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Correction of hypertension by normalization of endothelial levels of fibroblast growth factor and nitric oxide synthase in spontaneously hypertensive rats

PEDRO CUEVAS*, MARGARITA GARCÍA-CALVO†, FERNANDO CARCELLER*, DIANA REIMERS*, MERCEDES ZAZO†, BEGOÑA CUEVAS*, ISABEL MUÑOZ-WILLERY†, VICTORIA MARTÍNEZ-COSO*, SANTIAGO LAMAS†, AND GUILLERMO GIMÉNEZ-GALLEGO†‡

*Hospital Universitario Ramón y Cajal, Carretera de Colmenar, 28034 Madrid, Spain; and †Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas Velázquez, 144, 28006 Madrid, Spain

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ABSTRACT **Acidic and basic fibroblast growth factors (FGFs) share a wide range of diverse biological activities. To date, low levels of FGF have not been correlated with a pathophysiologic state. We report that blood vessels of spontaneously hypertensive rats are shown to be associated with a marked decrement in endothelial basic FGF content. This decrement correlates both with hypertension and with a decrease in the endothelial content of nitric oxide synthase. Restoration of FGF to physiological levels in the vascular wall, either by systemic administration or by *in vivo* gene transfer, significantly augmented the number of endothelial cells with positive immunostaining for nitric oxide synthase, corrected hypertension, and ameliorated endothelial-dependent responses to vasoconstrictors. These results suggest an important role for FGFs in blood pressure homeostasis and open new avenues for the understanding of the etiology and treatment of hypertension.**

Acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) are homologous polypeptides (55% amino acid sequence identity) that were first isolated on the basis of their ability to induce mitogenesis on murine BALB/c 3T3 fibroblasts. aFGF was also isolated as an endothelial cell growth factor. It has become progressively evident that FGFs are mitogens of remarkably broad mitogenic activity. In fact, they induce cell division in almost all the mesoderm- and neuroectoderm-derived cell lines in culture. It has also been shown that these proteins share diverse hormone-like activities, including vasodilation. So far, no clear-cut differences have been found in the biological spectrum of activities of these polypeptides. FGFs have been also called heparin binding growth factors because of their characteristic high affinity for heparin and heparan sulfate (for reviews, see refs. 1–3). It has been shown in rats and rabbits that FGFs cause an acute vasodilation when they are administered systemically at the level of subnanomoles per kilogram of body weight (4). FGF-induced vasorelaxation is partially abrogated by inhibitors of the synthesis of nitric oxide (NO). Endothelial-derived NO is one of the most important regulators of vascular tone (5). The synthesis of NO is catalyzed in endothelial cells by a distinct, constitutive NO synthase (ecNOS; ref. 6).

The spontaneously hypertensive (SH) rat strain was developed by Okamoto and Aoki (7) by mating a couple of SH rats from the normotensive Wistar–Kyoto (WKY) strain. In SH rats, hypertension develops quite abruptly between weeks 5 and 20 and worsens as they age. The developmental course of hypertension makes SH rats an adequate model of essential hypertension (8, 9). SH rats have been, consequently, the object of numerous studies. Deep neuroendocrine alterations that could cause blood pressure elevation have been described in these rats. Thus, alterations in the levels of vasoactive intestinal polypeptide, vasopressin, adrenergic amines, and angiotensin II have been reported (10–14). On the other hand, vascular hypertophy or hyperplasia, histological changes also related to the development of hypertension, are clearly evident in these rats 3 months after birth (8). Consequently, hypertensive mechanisms in SH rats have been attributed to both neurogenic and nonneurogenic factors (8). Nevertheless, the initial mechanism that triggers the accumulation of all such anomalies has not been clearly shown. We explored whether the vasodilatory activity of FGF could ameliorate the hypertensive condition of SH rats. Treatment of these rats with FGFs showed that these polypeptides have a long-term regulatory effect on arterial blood pressure in addition to the acute one previously described (4). Our results also demonstrate that hypertension in SH rats develops as their endothelium becomes depleted of FGF. A close, direct correlation between endothelial FGF and ecNOS levels was subsequently observed. Finally, replenishment of the endothelium-associated FGF pool corrected hypertension and reduced the increased sensitivity to phenylephrine of mesenteric resistance vessels. This correction was accompanied by restoration of ecNOS levels within the endothelium.

MATERIALS AND METHODS

Animals. SH rats were purchased from Iffa Credo. Normotensive WKY rats were obtained from the animal facilities in one of our institutions. During the adaptation period and after the experiments, the rats were kept in a temperature-controlled room under continuous 12-hr cycles of light and darkness (light period 7 a.m. to 7 p.m.) with free access to standard chow food and tap water.

aFGF. aFGF was prepared and its purity controlled as described (4, 15). Digested aFGF was prepared by treating 50 μg of aFGF in 250 μl of 10 mM sodium phosphate, pH 7.2/1.5 M NaCl with a mixture 1:1 of trypsin and V-8 endoproteases (Boehringer Mannheim, sequencing grade) for 24 hr at 25°C. Proteases were added at the beginning and after 6 hr of digestion (5 μg of protein each time). SDS/PAGE of the digested protein did not show any detectable protein band.

Blood Pressure Measurement. Long-term pressure changes were monitored by measuring systolic and diastolic blood pressures by the tail-cuff method without anaesthesia using an

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Abbreviations: FGF, fibroblast growth factor; aFGF, acidic FGF; bFGF, basic FGF; ecNOS, endothelial cell constitutive NO synthase; MAP; mean arterial blood pressure; SH, spontaneously hypertensive; WKY, Wistar–Kyoto.

‡To whom reprint requests should be addressed.

ad hoc pressure monitor (Letica Scientific Instruments, Barcelona; model LE500). All measurements were carried out at the same time of the day and by the same person, according to described procedures (16). Briefly, rats were prewarmed at 50°C for 3–5 min in a warm box and then introduced in an acrylic restraining cage placed on top of a heating plate at 37°C. Measurements started when the rats rested quietly in the cage once they got used to the new environment (5–10 min). Ten consecutive blood pressure determinations were recorded and were averaged after discarding the highest and lowest values. Mean arterial blood pressure was estimated from systolic and diastolic pressures. The values of the mean arterial pressure estimated both directly and by the tail-cuff method differed by less than 10% (4).

Immunological Procedures. Immunostaining of histological sections was carried out as described (17). Anti-bFGF serum (titer 1:10,000, as determined by ELISA immunoassay) was prepared according to procedures already described (18). No immunoreactivity was observed when this serum was preincubated overnight with an excess of bFGF (3.4 μg of growth factor per μl of serum, approximately a 3.5-fold molar excess of growth factor to immunoglobulin) at 4°C for 24 hr, whereas the immunoreactivity remained unaffected when the treatment was carried out with aFGF. The preparation of anti-aFGF (titer 1:25,000, as determined by ELISA) has been already described (18). In the experiments reported here anti-aFGF antibodies were separated from the serum by chromatography onto protein-A Sepharose. Immunoglobulin α-chain was eluted from the column with 0.1 M glycine (pH 3) after extensive washing with 0.1 M sodium phosphate (pH 8), dialyzed two times for 24 hr against 100 volumes of 0.1 M ammonium bicarbonate, and lyophilized. These antibodies did not react above the background level with bFGF in ELISA immunoassays, and as in the case of anti-bFGF serum, they were blocked by aFGF but remained unaffected by bFGF when preincubated as described above. The anti-ecNOS antibody has been described elsewhere (19), and it does not crossreact with protein extracts of cells expressing inducible NOS. The number of positive and negative immunoreactive endothelial cells per cross section of thoracic aorta (6 μm thick) of each rat is the average of the counts in three different sections 60 μm apart from each other.

Gene Transfection. Plasmid:liposome complexes were prepared as described (20), using L-α-phosphatidylethanolamine (Sigma) and 3β[*N*-(*N'*,*N'*-dimethylaminoethane)-carbomoyl]-cholesterol that was synthesized as described (21). Plasmid pMAM*neo* was supplied by CLONTECH. To build plasmid pMAMaFGF, the aFGF gene was digested from plasmid pMG47 (15) with restriction endonucleases *Nco*I and *Hin*dIII and positioned under the control of the strong RSV-LTR promoter by inserting it between restriction sites *Nhe*I and *Sal*I of plasmid pMAM*neo*, after adding the appropriate linkers. Rats (18 months old and weighing 350–400 g) received 750 μg of DNA in a single 4-ml bolus through the external jugular vein, at a ratio of 1 μg to 11 nmol of liposomal lipid.

Effect of Phenylephrine on Isolated Mesenteric Arteries. Rats were killed by stunning followed by cervical dislocation. Vessels (2 mm long) with a mean internal diameter of 180–270 μm (third generation) were dissected in ice-cold Krebs solution from the superior mesenteric bed at 8–10 cm from the pylorus. To measure isometric tension, vessels were mounted in a 410 A myograph (Trading, Denmark) after prewarming at 37°C. After a 1-hr equilibration period in the myograph chamber, the internal circumference was set to 90% of that of the relaxed vessel with a transmural pressure of 100 mmHg (1 mmHg = 133 Pa). The vessels were kept for an additional hour under these conditions and were then stimulated for 2 min with prewarmed (37°C) Krebs solution in which NaCl was exchanged for KCl on an equimolar basis. They were then rinsed with prewarmed Krebs (37°C), and phenylephrine was added to induce contraction. Solutions in the myograph were continuously bubbled with a mixture of 95% O_2/5% CO_2.

RESULTS AND DISCUSSION

Long-Term Effect of Intravenous Administration of aFGF to SH Rats. Systemic administration of aFGF to anesthetized SH rats in early and late phases of hypertension (5- and 14-month-old, respectively) induced an acute decrease of their mean arterial blood pressure (MAP) in a dose-dependent manner similar to that described for normotensive rats (not shown; ref. 4). This effect was also transient, the pressure returning to its basal levels 1 hr after injection of the protein. However, we observed that 24 hr after aFGF administration, MAP averaged ≈30 mmHg less than pretreatment levels in both the 5- and 14-month-old SH rats. The effect is illustrated in Fig. 1*A*. This long-term MAP decrease was not observed in the normotensive WKY parent rat strain (Fig. 1*B*). Similar results were also obtained with bFGF (not shown). The FGF preparations used in our experiments contained a single polypeptide by SDS/PAGE, reversed-phase HPLC, and amino acid analysis criteria. The long-term hypotensive properties of the preparation disappeared when it was either heated (not shown) or treated with a mixture of high purity trypsin and V8 endoproteases (Fig. 1*A*). Thus, the effect seems specific of FGF.

Endothelial Levels of FGF in SH Rats. It has been shown that circulating levels of FGFs decrease rapidly after a systemic bolus because they are rapidly sequestered by heparin-like molecules in the luminal surface of the endothelium, a tissue in which bFGF accumulates at considerable levels in physiological conditions (22–24). Although aFGF is not expressed at detectable levels in endothelial cells, it is also capable of stimulating their growth (1–3). Thus, it is possible that its systemic injection could compensate for a potential bFGF deficit in SH rats. Several histological anomalies in the endothelial cells of the femoral and thoracic aorta arteries from SH rats have been reported (25, 26). We therefore looked for potential alterations in the endothelium-associated bFGF pool of these rats.

We observed that the endothelium of the thoracic aortas from SH rats in the early established phase of hypertension (5-month-old; average MAP of 171 ± 8 mmHg versus 120–130 mmHg in normotensive rats; $n = 6$) scarcely stained with

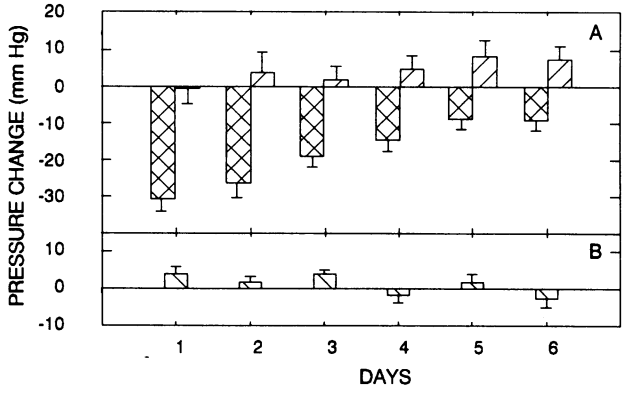

FIG. 1. Long-term changes in the MAP of SH and normotensive rats after a systemic bolus of aFGF. (*A*) MAP changes in 14-month-old SH rats receiving aFGF treated with endoproteases (hatched bars; $n = 16$) and untreated protein (crosshatched bars; $n = 23$), respectively. (B) MAP changes in normotensive rats receiving untreated aFGF. The average MAP before the treatment was 190 ± 12 mmHg for SH and 127 ± 12 mmHg for normotensive rats, respectively. Pressure changes in each rat are referred to pretreatment MAP values. Vertical bars represent mean + SEM. aFGF (2.6 μg) was systemically administered to the rats in PBS/heparin buffer, as described (4).

anti-bFGF antibodies (Fig. 2*A*), whereas immunostaining of the endothelium incubated with anti-bFGF antibodies is considerably more abundant and homogeneous in 4-week-old SH rats that are starting to develop hypertension at its first stages (Fig. 2*B*; ref. 8). No significant differences were observed between 5- and 10-month-old SH rats in the course of hyper-

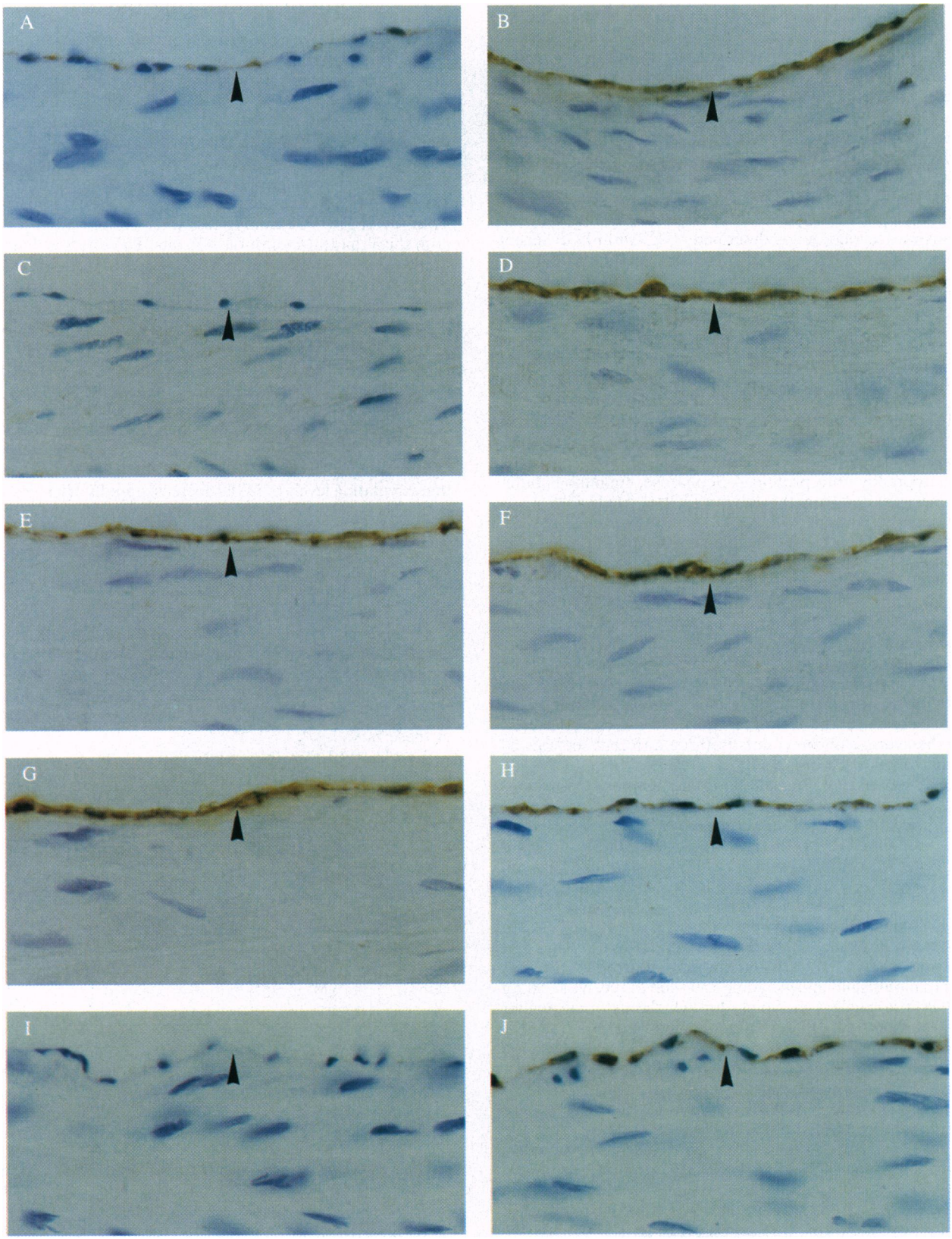

FIG. 2. Immunohistochemical detection of FGF and ecNOS in cross sections of thoracic aortas from normotensive and SH rats. The strain, age, treatment, and antibody used for the samples shown were as follows (respectively): (*A*) SH, 5 months, none, bFGF; (*B*) SH, 4 weeks, none, bFGF; (*C*) SH, 18 months, none, bFGF; (*D*) WKY, 18 months, none, bFGF; (*E*) SH, 18 months, aFGF bolus, aFGF; (*F*) SH, 18 months, aFGF gene transfer, aFGF; (*G*) WKY, 18 months, none, ecNOS; (*H*) SH, 4 weeks, none, ecNOS; (*I*) SH, 18 months, none, ecNOS; (*J*) SH, 18 months, aFGF gene transfer, ecNOS. Aorta cross sections were 6 μm thick. Arrowheads point to the endothelial cell monolayer. (×1100.)

tension or in the evolution of the endothelium staining with anti-bFGF antibodies. About 30% of the endothelial cells per section of thoracic aorta from such rats did not show any associated immunoreactivity with anti-bFGF antibodies (Fig. 2). Nevertheless, as the rats grew older and hypertension progressed to later stages, the number of nonimmunoreactive cells increased up to 61% in 14-month-old rats and to 90% in 18-month-old rats (average MAP = 185 ± 4 and 222 ± 5 mmHg, respectively; $n = 6$). A representative section of the thoracic aorta from 18-month-old rats incubated with anti-bFGF antibodies appears in Fig. 2*C*. Disappearance of bFGF immunoreactivity in endothelial cells in SH rats was associated with irregular shapes of the nucleus and cytoplasm swelling, a finding which suggests an alteration in the normal cell physiology. Immunostaining of the endothelium with anti-bFGF antibodies was quite intense and practically uniform in the thoracic aortas of normotensive WKY rats during their entire lifespan (Fig. 2*D*). Similar findings were obtained in resistance blood vessels from the brain of SH rats.

The effect of aFGF administration on the potential modifications of endothelial FGF content in SH rats was studied next. Fig. 2*E* shows that, 24 hr after receiving the systemic bolus of aFGF, the overall immunostaining intensity of the endothelium from the thoracic aortas of 18-month-old SH rats appeared practically indistinguishable from that of normotensive ones (Fig. 2*D*), with use of anti-aFGF and anti-bFGF antibodies, respectively. Thus, treatment with aFGF seems able to replenish the FGF endothelial pool. Fig. 3 shows the evolution of the endothelial aFGF content of 14-month-old SH rats during the 6 days subsequent to the systemic aFGF administration. In concordance with these data, the decay in their endothelial aFGF content closely and inversely correlates with the pattern of blood pressure recovery to pretreatment levels in same-age SH rats (Fig. 1*A*).

Regeneration of the Endothelial FGF Levels and Normalization of Blood Pressure by Transfection of the aFGF Gene. It has been shown that endothelial cells are efficiently transfected *in vivo* with foreign genes by a single systemic injection of expression plasmid:cationic liposome complexes (27). Thus, we tested whether we could regenerate the FGF pool associated to the endothelium by transfection with the pMAMaFGF expression plasmid in rats with undetectable levels of bFGF. The employment of aFGF instead of bFGF allows a precise monitoring of the transfection effects because (*i*) there is no aFGF in the endothelium and (*ii*) bFGF is not recognized by the aFGF antibodies (see *Material and Methods*). As shown in Fig. 4*A*, a significant blood pressure decrease was observed in SH rats transfected with pMAMaFGF plasmid. This blood pressure reduction was absent in those that received pMAM*neo* plasmid as control. Six days after the transfection treatment, once the arterial blood pressure of the rats became stable, three of the rats were randomly killed for immunohistochemistry, whereas the other four received a second liposome-aided aFGF-gene transfection treatment. Control rats were also sacrificed at this moment and two of these were randomly separated for immunohistochemistry. The results of the immunohistochemistry show that aFGF in the endothelium of rats transfected with plasmid pMAMaFGF (Fig. 2*F*) reached expression levels similar to both normotensive rats and to those which had received an aFGF systemic bolus (Fig. 2 *D* and *E*). No immunoreactivity was detected in animals injected with pMAM*neo*. We did not observe a further arterial blood pressure decrease in the rats receiving the second transfection treatment (mean decrement between days 7 and 15 with respect to the pressure values previous to the first treatment = 51 ± 0.4 mmHg). The MAP of those rats increased slowly; however, 134 days after transfection it was still below pretreatment levels (Fig. 4*B*), and 48% of the endothelial cells from the thoracic aortas of these rats (≈22 months old when they were killed) still stained positively for aFGF (not shown). Histological studies of arteries, other potential tissues of aFGF-gene transfection (liver, kidney, heart, and lungs; ref. 27), and organs where FGFs are predominantly cleared (liver and kidney; refs. 22–24 and 28) did not show signs of histopathological lesions in these rats. Thus, the *in vivo* toxicity of the aFGF gene transfection by systemic injections of expression plasmid:cationic liposome complexes seems minimal. These results are in agreement with the lack of effect observed after a continuous 3-day intravenous administration of bFGF (28).

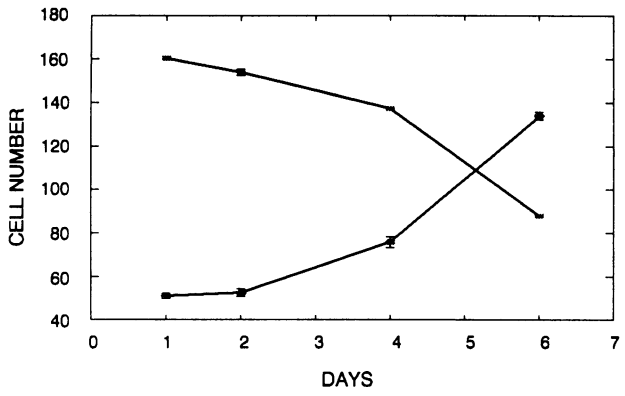

FIG. 3. Evolution of the aFGF content in the endothelium of the thoracic aortas from SH rats after systemic administration of aFGF. Negatively (●) and positively (■) immunostained endothelial cells, respectively, per section of thoracic aorta incubated with anti-aFGF antibodies from 14-month-old SH rats killed at different times after the injection of a systemic bolus of 2.6 μg of aFGF. Results represent pooled data from four rats. The number of positive and negative immunoreactive cells per section of thoracic aorta in each rat is the average of the counts in three different cross sections (6 μm thick), 60 μm apart from each other. Vertical bars represent SEM. Where error bars are not visible, they are smaller than the data point.

Effect of the Regeneration of the Endothelial Pool of FGF on the Levels of ecNOS in SH Rats. Low levels of ecNOS have been encountered in patients with pulmonary hypertension (29), disruption of the gene encoding ecNOS causes hypertension (30), and depletion of FGF in the medium causes a decrease in the levels of ecNOS in cultured endothelial cells (31). Therefore, we studied whether alterations in the mechanism of NO synthesis could correlate with FGF deficiencies in SH rats. Immunohistochemistry with anti-ecNOS antibodies shows a uniform staining of the endothelium of old (Fig. 2*G*) and young normotensive rats (not shown). A nearly uniform staining pattern is also observed in 4-week-old SH rats (Fig. 2*H*). However, scant staining, comparable with that shown in Fig. 2*A*, was detected in 5-month-old SH rats (not shown). The percentage of unstained cells remained approximately the same in 5- and 10-month-old animals (27% and 28%, respectively) and increased as the rats grew older (66% and 91% in 14- and 18-month-old rats, respectively). Fig. 2*I* illustrates the practical absence of immunoreactivity with anti-ecNOS antibodies in the endothelium of the thoracic aorta from 18-month-old SH rats. However, ecNOS abundance appeared restored to nearly normal levels (Fig. 2*G*) 6 days after transfection with plasmid pMAMaFGF (Fig. 2*J*) and 24 hr after the systemic administration of 2.6 μg of aFGF (not shown). The correlation coefficient ($r = 0.99$) and regression line determinants (slope = 0.94; ordinate at the origin = 2.5) of the percentage per cross section of thoracic aorta of unstained cells using anti-ecNOS antibodies plotted against that of the unstained ones with anti-bFGF, of SH rats of different ages (10 to 18 months old; $n = 6$ per age), show that a strict direct dependence indeed exists between both groups of data. Thus, these observations suggest that the decrease in ecNOS levels with age closely parallels bFGF content in SH rats. The same

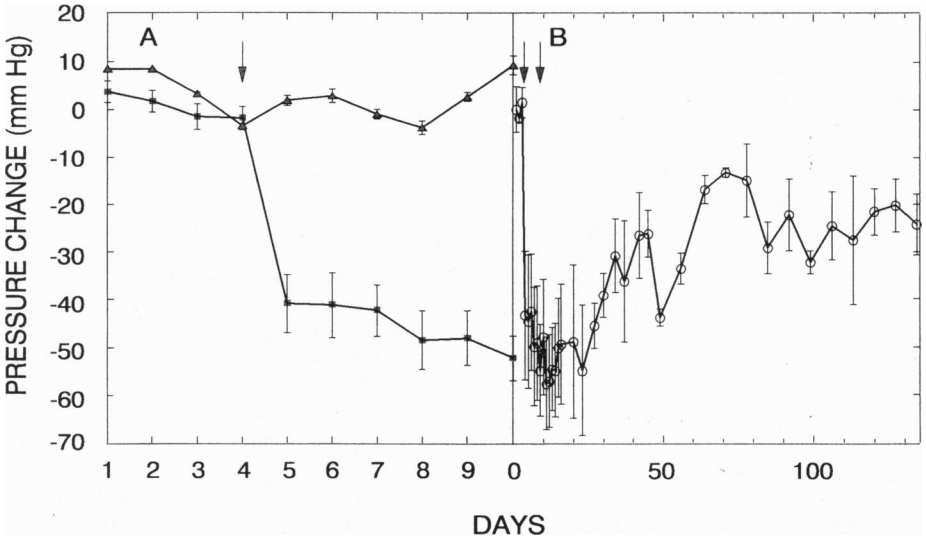

FIG. 4. Effect of aFGF gene transfection in SH rats. (*A*) MAP changes in 18-month-old SH rats after a systemic injection of pMAMaFGF (n = 7; ■) and control pMAM*neo* (n = 6; ▲) plasmid:cationic liposome complexes. The evolution of MAP in the four rats receiving a second pMAMaFGF plasmid transfection treatment is summarized in *B*. Transfection treatments were carried out where indicated by arrows. The average MAP of the rats before the treatment was 233 ± 16 mmHg. Pressure changes in each rat are referred to pretreatment MAP values. Vertical bars represent SEM. Where error bars are not visible, they are smaller than the data point.

type of correlation is maintained between the number of endothelial cells stained by anti-ecNOS and anti-aFGF antibodies, respectively, in thoracic aortas of 18-month-old SH rats 6 days after the transfection with plasmid pMAMaFGF (Table 1).

Endothelial dysfunction in SH rats has been known to be involved in their anomalous vasoreactivity (32). Hence, we next studied, in 16- to 18-month-old animals, if treatment with aFGF would be able to restore this vascular derangement. To this end, we chose third order mesenteric resistance arteries, because the contribution of large conduit arteries to the regulation of peripheral vascular resistance is relatively minor. Whereas SH rats showed a pattern of complete instability in the response to phenylephrine (Fig. 5, lower trace), mesenteric resistance vessels from aFGF-treated SH rats contracted less intensely and maintained a stable pattern along time (Fig. 5, upper trace). Thus, the increased sensitivity to adrenergic amines of SH rat vessels (33) seems suppressed by a treatment that promotes restoration of ecNOS immunoreactivity.

The trophic activity of FGFs on endothelial cells may represent a potential mechanistic substrate for the long-term blood pressure normalization observed in SH rats upon rebuilding of endothelial FGF pool (2). The data reported here suggest that correction of hypertension in SH rats could be mediated, at least in part, by the restoration of ecNOS levels once the normal endothelial physiology is attained, possibly because of the regeneration of its associated pool of trophic factors. However, the participation of other mechanisms in the correction of hypertension by FGFs cannot be ruled out, given the several pathways that participate in the regulation of vascular tone and the broad variety of cellular events triggered by trophic factors. Furthermore, involvement of other processes activated by a recovery of FGF levels in the liver, lungs, and kidney, organs where these proteins accumulate after systemic administration and genetic transfection, cannot be excluded either (22–24, 27, 28). Nevertheless, a mere accumulation of FGFs above physiological levels in those organs seems insufficient to account for the long-term changes on arterial blood pressure observed in SH rats because such an effect was not observed in normotensive rats. Thus, the blood pressure normalization occurring in SH rats probably hints toward a specific replenishment of FGF levels in otherwise anomalously FGF-depleted tissues, as could be the case of the endothelium. Both the ability of the aFGF-gene transfected cells to replenish the FGF endothelial pool and the accumulation in the endo-

Table 1. Immunostaining of endothelial cells after *in vivo* gene transfection

	No. of cells					
	Rat 1		Rat 2		Rat 3	
Section	+	−	+	−	+	−
			anti-aFGF			
1	180	30	173	25	178	27
2	176	27	169	31	186	33
3	183	30	179	22	180	35
			anti-ecNOS			
1	175	31	179	25	180	28
2	183	32	184	26	184	24
3	177	29	181	30	178	25

Number of endothelial cells per cross section of the thoracic aorta from 18-month-old SH rats positively (+) and negatively (−) immunostained with anti-aFGF and anti-ecNOS antibodies, 6 days after *in vivo* transfection with plasmid pMAMaFGF

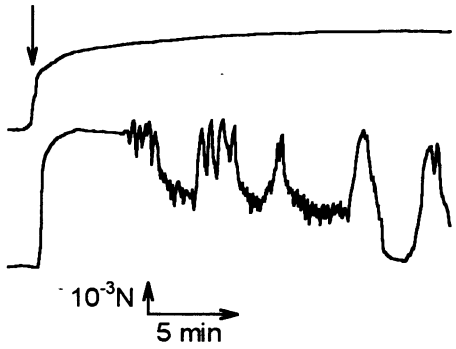

FIG. 5. Effect of systemic administration of aFGF on the response to phenylephrine of isolated mesenteric resistance arteries of SH rats. Phenylephrine (5×10^{-6} M) was added where indicated by the arrow. Upper trace, aFGF-treated rat; lower trace, untreated rat. An experiment, representative of four independent ones in four different animals, is depicted in each case. The vessel tension before phenylephrine addition was 0.38 mN (upper trace) and 1.78 mN (lower trace). Rats treated with aFGF received systemically 2.6 μg of the protein in PBS/heparin buffer, as described (4), 24 hr before the experiment.

thelium of the protein systemically injected demonstrate that glycosaminoglycan binding sites are present in endothelial cells of SH rats and would support the hypothesis of reduced bFGF synthesis in endothelial cells of SH rats. A low endothelial FGF-synthesis rate seems a new pathophysiological feature of these rats to be added to others previously described (10–14). The results reported here, and those of Kostyk *et al.* (31) in cultured endothelial cells, suggest that FGF may modulate ecNOS levels, thus contributing to regulate vascular tone.

Our results show that SH rats develop a severe deficit of FGF. These animals may, therefore, become an important tool to study the physiological roles of these proteins. In addition, the efficiency of the aFGF *in vivo* gene transfer herein reported suggests that this procedure may be of potential application to the treatment of several cardiovascular and neurologic pathophysiological conditions, which may benefit considerably from a steady administration of FGF.

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