Contribution of Ras GTPase/MAP Kinase and Cytochrome P450 Metabolites to Deoxycorticosterone-Salt–Induced Hypertension

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Abstract—We recently reported that norepinephrine and angiotensin II activate the Ras/mitogen-activated protein (MAP) kinase pathway through generation of a cytochrome P450 (CYP450) and lipoxygenase metabolites. The purpose of this study was to determine the contribution of Ras/MAP kinase to deoxycorticosterone acetate (DOCA)-salt–induced hypertension in rats. Administration of DOCA and 1% saline drinking water to uninephrectomized rats for 6 weeks significantly elevated mean arterial blood pressure (MABP) (166±5 mm Hg, n=19) compared with that of normotensive controls (95±5 mm Hg, n=7) (P<0.05). The activity of Ras and MAP kinase measured in the heart was increased in DOCA-salt hypertensive rats. Infusion of the Ras farnesyl transferase inhibitors FPT III (138 ng/min) and BMS-191563 (694 ng/min) significantly (P<0.05) attenuated MABP to 139±4 mm Hg (n=14) and 126±1 mm Hg (n=4), respectively. Moreover, infusion of MAP kinase kinase inhibitor PD-98059 (694 ng/min) also reduced MABP in hypertensive rats. Morphological studies of the kidney showed that treatment of rats with FPT III, which reduced Ras activity, minimized the hyperplastic occlusive arteriosclerosis and fibrinoid vasculitis observed in untreated hypertensive rats. In addition, the rise in CYP450 activity and MABP in hypertensive rats was prevented by the CYP450 inhibitor aminobenzotriazole (50 mg/kg) and was associated with a decrease in Ras and MAP kinase activity in the heart. These data suggest that the Ras/MAP kinase pathway contributes to DOCA-salt–induced hypertension and associated vascular pathology consequent to activation of CYP450. (Hypertension. 2000;35[part 2]:457-463.)

Key Words: Ras ■ MAP kinase ■ DOCA ■ pathology ■ cytochrome P450

Several vasoactive agents, including norepinephrine, angiotensin II (Ang II), and endothelin-1, promote arachidonic acid release from tissue lipids via activation of ≈1 lipases.1–3 Arachidonic acid is metabolized by cyclooxygenase into prostaglandins and thromboxane A2, by lipoxygenase into leukotrienes and HETE (5-, 12-, and 15-HETE), and by cytochrome P450 (CYP450) into epoxyeicosatrienoic acid and 12-, 19-, and 20-HETE.4–7 Some of the products of arachidonic acid metabolized by cytochrome P450 into epoxyeicosatrienoic acid and 12-, 19-, and 20-HETE.4–7 Some of the products of arachidonic acid, by activating Ras/MAP kinase, contribute to the increase in blood pressure in spontaneously hypertensive, deoxycorticosterone acetate (DOCA)-salt–induced, and Ang II–induced models of hypertension.17–19 These observations and the demonstration that a CYP450 inhibitor reduces Ras activation and the development of Ang II–induced hypertension19 suggest that the CYP450 metabolite 20-HETE raises blood pressure by activating the Ras/MAP kinase pathway. Whether the Ras/MAP kinase pathway is also involved in Ang II–independent models of hypertension is not known. One such model is hypertension induced by DOCA (an analogue of aldosterone) in rats drinking a 1% saline solution.20 Administration of DOCA causes retention of salt and water, increased plasma volume, and decreased renin secretion and consequently decreases Ang II levels.21 However, several other factors, such as catecholamine, vasopressin, and endothelin-1, also contribute to the development of DOCA-salt hypertension.22–28 Because these factors are known to increase arachidonic acid release and MAP kinase activity,1,3,20,30 it is possible that CYP450 metabolites of arachidonic acid, by activating Ras/MAP kinase, contribute to the increase in blood pressure in spontaneously hypertensive, deoxycorticosterone acetate (DOCA)-salt–induced, and Ang II–induced models of hypertension.17–19

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to the development of DOCA-salt hypertension in rats. To test this hypothesis, we investigated the effect of various inhibitors of Ras, MAP kinase kinase (MEK), and CYP450 on the development of DOCA-salt hypertension and/or on the associated vascular injury and hypertrophy.

**Methods**

**Materials**

DOCA, NaCl, sodium pentobarbital, lauric acid, and heparin were from Sigma Chemical Co; the farnesyl protein transferase inhibitors FPT III and BMS-191563 were from Calbiochem and a gift from Bristol-Myers Squibb, respectively; 2ML osmotic minipump from Alzet Corp; H-Ras monoclonal antibody from Santa Cruz Biotechnology; the CYP450 inhibitor ABT from Acros; and PD-98059, MAP kinase, and phosphospecific MAP kinase antibodies from New England Biolabs.

**Experimental Procedures**

**DOCA-Salt Hypertension**

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) weighing 200 g were kept at 24°C under controlled conditions of light (6 AM to 6 PM) and humidity (50%). The animals were fed standard rat chow (Ralston Purina Co) containing sodium (174 mEq/kg) and potassium (282 mEq/kg) and had free access to the food and water. All procedures were done in accordance with institutional guidelines for animal research. The rats were anesthetized with a ketamine-xylazine mixture. A small midline abdominal incision (2 cm) was made to ligate the left renal artery and vein and dissect out the left kidney. The abdominal incision was sutured and the animal was allowed to recover from anesthesia for 3 days. The animals were given standard chow and tap water containing 1% NaCl. Five animals were sham-operated without removal of the kidney and were given tap water containing 1% NaCl. The unpunecutomized animals were injected intramuscularly with a suspension of DOCA in sesame oil (30 mg/kg per week, 0.2 mL) or vehicle for 6 weeks as described.

**Blood Pressure Measurement**

Animals were anesthetized with sodium pentobarbital (60 mg/kg), and the left femoral artery was exposed surgically. A small incision was made in the femoral artery, and a catheter was inserted and connected to a pressure transducer (Grass model 7m, Grass Instruments) for blood pressure measurement. Blood pressure was recorded on a polygraph (model 7D; Grass polygraph). The MAP was expressed as mm Hg. After blood pressure measurement, the thoracic cavity was exposed and the left kidney and hearts were removed. The heart tissue was rapidly frozen in liquid nitrogen for the measurement of Ras and MAP kinase protein and activity levels, and the kidney was used for histological examination and measurement of CYP450 activity.

**CYP450 4A Activity**

CYP450 4A activity, measured as lauric acid 12-hydroxylase activity, was determined according to the method described earlier, with small modifications. Kidney protein extracts (200 μL, 0.5 mg) were mixed with 100 μL of buffer containing 0.4% NAD (wt/vol), 0.4% NADP (wt/vol), 0.75% glucose 6-phosphate (wt/vol), 1% BSA (wt/vol), 1% glucose 6-phosphate dehydrogenase (vol/vol), 0.05 mmol/L MgCl₂, 0.05 mmol/L Tris-HCl, pH 7.6, and the volume was adjusted to 1 mL with Tris-HCl (pH 7.6). The enzymatic reaction was initiated by the addition of 25 μL of [14C]lauric acid solution (5 mmol/L, 5000 dpm/nmol) and continued for 30 minutes at 37°C. [14C]lauric acid solution was prepared by diluting [14C]lauric acid (55 mCi/mmol, Amersham) and unlabeled lauric acid in ethanol. The reaction was stopped by adding 100 μL of 0.2N acetic acid. The metabolites were extracted twice in 3 mL of ethyl acetate, and the organic phase was dried under nitrogen. Samples were then resuspended and sonicated in 50 μL methanol and separated by thin-layer chromatography with hexane, ethyl ether, and acetic acid as the solvent system (2:8:0.1 vol/vol). Formation of 12-hydroxylauric acid was detected with Instant Imager (Packard Instruments).

**Measurement of Ras, MAP Kinase, and Phosphospecific MAP Kinase Levels by Western Blotting**

Frozen heart tissues were processed for Western blot analysis as described. Briefly, the tissues (200 mg) were powdered in liquid nitrogen with a mortar and pestle and homogenized in 1 mL of buffer (mmol/L: HEPES 20 [pH 7.5], β-glycerophosphate 20, sodium pyrophosphate 20, sodium vanadate 0.2, EDTA 2, sodium fluoride 20, benzamide 10, and DTT 1, and 20 μg/mL leupeptin) with a Dounce homogenizer. Cell debris was removed by centrifugation (14 000 g for 10 minutes at 4°C), and the supernatant was stored in aliquots at −70°C. Proteins (200 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. The blots were blocked with 3% BSA in TBS buffer (20 mmol/L Tris, 137 mmol/L NaCl, pH 7.6) at room temperature for 1 hour and then incubated for 2 hours with MAP kinase or Ras or phosphospecific MAP kinase antibodies (1:1000 dilution). The blots were developed with biotinylated secondary antibody and horseradish peroxidase, and signals were detected with ECL Western blotting detection reagents (Amersham).

**GST-RBD Preparation**

The plasmid pGEX encoding amino acids 1 to 149 of the Ras-raf binding domain (RBD) of Raf was used (kindly provided by Dr Senthilkumar Muthuswamy, Harvard University). The plasmid was transformed into Escherichia coli strain DH5α for protein expression. An overnight culture of bacteria (2 L) was grown at 37°C in LB medium containing 100 μg/mL ampicillin. Expression of GST-RBD protein was induced overnight at 30°C with 0.1 mmol/L isopropyl-1-thio-β-d-galactopyranoside at a cell density of ∼0.5 A₅₆₀. The bacterial culture was centrifuged at 5000g for 20 minutes and washed once with ice-cold buffer (25 mmol/L HEPES [pH 7.5], 150 mmol/L NaCl). The pellet was sonicated for 4 minutes (4 bursts of 1-minute duration) in 10 mL of lysis buffer (20 mmol/L HEPES [pH 7.5], 120 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin). The lysates were gently stirred for 30 minutes at 4°C and centrifuged at 10 000g for 30 minutes to remove the pellet. NP-40 was added to a final concentration of 0.5% to the supernatant containing crude GST-RBD, and the supernatant was then incubated with 500 μL of prewashed glutathione sepharose 4B beads (Pharmacia Biotech, Sweden) for 30 minutes at 4°C. The beads were isolated by centrifugation and washed 3 times with ice-cold PBS.

**Affinity Precipitation of Ras-GTP**

One milligram of proteins isolated from heart tissues was incubated with GST-RBD bound to 75 μL of glutathione sepharose beads on a rocker for 1 hour at 4°C. The beads were then washed 3 times with lysis buffer (100 mmol/L HEPES [pH 7.5], 1 mol/L NaCl, 1%
Figure 1. MABP of normotensive rats, rats drinking salt water (1% NaCl), uninephrectomized rats treated with DOCA (Neph+DOC), uninephrectomized rats drinking salt water (Salt+Neph), and uninephrectomized rats treated with DOCA-salt (Neph+DOC+Salt). Data are presented as mean±SEM (n=5 to 19). *Value significantly different from normotensive controls (P<0.05).

Histological Examination
The kidneys removed from the DOCA-salt hypertensive rats with or without treatment with FPT III were fixed in formalin and then divided into 3 parts by 2 mid-transverse sections. To ensure homogeneous sampling, the middle part, 0.5 cm thick, was selected for histological examination in all animals. The fixed sections were processed for paraffin embedding, and hematoxylin-eosin–stained sections were prepared by standard techniques. Two to 4 cross sections representative of the renal cortex, renal medulla, and pelvis were examined on each case. Semiquantitative assessment of the severity and extent of the arterial lesions was performed. The number and size of arteries affected per cross section were determined. The affected arteries were examined for the presence and absence of the following features: (1) fibrinoid necrosis; (2) alterations in the endothelial or smooth muscle cells, such as cellular hypertrophy, pyelosclerosis, single-cell necrosis, or cellular dropout; (3) hemorrhage in the media (hematomena); and (4) concentric mural hypertrophy with or without "onionskin" change.

Statistical Analysis
Values are reported as mean±SEM. The data were analyzed by 1-way ANOVA, and the difference between the means for multiple comparisons was determined with the Newman-Keuls test; a value of P<0.05 was considered statistically significant.

Results
Effects of FPT III, BMS-191563, PD-98059, and ABT on MABP in DOCA-Salt Hypertensive Rats
Administration of DOCA (30 mg/kg per week IM) and 1% saline drinking water to uninephrectomized rats for 6 weeks significantly increased MABP (166±5 mm Hg) compared with that of normotensive rats (95±5 mm Hg) (P<0.005) and rats treated with salt (108±2 mm Hg), salt plus nephrectomy (112±11 mm Hg), or DOCA plus nephrectomy (121±13 mm Hg) (Figure 1). Infusion of the Ras-farnesyl transferase inhibitors FPT III or BMS-191563 during the last 5 days of DOCA-salt treatment significantly reduced MABP to 139±4 and 126±1 mm Hg, respectively (P<0.05) (Figure 2). The inhibitor of MEK PD-98059 also reduced MABP in hypertensive rats to 120 mm Hg (Figure 2). Treatment of DOCA-salt animals with the CYP450 inhibitor ABT also reduced MABP to 97±7 mm Hg (P<0.05).

Effects of FPT III and ABT on Ras Activity in DOCA-Salt Hypertensive Rats
Protein extracts from the hearts of DOCA-salt hypertensive rats, normotensive controls, and hypertensive animals treated with inhibitors of Ras, MEK, and CYP450 were obtained. Ras protein levels were not altered with DOCA-salt treatment. Moreover, FPT III or ABT did not alter Ras protein levels in the hearts of DOCA-salt hypertensive rats (Figure 3A). CYP450 4A activity, measured as lauric acid hydroxylase activity, was elevated in the kidney of DOCA-salt hypertensive rats, and treatment with ABT attenuated this response (Figure 3B). It has been shown that Ras protein interacts with Raf and that the residues 51 to 131 of mammalian Raf-1 (called RBD) are sufficient to bind Ras-GTP but not Ras-GDP.36,37 RBD was expressed as a GST fusion protein and was purified with glutathione-sepharose beads (Figure 3C). The Ras activity, measured as Ras-GTP bound to the RBD of Raf, was increased in the DOCA-salt hypertensive rats. However, the Ras activity was decreased in the hearts of DOCA-salt hypertensive animals treated with FPT III or ABT (Figure 3D). Treatment of normotensive rats with these inhibitors did not show any significant changes in Ras protein and activity levels (data not shown).

Effects of FPT III, PD-98059, and ABT on MAP Kinase Activity in DOCA-Salt Hypertensive Rats
Ras stimulates Raf by promoting its association with the plasma membrane and activates the MEK/MAP kinase pathway.38 Hence, we studied the effects of Ras, MAP kinase, and CYP450 inhibitors on the MAP kinase activity. Western blot analysis using the MAP kinase antibody showed that similar levels of ERK1 and ERK2 were present in heart extracts from normotensive and DOCA-salt hypertensive animals. Moreover, treatment of hypertensive animals with inhibitors of Ras, MEK, and CYP450 did not decrease the levels of protein (Figure 4A). However, activation of MAP kinase, as measured by phosphospecific MAP kinase antibody, showed marked elevation of phosphorylated MAP kinase levels with DOCA-salt treatment. This MAP kinase activity was atten-
ated in the hearts of hypertensive animals treated with FPT III, PD-98059, or ABT (Figure 4B).

Effect of FPT III on the Morphological Changes in the Kidney of DOCA-Salt Hypertensive Rats

The kidneys from the uninephrectomized animals with DOCA-salt–induced hypertension, as expected, were enlarged. Microscopic examination of these kidneys demonstrated a generalized concentric hypertrophy of small arteries and multiple foci of fibrinoid necrosis in the small and large arteries. Foci of interstitial fibrosis, tubular atrophy, and chronic inflammation were also identified (Figure 5A). DOCA-salt hypertensive animals treated with FPT III in the last week of DOCA-salt treatment (5 weeks) did not exhibit any evidence of hyperplastic arteriosclerosis, arterial necrosis, or interstitial scarring (Figure 5B). These kidneys were essentially unremarkable, except for the presence of red blood cell casts in the renal tubules. The effects of BMS-191563 and PD-98059 on the morphological changes produced in the kidney of DOCA-salt hypertensive rats were not examined. The effect of CYP450 inhibition to minimize renal vascular hypertrophy in DOCA-salt hypertensive rats has recently been reported.18

Discussion

It was reported previously that the CYP450 metabolites of arachidonic acid, especially 20-HETE, activate the Ras/MAP kinase pathway,12 which in turn promotes vascular hyperplasia.14 The present study demonstrates that Ras/MAP kinase, probably activated by the CYP450 metabolite 20-HETE, contributes to the development of DOCA-salt hypertension and the associated renal hyperplastic occlusive arteriosclerosis and fibrinoid vasculitis in rats. In our study, administration of DOCA-salt to uninephrectomized rats produced a significant rise in MABP compared with that in animals with uninephrectomy, DOCA, or salt treatment alone. The increase in blood pressure in DOCA-salt hypertensive rats was associated with an increase in Ras activity in the heart, indicating involvement of Ras in the development of DOCA-salt hypertension. Ras is activated upon binding to the membrane, and this process is initiated by farnesylation of Ras by farnesyl transferase.39 Our finding that FPT III and BMS-191563, inhibitors of Ras farnesylation, reduced arterial blood pressure supports our contention that the development of DOCA-salt hypertension is dependent on the activity of Ras. The decrease in blood pressure in DOCA-salt hypertensive rats produced by FPT III was

Figure 3. Effects of inhibitors of Ras farnesyl transferase (FPT III and BMS-191563) and CYP450 (ABT) on activation of Ras in DOCA-salt hypertensive rats. A, Measurement of Ras protein levels. Western blotting of heart extracts obtained from control or DOCA-salt hypertensive rats treated with vehicle (VEH), FPT III, or ABT. Proteins (200 μg) were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The blots were probed with monoclonal Ras antibody (1:1000 dilution). B, Inhibition of CYP450 activity by ABT. Lauric acid hydroxylase activity, which reflects CYP4504A activity, was measured as the NADPH-dependent formation of 12-hydroxylauric acid in kidney lysates incubated with [14C]lauric acid as a substrate. C, Expression and purification of GST-RBD. Bacterial strain expressing GST-RBD fusion protein was induced with isopropyl-1-thio-β-D-galactopyranoside. Total proteins from bacterial lysates were incubated with prewashed glutathione sepharose 4B beads. The retained GST-RBD was separated by SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, and probed with GST antibody. The molecular mass markers are indicated on the left and GST-RBD and GST on the right. D, Measurement of Ras activation. Ras GTP was detected by GST-RBD in hearts as described in Methods. The experiments shown in A, B, C, and D were performed 3 to 4 times, and a representative blot of these experiments is presented.

Figure 4. Effects of inhibitors of Ras farnesyl transferase (FPT III), MEK (PD-98059), and CYP450 (ABT) on MAP kinase protein levels (A) and phosphorylation of MAP kinase (B). Protein extracts from hearts were obtained from control rats or DOCA-salt hypertensive rats treated with vehicle (Veh) or FPT III, PD-98059, or ABT. Proteins (400 μg) were separated on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The blots were probed with MAP kinase antibody (A) or phosphospecific antibody raised against the activation site of MAP kinase (B). These experiments were performed 3 times, and a representative blot is presented.
Figure 5. Effect of Ras farnesyl transferase inhibitor FPT III on the morphological changes in the kidney of hypertensive rats. A, Micrograph depicts hyperplastic proliferative lesions in the interlobular arteries in kidney obtained from DOCA-salt–induced hypertensive rats. One arterial segment (arrow) exhibits an “onionskin” pattern of arteriosclerosis. B, Kidney from rat with DOCA-salt–induced hypertension treated with Ras inhibitor (FPT III). Note the normal interlobular artery without evidence of proliferative vasculopathy.

Figure 6. Effect of Ras farnesyl transferase inhibitor FPT III on the morphological changes in the kidney of hypertensive rats. A, Micrograph depicts hyperplastic proliferative lesions in the interlobular arteries in kidney obtained from DOCA-salt–induced hypertensive rats. One arterial segment (arrow) exhibits an “onionskin” pattern of arteriosclerosis. B, Kidney from rat with DOCA-salt–induced hypertension treated with Ras inhibitor (FPT III). Note the normal interlobular artery without evidence of proliferative vasculopathy.
of DOCA-salt hypertensive rats could also be due to a decrease in MAP kinase activity. An increase in MAP kinase activity has been shown to mediate the effect of 20-HETE on vascular smooth muscle cell proliferation. Inhibition of CYP450 activity with CoCl₂ has been reported to decrease organ hypertrophy and renal injury and to attenuate the effect of endothelin-1 to stimulate vascular hyperplasia in DOCA-salt hypertensive rats. Therefore, it is possible that the CYP450 metabolite 20-HETE, generated in response to vasoactive agents, increases vascular tone and leads to the development of DOCA-salt hypertension in rats by causing vascular hypertrophy and hyperplasia.

In conclusion, the present study demonstrates that the Ras/MAP kinase pathway, probably activated by a metabolite of arachidonic acid formed via CYP450, most likely 20-HETE, promotes development of DOCA-salt hypertension and hyperplastic occlusive arteriosclerosis and fibrinoid vasculitis in rats.

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smaller than that caused by BMS-191563. This is probably due to the infusion of smaller amounts of FPT III.

The mechanism by which Ras promotes the development of DOCA-salt hypertension most likely involves activation of MEK and MAP kinase pathways, for the following reason. In DOCA-salt hypertensive rats, cardiac MAP kinase activity was increased, whereas it was reduced in animals treated with Ras inhibitors. Moreover, the MEK inhibitor PD-98059 also diminished the development of DOCA-salt–induced hypertension in rats. The effect of inhibitors of Ras and MEK to minimize the development of hypertension in DOCA-salt–treated rats is unlikely to be due to a nonspecific effect on the cardiovascular system, because they did not alter arterial blood pressure in normotensive rats. Whether the decrease in arterial blood pressure produced by the inhibitors of Ras and MEK in DOCA-salt hypertensive rats is due to a decrease in peripheral vascular resistance and/or cardiac output remains to be established.

We have reported that the Ras/MAP kinase pathway contributes to the elevation of MABP in Ang II–dependent hypertension in rats. However, in the DOCA-salt model, Ang II levels are markedly diminished, and other vasoactive agents, such as catecholamines, vasopressin, and endothelin-1, contribute to the development of hypertension. Because these agents have also been reported to increase MAP kinase activity in vascular smooth muscle cells, it is possible that activation of the Ras/MAP kinase pathway promotes the development of hypertension in this model. It has been reported that endothelin-1 promotes DOCA-salt hypertension by stimulating the production of 20-HETE because the endothelin A receptor blocker BMS-182874 reduces arterial blood pressure and the urinary excretion of 20-HETE. Moreover, CYP450 inhibitors also reduced arterial blood pressure in DOCA-salt hypertensive rats. It is possible that the Ras/MAP kinase pathway is activated by 20-HETE production by endothelin-1 and other vasoactive agents in DOCA-salt hypertension. Supporting this view is our finding that ABT, which inhibited CYP450 activity (Figure 3B) and decreased the production of 20-HETE in the kidney, reduced arterial blood pressure and decreased Ras and MAP kinase activities in the hearts of DOCA-salt hypertensive rats. The effects of the various inhibitors used in this study to block Ras, MAP kinase, and CYP450 activation did not correlate with their effect to reduce MABP. Therefore, it is possible that other signaling mechanisms might also contribute to DOCA-salt–induced hypertension.

It is well established that hypertension produced by prolonged treatment with DOCA-salt in uninephrectomized rats is associated with nephroclerosis, enlargement of the heart and kidney, and vasculitis. Hypertrophy is an important feature of hypertension, and structural changes in vessel wall contribute to an increase in vascular resistance. In the present study, DOCA-salt treatment in rats also caused concentric hypertrophy of small arteries, multiple foci of fibrinoid necrosis in small and large arteries, and tubular atrophy within the kidney. In DOCA-salt hypertensive rats treated with the Ras inhibitor FPT III, there was no evidence of morphological alterations, such as hyperplastic arteriosclerosis, arterial necrosis, or interstitial scarring. The effect of FPT III to ameliorate structural alterations in the vasculature...


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