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Developmental immunolocalization of heat shock protein 70 (HSP70) in epithelial cell of rat kidney

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Summary. During renal development the cells in the medulla are exposed to elevated and variable interstitial osmolality. Heat shock protein 70 (HSP70) is a major molecular chaperone and plays an important role in the protection of cells in the renal medulla from high osmolality. The purpose of this study was to establish the time of immunolocalization and distribution of HSP70 in developing and adult rat kidney. In addition, changes in HSP70 immunolocalization following the infusion of furosemide were investigated. In adult animals, the HSP70 was expressed in the medullary thin ascending limb of Henle's loop (ATL) and inner medullary collecting duct (IMCD). In developing kidney, HSP70 immunoreactivity was first detected in the IMCD of the papillary tip on postnatal day 1. From four to 14 days of age, HSP70 was detected in the ATL after transformation from thick ascending limb, beginning at the papillary tip and ascending to the border between the outer and inner medulla. The immunolocalization of HSP70 in both the ATL and IMCD gradually increased during two weeks. The gradual increase in HSP70 was associated with an increase in its mRNA abundance. However, furosemide infusion resulted in significantly reduced HSP70 immunolocalization in the IMCD and ATL. These data demonstrated that the expression of HSP70 was closely correlated with changes in interstitial osmolality during the development of the kidney. We suggest that HSP70 protects ATL and IMCD cells in the inner medulla from the stress of high osmolality and may be involved in the transformation of the ATL of the long loop of Henle during renal development.

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Introduction

Heat shock protein70 (HSP70) plays a major role in the adaptation of the kidney to high levels of NaCl and urea. The abundance of HSP70 is over 20-fold higher in the hypertonic renal medulla than in the isotonic cortex (Müller et al., 1996). Hypertonicity induces the immunolocalization of HSP70 in the inner medullary collecting duct (IMCD) cells (Rauchman et al., 1997). A number of experiments have demonstrated that cell survival at high levels of urea increases dramatically as a function of HSP70 immunolocalization. The increased immunolocalization of HSP70 induced by experimental hypertonicity (Santos et al., 1998) or by stable transfection of HSP70 cDNA (Neuhofer et al., 2001) promotes cell survival in the presence of a high level of urea, whereas the forced downregulation of HSP70 with antisense nucleotides renders cells more susceptible to urea toxicity (Neuhofer et al., 1999). During renal development in rodents, urinary osmolality rises from 300 mosmol/kg at birth to nearly 2000 mosmol/kg by three weeks of age (Beck et al., 1998). With the development of the urine-concentrating system and the associated increase in osmolality in the renal papilla, cells in this region are exposed to an environment of elevated and variable extracellular osmolality (Beck et al., 1998). Elevated extracellular osmolality in the developing kidney can disturb the intracellular metabolism and medullary cell growth, and may therefore be perceived as a cellular stress (Kültz and Chakravarty, 2001; Dmitrieva and Burg, 2005). Therefore, renal medullary cells require proper chaperones to adapt them to increasing hypertonic stress

during renal development.

The maturation of the loop of Henle and the development of a true inner medulla occur at the time of the development of a hypertonic medullary interstitium. At birth, all loops of Henle in the rat renal papilla have a configuration of short loops, and there is no medullary thin ascending limb of Henle's loop (ATL). During the first two weeks of life, the cuboidal epithelium of the thick ascending limb (TAL) is gradually transformed into the ATL by a process that starts just before the bend of the loop and proceeds toward the outer medulla. The transformation of the ATL epithelium occurs by the deletion of TAL cells by apoptosis (Kim et al., 1996). Apoptosis plays an important role in the maturation of the renal papilla. However, hypertonic stress causes an immediate increase in cellular ionic strength as a result of osmosis (Uchida et al., 1989; Neuhofer et al., 2002), which in turn causes apoptosis and the activation of p53 (Dmitrieva et al., 2000; Michea et al., 2000). Local hypertonicity and cell shrinkage may be important factors controlling apoptosis in the developing kidney. However, the mechanism underlying the regulation of apoptosis during tubule maturation is unknown. Little is also known about the function and regulation of HSP70 under in vivo conditions. In particular, there is no information about HSP70 in the developing kidney during the increase in osmolality in the renal papilla. Therefore, this study was designed to determine the immunolocalization of HSP70 during the development of the rat renal papilla and to determine the role of HSP70 during tubule maturation, including the loop of Henle.

Materials and methods

Animals and tissue preservation

Specific pathogen-free inbred male Sprague Dawley rats, routinely screened serologically for relevant respiratory pathogens, were purchased from Daehan Biolink Co. Ltd (Seoul, Korea). The rats were maintained in an animal facility under standard laboratory conditions for the experiments, and provided with water and standard chow ad libitum. All experimental procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of Korea.

The kidneys were obtained from embryonic day (E)16, E18, and E20, postnatal day (P)1, P4, P7, P14, and P21, and adult animals. To observe the effects of hypoosmolality on the immunolocalization of HSP70, furosemide (5 mg/kg; Yuhan Pharmacy, Seoul, Korea) was injected subcutaneously at 12 h intervals from immediately after birth, either for four days (four-day furosemide injection group) or for seven days (seven-day furosemide injection group). The rats were killed with an overdose of pentobarbital (50 mg/kg) 2 h after the last injection. The kidneys were preserved by *in vivo* perfusion through the heart or abdominal aorta. The animals were initially perfused briefly with phosphate-buffered saline (PBS; osmolality 298 mosmol/kg H_2O, pH 7.4) to rinse away the blood. This was followed by perfusion with a periodate–lysine–2% paraformaldehyde solution for 5 min. After fixation, the kidneys were removed and cut into 1-2 mm thick slices, which were further fixed by immersion in paraformaldehyde solution overnight at 4°C. Sections of tissue were cut transversely through the entire kidney on a vibratome (Technical Products International, Inc., USA) at a thickness of 50 μm, and processed for immunohistochemical analyses using a horseradish-peroxidase preembedding technique.

Antibodies

HSP70 immunoreactivity was detected using an affinity-purified monoclonal antibody (SPA-810, StressgenBioreagents Corp., MI). The TAL of the loop was identified using a rabbit polyclonal antibody directed against Na^+/K^+-ATPase ß1 (UBI, Lake Placid, NY). This antibody labels the basolateral plasma membranes of the TAL, the distal convoluted tubules, and the connecting tubule cells (Wetzel and Sweadner, 2001). The descending thin limb (DTL) of the loop of Henle was identified using a rabbit polyclonal antibody directed against aquaporin 1 (AQP1; Chemicon, Temecula, CA, USA). This antibody labels the apical and basolateral plasma membranes of the proximal tubules and DTL (Nielsen et al., 1993).

Immunohistochemistry

Vibratome sections (50 μm thick) through the entire kidney were taken from all animals and embedded in Poly/Bed 812 resin (Polysciences, Warrington, CA) sandwiched between polyethylene vinyl sheets. The vibratome sections were processed for immunohistochemistry using an indirect preembedding immunoperoxidase method. All sections were washed three times with 50 mM NH_4Cl in PBS. Before incubation with the primary antibody, the sections were pretreated with PBS containing 1% bovine serum albumin (BSA), 0.05% saponin, and 0.2% gelatin (solution A) for 4 h. They were then incubated overnight at 4°C with antibody directed against HSP70 (1:150) diluted in 1% BSA in PBS (solution B). The control samples were incubated in solution B lacking primary antibody. After three washes with solution A, the sections were incubated for 2 h in horseradish-peroxidase-conjugated donkey anti-mouse IgG Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in solution B. The tissues were rinsed first in solution A and then in 0.05 M Tris buffer (pH 7.6). For the detection of horseradish peroxidase, the sections were incubated in 0.1% 3,3'-diaminobenzidine in 0.05 M Tris buffer for 5 min, after which H_2O_2 was added to a final concentration of 0.01% and the incubation was continued for 10 min. After they had been washed with

0.05 M Tris buffer, the sections were dehydrated in a graded series of ethanol. Quantitative analysis of HSP70 immunoreactivity was performed using an image analyzer (Image Inside/ZHWA optical, Korea).

Double labeling

The flat-embedded vibratome kidney sections were processed for the immunohistochemical identification of HSP70 in the IMCD, ALT, and distal tubules of the cortex. Different areas of the renal medulla were excised and glued onto an empty block of Poly/Bed 812 resin. Three consecutive 1 μm sections were cut with an ultramicrotome (LKB-IV, Sweden) for double immunolabeling for AQP1 using a postembedding technique. The sections were treated for 5 min with a mixture of saturated sodium hydroxide and absolute ethanol (1:1) to remove the resin. After three brief rinses in absolute ethanol, the sections were hydrated with a graded series of ethanol and rinsed in tap water. The sections were then rinsed with PBS, incubated in normal donkey serum (Jackson ImmunoResearch Laboratories) for 1 h, and then incubated overnight at 4°C with antibody directed against HSP70. After they had been washed in PBS, the sections were incubated for 2 h in peroxidase-conjugated donkey anti-rabbit IgG (Fab fragment) and washed again with PBS. For the detection of AQP1 and Na^+/K^+-ATPase ß1, Vector SG (Vector Laboratories, Burlingame, CA) was used as the chromogen to produce a gray–blue color, which was easily distinguishable from the brown label produced by 3,3'-diaminobenzidine in the first immunolocalization procedure for HSP70 using the preembedding method. The sections were washed with distilled water, dehydrated with graded ethanol and xylene, mounted in Canada balsam (ProSciTech, Australia), and examined by light microscopy.

Immunoblot analysis

The renal cortices and medullas from five animals in each age group were homogenized in lysis buffer containing 20 mMTris-HCl, 1% Triton X-100, 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.02% sodium azide, 1 mM EDTA, 10 μM leupeptin, and 1 mMphenyl-methyl-sulfonyl fluoride. The homogenate was centrifuged at 3000 μg for 20 min at 4°C. After the protein concentration in the supernatant had been determined with the Coomassie method (Pierce, Rockford, IL), the samples were loaded (30 μg/lane) and separated electrophoretically on sodium dodecyl sulfate-polyacrylamide gels under reducing conditions. The proteins were transferred by electroelution to nitrocellulose membranes that had been blocked with 5% nonfat dry milk in PBS-T (0.1% Tween 20 in 0.01 M PBS, pH 7.4) for 30 min at room temperature, and the membranes were then incubated for 24 h at 4°C with affinity-purified anti-HSP70 antibody (1:150). The membranes were washed in several changes of PBS-T and incubated for 1 h with horseradish-peroxidase-conjugated goat anti-mouse IgG (1:1,000). After a final wash, the antibody label was visualized with an enhanced chemiluminescence system (Amersham ECL™, Amersham Life Sciences, Buckinghamshire, UK) at room temperature.

Ribonuclease protection assays

The kidneys were perfused through the abdominal aorta with ice-cold PBS to rinse out the blood. RNA was extracted from the kidney cortex, outer medulla, and inner medulla using TRIzol Reagent (Life Technologies, Grand Island, NY). A rat HSP70 cDNA corresponding to the human HSP70 cDNA was PCR amplified. This fragment was cloned into pCRII–TOPO (Invitrogen, Carlsbad, CA), and a ^{32}P-labeled antisense probe was made with SP6 RNA polymerase. The plasmid used for the synthesis of the rat ß-actin probe was purchased from Ambion (Austin, TX). A ribonuclease protection assay was performed using a commercial kit (Ambion). The radioactivity of the actin bands was quantified with a Phosphoimager (Molecular Dynamics, Sunnyvale, CA). In each reaction, HSP70 mRNA was measured together with ß-actin mRNA, and the abundance of the latter was used to correct for RNA loading. Data were collected from five animals in each group.

Statistical analysis

All data obtained from the quantitative analyses were analyzed with one-way ANOVA to determine statistical significance. Bonferroni's test was used for post hoc comparisons. P values below 0.05 or 0.01 were considered to be statistically significant.

Results

Immunolocalization of HSP70 in the adult rat kidney

Light microscopic assessment of 50 μm sections showed HSP70 immunolocalization in the outer cortex and especially in the inner medulla, where it was most strongly localized. There was no labeling in the outer stripe of the outer medulla, and the boundary between the outer and inner medullas was well defined (Fig. 1A). At higher magnification of the cortex, weak HSP70 immunoreactivity was only observed in the cortical thick ascending limb (cTAL; Fig. 1B). There was no labeling in the proximal tubules, connecting tubules, or cortical collecting ducts. In the border between the outer and inner medullas, strong HSP70 immunoreactivity was observed in the ATL, and there was an abrupt transition from the intensely labeled ATL to the unlabeled DTL, marking the border between the outer and inner medullas (Fig. 1C). Most of the strongly labeled tubular profiles were located in the IMCD and the thin limbs of the inner medulla (Fig. 1D, F).

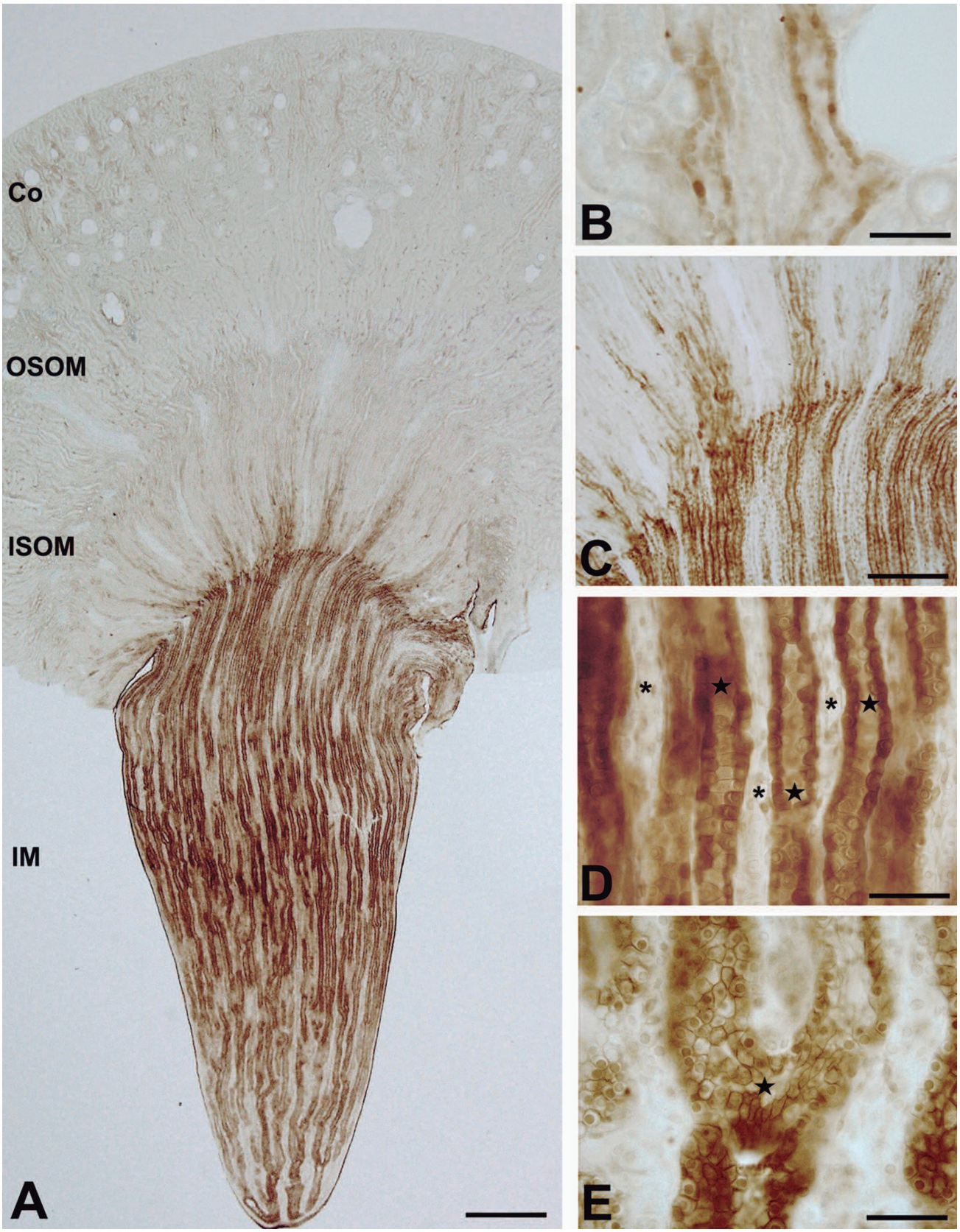

Fig. 1. Light micrographs of a 50 μm vibratome section from an adult rat kidney, illustrating immunoreactivity for heat shock protein(HSP) 70. HSP70 immunoreactivity is located in the inner medulla (IM) and outer cortex (Co). Note the well-defined border between the outer medulla and the inner medulla **(A)**. Higher magnifications of the outer cortex **(B)**, the border between the outer medulla and inner medulla **(C)**, and the middle **(D)** and distal parts **(E)** of the renal papilla. HSP70 protein is expressed in the inner medullary collecting ducts (stars) and weakly in the thin limb cells (asterisks) in the inner medulla **(D, E)**. Abbreviations: Co, cortex; OSOM, outer stripe of the outer medulla; ISOM, inner stripe of the outer medulla; IM, inner medulla. Scale bars: A, 165 μm; B, D, E, 25 μm; C, 50 μm.

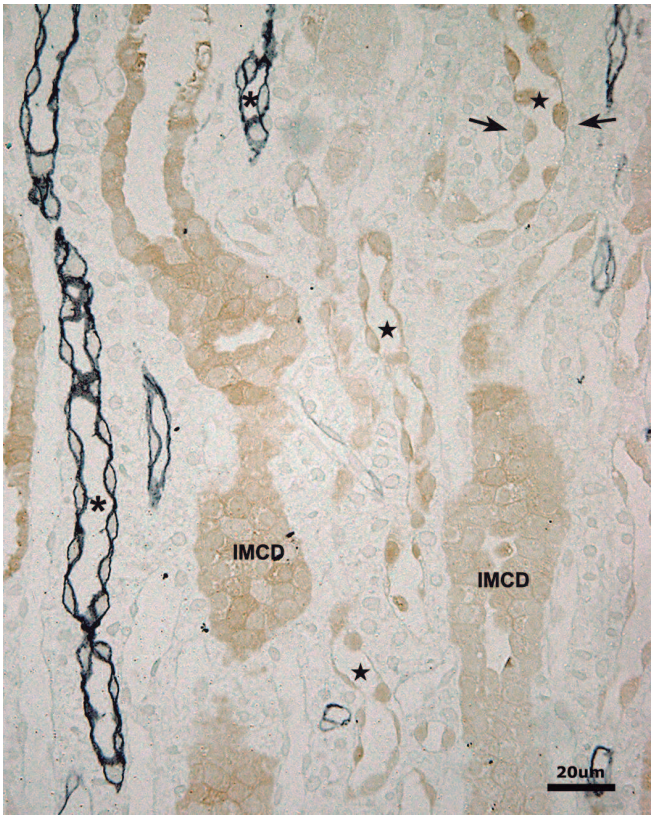

Fig. 2. Light micrograph of a 1 μm section from the middle part of the renal papilla of an adult rat, illustrating double immunostaining for AQP1 (blue) and HSP70 (brown). HSP70 immunoreactivity was present in the inner medullary collecting duct (IMCD) and ascending thin limb (stars). In contrast, no HSP70 immunoreactivity was observed in the AQP1-positive descending thin limb (asterisk). Note that HSP70 immunoreactivity in the ascending thin limb increases toward the proximal part of the renal papilla (arrows). Scale bar: 20 μm.

To determine whether HSP70 is expressed in the thin limb, a double immunolabeling technique with AQP1 was used. AQP1 immunoreactivity was confined to the plasma membrane of the proximal tubules and the DTL of the loop of Henle. At higher magnification of 1 Ìm sections, HSP70 immunoreactivity was observed in the IMCD and the AQP1-negative ATL, but there was no HSP70 immunolabeling in the AQP1-positive DTL (fig. 2). This result confirms that HSP70 is expressed in the ATL and cTAL but not in the DTL of Henle's loop.

Immunolocalization of HSP70 in the developing rat kidney

Immunoblot analysis of the inner medulla of the developing rat kidney revealed that HSP70 protein is expressed in the renal medulla during renal papillary growth. HSP70 was not detected in the fetal kidney, but a faint band was observed in the protein from the renal papilla on P1. Determination of the relative abundance of HSP70 protein by densitometry demonstrated a gradual increase in the first two weeks (P1 as a 100 % reference point, P4, 247.2±11.2, $p<0.05$; P7, 407.5±22.9, $p<0.05$; P14, 489.1±7.2, $p<0.05$) after birth (fig. 3A). In view of the dramatic changes in HSP70 immunolocalization after birth, we quantified the abundance of HSP70 mRNA using an RNase protection assay. The abundance of HSP70 mRNA gradually increased from E20 to P5 (F20 as a 100 % reference point, P1, 242.2±13.1, $p<0.05$; P3, 292.7±24.6, $p<0.05$; P5, 339.4±11.2, $p<0.05$). In particular, significant changes were observed one day after birth (fig. 3B). This result is consistent with the results of the immunoblotting and immunohistochemical studies, in which the inner medulla was first labeled, immediately after birth.

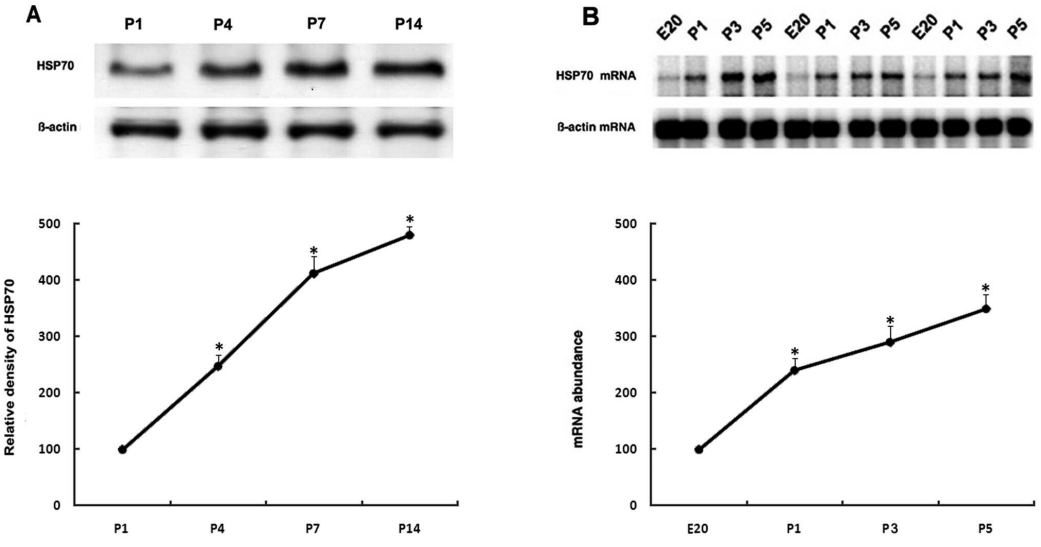

Fig. 3. Abundance of HSP70 protein **(A)** and mRNA **(B)** in the inner medulla of kidneys from fetuses and pups. Immunoblot analysis was performed to measure HSP70 and ß-actin proteins (top). The density of the HSP70 band was divided by the density of the corresponding ß-actin band to correct for protein loading. In each experiment, the corrected density was expressed relative to that of P1. HSP70 immunolocalization commenced after birth and increased gradually during development. RNase protection assays were performed to measure the mRNAs of HSP70 and ß-actin (top). The radioactivity of the HSP70 band was divided by the radioactivity of the corresponding ß-actin band to correct for RNA loading. In each experiment, the corrected radioactivity was expressed relative to that at E20. Values are means ± SE; n=5. *$p<0.05$ compared with P1 **(A)** or E20 **(B).**

Immunohistochemical analysis of the fetal kidney revealed that HSP70 immunoreactivity was undetectable in any of the tubular segments (data not shown). HSP70 immunoreactivity first appeared in the distal part of the renal papilla at P1 and ascended gradually to the future border between the outer and inner medullas during the three weeks after birth (Fig. 4). In the kidneys of P4 to P14, HSP70 immuno-reactivity was mainly located in the inner medulla. HSP70-positive cells first appeared in the growing papilla at the papillary tip, gradually increasing in number in an ascending manner (Figs. 4, 5).

To establish the exact sites of HSP70 immunostaining in the developing loop of Henle a double-immunolabeling procedure was used, in which the TAL and DTL were identified with antibodies directed against

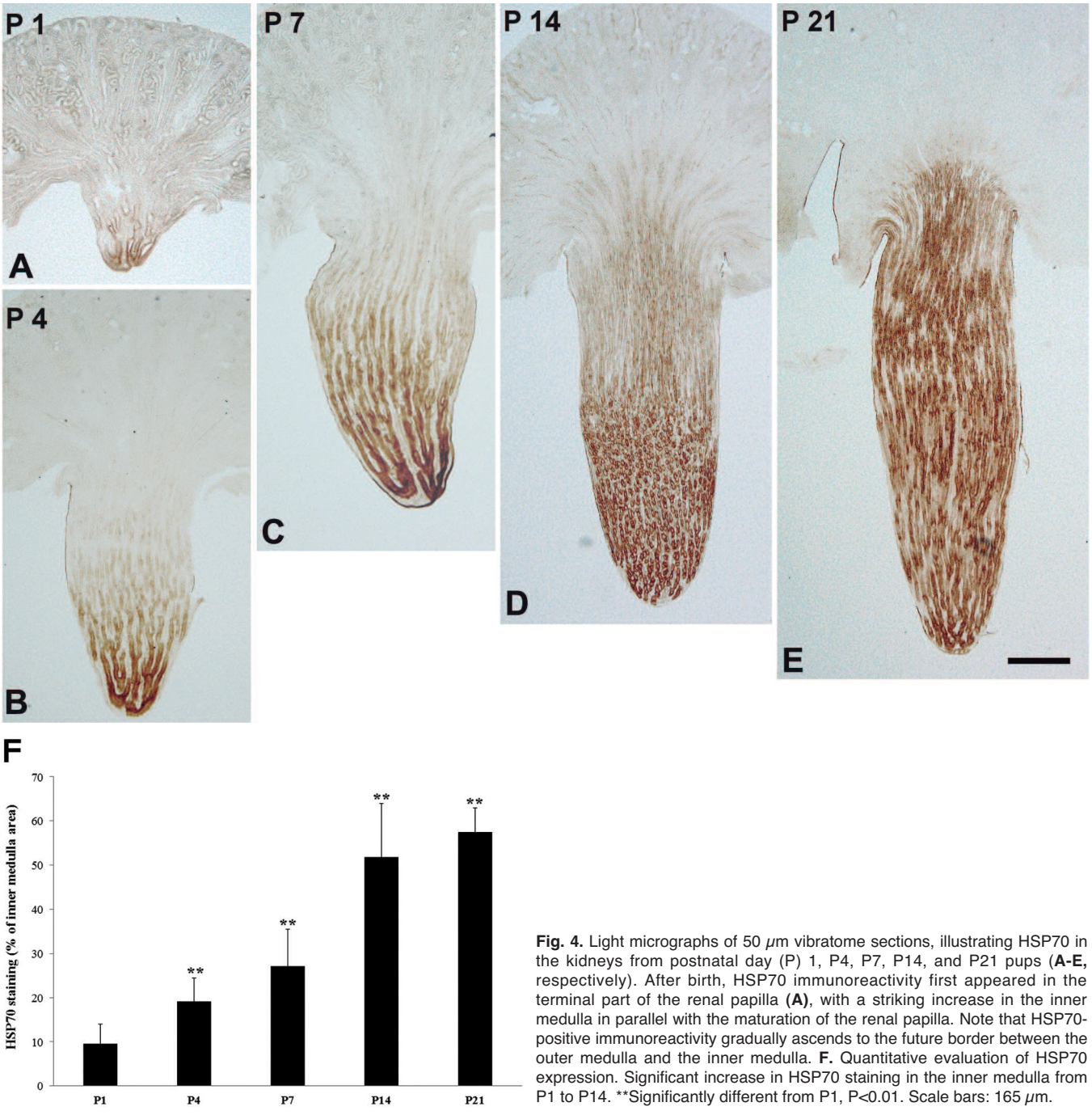

Fig. 4. Light micrographs of 50 μm vibratome sections, illustrating HSP70 in the kidneys from postnatal day (P) 1, P4, P7, P14, and P21 pups (**A-E,** respectively). After birth, HSP70 immunoreactivity first appeared in the terminal part of the renal papilla **(A)**, with a striking increase in the inner medulla in parallel with the maturation of the renal papilla. Note that HSP70-positive immunoreactivity gradually ascends to the future border between the outer medulla and the inner medulla. **F.** Quantitative evaluation of HSP70 expression. Significant increase in HSP70 staining in the inner medulla from P1 to P14. **Significantly different from P1, P<0.01. Scale bars: 165 μm.

the ß1 subunit of Na^+/K^+-ATPase and AQP1, respectively (Fig. 6). In the base of the renal papilla, HSP70 immunoreactivity was observed in the IMCD and AQP1-negative ATL. This distribution is similar to that seen in the adult kidney. There was no HSP70 immunoreactivity in the AQP1-positive DTL (Fig. 6A) or in the Na^+/K^+-ATPase-positive TAL (Fig. 6B). These data indicate that HSP70 is first expressed in the ATL after transformation of the TAL. At this stage the ascending limb is in the process of differentiating from Na^+/K^+-ATPase-positive cuboidal epithelium to Na^+/K^+-ATPase-negative flatter epithelium. In the transforming primitive TAL, HSP70-positive cells appeared at the papillary tip and gradually increased in number in an

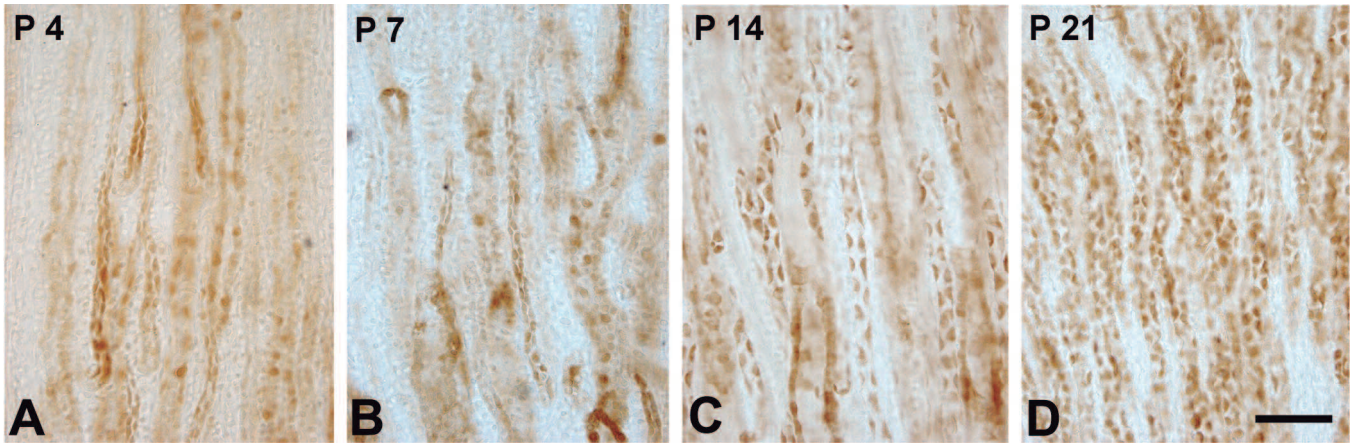

Fig. 5. Light micrographs of 50 μm vibratome sections, showing HSP70 immunoreactivity in the proximal portion of the renal papilla in the kidneys of P4 **(A)**, P7 **(B)**, P14 **(C)**, and P21 pups **(D)**. HSP70 immunoreactivity increased gradually in parallel with the number of ascending thin limbs, in association with the maturation of the renal papilla from P4 to P21. Scale bars: 25 μm.

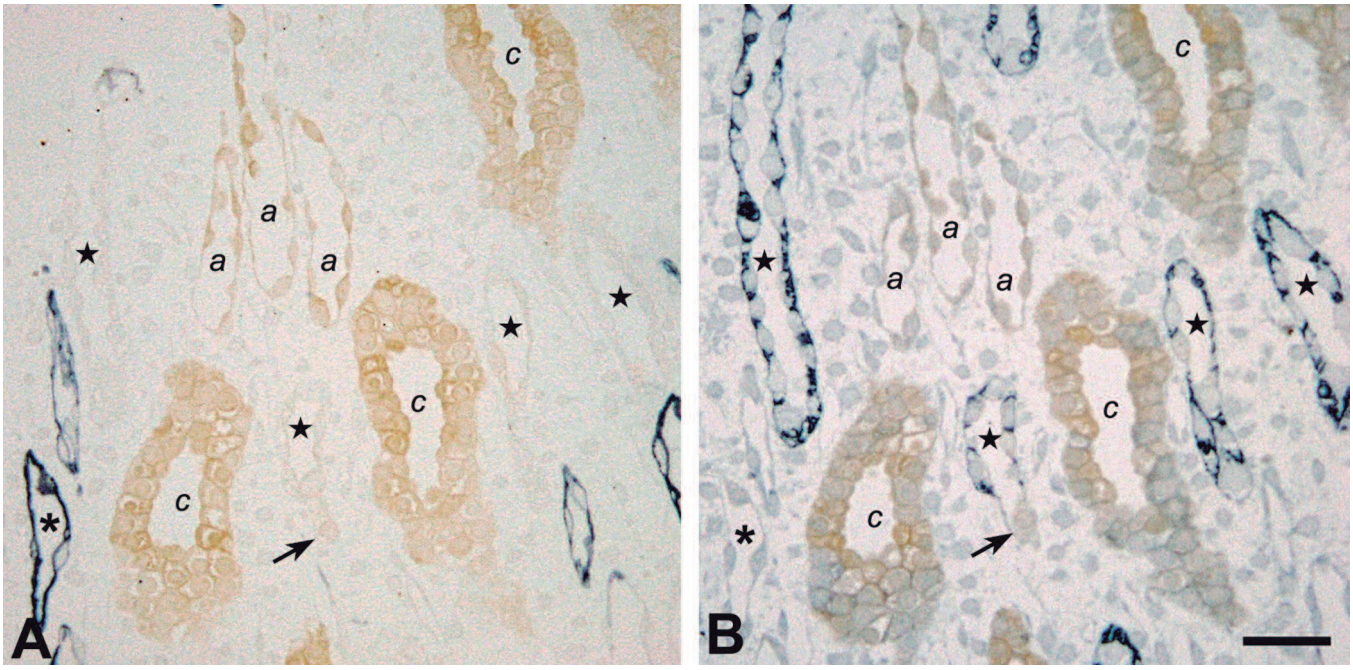

Fig. 6. Light micrographs of serial 1 μm sections from the distal papilla of a four-day-old pup, illustrating double immunostaining for HSP70 (brown) and AQP1 (blue) **(A)**, and for HSP70 (brown) and the ß1 subunit of Na^+/K^+-ATPase (blue) **(B)**. HSP70 was stained with a preembedding technique. AQP1 and the ß1 subunit of Na^+/K^+-ATPase were stained on plastic sections of the same tissue after the removal of the plastic by etching. HSP70 immunoreactivity was observed in the inner medullary collecting duct (c) and the ascending thin limb (a). No HSP70 immunoreactivity was observed in the AQP1-positive descending thin limb (asterisks) or in the Na^+/K^+-ATPase -positive thick ascending limbs (stars). Scale bars: 20 μm.

ascending manner (Figs. 5, 6). There was a gradual increase in HSP70 immunoreactivity in both the ATL and IMCD cells during postnatal development (Figs. 3, 4). In the kidney of P7 and P14, most of the TAL epithelia in the renal papilla were transformed to flatter epithelia. Thus, there were numerous HSP70-positive ATLs, which were newly formed in the renal papilla (Fig. 5).

Effect of furosemide on HSP70 immunolocalization

To investigate the role of tonicity in HSP70 immunolocalization in the developing renal medulla, furosemide was administered (10 mg/kg per day) from E1 to E7. Furosemide, a high-ceiling antidiuretic, lowers medullary interstitial tonicity. Immunoblot analysis revealed that the abundance of HSP70 protein in both P4 (100±7.4 versus 34.7±14.6; $p<0.05$) and P7 (193.7±29.3 versus 25.6±0.76; $P<0.01$) furosemide-infused animals was significantly lower than that in the corresponding control animals (Fig. 7). Immunohistochemistry confirmed that HSP70 immunoreactivity was markedly reduced in the furosemide-injected animals (Fig. 8B,D). In the treated animals, HSP70 was significantly downregulated in the IMCD. In particular, HSP70 immunoreactivity had disappeared from the ATL. HSP70 immunoreactivity was localized in the papillary surface epithelium, and this was also reduced in the furosemide-injected animals (Fig. 8C,D).

Discussion

The functional integrity of the kidney depends on its normal development, and on physiological cell turnover. Renal medullary cells are constantly exposed to steep osmotic gradients during renal development because of the urine-concentrating mechanism. Elevated extracellular osmolality disturbs the intracellular metabolism and medullary cell growth, and can consequently be perceived as cellular stress (Uchida et al., 1989; Neuhofer et al., 2002). Therefore, the renal medullary cells require proper chaperones during their adaptation to increasing hypertonic stress.

In this study, we confirmed that HSP70 is not expressed in the fetal rat kidney cells, but appears first in the renal papilla immediately after birth, with the establishment of the urine-concentrating mechanism, and gradually increases during development, as shown by both western blot analysis and immunohistochemistry. In the medullary collecting duct, HSP70 immunoreactivity increases progressively with depth into the inner medulla and coincides with the known increase in medullary tonicity during the first three weeks after birth. To examine the role of medullary tonicity as a possible mediator of the immunolocalization of HSP70, we infused furosemide into rat pups for four or seven days after birth. In the injected groups, HSP70 was significantly reduced in the IMCD. The amount of HSP70 protein also decreased in the injected groups relative to that in the control group. This result suggests that the activity of HSP70 is dependent on the variation in the tonicity of the renal papilla and has a protective role against osmotic stress under increasingly hypertonic conditions.

During the production of concentrated urine, the cells of the renal medulla are subjected to high concentrations of solutes, particularly NaCl and urea. The cells, both in vivo and in cell culture, adapt to these adverse conditions using a number of mechanisms, including the accumulation of a variety of organic osmolytes (Garcia and Burg, 1991) and the induction of heat shock proteins (Cohen et al., 1991). High concentrations of urea have caused apoptosis in in vivo studies (Dmitrieva et al., 2001). HSP70 can protect cells, tissues, organs, and animals from normally lethal heating, as well as from numerous disease states (Nagayama et al., 2001). In addition, HSP70 important role embryonic lens formation from development program (Evans et al., 2005). HSP70 functions as a molecular chaperone and reduces the stress-induced denaturation and aggregation of intracellular proteins

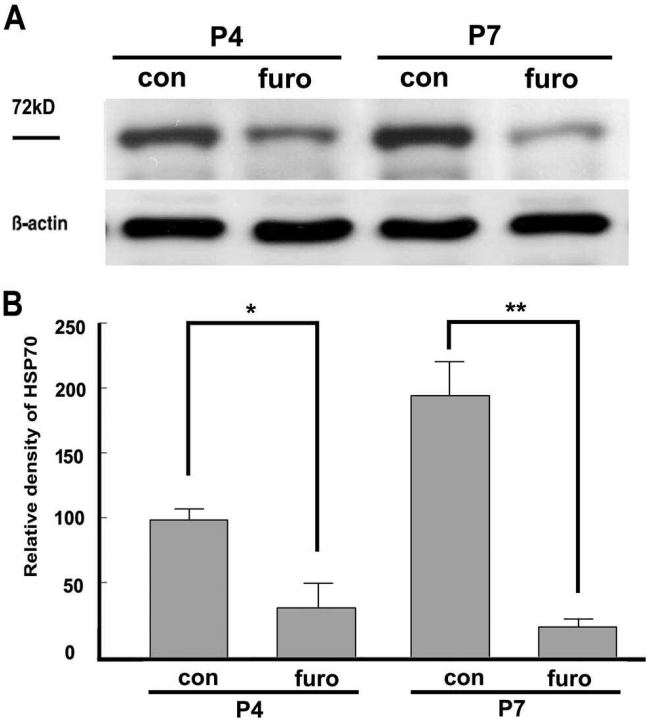

Fig. 7. Abundance of HSP70 protein in the inner medullas of P4 and P7 pups. Immunoblot analysis was performed to measure HSP70 and ß-actin proteins after the subcutaneous injection of saline (con) or furosemide (furo) (top). The density of the HSP70 band was divided by the density of the corresponding ß-actin band to correct for protein loading. In each experiment the corrected intensity was expressed relative to that of the P4 control. The amount of HSP70 protein in both the P4 and P7 furosemide-injected animals was significantly lower than that in the corresponding control animals. Values are means ± SE; n = 5. * $p< 0.05$, ** $p < 0.01$ versus rats treated with vehicle.

(Nagayama et al., 2001). The overexpression of HSP70 induced by hypertonicity or the transfection of cDNA results in a dramatic increase in cell survival at high concentrations of urea (Neuhofer et al., 2001, 2005). However, at birth, all of the loops of Henle in the rat renal papilla are configured as short loops, and there are

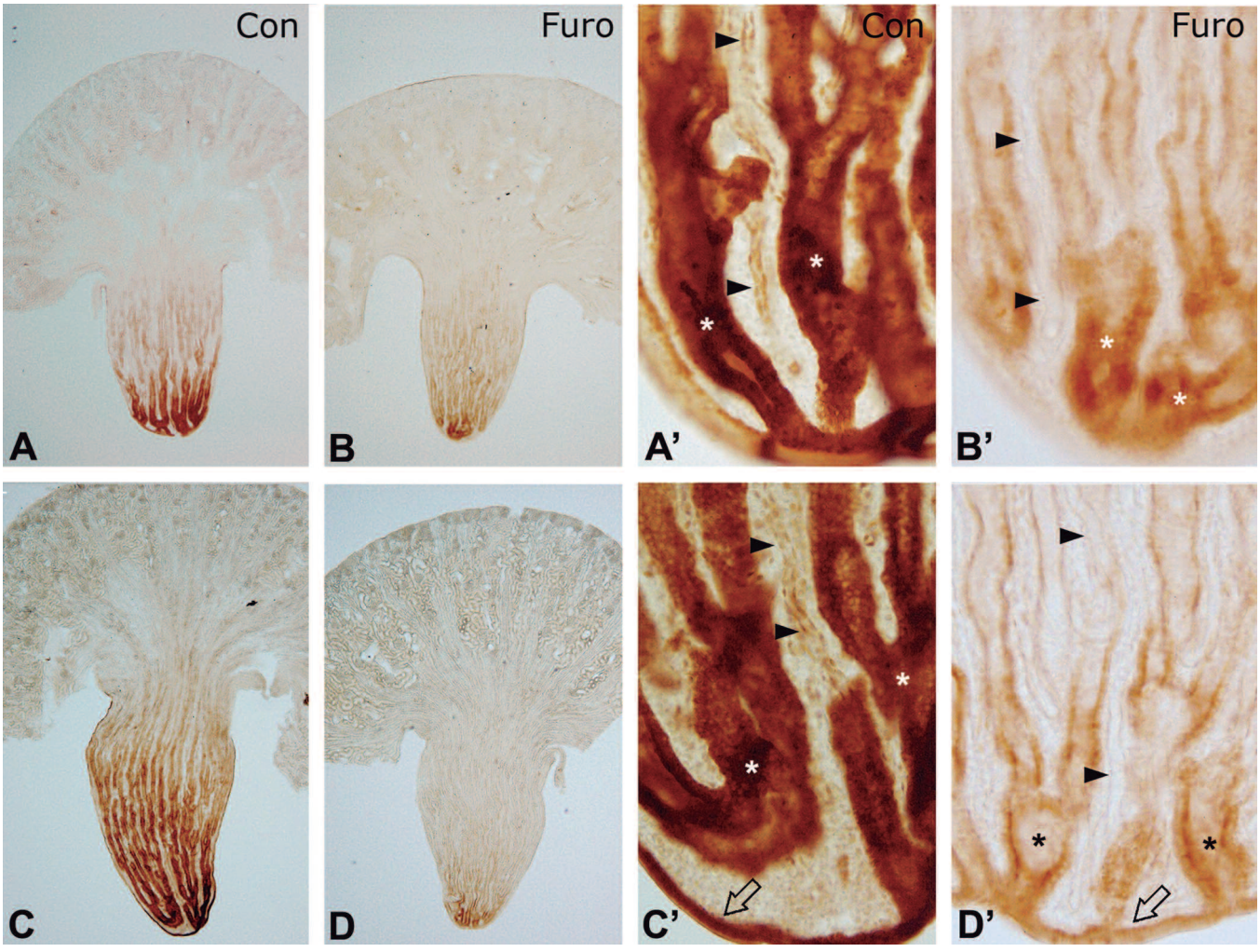

Fig. 8. Light micrographs of 50 μm sections from the kidneys of four-day-old **(A, A', B, and B')** and seven-day-old pups **(C, C', D, and D')** after the subcutaneous injection of saline **(A, A', C, and C')** or furosemide **(B, B', D, and D')**. **A', B', C', and D'.** higher magnifications of the distal part of the renal papilla. HSP70 immunoreactivity was markedly reduced in the furosemide-injected animals **(B, D)**. Quantitative evaluation of HSP70 expression at P4 **(E)** and P7 **(F)**. In the treated animals, HSP70 was downregulated in the inner medullary collecting ducts (asterisks). In particular, HSP70 immunoreactivity disappeared in the ascending thin limbs (arrowheads). Note that HSP70 immunoreactivity was localized in the papillary surface epithelium and that this was also reduced in the furosemide-injected animals (open arrows, **C'D'**). Significant increase in HSP70 staining in the inner medulla. *Significantly different from con, P<0.05.**Significantly different from con, P<0.01. Scale bars: A-D, 165 μm; A'-D', 25 μm.

no ATLs. In the developing loop of Henle, immature TALs in the renal papilla are transformed into ATLs by the apoptotic deletion of cells and the transformation of the remaining cells into a thin, flatter epithelium (Neiss, 1982; Kim et al., 1996). The lengthening of the renal papilla, which includes the long loops of Henle, is associated with the maturation of the urine-concentrating system. These changes lead to the development of a corticopapillary osmotic gradient. Hypertonic stress causes an immediate increase in cellular ionic strength as a result of osmosis (Kültz and Chakravarty, 2001), which in turn causes apoptosis and the activation of p53 (Dmitrieva et al., 2000; Michea et al., 2000). Local hypertonicity and cell shrinkage may be important factors controlling apoptosis in the developing kidney. Therefore, apoptosis plays an important role in the maturation of the renal papilla. However, how apoptosis is regulated during tubule maturation is unknown. In this study, HSP70 immunoreactivity was detected from P4, during the ATL transition from TAL in the future long loops of Henle in the renal papilla. The intensity of HSP70 immuno-reactivity in the ATLs increased in the initial part of the inner medulla during renal development. In neonatal rat kidney cells, immunostaining for HSP70 was also observed in the transforming ascending limb. This result suggests that HSP70 plays an important role in protecting the remaining cells from apoptosis in the transforming ascending limb of the long loop of Henle.

Previous studies demonstrated that cyclooxygenase-(COX-2) interfere HSP70 expression in cultured inner medullary collecting duct 3 (IMCD3) cells (Neuhofer et al., 2004). In this study, COX-2 inhibition impaired the tonicity-induced up-regulation of HSP70 expression and rendered the cells susceptible to high urea concentrations. In addition, CoX-2 inhibition induced papillary apoptosis and osmosensitve genes such as Na- and Cl-dependent betaine transporter (BGT1) in renal papilla of water-deprived rats. Interestingly, the osmotic induction of the genes involved in osmolyte accumulation and of HSP70 is mediated by TonEBP/NFAT5, a transcriptional activator that is essential for the expression of these target genes in reponse to hyprtonicity (Woo et al., 2002).

Recently, Han et al. (2004) reported their analysis of the immunolocalization of the tonicity-enhancer binding protein (TonEBP) and its target gene in the developing rat kidney. HSP70 immunolocalization is directly stimulated by TonEBP in the renal medulla (Woo et al., 2002). Knockdown of TonEBP leads to the reduced immunolocalization of HSP70 (Na et al., 2003). TonEBP regulates the protection of the renal medulla cells from high osmolality by enhancing the immunolocalization of HSP70. In the developing rat kidney, TonEBP immunolocalization is first detected in the renal medulla at E16 and increases gradually throughout papillary development (Han et al., 2004). This immunolocalization pattern is similar to that observed in our study, except for the starting time, insofar as the immunolocalization of TonEBP in the renal medulla commences several days earlier than HSP70 immunolocalization. These data suggest that the activity of TonEBP is required for the immunolocalization of HSP70, which protects renal medullary cells against the steeply increasing medullary tonicity during papillary development.

In this study we tried to establish the time of immunolocalization and distribution of HSP70 in developing and adult rat kidney. In conclusion, the observed pattern of HSP70 immunolocalization supports the idea that HSP70 plays an important role in the ability of renal medullary cells to adapt to osmotic stress during renal development in neonatal rats. It has been suggested that high osmotic stress leads directly to increases in HSP70 gene transcription, to protect the cells against osmotic stress in the renal medulla during renal development. There is now convincing evidence that HSP70 immunolocalization in neonatal kidney cells is involved in the differentiation of the ATL.

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