Tubular mitochondrial alterations in neonatal rats subjected to RAS inhibition

Daina Lasaitiene,1 Yun Chen,1 Vida Mildziene,5,6 Zita Nauciene,5,6 Birgitta Sundelin,4 Bengt R. Johansson,3 Masato Yano,7 and Peter Friberg2

1Department of Physiology, Institute of Physiology and Pharmacology, 2Department of Clinical Physiology, and 3The Electron Microscopy Unit, Institute of Anatomy and Cell Biology, University of Gothenburg; 4Department of Pathology and Cytology, Karolinska Hospital, Stockholm, Sweden; 5Institute for Biomedical Research, Kaunas University of Medicine, Kaunas; 6Environmental Research Center, Faculty of Nature Science, Vytautas Magnus University, Kaunas, Lithuania; and 7Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

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Lasaitiene, Daina, Yun Chen, Vida Mildziene, Zita Nauciene, Birgitta Sundelin, Bengt R. Johansson, Masato Yano, and Peter Friberg. Tubular mitochondrial alterations in neonatal rats subjected to RAS inhibition. Am J Physiol Renal Physiol 290:F1260–F1269, 2006—Pharmacological interruption of the angiotensin II (ANG II) type 1 receptor signaling during nephrogenesis in rats perturbs renal tubular development. This study aimed to further investigate tubular developmental defects in neonatal rats subjected to ANG II inhibition with enalapril. We evaluated tubular ultrastructural changes using electron microscopy and estimated spectrophotometrically or activity or concentration of succinate dehydrogenase (SDH), cytochromes a and c, which are components of mitochondrial respiratory chain, on postnatal days 2 and 9 (PD2 and PD9). Renal expression of sodium-potassium adenosinetriphosphatase (Na+–K+–ATPase) and two reflectors of mitochondrial biogenesis [mitochondrial transcription factor A (TFAM) and translocase of outer mitochondrial membrane 20 (TOM20)] were also studied using Western immunoblotting and immuno histochemistry. Enalapril disrupted inner mitochondrial membranes of developing cortical and medullary tubular cells on PD2 and PD9. These findings were paralleled by impaired mitochondrial respiratory function, as revealed from the changes in components of the mitochondrial respiratory chain, such as decreased cytochrome c level in the cortex and medulla on PD2 and PD9, decreased cytochrome a level in the cortex and medulla on PD2, and diminished cortical SDH activity on PD2 and PD9. Moreover, tubular expression of the most active energy-consuming pump Na+–K+–ATPase was decreased by enalapril treatment. Renal expression of TFAM and TOM20 was not altered by neonatal enalapril treatment. Because nephrogenesis is a highly energy-consuming biological process, with the energy being utilized for renal growth and transport activities, the structural-functional alterations of the mitochondria induced by neonatal enalapril treatment may provide the propensity for the tubular developmental defect.

renal development; cytochrome c; cytochrome a; succinate dehydrogenase; Na+–K+–adenosinetriphosphatase

IT IS DELETERIOUS to give angiotensin-converting enzyme (ACE) inhibitors or angiotensin II (ANG II) type 1 (AT1) receptor blockers during pregnancy, because they can cause fetal death and oliguria/anuria in the newborns (22, 26). The explanation behind this effect may have its background in an experimental model of neonatal pharmacological inhibition of ANG II formation or AT1 receptor stimulation that causes irreversible kidney damage (6, 33). In such a model, perturbed medullary tubular development is among the earliest changes, leading to a marked impairment in urinary concentrating ability and a disturbed reabsorption of sodium in adult rats (12, 21). Sodium reabsorption is the predominant transport-related energy-demanding process (11). Mitochondria provide adenosine 5′-triphosphate (ATP), which is synthesized by the respiratory chain (RC), embedded in the inner mitochondrial membranes (11, 30). Five larger complexes (I–V) and two mobile smaller complexes ("carrier"), i.e., ubiquinone and cytochrome c, are involved in energy transformation (30).

Although the renin-angiotensin system (RAS)-mediated kidney development has been extensively studied, the subcellular mechanisms underlying the structural and functional tubular abnormalities are still enigmatic. The aim of the present study was to characterize ultrastructural changes in the developing kidneys of neonatal rats subjected to ANG II inhibition by enalapril, focusing on the tubular system. We have demonstrated that enalapril disrupts inner mitochondrial membranes of the developing cortical and medullary tubular cells, raising the hypothesis of a potential decline in the respiratory function of mitochondria and, hence, the affected energy supply. Therefore, we further measured essential components of the RC, including cytochromes a (complex IV) and c (carrier) and succinate dehydrogenase (SDH; complex II), which are the markers of mitochondrial activity in the tissue (3, 23). Given the coupling of mitochondrial energy production to its consumption by Na+–K+–ATPase, we also studied the abundance of Na+–K+–ATPase in the developing kidneys exposed to enalapril treatment. During kidney development, the mitochondria replicate their DNA and proliferate, synthesizing and importing various mitochondrial components and acquiring the characteristic structural organization, a process known as mitochondrial biogenesis (5, 18, 24). To test whether neonatal enalapril treatment affected renal mitochondrial biogenesis, we studied expression of mitochondrial transcription factor A (TFAM), which is essential for replication of mitochondrial DNA, and translocase of outer mitochondrial membrane 20 (TOM20), which is essential for protein import into the mitochondria (4, 10, 20, 35).

MATERIALS AND METHODS

General procedures. Female Wistar rats (B&K Universal, Sollentuna, Sweden) were transported to our facility on their 14th day of pregnancy. They were observed carefully for determination of the day of delivery. Weight-matched pups were divided into two groups...
receiving daily intraperitoneal injections of either enalapril maleate (10 mg/kg; Sigma-Aldrich Sweden) or isotonic saline vehicle in equivalent volumes of 10 ml/kg from postnatal day (PD) 0 (within 12 h after birth) to PD2 or PD9. Rats had free access to standard rat chow and tap water and were kept in rooms with a controlled temperature of 24°C and a 12:12-h dark (6:00 PM–6:00 AM)-light cycle throughout the study. The local ethics committee in Gothenburg approved all experiments.

For electron microscopy, four pups from the enalapril- and saline-treated groups were killed on PD2 and PD9. For studies of renal expression of the RC components (n = 24–27/group, PD2; n = 6–8/group, PD9) and the amounts of TFAM, TOM20, and Na+/K+-ATPase (n = 7–8/group, PD9), pups were killed on PD2 or PD9.

Electron microscopy. After rapid removal, the whole kidneys were prefixed briefly in cold fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.02% sodium azide in 0.05 M sodium cacodylate (pH 7.2) and dissected into cortex and medulla. Cortex and medullas were cut into 1-mm³ tissue blocks, which were immersion-fixed in the same fixative for 24 h at 4°C. They were postfixed in 1% OsO₄ plus 1% potassium ferrocyanide for 2 h at 4°C followed by staining en bloc with 0.5% uranyl acetate. After dehydration, the specimens were embedded in epoxy resin (Agar 100) and cured by heat. Ultrathin tissue sections, ~50 nm, were obtained with an ultramicrotome (Ultracut E, Reichert, Austria) fitted with a diamond knife. Sections were contrasted with lead citrate and uranyl acetate and examined with a Zeiss 912AB electron microscope equipped with a MegaView III camera (Soft Imaging Systems, Münster, Germany) for digital image capture.

Spectrophotometric analysis. Both kidneys from each rat were dissected into cortex and medulla, snap frozen in liquid nitrogen, and kept at −80°C until processed for the measurements. To achieve a sufficient amount of the renal tissue at PD2, dissected cortices and medullas from three pups were pooled into one sample. Cortices and medullas were homogenized (homogenizer Kika Laboratortechnik T25 basic 20S) in 1 ml of buffer containing 130 mM KCl, 20 mM Tris·HCl, 5 mM KH₂PO₄, 0.1% Triton X-100, and 1 mM MgCl₂ (pH 7.2). The amounts of cytochromes and activity of SDH (complex II) were determined at the same day. The protein concentration in the homogenates was determined using the Biuret method (9).

The amounts of cytochromes a and c were determined spectrophotometrically (Hitachi-557) by recording spectral differences between their reduced and oxidized forms (29). To achieve this, we added 0.6 ml of the homogenate to spectrophotometric cuvettes containing 0.6 ml of incubation medium. In the sample cuvette, the cytochromes were reduced by addition of sodium dithionite. In the reference cuvette, the cytochromes were oxidized by addition of 0.3% hydrogen peroxide. The spectral differences were recorded between 650 and 520 nm, and the contents (C) of cytochromes were calculated as C = 1,000ΔA/cm−1 for cytochrome c, and ΔA at 650 and 605 nm and ε = 16.5 mM−1 cm−1 for cytochrome a (29).

The activity of SDH was assayed spectrophotometrically by the reduction rate of ubiquinone (CoQ) at 37°C (27). To achieve this, we added 15 μl of the homogenate to 0.96 ml of the medium (the same one used for homogenization except for Triton X-100) containing 15 mM succinate and 100 μM 2,6-dichlorophenolindophenol. After an equilibration of temperature (37°C), the reaction was started by adding 10 μl of 5 mM CoQ solution in ethanol (final concentration: 41 μM CoQ). The rate of reaction was determined

![Image](http://ajprenal.physiology.org/)

Fig. 1. Ultrastructure of developing cortical distal nephron at postnatal day (PD) 2 (A and C, saline; B and D, enalapril). In control rat, epithelial cells are rich in basal infoldings (A, arrowhead), whereas in the enalapril-treated rat, epithelial cells lack basal infoldings and have grossly affected mitochondria (B, arrowhead). Enalapril treatment causes disruption of mitochondrial cristae and disintegration of outer mitochondrial membrane (D, arrows and double arrow, respectively).
as the decrease in absorbance at 600 nm and was calculated using \( \epsilon = 21 \text{mM}^{-1}\cdot\text{cm}^{-1} \).

**Immunohistochemistry.** Kidneys (PD9) were fixed in 4% paraformaldehyde, dehydrated in alcohols, and embedded in paraffin. Sections (4 \( \mu \)m) were cut and mounted on glass slides. Before immunostaining, sections were heated at 60°C for 30 min, deparaffinized, rehydrated, and boiled in citric acid buffer (0.01 M, pH 6.0) for 30 min. After overnight incubation (at 4°C) with a monoclonal mouse anti-chicken Na\(^+\)-K\(^+\)-ATPase \( \alpha_1 \)-subunit antibody (\( \alpha_6 \), dilution 1:100; Developmental Studies Hybridoma Bank; antibody is cross-reactive against rat Na\(^+\)-K\(^+\)-ATPase \( \alpha_1 \)-subunit), sections were depleted of endogenous peroxidase activity and incubated with peroxidase-linked F(ab\(^2\)) fragment of sheep anti-mouse IgG (1:100; Amer-

sham, Uppsala, Sweden). Immunoreactivity was visualized using 3-amino-9-ethyl-carbazole (Sigma Chemical, St Louis, MO) containing hydrogen peroxide, and counterstaining was performed with Mayer’s hematoxylin. The antibodies were diluted in phosphate-buffered saline (PBS) containing 1% of bovine serum albumin (BSA). Negative control samples were obtained by replacement of primary antibody with 1% BSA.

**Western immunoblotting.** Dissected renal cortexes and medullas from 9-day-old rats were homogenized in buffer containing 250 mM sucrose, 10 mM HEPES-Tris (pH 6.95), and protease inhibitors (Complete Mini; Roche). Protein concentration was measured using a Bio-Rad protein assay kit. Aliquots (100 \( \mu \)g of proteins) were solubilized in Laemmli sample buffer and separated, under reducing conditions, using a 10% SDS-PAGE gel. **Fig. 2.** Ultrastructure of developing proximal tubules at PD2 (A, C, and E, saline; B, D, and F, enalapril). Microvilli in the proximal tubules are well preserved (A and B, arrows). In the enalapril-treated rat, epithelial basal infoldings are missing (D) and mitochondrial cristae are mildly affected (F, arrow).
conditions, by electrophoresis on 4–15% Tris-HCl gradient gel (Bio-Rad, Hercules, CA). Proteins were transferred to a polyvinylidene difluoride membrane (Amersham, Uppsala, Sweden). After being blocked in 5% nonfat milk with PBS plus 0.1% Tween 20 (PBS-T), the membrane was incubated with primary α1-subunit-specific anti-Na\(^+\)-K\(^+\)-ATPase (α6F; 1:500), polyclonal rabbit anti-mouse TFAM [1:1,000; kind gift of Dr. N. G. Larsson; antibody is cross-reactive against rat TFAM (20)], or β-actin (1:5,000; Sigma) diluted in 5% nonfat dry milk in PBS-T for 1 h. The proteins were detected using peroxidase-linked F(ab)\(_2\) fragment of sheep anti-mouse IgG (1:1,000; Amersham) or goat anti-rabbit IgG (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), respectively, and the ECL detection system (Amersham). The specificity of primary antibodies was shown by their omission. Bands were visualized using a Fuji LAS-1000 cooled charge-coupled device camera/dark box, employing Image Reader LAS-1000 version 1.1 software, and the density of the bands was analyzed with the help of Image Gauge software version 3.45.

### Results

#### Ultrastructure

In control kidneys, the ultrastructure of nearly all tubular mitochondria was well preserved at both time points (Figs. 1C, 2E, 3C, 4E, and 5C). In enalapril-treated kidneys, discernible mitochondrial changes were observed in the tubules of distal and proximal nephron on PD2 and PD9. The mitochondria of distal and collecting tubules were swollen and had fewer cristae (Figs. 1D, 3D, 5D, and 6D). In severely disorganized mitochondria, no remnants of the cristae were observed and the outer mitochondrial membrane was disintegrated (Fig. 1D). Mitochondrial changes were milder in the proximal tubules (Figs. 2F and 4F). In both treatment groups, the microvilli of proximal tubules were well preserved (Figs. 2, A and B, and 4, A and B).

In control kidneys, basal infoldings in the epithelial cells of distal, proximal, and collecting tubules were pronounced on PD2 and PD9 (Figs. 1A, 2C, 3A, 4C, 5A, and 6A). Conversely, these epithelial basal infoldings were absent in distal, proximal, and collecting tubules from enalapril-treated rats (Figs. 1B, 2D, 3B, 4D, 5B, and 6B).

**Components of the mitochondrial respiratory chain.** To investigate whether these structural abnormalities would lead to changes in respiratory function of mitochondria, we determined different components of the mitochondrial RC. In general, our results showed higher mitochondrial activity (higher amounts of cytochromes a and c and higher SDH activity) in the cortex compared with medulla in both treatment groups on PD9 (Figs. 7–9), suggesting a preference for the aerobic energy supply in renal cortical tissue. These findings are in line with earlier experiments performed on adult rats (14) and may reflect lower metabolic activity and/or lesser density of medullary mitochondria.
The activity of cortical SDH from enalapril-treated rats was decreased by 16% on PD2 and by 31% on PD9 (Fig. 7). The renal amount of cytochrome c was decreased by enalapril treatment on PD2 and PD9, being lower by -24–32% in the cortex and by 22–36% in the medulla from enalapril-treated rats (Fig. 8). Enalapril treatment decreased the cortical amount of cytochrome a by 30% and the medullary amount by 27% only on PD2 (Fig. 9).

Expression of TFAM and TOM20. Renal expression of TFAM and TOM20 proteins was not changed by neonatal enalapril treatment (Figs. 10 and 11).

Expression of Na\(^+\)-K\(^+\)-ATPase. Antibody to \(\alpha_1\)-subunit of the Na\(^+\)-K\(^+\)-ATPase revealed a band at ~96 kDa. This band disappeared when the primary antibody was omitted (Fig. 12A). The amount of the \(\alpha_1\)-subunit protein in the cortex, but not medulla, from enalapril-treated rats was decreased on PD9 (Fig. 12B). Immunohistochemical staining showed that the signal for \(\alpha_1\)-subunit of the Na\(^+\)-K\(^+\)-ATPase was confined to the basolateral membrane of epithelial cells in the cortical and medullary tubules from control and enalapril-treated kidneys (Fig. 12, C–F). Enalapril-treated kidneys demonstrated weaker cortical labeling for \(\alpha_1\)-subunit protein (Fig. 12D).
The present study shows that ACE inhibition in the neonatal rat has a detrimental effect on tubular mitochondrial structure and function during renal morphogenesis. Enalapril-induced structural mitochondrial alterations were present along the proximal and distal part of the nephron, with more prominent changes observed in its distal part. Enalapril-induced structural mitochondrial alterations were paralleled by the impaired mitochondrial respiratory function, as evident from the decreases in the amounts of cytochromes \( a \) and \( c \) and in the activity of SDH. As a possible consequence, decreased levels of the \( \alpha_1 \)-subunit of Na\(^+\)-K\(^+\)-ATPase were found in the cortex of enalapril-treated rats. These findings provide strong explanatory support for our previous observation of reduced sodium reabsorption and urine-concentrating ability in adult rats subjected to neonatal ACE inhibition (12).

Rats are born with immature kidneys, and renal tubules undergo postnatal structural-functional differentiation (1). ANG II plays an important role in the tubular development, eliciting a growth-promoting effect on the tubular cells of the distal and proximal part of the nephron (36, 37). Tubulogenesis is correlated with the differentiation of renal cell functions, such as active sodium reabsorption and urine-concentrating ability, and this is associated with the increase in the area of the basolateral cell membranes as well as the amount of Na\(^+\)-K\(^+\)-ATPase in various nephron segments (13, 19, 28). In addition to the increase in the basolateral membrane area, tubular mitochondrial biogenesis occurs (5, 18). The mitochondria increase in size and number, and they experience profound postnatal changes in the organization of their inner membranes, together with the increase in the activity of the RC enzymes (5). This implies that the source of ATP increases considerably. Our results show that neonatal enalapril treatment disrupted the structural integrity of inner mitochondrial membranes in the developing tubular cells. The highly convoluted foldings, or cristae, of the inner mitochondrial membrane greatly expand its surface area, enhancing the ability to generate ATP (25). Therefore, enalapril-induced disruption of mitochondrial cristae will inevitably lead to impaired ATP generation. As a corollary, we found that enalapril treatment affected cytochromes \( a \) and \( c \) and SDH. A more pronounced effect was observed in the enalapril-treated cortex, where both the amounts of cytochrome \( a \) (only PD2) and \( c \) (PD2 and PD9) and the activity of SDH (PD2 and PD9) decreased by \(~16–30\%\) on PD2 and by 31–32\% on PD9. In the enalapril-treated medulla, only the amounts of cytochrome \( a \) (PD2) and \( c \) (PD2 and PD9) were substantially lower (22–36\%). Cytochromes \( c \) and \( a \) and SDH are essential components of the RC that are embedded in the inner mitochondrial membrane and catalyze the transport of electrons along the RC during oxidative phosphorylation, i.e., mitochondrial ATP synthesis (30, 32). Cytochrome \( c \) serves as a mobile electron carrier in the mitochondrial RC that passes...
electrons from cytochrome $bc_1$ (complex III) to cytochrome oxidase (complex IV), whereas cytochrome $a$ is one of the components of complex IV that catalyzes the transfer of electrons from cytochrome $c$ to molecular oxygen. Electron transfer along the RC may be retarded in enalapril-treated kidneys because of the decreased amounts of cytochromes $c$ and $a$. SDH (complex II) is an integral enzyme of the inner mitochondrial membrane with a covalently bound prosthetic (flavin adenine dinucleotide) group (15, 17, 30). The reduced activity of SDH in the cortex of enalapril-treated neonatal rats, as found in this study, may interfere with mitochondrial ATP

Fig. 6. Ultrastructure of developing medullary collecting duct at PD9 (A and C, saline; B and D, enalapril). The enalapril-treated rat demonstrates lack of epithelial basal infoldings (B, arrowhead) and disappearance of mitochondrial cristae (D, arrows).

**Fig. 7.** Activity of succinate dehydrogenase (SDH) in kidneys from 2- and 9-day-old rats treated with saline or enalapril. A decrease in cortical SDH activity is observed in enalapril-treated rats on PD2 and PD9. Values are means (SD); $n = 8–9$/group, PD2; $n = 6–8$/group, PD9. *$P < 0.05$ vs. saline vehicle.

**Fig. 8.** Amount of cytochrome $c$ in kidneys from 2- and 9-day-old rats treated with saline or enalapril. The amount of cytochrome $c$ is decreased in the cortex and medulla from enalapril-treated rats on PD2 and PD9. Values are means (SD); $n = 8–9$/group, PD2; $n = 6–8$/group, PD9. *$P < 0.05$ vs. saline vehicle.
synthesis given that SDH is an interconnector of the RC and Krebs cycle, which occurs in mitochondrial matrix and provides electrons to the RC, thus fueling ATP production (15, 17).

ATP utilization by the developing kidney is needed not only for its growth but also for the transport of solutes and water (2, 11). Sodium reabsorption is the predominant transport-related energy-consuming process, with the Na\(^{+}\)-K\(^{+}\)-ATPase being the most abundant and active energy-consuming pump (11). The Na\(^{+}\)-K\(^{+}\)-ATPase polarized to the basolateral membrane is the major driving force for sodium reabsorption, and it actively extrudes sodium from the tubular cells, providing the electrochemical gradient for transportation of sodium from the tubular lumen to the interstitial space (7). Neonatal enalapril treatment decreased cortical expression of \(\alpha_{1}\)-subunit of Na\(^{+}\)-K\(^{+}\)-ATPase in the developing kidney. Interestingly, decreased expression of Na\(^{+}\)-K\(^{+}\)-ATPase coincided with the lack of basal infoldings in the basolateral membrane of tubular cells. One may surmise that mitochondrial ultrastructural and biochemical changes in the developing rat kidneys induced by enalapril are secondary to a reduction in arterial pressure and thereby decreased tissue oxygen supply. However, this assumption appears to be less probable given the greater resistance of the immature nephron to hypoxia (8). Furthermore, if enalapril-induced hypoxia was the leading cause of mitochondrial damage, the aberrant mitochondria would have dominated in the proximal tubules, which have relatively little glycolytic capacity, making them dependent on aerobic mitochondrial metabolism for ATP synthesis (31). However, rather well-preserved microvilli in proximal tubules of ANG II-inhibited rats indirectly reflect a good state of proximal tubular epithelium. Therefore, our findings suggest that hemodynamic changes may not be the primary cause for the observed tubular mitochondrial changes in the enalapril treated rats and propose a novel, nonischemic cause of mitochondrial damage. One might argue that the tubular mitochondrial changes caused by enalapril treatment could be attributed to the specific effect of the drug. We addressed this issue in a pilot study in which kidney ultrastructure was analyzed in neonatal rats after administration of the AT\(_{1}\) receptor blocker losartan. Notably, our findings showed that both neonatal AT\(_{1}\) receptor blockade and ACE inhibition cause comparable tubular mitochondrial changes (data not shown), thus demonstrating an important role for the RAS in structural organization of tubular mitochondria. It is possible that a lack of growth-promoting effect of ANG II on tubular epithelial cells leads to the secondary changes of tubular mitochondria. However, the levels of TFAM and TOM20 proteins, known to be the key players in mitochondrial DNA transcription and protein import into the mitochondria, are decreased in kidneys from 9-day-old rats treated with enalapril. Importantly, changes in basolateral membrane surface area are directly proportional to alterations in Na\(^{+}\)-K\(^{+}\)-ATPase activity (16, 34). Bearing this information in mind, we suggest that sodium loss that is observed in rats subjected to neonatal ACE inhibition (12) may be partly associated with the downregulation of Na\(^{+}\)-K\(^{+}\)-ATPase pump in the tubular epithelial cells.

Fig. 9. Amount of cytochrome \(a\) in kidneys from 2- and 9-day-old rats treated with saline or enalapril. The amount of cytochrome \(a\) is decreased in the cortex and medulla from enalapril-treated rats on PD2. Values are means (SD); \(n = 8–9/\text{group}, \text{PD2}; n = 6–8/\text{group, PD9. } *P < 0.05 \text{ vs. saline vehicle.}

Fig. 10. Expression of mitochondrial transcription factor A (TFAM) protein in kidneys from 9-day-old rats treated with saline or enalapril. Densitometric analysis of the \(\sim 25\)-kDa bands shows no significant difference in renal expression of TFAM protein between saline- and enalapril-treated rats. Values are means (SD); \(n = 7–8/\text{group.}

Fig. 11. Expression of translocase of outer mitochondrial membrane 20 (TOM20) protein in kidneys from 9-day-old rats treated with saline or enalapril. Densitometric analysis of the \(\sim 20\)-kDa bands shows no significant difference in renal expression of TOM20 protein between saline- and enalapril-treated rats. Values are means (SD); \(n = 7–8/\text{group.}

Fig. 12. Amount of cytochrome \(a\) in neonatal kidneys

Expression of TOM20 in neonatal kidneys

Expression of TFAM in neonatal kidneys
mitochondria, respectively (5, 10, 24), were not changed in ACE-inhibited developing kidneys. This finding suggests that neonatal ACE inhibition did not affect mitochondrial biogenesis. Additional studies are needed to elucidate the precise mechanisms behind mitochondrial abnormalities induced by neonatal ACE inhibition.

In summary, the main conclusion of the present study is that enalapril induces changes in the mitochondrial structure and function in the developing rat kidney. We assume that compromised bioenergetic function of the mitochondria may provide the propensity for the tubular developmental defect in the ACE-inhibited newborn rats.

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