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The contribution of nitric oxide to renal vascular wall thickening in rats with L-NAME-induced hypertension

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Abstract We investigated the mechanisms of renal vascular wall thickening in a rat model of *N*-nitro L-arginine methyl ester (L-NAME)-induced hypertension. To separate the effects of L-NAME-induced hypertension from other effects of nitric oxide (NO) inhibition, we created two models of L-NAME-induced hypertension: both had the same blood pressure level but NO inhibition was moderate in one group (group M) and severe in the other (group S). Urinary excretion of nitrates and nitrites was lower in group S than in group M. Wall thickening and lipid deposition in renal vessels were significantly greater in group S than in groups M. Simple and multiple regression analyses indicated that renal vascular wall thickening was more strongly correlated with lipid deposition than with blood pressure. The number of vessels positive for staining with Sudan black B was negatively correlated with urinary NO excretion. Expression of fibronectin and transforming growth factor-β was greater in the Sudan black B-positive than in the Sudan black B-negative vessels, suggesting that extracellular matrix production was increased in vessels with lipid deposition. Lipid deposition and increased production of extracellular matrix may contribute to renal vascular wall thickening in L-NAME-induced hypertension. Some mechanisms independent of hypertension play important roles in vascular wall thickening induced by NO inhibition.

Key words Renal vascular wall thickening · Lipid deposition · NO inhibition · Extracellular

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Introduction

Nitric oxide (NO), which is synthesized from L-arginine by members of the family of nitric oxide synthase [15], is important in the regulation of blood pressure [13, 16]. Chronic inhibition of endothelial NO production by *N*-nitro L-arginine methyl ester (L-NAME) induces systemic hypertension in rats [20]. This model of hypertension is characterized by renal vascular wall thickening [1, 9, 17, 19]. Severe systemic hypertension is one mechanism of vascular wall thickening in L-NAME-induced hypertension, but other mechanisms may operate: for example. NO inhibition itself may contribute to the development of vascular wall thickening as NO suppresses the proliferation of smooth muscle cell [6].

Recently, Bouriquet et al. observed lipid disposition in renal preglomerular vessels in a rat model of L-NAME-induced hypertension [2]. NO inhibition has also been found to increase the uptake of low-density lipoprotein (LDL) by the aortic walls and the heart [3], and it is thus possible that vascular lipid deposition may be involved in the development of vascular wall thickening in L-NAME-induced hypertension. Phenotypic changes in smooth muscle cells induced by the LDL uptake may contribute to vascular wall thickening via increased production of extracellular matrix; this mechanism has been demonstrated in atherosclerotic lesions [18].

We investigated the role of NO inhibition in vascular wall thickening in a rat model of L-NAME-induced hypertension. To separate the effects of decreased NO production on the vascular walls from that of hypertension

induced by NO inhibition, we created two models of L-NAME-induced hypertension, in which the degree of hypertension was similar but the degree of NO inhibition differed.

Materials and methods

After a 5-day equilibration period, 5-week-old male SD rats (SLC, Hamamatsu, Japan) were divided into two groups. Rats with moderate NO inhibition (group M, *n*=10) were fed 18 g/day of a 23% protein diet and received 35 ml/day of deionized water with 60 mg/dl of L-NAME. Rats with severe NO inhibition (group S, *n*=11) were fed 18 g/day of a 6% protein diet and received 35 ml/day of deionized water with 7.5mg/dl of L-NAME. Blood pressure was measured by the tail-cuff method (UR-1000, Ueda Co., Tokyo, Japan) at 0, 1, 2, 3 and 4 weeks. Blood samples and 24-h urine samples were obtained after an overnight fast after 4 weeks. Both kidneys were then removed with rats under intraperitoneal pentobarbital sodium-induced anaesthesia (40 mg/kg body weight). Urine and plasma samples were stored at −30° C until analysed. The left kidney was fixed in 4% paraformaldehyde for light microscopy. The right kidney was snap-frozen in *n*-hexane cooled in dry ice-acetone and stored at −70° C for immunofluorescent microscopy and Sudan black B staining (NO_x).

The urinary nitrate+nitrite (NO_x) concentrations was measured using the Griess reagent after nitrates had been quantitatively converted to nitrites by nitrate reductase [7]. Plasma levels of total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were measured enzymatically. Plasma and urinary levels of creatinine were measured by the Jaffe reaction.

To quantify vascular wall thickening the left kidney was fixed in 4% paralformaldehyde. Paraffin-embedded specimens were cut into 2-μm-thick sections and stained with periodic acid–Schiff and Masson trichrome stain. We examined 200 vessels in the cortical layer, including the arcuate arteries, interlobular arteries and arterioles; for each animal we graded renal arterial and arteriolar wall thickening on a 4-point scale as follows: 0, no thickening; 1+, mild vascular wall thickening; 2+, moderate thickening, 3+, severe thickening with severe luminal stenosis or occlusion. We investigated differences between the two groups in the mean group scores for vascular wall thickening and the frequency of vessels with grade 3 wall thickening.

Kidney samples cut into 8-μm-thick sections were stained with Sudan black B. Sections were washed in distilled water, exposed to 50% ethanol for 5 min, and then stained with a filtered solution of Sudan black B (Wako Pure Chemial Industries, Osaka, Japan) in 70% ethanol for 20 min. After being washed in 50% ethanol, sections were counterstained with nuclear fast red and mounted in a glycerol solution. Approximately 100 arcuate arteries or interlobular arteries from each specimen were examined to calculate the number of Sudan black B-positive vessels.

We examined the topographical relations between vascular lipid deposits and vascular localization of α-smooth muscle actin (α-SMA), fibronectin (FN), transforming growth factor-β1 (TGF-β), and apolipolprotein B (ApoB) in four pairs of consecutive 4-μm and 8-μm cryostat sections. The 4-μm-thick sections were examined by immunofluorescent microscopy, and the 8-μm-thick sections were used for Sudan black B staining. For immunofluorescent microscopy, 4-μm cryostat sections were placed on glass slides and stained with mouse monoclonal FITC-conjugated anti-α-SMA antibody (Sigma, St. Louis, Mo.), rabbit polyclonal anti-rat FN antibody (Chemicon International, Temecula, Calif.) rabbit polyclonal anti-human TGF-β antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) or goat polyclonal anti-ApoB antibody (Rockland, Gilbertsville, Pa.). Sections were exposed to these antibodies for 30 min at 37° C. Sections exposed to primary antibodies, except for the FITC-conjugated antibody, were incubated with FITC-labelled anti-rabbit (Organon Teknika Corporation, Durham, N.C.), anti-mouse (Organon Teknika, Durham, N.C.), or anti-goat antibody (Sigma), depending on the primary antibody used.

For immunoelectron microscopy, kidney tissues were cut into small pieces and fixed by immersion in periodate lysin-paraformaldehyde fixative [12].They were embedded in Lowicryl K4M at −30° C and were polymerized by ultraviolet light (Model B-100A Black-Ray Ultraviolet Lamp UVP, Upland, Calif.) at −30° C for 48 h. Immunogold staining was performed as described previously [23]. Briefly, thin sections of Lowicryl K4M-embedded tissue were mounted on 300-mesh nickel grids and stained with mouse monoclonal anti-α-SMA antibody (Sigma), rabbit polyclonal anti-rat FN antibody (Chemicon International), or goat polyclonal anti-ApoB antibody (Rockland, Gilbertsville, Pa.) for 60 min, followed by 10-nm gold-labelled anti-rabbit, anti-mouse, or anti-goat antibody (Sigma), depending on the primary antibody used. The specimens were then stained with uranyl acetate for 5 min and examined with a JEOL 100 CX (Nihon Denshi, Tokyo, Japan).

Data are presented as mean±SD. Differences between groups M and S were assessed by the unpaired Student's *t*-test. Correlations between the degree of vascular wall thickening, the frequency of Sudan black B-positive vessels, and other analytical data were determined by linear regression analysis. Multiple regression analysis was performed to assess the influences of vascular lipid deposition, blood pressure, urinary NO_x excretion, and the plasma level of total cholesterol on vascular wall thickening. A *P*-value <0.05 was accepted as indicating statistical significance.

Results

The mean blood pressure was significantly higher at 1, 2, 3 and 4 weeks after L-NAME treatment than the baseline blood pressure in both groups. There was no significant difference in the mean blood pressure between groups at any time point. Urinary NO_x excretion was lower in groups M and S than in age-matched normal SD rats (6.0±1.0 μmol/day; $P<0.001$), and the decrease in urinary NO_x excretion was greater in group S than in group M (Table 1). Creatinine clearance was significantly lower in group S than in group M. Plasma levels of total cholesterol and HDL cholesterol were significantly higher in group S than in group M. There was no significant difference in the plasma level of triglycerides between groups.

Table 1 Clinical and laboratory data (mean±SD) at 4 weeks

	Group M[a] (*n*=10)	Group S[a] (*n*=11)	*P*
Blood pressure (mm Hg)	151±26.8	154±19.6	NS
Urinary NO_x (μmol/day)	1.54±0.98	0.56±0.44	<0.05
Creatinine clearance (L/day)	1.64±0.72	1.09±0.48	<0.05
Total cholesterol (mg/dl)	66.2±16.0	89.6±21.2	<0.01
HDL cholesterol (mg/dl)	23.1±9.34	31.7±9.64	<0.001
Triglycerides (mg/dl)	71.1±30.4	67.1±50.2	NS

[a] Group M, moderately suppressed NO; group S, severely suppressed NO

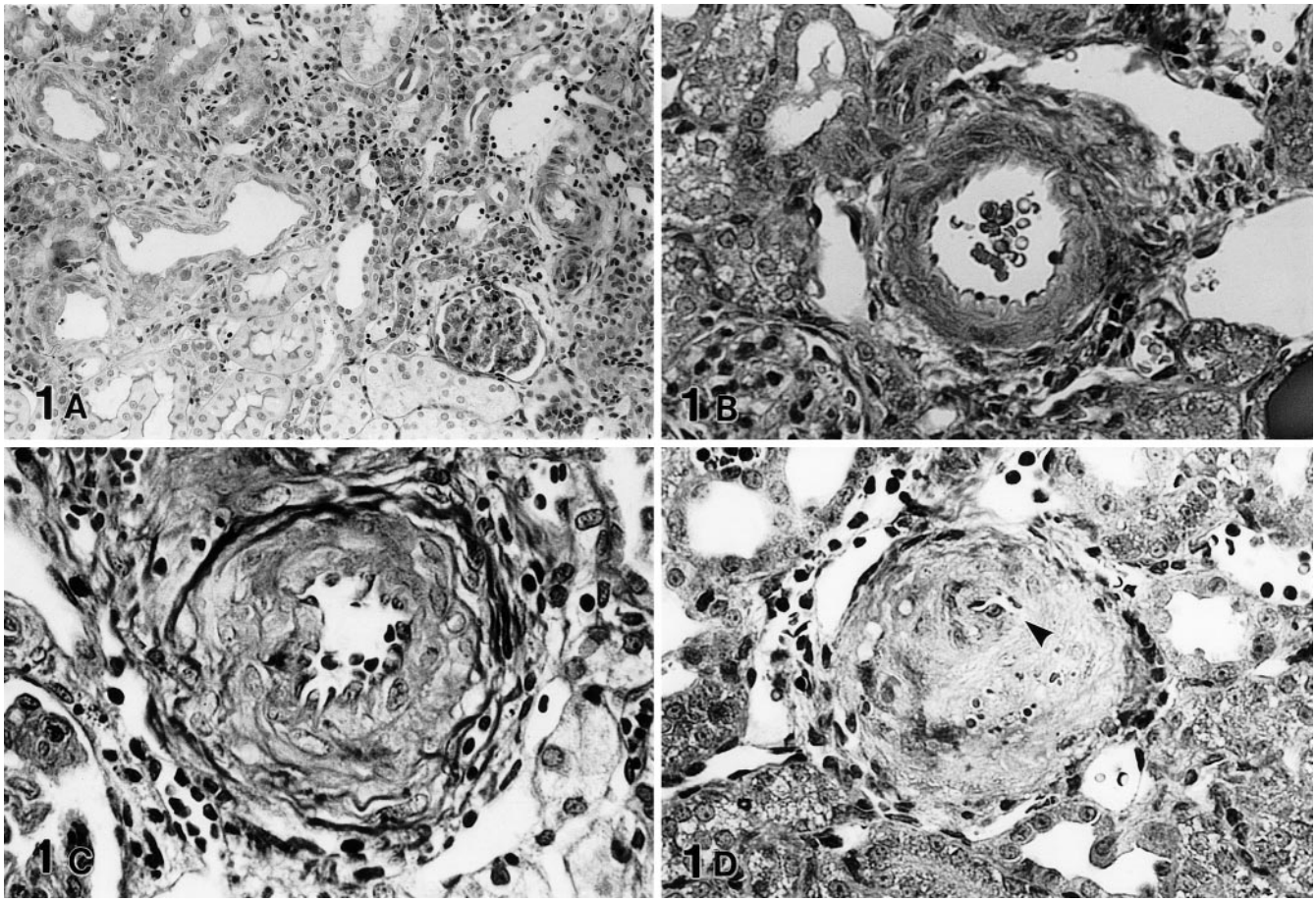

Fig. 1A–D Light micrographs showing the renal cortex from a rat with severe NO suppression. **A** Focal tubular atrophy and glomerular collapse were also observed. **B** Mild, **C** moderate and **D** severe arterial wall thickening with severe luminal stenosis (*arrowhead*) were observed. **A** Periodic acid-Schiff, ×100. **B**, **C**, **D** Masson trichrome, ×400

Table 2 Simple regression analysis of the correlations between renal vascular changes and other parameters in all rats (n=21)

Parameter	Score for renal vascular wall thickeninig		Frequency of Sudan black B-positive vessels	
	r	*P*	*r*	*P*
Blood pressure	0.531	<0.05	0.266	NS
Total cholesterol	0.478	<0.05	0.627	<0.01
HDL cholesterol	0.372	NS	0.498	<0.05
Triglycerides	–0.111	NS	–0.114	NS
Urinary NO_x	–0.553	<0.05	–0.557	<0.05
Score for renal vascular wall thickening	–	–	0.722	<0.001
Frequency of Sudan black B-positive vessels	0.722	<0.001	–	–

Mild, moderate, and severe arterial wall thickening (Fig. 1B–D) was noted in renal arterial vessels in rat from both groups. The score for renal vascular wall thickening was significantly higher in group S (1.80±0.34) than in group M (1.41±0.16; P<0.01). Vessels with severe vascular wall thickening were observed in 1.8±2.1% of group M rats, as opposed to 22.6±22.6% of group S rats (P<0.05). Focal tubular atrophy and focal glomerular collapse were observed in both groups (Fig. 1A), but were more pronounced in group S.

In some renal vessels in both groups, vascular walls showed marked staining with Sudan black B (Fig. 2). Some vessels in group S rats were entirely encircled by Sudan black B-positive lesions (Fig. 2D). The frequency of Sudan black B-positive vessels was significantly greater in group S (35.4±15.5%) than in group M (9.4±5.3%; P<0.001). Tubular cells showed positive staining with Sudan black B, while glomerula cells were negative for Sudan black (Fig. 2A).

Simple regression analysis showed that the score for renal vascular wall thickening was significantly correlated

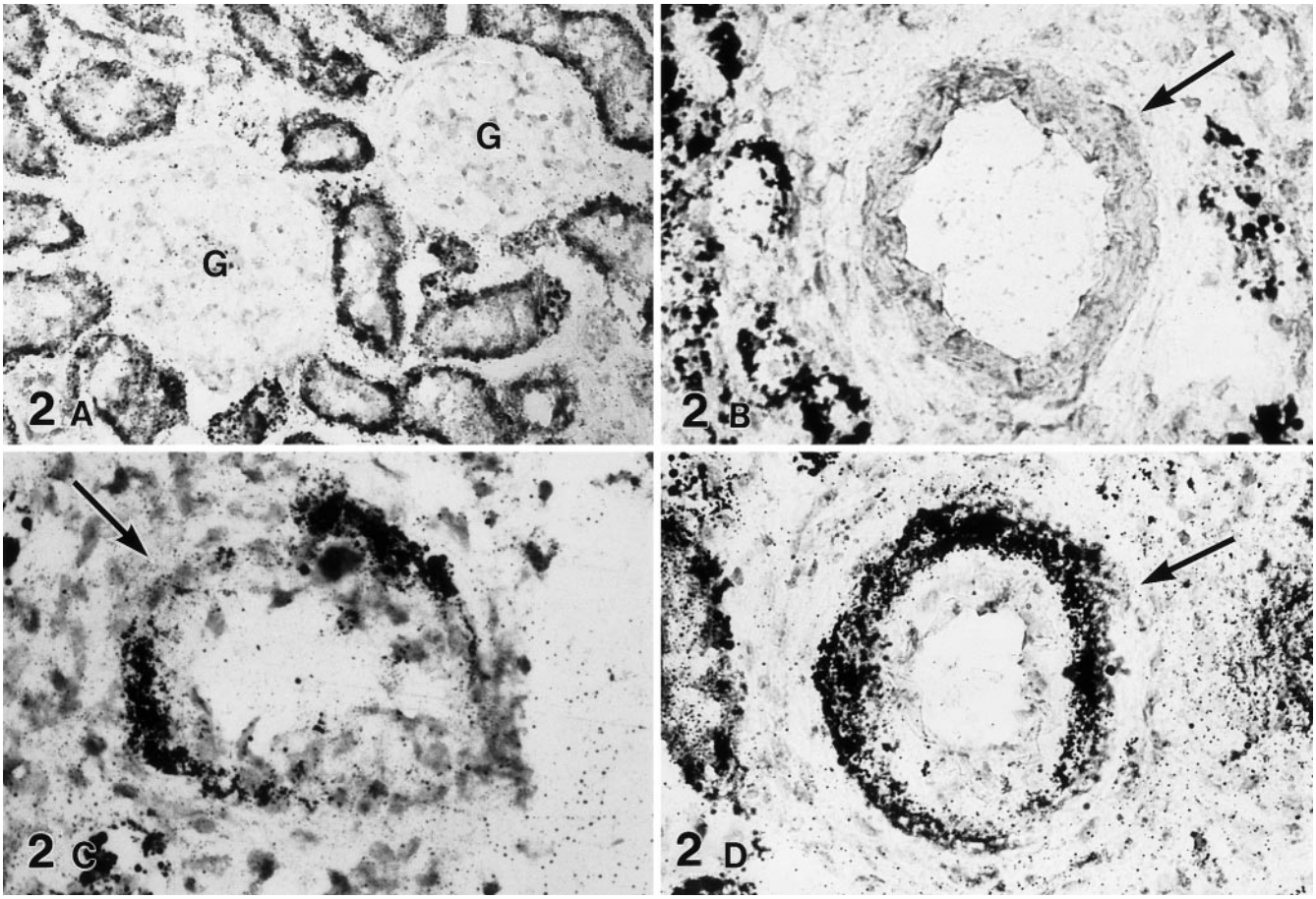

Fig. 2A–D Sudan black B staining of renal vessels from a rat with severe NO suppression (**B**, **D**) and a renal vessel from a rat with moderate NO suppression (**C**). Sudan black B-negative vessels (**B**, *arrow*) and Sudan black B-positive vessels (**D**, *arrow*) were observed in the same section. Lipid deposition in vascular walls was weaker in the rat in group M (**C** *arrow*) than in the rat in group S. Tubular cells, but not glomerular cells, were labeled with Sudan black B (**A**). *G* glomerulus. **A** ×100 **B, D** ×200 **C** ×400

Table 3 Multiple regression analysis of factors affecting vascular wall thickening (*n*=21). Multiple correlation= 0.8073 (*B* standardized regression coefficients, *P* predicted value)

Variables		B	P
Dependent	Independent		
Vascular wall thickening	Frequency of Sudan black B-positive vessels	0.0110	0.0083
	Blood pressure	0.0047	0.0277
	Urinary NO_x excretion	–0.0350	0.5875
	Plasma total cholesterol	–0.0001	0.9776

with the systemic blood pressure, the plasma level of total cholesterol, and the frequency of Sudan black B-positive vessels (Table 2). Vascular wall thickening was negatively correlated with urinary NO_x excretin. The frequency of Sudan black B-positive vessels was positively correlated with the plasma level of total cholesterol and HDL cholesterol. The frequency of Sudan black B-positive vessels was negatively correlated with urnary NO_x excretion. There was no significant correlation between the frequency of Sudan black B-positive vessels and the systemic blood pressure. Multiple regression analysis showed that renal vascular wall thickening in rats with L-NAME-induced hypertension was more strongly correlated with renal vascular lipid deposition than with the blood pressure (Table 3).

Positive staining for ApoB was observed in the vascular walls of Sudan black B-positive vessels; the distribution of ApoB was consistent with the distribution of Sudan black B-positive lesions (Fig. 3A, B), indicating that the lipids deposited in the renal vascular wall included LDL. Renal vessels showed strongly positivity for anti-α-SMA in both groups. Neither glomeruli nor tubules were labelled with anti-α-SMA. Anti-α-SMA positivity was weaker in Sudan black B-positive lesions than in Sudan black B-negative vessels (Fig. 4A–D). FN staining

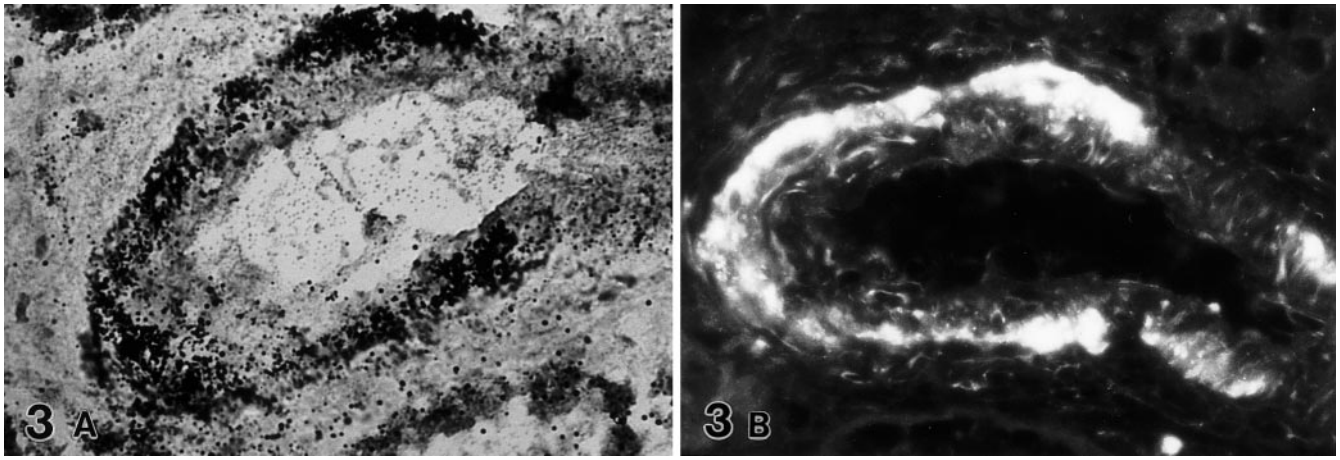

Fig. 3 Light micrographs showing **A** Sudan black B staining and **B** immunostaining with anti-ApoB antibody in consecutive cryostat sections from the kidney of a rat with severe NO suppression. Intravascular localization of ApoB was consistent with the localization of Sudan black B-positive lesions. ×200

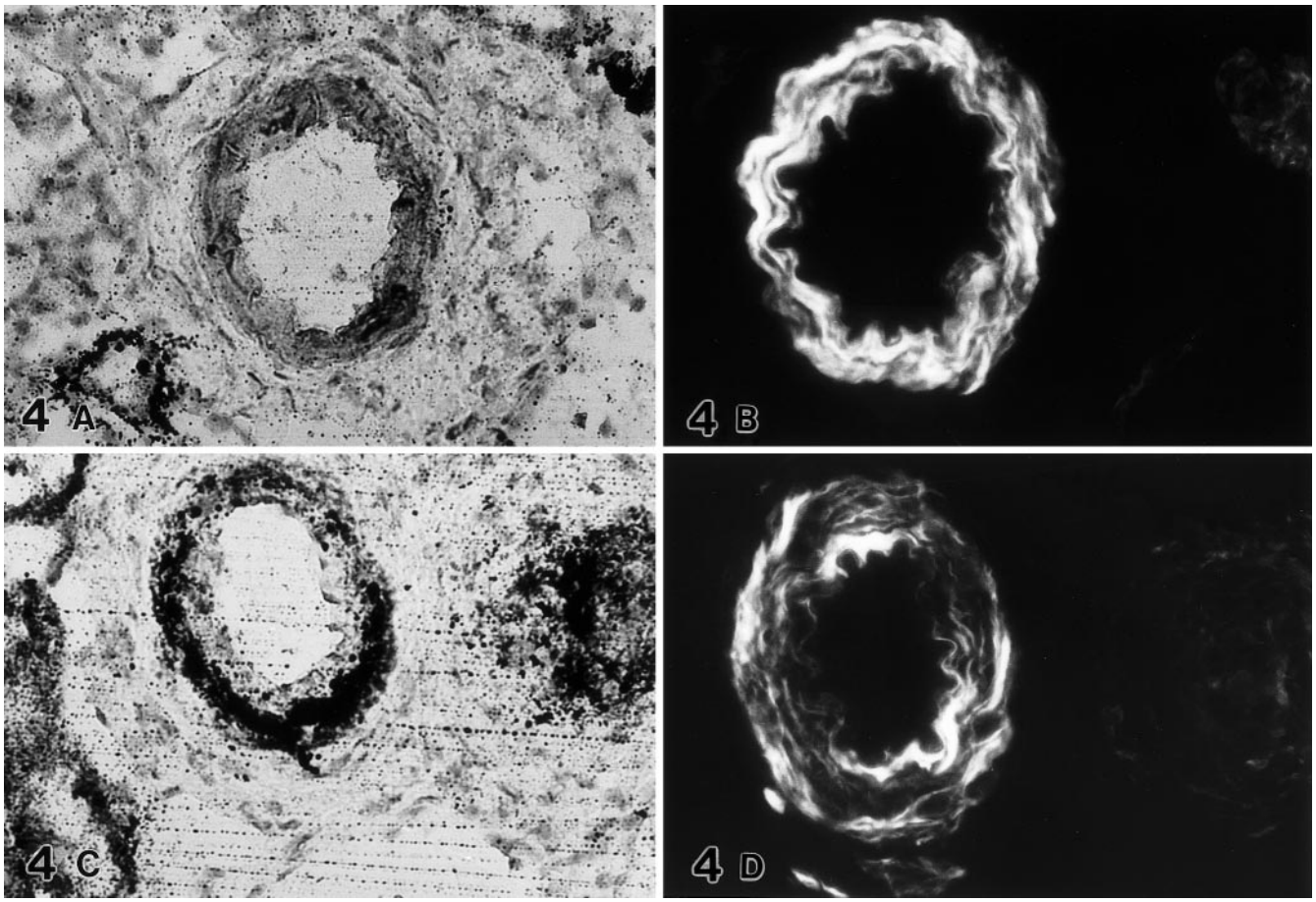

Fig. 4 Light micrographs showing Sudan black staning (**A**, **C**) and immunostaining with anti-α-SMA (**B**, **D**) in consecutive cryostat sections from the kidney of a rat with severe NO suppression. The Sudan black B-negative vessel (**A**) showed strong labeling with α-SMA (**B** same vessel as **A**). The Sudan black B-positive vessel (**C**) showed weak labeling with α-SMA (**D** same vessel as **C**). ×200

was observed in the glomerular mesangial area, the interstitium and the vascular wall in both groups. FN positivity was stronger in Sudan black B-positive vessels than in Sudan black B-negative vessels (Fig. 5A–5D). Weak labelling with anti-TGF-β was observed in vascular walls in both groups. Anti-TGF-β positivity was stronger in Sudan black B-positive lesions than in the Sudan black B-negative vessels (Fig. 6A–D).

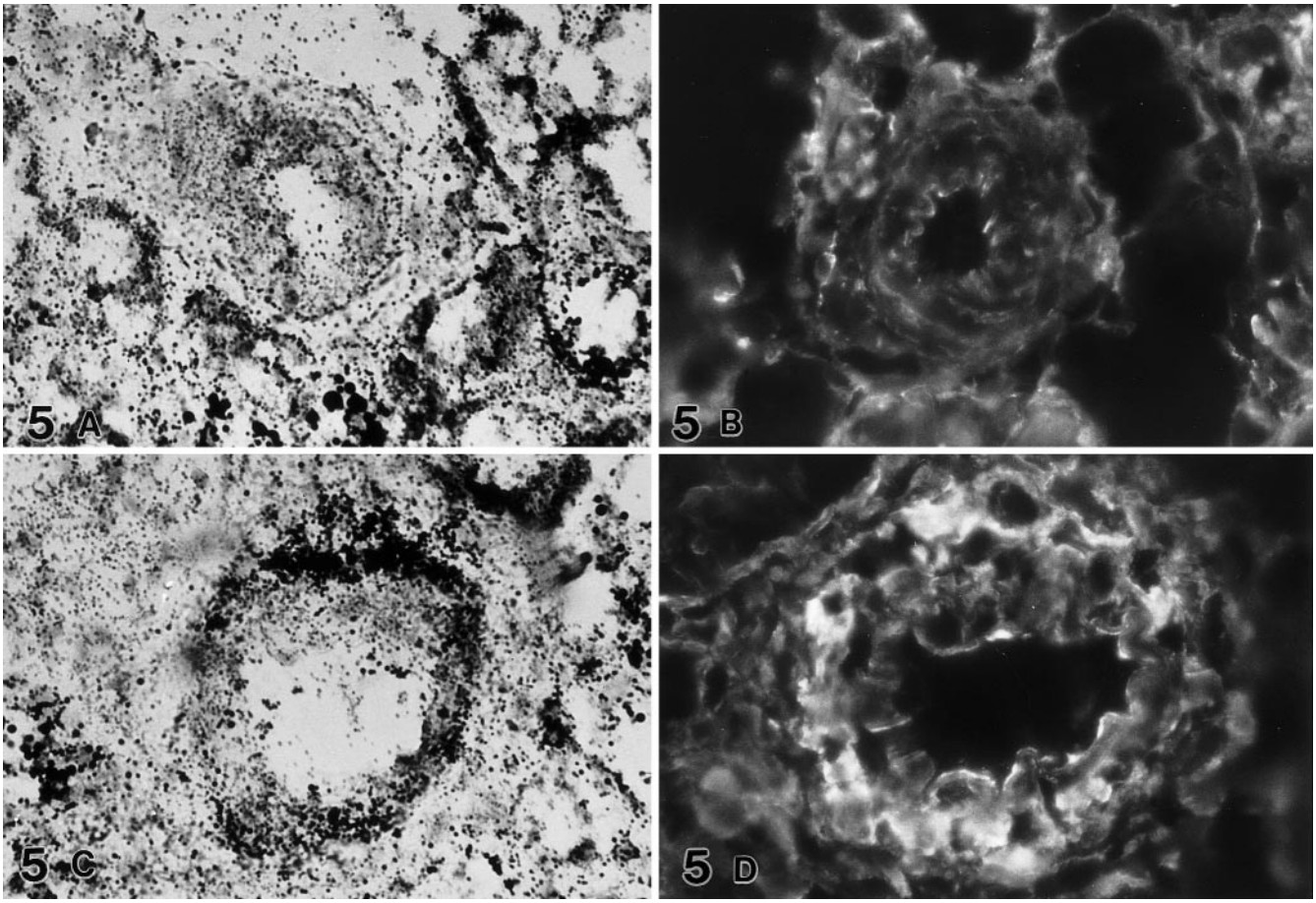

Fig. 5 Light micrographs showing Sudan black B staining (**A**, **C**) and immunostaining with anti-FN antibody (**B**, **D**) in consecutive cryostat sections from the kidney of a rat with severe NO suppression. The Sudan black B-negative vessel (**A**) showed weak labeling with FN (**B** same vessel as **A**). The Sudan black B-positive vessel (**C**) showed stronger immunolabeling with FN (**D** same vessel as **C**). ×200

Immunoelectron microscopy showed marked α-SMA labelling in the smooth muscle cell cytoplasm in kidneys from normal rats (Fig. 7A). FN and ApoB were not detected in renal vascular walls from normal rats. Less strong anti-α-SMA positivity was observed in smooth muscle cells from rats in both groups with L-NAME-induced hypertension, particularly at the endothelial side of smooth muscle cells (Fig. 7B). FN was observed around smooth muscle cells (Fig. 7C), and weak ApoB staining was present in the smooth muscle cell cytoplasm (Fig. 7D) in kidney specimens from hypertensive rats.

Discussion

In a preliminary study, we found a correlation between the degree of vascular wall thickening and the dose of L-NAME administered; administration of a higher dose of L-NAME was also associated with a higher blood pressure. It was not clear whether vascular wall thickening was induced by suppressed NO production or by high blood pressure, and we therefore carried out a trial to dissociate the effects of suppressed NO production and high blood pressure, finding that a low-protein diet enhanced the L-NAME-induced increase in blood pressure. A 23% protein diet plus 60 mg/dl L-NAME and a 6% protein diet plus 7.5 mg/dl L-NAME induced similar degrees of hypertension with different levels of NO inhibition. In the current study we used these models of hypertension with suppressed NO production, and we expected, blood pressures were the same in both groups of hypertensive rats but the degree of NO inhibition (evaluated by urinary NO_x excretion) differed. It is not clear why there was no difference in blood pressure between rats with severe NO inhibition and rats with moderate NO inhibition. The low-protein diet in group S may have affected blood pressure via factors other than NO production [4, 19].

Vascular wall thickening was more severe in rats with severe NO inhibition than in rats with moderate NO inhibition. Although the semiquantitative histological method used in this study might not be very convincing, we tried to offset the weakness of this method by increasing the number of vessels studied. There was a significant negative correlation between renal vascular wall thickening and urinary NO_x excretion. These observatios suggest that NO inhibition itself may contribute to thickening of the renal vascular wall.

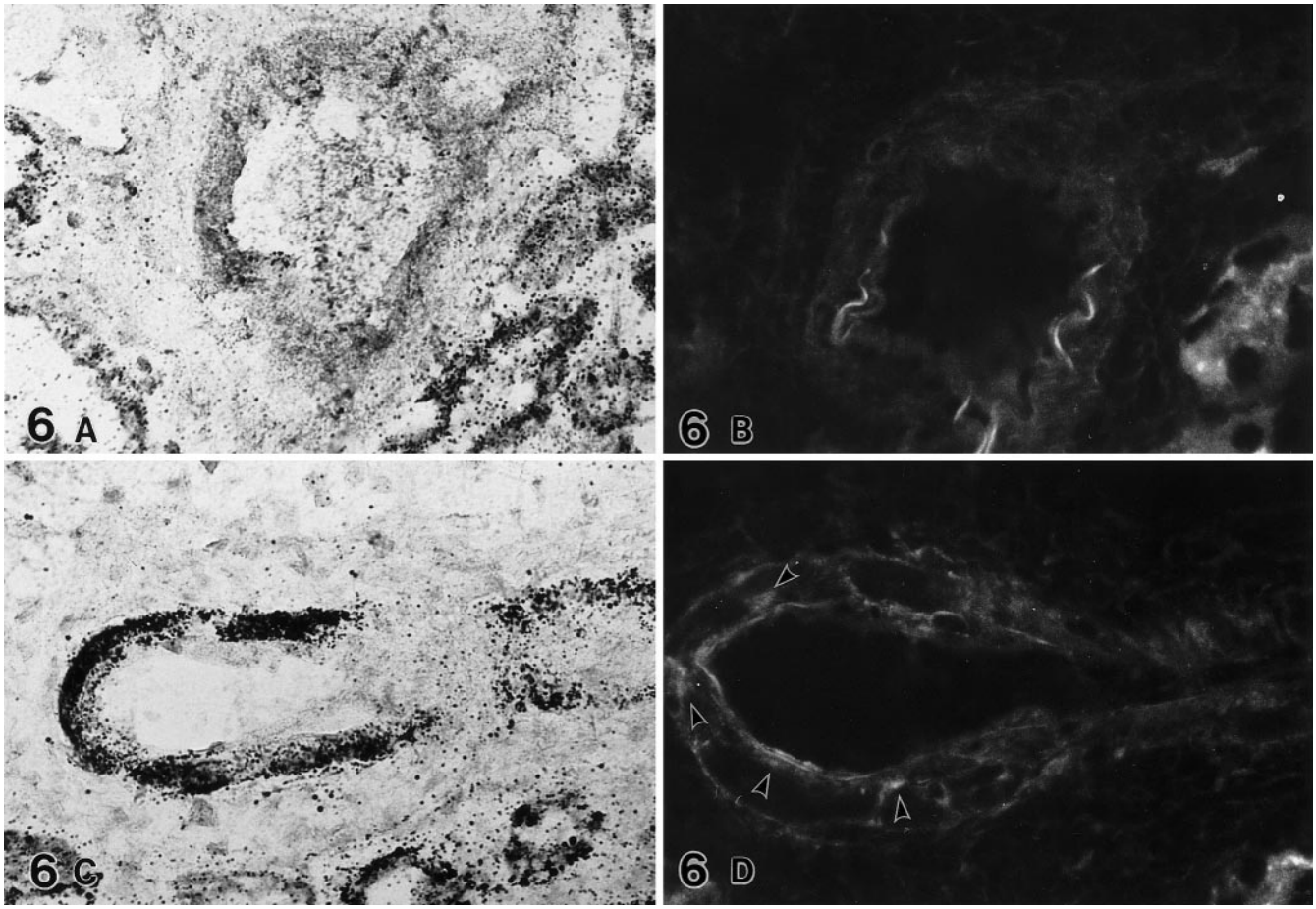

Fig. 6 Light micrographs showing Sudan black B staining (**A**, **C**) and immunostaining with anti-TGF-β antibody (**B**, **D**) in consecutive cryostat sections from the kidney of a rat with severe NO suppression. The Sudan black B-negative vessel (**A**) showed weak labelling with TGF-β (**B** same vessel as **A**). The Sudan black B-positive vessel (**C**) showed increased immunolabelled with TGF-β (**D** *arrowheads*, same vessel as **C**). ×200

Lipid deposition was observed in renal vessels in rats with L-NAME-induced hypertension, which was previously reported by Bouriquet et al. [2]. Linear regression analysis demonstrated that renal vascular wall thickening was strongly correlated with lipid deposition in renal vascular walls; multiple regression analysis showed that renal vascular wall thickening was more strongly correlated with lipid deposition than with the blood pressure level. These observations suggest that lipid deposition has a greater influence on vascular wall thickening than an increase in blood pressure in the presence of L-NAME-induced hypertension.

Lipid deposition in the vascular walls was significantly correlated with urinary excretion of NO_x, but not with blood pressure. The frequency of Sudan black B-positive vessels was significantly greater in rats with severe NO inhibition. The localization of ApoB corresponded to the distribution of Sudan black B-positive lesions, suggesting that the lipid detected by Sudan black B staining may have been LDL. Cardona-Sanclemente et al. have observed increased uptake of LDL by the aortic walls and the heart in L-NAME-treated rats [3]. These findings suggest that inhibition of NO synthesis may increase LDL uptake by renal vascular walls, resulting in an increase in Sudan black B-positive vessels.

In the present study, labelling for α-SMA was decreased in Sudan black B-positive vessels. Severe NO inhibition was associated with decreased expression of α-SMA. Ono et al. also observed decreased α-SMA staining in the injured afferent arterioles, with fibrinoid degeneration in L-NAME-treated rats [14]. The mechanisms that suppress the α-SMA expression in L-NAME-treated rats are not clear. Kawada et al. [10] showed that NO suppressed α-SMA expression in rat hepatic stellate cells, so that it is possible that the reduction in α-SMA positivity in L-NAME-treated rats may not have been caused by the direct effects of decreased level of NO. The phenotypic change from the contractile to the synthetic state in smooth muscle cells is associated with the production of several growth factors and extracellular matrix [11, 18, 21]. The decreased expression of α-SMA in vascular walls with lipid deposition suggests that lipid deposition induced phenotypic changes in smooth muscle cells, which has been demonstrated in atherosclerotic lesions [5, 8, 22]. Staining for FN and TGF-β was greater in Sudan black B-positive vessels than in Sudan black B-negative vessels. The increases in TGF-β and components of the extracellular matrix, in-

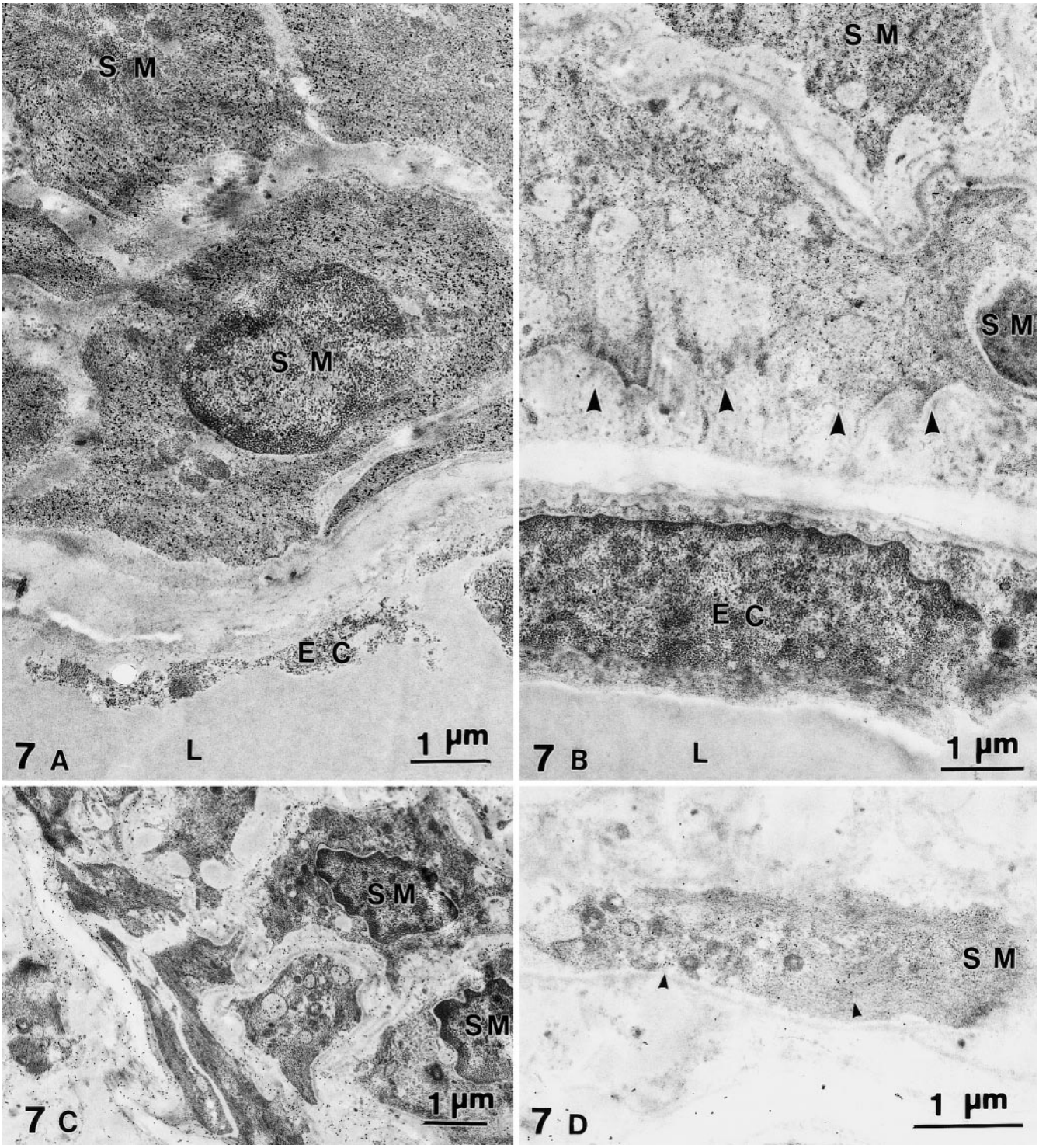

Fig. 7A–D Electron microscopic localization of α-SMA, FN and ApoB in renal vascular walls from normal rats and rat with severe NO suppression using immunogold staining. **A** In the normal rat, smooth muscle cell cytoplasm showed marked α-SMA labelling. ×13000. **B** In severe NO suppression, the number of gold particles was decreased, indicating decreased expression of α-SMA in the smooth muscle cell cytoplasm, particularly at the endothelial side of smooth muscle cells (*arrowheads*). ×13000. **C** FN labelling was observed around smooth muscle cells in the rat with severe NO suppression. ×10000. **D** Weak ApoB labelling was observed in the smooth muscle cell cytoplasm (*arrowheads*). (*EC* endothelial cell, *L* lumen, *SM* smooth muscle cell). ×17000

cluding FN, may have contributed to vascular wall thickening.

The present findings suggest that renal vascular wall thickening in rats with L-NAME-induced hypertension is induced not only by an increased in blood pressure but also by decreased NO synthesis. They also suggest that lipid deposition plays an important part in the development of vascular wall thickening. Inhibition of NO synthesis may stimulate LDL uptake by smooth muscle cells and may also stimulate the phenotypic change from a

contractile to a synthetic state, which induces increased production in extracellular matrices.

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