Relevance of renal-specific oxidoreductase in tubulogenesis during mammalian nephron development

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Kanwar, Yashpal S., Qiwei Yang, Yufeng Tian, Sun Lin, Jun Wada, Sumant Chugh, and Satish K. Srivastava. Relevance of renal-specific oxidoreductase in tubulogenesis during mammalian nephron development. Am J Physiol Renal Physiol 282: F752–F762, 2002. First published October 30, 2001; 10.1152/ajprenal.00181.2001.—Renal-specific oxidoreductase (RSOR), an enzyme relevant to diabetic nephropathy, is exclusively expressed in renal tubules. Studies were initiated to determine whether, like other tubule-specific proteins, it selectively modulates tubulogenesis. Northern blot analyses revealed a ~1.5-kb transcript, and RSOR expression was detectable in mice embryonic kidneys at day 13, gradually increased by day 17, and extended into neo- and postnatal periods. RSOR mRNA and protein expression was confined to proximal tubules, commencing at gestational day 17 and increasing subsequently, but remained absent in glomeruli and medulla. Treatment with RSOR antisense oligodeoxynucleotide resulted in a dose-dependent dysmorphogenesis of metanephric explants harvested at gestational day 13. The explants were smaller and had expanded mesenchyme, and the population of tubules was markedly decreased. The glomeruli were unaffected, as assessed by mRNA expression of glomerular epithelial protein 1 and reactivity with wheat germ agglutinin. Antisense treatment led to a selective reduction of RSOR mRNA. Immunoprecipitation also indicated a selective translational blockade of RSOR. These findings suggest that RSOR is developmentally regulated, exhibits a distinct spatiotemporal distribution, and probably plays a role in tubulogenesis.

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delineate its role in tubulogenesis vs. glomerulogenesis.

MATERIALS AND METHODS

Animals. ICR mice (Harlan Sprague Dawley, Indianapolis, IN) were used for paired male and female mating, and appearance of the vaginal plug was designated day 0 of gestation. The mouse embryos were harvested at days 13, 17, and 19 (newborn) of gestation, and metanephroi were isolated. In addition, kidneys from 1-, 2-, and 3-wk-old mice were procured.

Gene expression of RSOR in developing kidneys by Northern blot analyses and in situ hybridization tissue autoradiography. RNA from embryonic kidneys at various stages of gestation and from kidneys of 1- and 3-wk-old mice was extracted by the guanidinium isothiocyanate-CsCl centrifugation method (8). Equal amounts of RNA were glyoxalated and subjected to 1% agarose gel electrophoresis in 10 mM sodium phosphate buffer, pH 7.0. A Northern blot was prepared by transferring the RNA to a nylon filter membrane (Amersham, Arlington Heights, IL). The membrane blot was hybridized with [α-32P]dCTP-labeled mouse RSOR cDNA. The membrane filter was washed under high-stringency conditions with 0.1× saline-sodium citrate (SSC)-0.1% SDS at 60°C, and autoradiograms were prepared. The same blot was also hybridized with a β-actin probe.

For in situ hybridization studies, the RSOR cDNA was subcloned into pBluescript KS(+) and used as a template for generating sense and antisense riboprobes by using the Riboprobe In Vitro Transcription System (Promega, Madison, WI). The riboprobes were synthesized by incorporating [α-32P]UTP (Amersham) using T3 and T7 RNA polymerase and the linearized 3′ end of the cDNA. The radiolabeled riboprobes were subjected to limited alkaline hydrolysis to yield <150-bp polynucleotide fragments, which were then used for in situ hybridization with the kidney tissue sections. Embryonic (days 13 and 17 of gestation), newborn, and 1-, 2-, and 3-wk-old mouse kidneys were immersed in 4% paraformaldehyde in PBS, pH 7.0, for 2 h at 24°C. The tissue sections were then dehydrated and embedded in paraffin. Tissue sections (3 μm thick) were prepared and mounted on glass slides coated with Vectabond (Vector Laboratories, Burlingame, CA). The sections were then deparaffinized, hydrated, treated with 0.2 N HCl, deproteinized by proteinase K treatment, and acetylated with 0.1 M triethanolamine and 0.25% acetic anhydride. After the sections were washed with 2× SSC, they were prehybridized with a solution containing 50% formamide, 10% dextran sulfate, and Denhardt’s solution in 0.3 M NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 10 mM Tris·HCl, pH 8.0, at 50°C for 2–3 h. Then the sections were hybridized with riboprobes included in the above solution at 50°C for 15 h. After hybridization, the tissue sections were washed with 50% formamide containing tRNA (0.5 mg/ml) in 2× SSC, treated with RNase A, and rewarshed with 0.1× SSC at 50°C. The sections were then dehydrated, air dried, and coated with NTB2 photographic emulsion (Eastman Kodak, New Haven, CT), and autoradiograms were prepared after 2–3 wk of exposure in the dark at 4°C.

RSOR protein expression in developing kidneys by immunofluorescence microscopy. Kidneys of embryos at days 13 and 17 of gestation and of newborn and 1-, 2-, and 3-wk-old mice were snap-frozen in chilled isopentane and embedded in OCT compound (Miles Laboratories, Elkhart, IN). Cryostat sections (4 μm thick) were prepared and air dried. Sections were washed with 0.01 M PBS, pH 7.4, and incubated with polyclonal anti-RSOR antibody (1:100 dilution) for 30 min in a humidified chamber at 37°C. After they were washed with PBS, sections were reincubated with goat anti-rabbit IgG antibody, conjugated with fluorescein isothiocyanate, for 30 min. The sections were rewashed with PBS, covered with a drop of buffered glycerol, mounted on coverslips, and examined with an ultraviolet microscope equipped with epifluorescence microscopy.

Antisense experiments. A sense-, a nonsense-, and an antisense-phosphorothioated oligodeoxynucleotide (ODN) were synthesized by an automated solid-phase synthesizer (Biotech Facility, Northwestern University) and purified by high-performance liquid chromatography. The antisense ODN sequence was selected from the 5′ end of the RSOR as follows: 5′-GGAGTGCGCCTTCTGTGACGAGAATCAGTCCACGTCGTTG-3′. The sequence of nonsense ODNs was as follows: 5′-TAATGATAGTAATGATAGTAATGATAGTAAT-3′ and 5′-GATCGATCGATCGATCGATCGATCGATCGATCGATCGATG-3′. Neither ODN (antisense and nonsense) exhibited any significant homology with other mammalian nucleotide sequences available in the GenBank database, and their specificity was determined by S1 nuclease protection assay, as described in our previous publications (19, 30).

A total of ~750 embryonic kidneys at day 13 of gestation were harvested and maintained in culture for 1–4 days. The details of the metanephric culture have been described previously (19, 30). Briefly, the explants were placed on top of a 0.8-μm Nucleopore filter (Millipore, Bedford, MA) and transferred to a petri dish containing serum-free culture medium. The medium consisted of equal volumes of Dulbecco’s modified Eagles’ medium and Ham’s nutrient mixture F-12, supplemented with iron-poor transferrin (50 μg/ml) and streptomycin and penicillin (100 μg/ml). The ODNs were added to the culture media daily at concentrations ranging from 0.5 to 1.5 μM for 1–4 days. At these concentrations, the ODNs usually retain their transnational blockade specificity with no discernible cytotoxic effects (7, 19, 30). About 250 explants per variable, i.e., sense, antisense, and nonsense, were processed for light microscopy, quantitative RT-PCR analyses, and immunofluorescence and immunoprecipitation (IP) studies. Another ~300 untreated explants, serving as control, were also processed for various studies. For light microscopy, the Epon-embedded explants were sectioned at the midplane, such that the section included a maximum number of ureteric bud iterations, both poles, and the hilum of the embryonic kidney.

Competitive RT-PCR analyses of antisense ODN-treated embryonic kidneys. Competitive RT-PCR analyses were carried out to assess the effect of antisense ODNs on RSOR mRNA expression; the technical details of this method have been described previously (19, 30). Briefly, total RNA was isolated from 50 explants per variable by the acid guanidinium isothiocyanate-phenol-chloroform extraction method (9). Extracted RNAs were treated with RNase-free DNase (Boehringer Mannheim, Indianapolis, IN) and then precipitated with ethanol. About 50 μg of total RNA, from each variable, were subjected to first-strand cDNA synthesis using Maloney’s murine leukemia virus-RT and oligo(dT) as a primer. The cDNAs from different variables were suspended in 50 μl of autoclaved water and kept at −70°C.

For the analyses of RSOR mRNA expression by RT-PCR, the respective sense and antisense primers were as follows: 5′-TGTTGGGACGAGATTCTGACGCC-3′ and 5′-GAGCCGGATAGAAGGATGTGAAGTCCACGTCGTTG-3′. Neither the respective antisense and sense primers were as follows: 5′-GACGGCTGAGATCGGATCGCTGAGAATCAGTCCACGTCG TTG-3′ and 5′-ATGGCCACTGCGCGATCG-3′(28). Using these primers, the expected sizes of the
PCR products are 430 bp for RSOR and 461 bp for β-actin, and their nucleotide sequences were confirmed by the dyeoxy chain termination method (23). To generate a competitive DNA template for RSOR, the primers were added to the minigene construct previously prepared in our laboratory (17). With the use of this modified competitive DNA template, the expected size of the PCR product is 211 bp. This minigene construct contains the primer sequences for β-actin with an expected size of the PCR product of 224 bp. Another control used in the antisense experiment included the determination of mRNA expression of glomerular epithelial protein-1 (GLEPP-1), which is expressed exclusively in the renal glomerular podocytes and is absent in the tubules (27). For GLEPP-1, the respective sense and antisense primers were as follows: 5′-GGGCTGTGTTGCAATGAGGT-3′ and 5′-CAACTAGCAAAGGGCCTGAAAG-3′. With the use of these primers, the expected size of the PCR product from embryonic renal cDNA is 467 bp. These primers were also added to the minigene construct to generate the competitive DNA template. The expected size of the PCR product using the competitive DNA template and GLEPP-1 primers is 271 bp.

For quantitative RT-PCR analyses, fixed amounts of cDNAs (1 μg in 1 μl) from antisense and nonsense ODN-treated explants and serial logarithmic dilutions of the competitive template DNA (500 ng/μl) of RSOR were coamplified (12). The reaction mixture included 5 μl of 10× PCR buffer, dNTPs at 250 μM each, sense and antisense primers at 10 μM, and 1 U of Taq polymerase (Perkin-Elmer, Norwalk, CT) in a total volume of 50 μl. The amplification reaction was carried out for a total of 30 cycles in a DNA Thermal Cycler (Perkin-Emer), with each cycle consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The PCR products of wild-type and mutant (competitive DNA template) renal RSOR were subjected to 2% agarose gel electrophoresis and photographed using an instant positive/negative film (Polaroid, Cambridge, MA). The negatives were analyzed by a scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA), and the relative area under the traces was computed. Similarly, the wild-type and mutant β-actin and GLEPP-1 PCR products were analyzed. The ratios of wild-type to mutant PCR DNA product densitometric readings were plotted using a logarithmic scale on the ordinate (y-axis) against the logarithmic dilutions of the competitive template DNA on the abscissa (x-axis).

Expression of de novo synthesized RSOR in antisense ODN-treated renal explants. IP studies were performed on renal explants treated with various ODNs, i.e., antisense, sense, and nonsense, to assess the translational blockade of RSOR. Treated explants were maintained in the organ culture system and labeled with [35S]methionine (0.25 mCi/ml) for 12 h before the termination of culture. They were rinsed with the culture medium. They were then extracted with 2 ml of IP buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.02% NaN3, 0.25 mM dithiothreitol, 1% Triton X-100, 10 mM N-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride) by vigorous shaking for 4 h at 4°C. The extract was microfuged, and the supernatant was saved. Total incorporated radioactivity was determined in the supernatant after trichloroacetic acid precipitation. Samples with equal amounts of radioactivity (~5 × 105 dpm) in a volume of 500 μl were used for IP, as previously described (19, 30). Polyclonal rabbit anti-RSOR antibody (10 μl) was added and gently swirled at 4°C in an orbital shaker for 12 h at 4°C. The antigen-antibody complexes were briefly microfuged and transferred to a fresh Eppendorf tube. After addition of 10 μg of protein A-Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ), in a volume of 100 μl of the IP buffer, the complexes were further incubated for 1 h at 4°C. They were then centrifuged for 1 min at 10,000 g, and the pellets were washed extensively with the IP buffer. Pellets containing the complexes were suspended in 20 μl of sample buffer (4% SDS, 150 mM Tris-HCl, pH 6.8, 20% glycerol, 0.125% bromphenol blue, 1 μl of β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). Aliquots of the samples were boiled and subjected to 10% SDS-PAGE. The gels were fixed in 10% acetic acid and 10% methanol, treated with 1 M salicylic acid, and vacuum dried, and autoradiograms were prepared.

Tissue expression of RSOR, wheat germ agglutinin, and GLEPP-1 in antisense-treated metanephric explants. Tissue expression of RSOR in tubules of antisense and sense or nonsense ODN renal embryonic explants was assessed by immunofluorescence microscopy as described above. To evaluate the concentration of nascent glomeruli in the antisense ODN-treated explants, expression of wheat germ agglutinin (WGA) and GLEPP-1 was assessed, since both are regarded as markers for the podocyte and the latter is a highly differentiated cell of the renal glomerulus. The adjacent serial sections were stained with anti-RSOR and WGA conjugated with rhodamine (Sigma Chemical, St. Louis, MO) at a dilution of 1:100 and photographed as described above. The cryostat sections from the midplane of the explant included a maximum number of ureteric bud iterations, and tubules and glomeruli were examined. Finally, the mRNA expression of GLEPP-1 was determined in the RSOR antisense-treated explant by in situ tissue autoradiography. The 467-bp product generated above by PCR was used to prepare the GLEPP-1 riboprobe. Conditions for hybridization of the GLEPP-1 riboprobe and preparation of the autoradiograms were essentially as described for the RSOR.

RESULTS

RSOR mRNA expression by Northern blot analyses. A single ~1.5-kb mRNA transcript in kidneys harvested from embryos at days 13 and 17 of gestation and newborn and 1- and 3-week-old mice was observed (Fig. 1A). The mRNA expression of RSOR was detectable at day 13 of gestation when ~25 μg of total RNA, isolated from ~100 explants, were used. The RSOR mRNA expression steadily increased during the various stages of gestation and was notably accentuated during neo- and postnatal stages, suggesting that it is developmentally regulated. No other transcript was seen throughout development, indicating that no other related isoform, e.g., aldose reductase or aldehyde reductase, is expressed during embryonic development. The mRNA expression of β-actin was constant throughout the embryonic, neonatal, and postnatal periods in mouse kidneys (Fig. 1B).

Expression of RSOR by tissue in situ autoradiography and immunofluorescence microscopy. Because RSOR is developmentally regulated, in situ hybridization and immunofluorescence studies were performed to examine its spatiotemporal distribution in embryonic and neonatal kidneys (Fig. 2). At day 13 of gestation, the expression was not distinct, and a very mild reactivity was observed in the ureteric bud branches in some of the metanephric explants (Fig. 2, A and G). At day 17, expression of RSOR was seen in the cortical tubules in the form of clusters, and the message was absent in the medulla (Fig. 2, B and H). In the new-

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that at day 17 born, the pattern of mRNA expression was similar to \( /H9251 \) -32P-dCTP-radiolabeled renal-specific capillary transferred to nylon filter membranes, and hybridized with \( [\alpha^{32}P] \)dCTP-labeled renal-specific oxidoreductase (RSOR) and \( \beta \)-actin cDNA probes. A transcript of \( 1.5 \) kb (arrow) for mouse renal RSOR shows mRNA expression progressively increasing in kidneys during gestation, with a steep rise during the postnatal period. Expression of \( \beta \)-actin remains constant. 13d and 17d, Embryonic days 13 and 17; NB, newborn; 1W and 3W, 1- and 3-wk-old mice.

Fig. 1. Northern blot analyses of mRNA expressed in mouse kidney during fetal, neonatal, and postnatal periods. Total RNAs were denatured with glyoxal, subjected to 1% agarose gel electrophoresis, capillary transferred to nylon filter membranes, and hybridized with [\alpha^{32}P]dCTP-radiolabeled renal-specific oxidoreductase (RSOR) and \( \beta \)-actin cDNA probes. A transcript of \( 1.5 \) kb (arrow) for mouse renal RSOR shows mRNA expression progressively increasing in kidneys during gestation, with a steep rise during the postnatal period. Expression of \( \beta \)-actin remains constant. 13d and 17d, Embryonic days 13 and 17; NB, newborn; 1W and 3W, 1- and 3-wk-old mice.

immunofluorescence studies were performed on the metanephric explants treated with nonsense and antisense ODN to confirm that the development of nascent tubules and not of the glomeruli or the precursor of collecting ducts, i.e., ureteric bud branches, was affected. The studies included the evaluation of the expression of RSOR and WGA, a marker of glomerular podocytes. Cryostat tissue sections stained with WGA did not reveal any decrease in its reactivity with the glomeruli in explants treated with 1.5 \( \mu \)M RSOR antisense ODN compared with those treated with nonsense/sense ODN (Fig. 5, F vs. B, and Fig. 5, H vs. D). Also, enumeration of WGA-stained glomeruli did not reveal any notable decrease in their population in the antisense ODN-treated explants. A marked decrease in the number of tubules was observed in the antisense ODN-treated explants using anti-RSOR antibody, which stains the tubules (Fig. 5, E vs. A, and Fig. 5, G vs. C).

**RSOR and GLEPP-1 mRNA and protein expression studies (antisense experiments).** To assess the transcriptional and translational RSOR-specific blocking activities of antisense ODN, competitive PCR, IP, and in situ hybridization studies were performed. Competitive RT-PCR was chosen to circumvent the difficulties related to minute amounts of mRNA available for Northern blot analyses from embryonic explants harvested at day 13 of gestation. Within the range of \( 10^{-1} \sim 10^{-5} \) serial logarithmic dilutions of the competitive (mutant) template DNA dilution, the bands of wild-type and mutant DNA were discernible (Fig. 6, A–C, lanes 1–8), enabling densitometric analyses to obtain a ratio of the intensities of the bands. The graphic plots have been published previously (19, 30), and only the original data, i.e., electrophoreograms, are included to indicate the intensity of the bands. In
Fig. 2. In situ autoradiogram depicting spatiotemporal RSOR mRNA expression in kidneys harvested at various stages of embryonic development and postnatal periods. At day 13 (A and G), mRNA expression is barely detectable. At day 17 (B and H), mRNA expression is seen in clusters of cortical tubules. No expression is seen in the medulla. In newborn (C and I), expression progressively increases but remains clustered in some of the cortical tubules. In 1-wk-old (D and J), 2-wk-old (E and K), and 3-wk-old (F and L) mice, there is a dramatic increase in the RSOR mRNA expression. Although expression is confined to the cortex, it is somewhat diffuse and is absent in the glomeruli (arrowheads) as well as in the medulla. U, ureteric bud branches. Bi-directional arrows in B, D, and I show span of the renal cortex.
Fig. 3. Immunofluorescence photomicrographs depicting RSOR protein expression in mouse kidney in various fetal stages and in the postnatal period. Similar to mRNA expression, at day 13 (A and G), RSOR protein expression is detectable to a very mild degree in ureteric bud branches and nascent tubules. At day 17 (B and H), RSOR mRNA is expressed in the cluster of cortical tubules, and mild reactivity is also seen in the medullary tubules. In newborn kidneys (C and I), expression, although accentuated, is clustered in some of the cortical tubules, whereas expression in medullary tubules is considerably decreased. In kidneys from 1-wk-old (D and J), 2-wk-old (E and K), and 3-wk-old (F and L) mice, anti-RSOR reactivity is markedly increased, and it is readily seen to be confined to most of the cortical tubules, while expression in the medulla is almost negligible. RSOR expression in the renal glomeruli (arrowheads) is absent. t, tubules with anti-RSOR reactivity; t’, tubules without RSOR reactivity. Bidirectional arrows in B–D denote span of the renal cortex.
the nonsense ODN-treated (control) explants, a ratio of 1 for the reductase mRNA was obtained at dilutions of \(10^{-3}\)–\(10^{-4}\) of the competitive (mutant) DNA (Fig. 6, A, lanes 3 and 4). In the antisense ODN-treated explants, a ratio of 1 for reductase mRNA was obtained at dilutions of \(10^{-5}\)–\(10^{-6}\) of the competitive DNA (Fig. 6, A, lanes 5 and 6), suggesting a decrease on the order of 2 logs of the mRNA expression in the antisense ODN-treated explants. For \(\beta\)-actin and GLEPP-1, a ratio of 1 was obtained at dilutions of \(10^{-3}\)–\(10^{-4}\) in the antisense ODN-treated groups, which is similar to their corresponding controls (Fig. 6, B and C, lanes 3 and 4), suggesting no decrease in their mRNA expression.

For translational blockade studies, antisense and nonsense/sense ODN-treated explants were radiolabeled with \([\text{35S}]\)methionine, and extracts were immunoprecipitated with anti-RSOR antibody. Under reducing conditions, the SDS-PAGE autoradiographic analyses of immunoprecipitated proteins from control explants revealed a major \(~33\)-kDa band, corresponding to the molecular weight of RSOR described previously by Western blot procedures (31) (Fig. 6F, lane 1). Minimal reduction in the autoradiographic intensity of the \(~33\)-kDa band was noted in the sense/nonsense-treated groups compared with the control (Fig. 6F, lane 2). However, a significant reduction in the intensity of the \(~33\)-kDa band was observed in the antisense ODN-treated explants (Fig. 6F, lane 3), suggesting a blockade in the translation or de novo synthesis of RSOR.

To further confirm that the RSOR antisense ODN treatment selectively affected the tubular, and not the glomerular, mRNA, in situ autoradiography for GLEPP-1 mRNA was performed. No significant differences in the mRNA expression or population of the renal glomeruli were observed between the antisense ODN and the control group (Fig. 6, E vs. D), suggesting that the expression of GLEPP-1, a marker of glomerular podocytes, was unaffected.

**DISCUSSION**

**RSOR** belongs to a family of aldoketo reductases (AKRs), which are monomeric oxidoreductases with molecular weight ranging from 35 to 40 kDa and in-
clude >60 members (2, 14). They are expressed in a wide variety of tissues, where they catalyze the NADPH-dependent reduction of various aliphatic and aromatic aldehydes and ketones. The exact substrate for RSOR (GenBank accession no. AF197127) is unknown, but tentatively it is classified as aldehyde reductase-6. Although various members of the AKR are expressed in the kidney, in addition to other organ systems, the information that relates to their developmental expression is limited. RSOR-related enzymes, i.e., aldehyde reductase (AKR1A) and aldose reductase (AKR1B), have been isolated and characterized from the placenta (10, 29). Because the placenta has a dual origin, i.e., chorion frondosum from the fetus and decidua basalis from the mother, it is likely that these enzymes may be expressed in fetal tissues as well. In this regard, the mRNA expression of AKR1B has been studied in human fetal tissues, although no information is available in the literature as to the spatiotemporal expression of AKR1A during embryonic development. AKR1B is mildly expressed in certain fetal tissues, including fetal brain, lung, liver, eye, and kidney (6). Beyond AKR1B expression in fetal tissues, no further functional studies have been performed. However, further detailed studies describing spatiotemporal expression of AKR1B have been described in the rat eye (3), and interest in this area may be due to its relevance in one of the common complications of diabetes, i.e., cataractogenesis. At day 13 of murine gestation, there is a relatively high degree of mRNA expression in the optic cup compared with other organs. The level of expression continues to be high in subsequent stages but is confined to the germinative zone, the latter being the source of cells that become lens fibers. Because of such a spatiotemporal expression, AKR1B has been implicated in lens fiber morphogenesis (3). In the rat kidney, AKR1B mRNA expression is mostly detectable at a very late stage of gestation and is mainly seen in medullary tips of the newborn kidney. Its expression rises rapidly and, by postnatal day 12, reaches levels

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comparable to those in the adult kidney (4, 25). Because AKR1B expression in the kidney is a late occurrence, it probably has a lesser role in the renal morphogenesis.

RSOR is exclusively expressed in the fetal kidney and exhibits strict spatiotemporal distribution. The mRNA expression is detectable at day 13 of mouse gestation. Thereafter, it steadily increases during the later stages of gestation and reaches levels comparable with those in the adult kidney by 3 wk of postnatal life (Fig. 1). Because RSOR expression is detectable just after the commencement of nephrogenesis, it is conceivable that it may have a role in metanephrogenesis similar to other macromolecules that are expressed at that stage of embryonic development (5, 26). Some of the molecules that are known to regulate nephrogenesis are exclusively expressed in the tubules, and they selectively modulate tubulogenesis (16, 32). Because RSOR is exclusively expressed in the tubules at the onset of nephrogenesis and is confined to the proximal tubules (Figs. 2 and 3), it would be of interest to investigate whether it affects tubulogenesis and/or glomerulogenesis. So far, the molecules that have been described to exclusively modulate tubulogenesis in-
include extracellular matrix proteins, i.e., tubulointerstitial nephritis antigen and an integral membrane protein, SGLT-1 (16, 32). Here, a relevant issue therefore would be whether a cytosolic protein such as RSOR can modulate tubular development. Also, because it is difficult to assume that a given molecule is relevant to nephrogenesis or tubulogenesis simply because it is developmentally regulated, one has to prove this by performing in vitro or in vivo gene disruption experiments.

To address these issues, in vitro antisense ODN experiments were performed. This technology has yielded critical information in various developmental processes, provided appropriate controls are included in a given experiment (5). In cell culture systems, a high dose of various antisense ODNs (>10 μM) can be used, whereas in an organ culture system such as that of the kidney, this dose is quite toxic; i.e., one may observe foci of necrosis or apoptosis in antisense-treated explants. In view of this fact, the experiments were performed at 0.5–1.5 μM, which yielded dose-dependent alterations in the metanephric explants, and no discernible toxicity was observed (Fig. 4). The alterations in the metanephroi included expansion of the mesenchyme and a notable loss of tubules, while the population of glomeruli was unaffected. This loss of tubules and intact population of glomeruli could be confirmed by various glomerular markers, including WGA staining (Fig. 5) and GLEPP-1 in situ tissue hybridization (Fig. 6). Although these findings may suggest that antisense ODNs selectively affect tubulogenesis, the important question would be whether these effects specifically target RSOR. The fact that competitive RT-PCR revealed a 10- to 100-fold decrease in the mRNA expression of RSOR would suggest that the antisense ODN effects specifically target the RSOR gene (Fig. 6). Further support for these findings is derived from the translational blockade studies, in which a marked decrease in the intensity of the ~33-kDa band, corresponding to the protein size of RSOR, was observed in explants treated with antisense ODN, while the band of explants treated with nonsense ODN was unaffected. Thus a comparable decrease in mRNA and protein expression would suggest that the antisense ODN effects are specifically targeted at the RSOR, which resulted in a decrease in the population of tubules. Also, largely intact glomerulogenesis would support the fact that the effects specifically target the tubules. The deficiency of tubules in the antisense-treated explants may be related to the failure in their differentiation and maturation from the nephron progenitor elements. Also, it may in part be due to the atrophy or regression of the tubules that have previously matured and are present at day 13 of gestation in mice metanephric explants. The selective deficiency of tubules in the treated explants may be due to the ready uptake of the antisense ODNs by the tubular cells compared with that by the glomeruli. However, this may be unlikely, since the nonsense-treated glomeruli did not show much discernible change, and moreover, the alterations observed with antisense treatment were dose dependent.

Interestingly, the effects described above are reminiscent of the previously reported results for SGLT-1, which exhibits similar spatiotemporal expression in the kidney during embryonic life (32, 33). Because these two macromolecules have similar spatiotemporal expression and both are involved in glucose energy metabolism, it is conceivable that the glycolytic pathway plays a critical role in tubular maturation compared with its relevance in glomerular development. This is more of a speculation at present, and further studies on the expression of these molecules during development in kidneys exposed to high glucose concentrations are required. In this regard, it is known that high glucose ambience can induce dysmorphogenesis of the embryonic kidney in vitro culture systems (17). Also, it is worth mentioning that hyperglycemia, besides causing diabetic nephropathy (18, 21), is also known to cause renal developmental abnormalities in 2–3% of offspring of poorly controlled juvenile diabetes, which may be part of the ‘caudal regression syndrome’ or may solely involve the urogenital system (11).

In summary, the results of this investigation suggest that RSOR, an enzyme involved in glucose metabolism, is developmentally regulated and modulates renal tubulogenesis in an in vitro organ culture system. Finally, it is anticipated that the results of this investigation would provide an impetus for future in vivo genetic manipulation studies, i.e., transgenic and knockout mice experiments, to elucidate the role of RSOR in renal development under normoglycemic and hyperglycemic states.

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