of the kidney and urinary tract (Nishimura et al., 1999).

Several lines of evidence suggest that Angiotensin II plays an important role in the complex process of renal organogenesis. The renin–angiotensin system is up-regulated during renal development. In the rat, kidney organogenesis starts between E13–14 and last for 2 weeks after birth. The rat kidney is immature at birth, the less mature glomeruli are found near the renal capsule and the most mature in the juxtamedullary area. Angiotensin AT_2 receptors are expressed abundantly during fetal development and are markedly down-regulated after birth, whereas the abundance of AT_1 receptors increases as maturation proceeds (Ciuffo et al., 1993; Norwood et al., 1997; Garcia-Villalba et al., 2003; Vazquez et al., 2005). Inhibition of the renin–angiotensin system might produce renal abnormalities including abnormal renal vessels, failure to develop the renal pelvis and tubular atrophy associated with expansion of the interstitium (Guron et al., 1997, Wolf, 2002; Chen et al., 2004; Lasaitiene et al., 2004, 2006; Akil et al., 2005).

The present study demonstrates changes at the histological level and in the pattern of receptor localisation in rats born from mothers treated with Angiotensin II or its competitors during late pregnancy. While most studies evaluated the effect of Losartan or PD123319 at pharmacological doses (10–30 mg/kg/day) (Lakó-Futó et al., 2003; Chen et al., 2004; Lasaitiene et al., 2004; Akil et al., 2005), in the present study we used lower doses (1 mg/kg/day) to evaluate the effects of a mild treatment during the last week of pregnancy. Four different groups were compared, named as control, treated with saline vehicle, Angiotensin II, Losartan and PD123319.

The first and most simple conclusion of the present study is that a treatment during late pregnancy does affect kidney development of the pups, even at low doses as the ones used here. Although treatments lasted for 1 week before birth, we observed important effects on kidney development both at the histological and receptor localisation level in 1-week-old animals. These observations indicated that the damage persisted even when there was no longer exposure to the drugs.

It is now well established that Angiotensin II is a renal growth factor that modulates cell growth and differentiation (Wolf, 2002; Zhang et al., 2004; Gribouval et al., 2005; Lasaitiene et al., 2006). Following treatment during late pregnancy with Angiotensin II, kidneys from newborn animals appeared as more mature than those of control animals, with a nephrogenic zone containing lower number of immature glomeruli. The medulla exhibited very well developed tubules in coincidence with a higher binding localised to this area. Comparing to control animals, binding was increased in all studied areas, corresponding mainly to the AT_2 subtype. At 1-week-old, the nephrogenic zone contained fewer developing structures, while medullary rays were more defined than in control animals. Regarding

receptor localisation, autoradiography showed that angiotensin AT_2 receptors localised differentially in Angiotensin II-treated than in control animals in the medulla. While in control animals AT_2 binding was present in the OSOM, in Angiotensin II-treated animals it was localised to the ISOM. To our knowledge, few studies have been performed with treatment during late pregnancy. A recent report by Yuan et al. (2003) informed changes in renal medullar vasa recta following treatment with Angiotensin II treatment at non-hypertensive doses (5 ng/kg/min, IV) to adult male rats. Our present data agree with these observations since we observed an enhancement of tubular development in animals treated with Angiotensin II and with observations indicating that Angiotensin II exerts growth-stimulatory effects on tubular cells (Wolf, 2002). Moreover, Angiotensin II induces synthesis of collagen type IV in tubular cells (Desjardins and Bendayan, 1991), a necessary prerequisite for successful basement membrane formation, an effect mediated by AT_1 receptors.

We observed that treatment with Losartan also contributed to severe renal abnormalities. In newborn and 1-week-old animals, glomeruli appeared with altered structures and a lower number of developed glomeruli was observed. Some glomeruli exhibited enlarged Bowman's space, in concordance with lower [^{125}I]Angiotensin II binding in the cortex. As compared to control animals, tubule development was poor while convoluted tubules were highly developed. This observation agrees with changes in receptor localisation, since AT_2 binding present in the OSOM in control animals was absent in Losartan-treated animals at stages newborn and 1-week-old.

Blockade of angiotensin AT_2 receptors with PD123319, led to an enlarged and compact nephrogenic zone with increased number of developing glomeruli, while a low number of glomeruli was present at the juxtamedullary area, indicating an interruption on the development. Autoradiography of newborn kidneys showed a considerable loss of AT_1 binding in the kidney cortex as compared to control animals, since angiotensin AT_2 receptor localisation was quite similar to the total binding. Thus, the loss of binding corresponds to the lack of glomeruli in the cortex. Histological analysis of 1-week-old animals showed very few mature glomeruli, in coincidence with the loss of AT_1 binding in the cortex. Similarly, the medulla appeared to be disorganised.

Taken together, the present data suggest that both angiotensin AT_1 and AT_2 receptors are involved in the development of the nephron, and Angiotensin II provides signals through both receptors. Blockade of AT_1 receptors during the last week of pregnancy in the rat causes an important alteration of the whole kidney structure, maybe because of the growth promoting actions of AT_1 receptors. Blockade of angiotensin AT_2 receptors during the last week of pregnancy in the rat seems to arrest glomerular development and stop the normal progression of glomerulogenesis. In spite that low doses of Angiotensin II antagonists (Losartan or PD123319) were used, treatments were performed during a critical period of kidney organogenesis, suggesting a specific effect. Although, we cannot exclude the possibility of an adverse effect of Losartan treatment.

These observations confirm previous assumptions that Angiotensin II growth-stimulatory effect through AT_1 receptors may be counterbalanced by angiotensin AT_2 receptors-mediated apoptosis and growth inhibition. The present study makes a contribution to understand the role of Angiotensin II receptors during kidney organogenesis and supports the hypothesis of an inter-regulation between both Angiotensin II receptor subtypes.

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