Abstract—Hypertension is a cardiovascular disorder that appears in more than half of the patients with Williams–Beuren syndrome, hemizygous for the elastin gene among 26 to 28 other genes. It was shown that the antihypertensive drug minoxidil, an ATP-dependent potassium channel opener, enhances elastic fiber formation; however, no wide clinical application was developed because of its adverse side effects. The Brown Norway rat was used here as an arterial elastin–deficient model. We tested 3 different potassium channel openers, minoxidil, diazoxide, and pinacidil, and 1 potassium channel blocker, glibenclamide, on cultured smooth muscle cells from Brown Norway rat aorta. All tested potassium channel openers increased mRNAs encoding proteins and enzymes involved in elastic fiber formation, whereas glibenclamide had the opposite effect. The higher steady-state level of tropoelastin mRNA in minoxidil-treated cells was attributable to an increase in both transcription and mRNA stability. Treatment of Brown Norway rats for 10 weeks with minoxidil or diazoxide increased elastic fiber content and decreased cell number in the aortic media, without changing collagen content. The minoxidil-induced cardiac hypertrophy was reduced when animals simultaneously received irbesartan, an angiotensin II–receptor antagonist. This side effect of minoxidil was not observed in diazoxide-treated animals. In conclusion, diazoxide, causing less undesirable side effects than minoxidil, or coadministration of minoxidil and irbesartan, increases elastic fiber content, decreases cell number in the aorta and, thus, could be suitable for treating vascular pathologies characterized by diminished arterial elastin content and simultaneous hypertension. (Hypertension. 2013;62:00-00.)

Key Words: aorta ■ elastic tissue ■ elastin ■ potassium channels ■ rats

Haploinsufficiency of elastin in patients with Williams–Beuren syndrome leads, in more than half of cases, to development of supravalvular aortic stenosis and hypertension.1 Moreover, Eln−/− mice, a model for supravalvular aortic stenosis disease, have a higher arterial pressure (∆25–30 mm Hg) than their wild-type counterparts.2 These cardiovascular features are clearly linked to the decreased elastin synthesis in the aorta during development. Thus, it would be of interest to find molecules able to enhance elastin synthesis to treat this condition.

We previously showed that the Brown Norway (BN) rat, a normotensive inbred strain, presents the lowest content of elastin in the aorta compared with 6 other inbred rat strains.3–5 We also demonstrated that, compared with the LOU rat, the elastin deficit in the thoracic aorta of the BN rat is partly caused by a decrease in the synthesis of tropoelastin (TE), the soluble precursor of elastin. However, elastin gene polymorphism accounts for only 3.9% of the elastin content variance in F2 BNxLOU rats.7 After a genome-wide search for quantitative trait loci influencing the aortic elastin content in an F2 population derived from BN and LOU rats, we identified on chromosomes 2 and 14, 3 quantitative trait loci specifically controlling elastin levels: Ael1, Ael2, and Ael3.6 The polymorphic marker, D2Wox26, corresponding to the maximum logarithm of odds score value of Ael1, is situated within the gene encoding for the Na+/K+-ATPase α1 subunit. In addition, several other genes encoding for potassium channels are contained in Ael1 (Kcnmb2, Hcn1) and Ael2 (Hcn3, Kcnn3, Kcnd3, Kcn2, Kcn3, and Kcna10). This result supports the hypothesis that the deficit of aortic elastin content in the BN rat could be explained by anomalies in intracellular potassium concentration ([K+]i).
Minoxidil (Mx), an ATP-dependent K⁺ (K<sub>ATP</sub>) channel opener used as a vasodilator, was previously shown to increase elastin synthesis and hair growth. However, potassium channel openers, which induce a decrease in [K⁺], are an emerging and promising family of therapeutic agents in diverse disease conditions associated with metabolic disorders.

In the present study, we used the BN rat as an arterial elastin-deficient model to analyze the effect of K⁺ channel openers on elastin synthesis, treating cultured BN vascular smooth muscle cells (vSMCs) with different K⁺ channel openers and 1 potassium channel blocker and treating young BN rats with K⁺ channel openers.

Materials and Methods

Animals
Male inbred BN/Orl Rj rats were supplied by Janvier (Le Genest-St-Isle, France). The studies were performed under authorization no. C75-18-03 of the “Direction Départementale des Services Vétérinaires de Paris,” France. Animal care and experimental protocols were approved by the Animal Ethics Committee of Inserm/Paris Diderot University-University Paris 7, authorization no. 2012–15/698-0082.

vSMC Culture and In Vitro Experiments
vSMCs from aorta of 6-week-old BN rats were isolated and cultured as previously described. In all experiments reported here, confluent cultures at passage 4 were used. Cells were deprived of fetal calf serum for 24 or 48 hours and preincubated with 100 μmol/L MxS for 24 hours before their incubation for 8, 24, 32, or 50 hours, with 60 μmol/L 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), an inhibitor of RNA synthesis. RNAs were extracted from cells with the E.Z.N.A. Total RNA Kit I. mRNAs were extracted from cells with the E.Z.N.A. Total RNA Kit I. mRNAs were evaluated by real-time polymerase chain reaction.

In Vivo Experiments
In vivo experiments were performed with 3-week-old BN rats; they were housed individually and treated for 10 weeks with Mx or Dx (Molekula, UK). We also treated rats with irbesartan (Irb; Sanofi-Aventis, France), an angiotensin II-receptor antagonist, to obtain a decrease in blood pressure similar to that obtained at the beginning of the treatment with Mx and Dx. Because Mx is known to cause cardiac hypertrophy, partly as a consequence of the activation of the renin–angiotensin system, we coadministered Irb with Mx (Mx+Irb group) to 8 rats. All drugs were diluted in the drinking water. The measured drug intake (mg/kg per day) of Mx was 19.8±0.9, of Dx, 19.6±1.0, of Irb, 9.4±0.3, and of Mx+Irb, 20.4±0.7 to 3.8±0.1. The dose of Irb added to Mx was chosen to inhibit the effects of angiotensin II with no additional decrease in blood pressure.

Steady-state levels of mRNA of tropoelastin (TE; A), fibrillin-1 (B), and fibrillin-5 (C) were measured in vascular smooth muscle cells incubated without (■) or with MxS (■) or Dx (■) or pinacidil (■) for 24 hours at different concentrations. *P<0.05; **P<0.01 compared with the corresponding control condition.

Results

Potassium Channel Openers Increase vSMC mRNAs of Proteins and Enzymes Involved in Elastic Fiber Formation
Cultured vSMCs from BN rat aorta were incubated with 2 selective K<sub>ATP</sub> channel openers, MxS or Dx, and mRNAs encoding for proteins composing elastic fibers, ie, TE, fibrillin-1, and fibrillin-5, were quantified. Lysyl oxidases, LOX and LOXL1, are extracellular copper-dependent enzymes that catalyze the cross-linking of TE monomers and contribute to the formation of insoluble elastic fibers. LOX and LOXL1 mRNAs were thus also quantified. After 24 hours of treatment, MxS increased TE, fibrillin-1, fibrillin-5, LOX, and LOXL1 mRNAs with the same concentration-dependent expression pattern. The maximal increases were detected...
at the 5 μmol/L dose after 24 hours of treatment (Figures 1 and 2). Dx also increased TE, fibrillin-1, fibulin-5, LOX, and LOXL1 mRNAs. The maximal increases were detected at 5 and 50 μmol/L doses at 24 hours (Figures 1 and 2). Pinacidil, a general K+ channel opener, also induced an increase in mRNAs of proteins and enzymes involved in elastic fiber formation after 24 hours of treatment at 1 μmol/L (Figures 1 and 2). In keeping with this, treatment with glibenclamide, a K<sub>ATP</sub> channel blocker, markedly decreased the expression of these mRNAs with a similar decrease at 24 and 48 hours with 10 μmol/L (Figure 3).

Taken together, these results show for the first time that all potassium channel openers tested increase mRNAs encoding for several structural proteins and enzymes involved in elastic fiber formation.

MxS Increases Elastin Gene Transcription and TE mRNA Stability

The steady-state level of a specific mRNA results from the gene transcription rate and the degradation of mRNA. First, we investigated whether MxS had an effect on elastin gene transcription. We quantified TE pre-mRNA according to the methodology of Swee et al., quantifying TE intron 35 (Figure 4A) and showed that MxS increased the quantity of TE pre-mRNA. The maximum increase was 67% with 0.5 μmol/L MxS. Thus, MxS increases elastin gene transcription. We then investigated the effect of MxS on TE mRNA stability (Figure 4B). At every time point, TE mRNA quantity was increased in vSMCs incubated with 100 μmol/L MxS, and we deduced that the half-life is increased by 27.3% compared with the control (4.2 versus 3.3 hours). In addition, TE mRNA was maintained at a detectable level for >50 hours after the addition of DRB when cells were incubated with MxS, whereas it was completely undetectable after 8.4 hours in control conditions. MxS also increased the half-life of other mRNAs (β-actin: +45.5%, GAPDH: +207.9%, LOX: +64.8%, data not shown).

In Vivo Treatment of BN Rats With Mx or Dx Increases the Thickness of Elastic Lamellae in Aortic Media

We treated young growing BN rats for 2.5 months with the potassium channel openers Mx and Dx; Irb-treated rats were used for comparison. Mx, as well as Irb and Dx, led to a decrease in blood pressure at the beginning of the treatment (data not shown). However, there were no longer any significant variations in blood pressure, heart rate, body weight, or body length at the end of the experimental period in treated animals compared with controls (Table).

Histological studies were performed on infrarenal abdominal aorta. Orcein staining showed that Mx and Dx increased the thickness of all elastic lamina. Interlamellar elastic fibers also seemed thicker in Mx and Dx groups (Figure 5A).

Quantitative analyses of histological sections confirmed that Mx did indeed increase the thickness of internal elastic lamina (IEL), elastic lamellae (EL), and external elastic lamina (EEL) by 19.8%, 24.8%, 27%, respectively, and Dx, by 17.3%, 30.9%, and 9.9%, respectively (Figure 5B). Irb had no effect on the thickness of IEL, EL, and EEL. Gallego’s staining also confirmed that Mx increases elastic lamellae thickness; interlamellar elastic fibers also seemed thicker compared with the control group (Figure 5C). Transmission electron microscopy showed that the IEL and EL whose thickness had been increased by Mx were as smooth and regular as IEL and EL of the control group, suggesting a good organization of the newly synthesized elastic fibers.

Using histological sections, we also showed that there was no significant difference in medial thickness (Control, 43.1±5.4 μm; Irb, 37.9±6.8 μm; Mx, 43.2±4.6 μm; Dx, 45.8±5.1 μm) or in the number of EL (Control, 4.0±0.3; Irb, 4.1±0.3; Mx, 4.2±0.2; Dx, 4.0±0.3) between groups. Interestingly, there were less vSMCs in the media of Mx (909±136 nuclei/mm²; P<0.001 versus control and Irb groups) and Dx (1138±181 nuclei/mm²; P<0.01 versus control group; P<0.05 versus Irb group) groups compared with control (1562±236 nuclei/mm²) and Irb (1443±238 nuclei/mm²) groups. Irb had no effect on cell density compared with the control group.

Mx and Dx Induce an Increase in Insoluble Elastin Content in BN Rat Aorta

Insoluble elastin content expressed as mg/cm of aorta was increased by 31.6% and 11.0% by Mx and Dx treatment, respectively (Figure 6A). The same parameter expressed as a percentage of the thoracic aorta dry weight was also increased by Mx and Dx (Figure 6B). Irb did not modify the percentage of elastin compared with controls, and Irb coadministered

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Minoxidil-sulfate (MxS), diazoxide (Dx), and pinacidil increase LOX and LOXL1 mRNAs. Steady-state levels of mRNA of LOX (A) and LOXL1 (B) were measured in vascular smooth muscle cells incubated without (□) or with MxS (■) or Dx (■) or pinacidil (■) for 24 hours at different concentrations. *P<0.05; **P<0.01 compared with the corresponding control condition.
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with Mx (Mx+Irb) did not affect the increase in insoluble elastin content caused by Mx. In contrast, Mx, Mx+Irb, Dx, or Irb did not affect the percentage of collagen compared with the control group (Figure 6C). Mx decreased the percentage of cell proteins in the aorta by 24.8%, whereas Irb and Dx did not. Irb coadministered with Mx attenuated, in part, the decrease in cell proteins caused by Mx (Figure 6D).

We show thus for the first time, that, in vivo, Dx, like Mx, increases insoluble elastin content but not collagen in rat aorta, independently of blood pressure.

Mx, but Not Dx, Induces Expansive Aortic Remodeling and Eccentric Cardiac Hypertrophy Without Changes in Aortic or Cardiac Function

Echocardiographic studies showed that systolic and diastolic ascending aortic diameters were greater in the Mx and Mx+Irb groups than in control and Irb groups, despite similar blood pressure. In contrast, Dx treatment did not change ascending aortic diameter. Ex vivo measurements confirmed that Mx- and Mx+Irb-treated animals had an increased aortic circumference (Table).

To evaluate the functional properties of the thoracic and abdominal aorta of treated rats, we measured pulse wave velocity between the aortic arch and the iliac bifurcation of every rat but we observed no significant variation of this parameter (Table).

It is well known that Mx induces eccentric left ventricular (LV) hypertrophy and expansive remodeling. Echocardiography (Table) showed that left atrial diameter and LV wall thickness were not changed in BN rats by either Mx or Dx. Mx increased LV end-diastolic and end-systolic diameters with calculated LV mass, as compared with the control group, which characterized eccentric LV hypertrophy. There was no change in LV systolic function. LV posterior wall thickness tended to decrease in the Irb group compared with controls (P=0.07) and was thinner in the Mx+Irb group than in the control group with no significant LV hypertrophy. The Irb group showed a mild alteration in LV systolic function, but when Irb was associated with Mx, no change in LV function was evidenced. Dx had no effect on LV morphology and function. Ex vivo heart weight values confirmed the in vivo data. The heart dry-to-wet weight ratio was not changed by any treatment used.

Discussion

Using vSMCs isolated from the aorta of the BN rat, we have shown that MxS and Dx, selective KATP channel openers, increase the steady-state levels of mRNAs encoding for several proteins composing elastic fibers (TE, fibrillin-1, and fibrillin-5). We also showed here for the first time that MxS increases the transcription of the elastin gene and TE mRNA stability. In addition, the fact that pinacidil, a general potassium channel opener, increased TE mRNA strongly suggests a generalized positive effect of potassium channel openers on elastic fiber synthesis. These drugs also increase the steady-state level of mRNAs encoding for LOX and LOXL1 and, in vivo, the elastic fiber content in the BN aorta.

Figure 3. Glibenclamide decreases mRNAs encoding proteins involved in elastic fiber synthesis. Steady-state levels of mRNA of tropoelastin (TE; A), fibrillin-1 (B), fibrillin-5 (C), LOX (D), and LOXL1 (E) were measured in vascular smooth muscle cells incubated without (●) or with (□) glibenclamide for 24 hours at different concentrations. **P<0.01; ***P<0.001 compared with the corresponding control condition.

Figure 4. Minoxidil-sulfate (MxS) increases elastin gene transcription and tropoelastin mRNA stability. A. Tropoelastin (TE) pre-mRNA level in vascular smooth muscle cells (vSMCs) incubated (●) or not (□) with MxS for 24 hours at different concentrations. B, TE mRNA level quantified in vSMCs as a function of time after the addition of 60 μmol/L DRB, in absence (●) or in presence (▲) of 100 μmol/L MxS. TE mRNA levels are normalized to total RNA levels and expressed as relative expression, 1 being the level at t0. **P<0.01; ***P<0.001 compared with the control condition.
Previously, it had been only demonstrated that an Mx-induced K+ efflux increased elastin synthesis in vSMCs and skin fibroblasts at a pretranslational level.\(^8\)–\(^10\) Indeed, the decrease in intracellular potassium concentration ([K\(^+\)]) leads to hyperpolarization of the membrane and a reduction in Ca\(^{2+}\) influx through the voltage-gated Ca\(^{2+}\) or Ca\(^{++}\) channels,\(^15\) causing a decrease in [Ca\(^{2+}\)] or [Ca\(^{++}\)] and an increase in elastin synthesis.\(^8\)–\(^11\),\(^19\) On the contrary, an increase in [K\(^+\)] causes depolarization of the plasma membrane and the opening of voltage-dependent calcium channels, leading to an increase in [Ca\(^{2+}\)] or [Ca\(^{++}\)] and a decrease in elastin synthesis.\(^20\)

In addition to their stimulatory effect on elastin synthesis, MxS and Dx increase levels of mRNAs encoding for fibrillin-1, fibrillin-5, LOX, and LOXL1, other proteins involved in elastic fiber formation. Mx also stimulates LOX expression in skin fibroblasts.\(^21\) These results are consistent with the electron microscopic observations of well-formed elastic fibers in the aorta of Mx-treated BN rats: a deficiency in one of these elastic fiber components leads to defective elastic fiber formation.\(^22\)–\(^25\) Normal elastic fiber genesis is further supported by the homogeneous increase in elastic fiber thickness observed at the histological level in Mx- and Dx-treated rats. However, the number of EL remained unchanged. The significant increase in insoluble elastin content in Mx- and Dx-treated animals additionally demonstrated that the cross-linking

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Body weight, body length, and systolic blood pressure were measured, and hemodynamic parameters were analyzed. Morphological parameters of the ascending aorta and heart, and functional cardiac parameters of control rats and rats treated with Irb, Mx, Mx+Irb, or Dx were recorded by echography. AA indicates ascending aorta; BP, blood pressure; Epw, early diastolic expansion velocity of left ventricular posterior wall; IV, interventricular; LV, left ventricle; LVESD, left ventricle end-systolic diameter; and Spw, systolic maximal velocity of the posterior wall. *\(P<0.05; \**P<0.01; †P=0.06 compared with the control group. ‡P<0.05; ‡‡P<0.01 compared with the Irb group. §P<0.05 compared with the Mx group. ||Ex vivo measurement.
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process is efficient because the cross-links confer the resistance of elastin to the chemical treatment used for its purification. We may hypothesize that this increase in the elastin content in the aorta induces an increase in aortic elasticity. However, using an indirect evaluation of aortic elasticity via the measurement of pulse wave velocity, we were not able to demonstrate any increase in arterial elasticity in treated animals with increased aortic elastin content. The onset of IEL ruptures in the abdominal aorta of the BN rats after the age of 7 to 8 weeks were not quantified in the present study but they may counteract the effect of increased elastic fiber content in the treated animals.26

Arterial wall function is also determined by another essential component of the extracellular matrix, the collagens. However, the effect of Mx on collagen synthesis seems to be species specific and cell type dependent.8,21,27–29 In our experimental conditions, the treatment of BN rats with Mx or Dx did not change the relative content (%) of collagens in the media or modify medial thickness. Moreover, Mx and Dx caused a decrease in vSMC number; this decreased aortic cellularity in Mx-treated rats was confirmed by the measurement of cell protein concentration in the 0.3% sodium dodecyl sulfate extract of each aorta. This is consistent with the previous demonstration that Mx inhibits vSMC proliferation and migration both in vitro and in vivo, the latter attested by a decrease in neointimal formation after balloon catheter injury.30 Taken together, these data show that Mx and Dx increase elastin content and elastic fiber thickness, without aortic hypertrophy.

Irb, used at a dose that led to a decrease in systolic arterial pressure similar to that induced by Mx and Dx at the beginning of the treatment, did not induce any modification in the aortic wall composition: the relative contents of elastin, collagens, and cell proteins were similar in the aortas of Irb-treated and control BN rats. Furthermore, Irb, added to Mx at a dose that did not induce any additional decrease in blood pressure, did not alter the effect of Mx on arterial wall composition. Thus, the elastogenic effect of potassium channel openers is linked with the decrease in [K+], and not with the variation in blood pressure.

Echocardiographic studies showed that Mx induced eccentric cardiac hypertrophy characterized by an increased LV diameter with no change in wall thickness. The dry-to-wet heart weight ratio was unchanged, indicating that the increase in heart weight was attributable to increases in cellular and extracellular matrix contents and not only to edema. Irb reduced the Mx-induced cardiac hypertrophy, consistent with an activation

Figure 5. In vivo treatment of BN rats with minoxidil (Mx) or diazoxide (Dx) increases the thickness of elastic fibers in aortic media. A, Orcein staining of elastic fibers in transverse sections of the media of abdominal aorta of control rats and rats treated with irbesartan (Irb), Mx, or Dx. Arrows indicate interlamellar elastic fibers. B, The thickness of internal elastic lamina (IEL), elastic lamellae (EL), and external elastic lamina (EEL) was measured in orcein-stained sections of abdominal aorta from control rats (■) and rats treated with Irb (□), Mx (■), or Dx (■). *P<0.05 compared with controls. †P<0.05; ††P<0.01 compared with the Irb group. C, Gallego’s staining of elastic fibers in the abdominal aorta of control rat and rats treated with Mx (a and b). Observation by transmission electron microscopy of the similar samples at magnification ×7000 (c and d) or ×12000 (e and f) of media, showing endothelial cells (EC), IEL, EL, and vascular smooth muscle cells (vSMC; c–f). Scale bar, 10 μm.
KATP channels, formed by SUR2A and Kir6.2 are not, or only are activated by Dx in the absence of ADP, whereas cardiac cells, which are complexes of SUR2B and Kir6.2 subunits, be thus suitable for treating vascular pathologies characterized by diminished arterial elastin content and increased vSMC proliferation.

**Perspectives**

The present work demonstrated a positive effect of several different potassium channel openers on elastic fiber synthesis. Dx and coadministration of Mx and Irb may be novel therapeutic candidates to increase elastin content in patients with vascular disorders attributable to a lack of elastin. A clinical trial is currently underway, treating young patients with Williams–Beuren syndrome with Mx (NCT00876200). Further analysis of signaling pathway(s) linking potassium and elastin synthesis may provide new elastogenic candidates.

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We thank Gilles Faury for helpful discussion, Martine Muffat-Joly for measurement of blood pressure, Olivier Thibaudieu (IFR02, Paris) for his technical assistance, and Mary Osborne-Pellegrin for help in editing.

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**Disclosures**

None.

**References**


Novelty and Significance

What Is New?

- Not only minoxidil but also all potassium channel openers increase in vitro and in vivo elastin synthesis.
- mRNAs encoding several proteins and enzymes involved in elastic fiber formation are also increased by potassium channel openers.
- In vivo, diazoxide has fewer side effects than minoxidil.

What Is Relevant?

- Besides their antihypertensive effect, the potassium channel openers increase elastin quantity and decrease cell number in the aorta. These 3 effects could be beneficial in patients with Williams–Beuren syndrome, for those who have stenoses and hypertension.

Summary

All tested potassium channel openers increase elastin, proteins, and enzymes involved in the elastic fiber formation, both in vitro and in vivo. Irbesartan reduces the minoxidil-induced cardiac hypertrophy, a side effect of minoxidil not observed in diazoxide-treated animals. Treatment of elastin-deficient patients with potassium channel openers could reduce the risk of stenosis and development of hypertension.
Potassium Channel Openers Increase Aortic Elastic Fiber Formation and Reverse the Genetically Determined Elastin Deficit in the BN Rat

Séverin Slove, Morgane Lannoy, Jacques Behmoaras, Mylène Pezet, Natacha Sloboda, Patrick Lacolley, Brigitte Escoubet, Julia Buján and Marie-Paule Jacob

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Potassium channel openers increase aortic elastic fiber synthesis and reverse the genetically determined elastin deficit in the BN rat

Séverin Slove1,2, Morgane Lannoy1,2, Jacques Behmoaras1, Mylène Pezet3, Natacha Sloboda4, Patrick Lacolley4, Brigitte Escoubet3,5,6, Julia Buján7, Marie-Paule Jacob1.

Supplement data - Materials and Methods

1- Echography and hemodynamics studies

Blood pressure measurements
Non-invasive measurements of systolic arterial pressure and heart rate were performed in the first week after the beginning, and at the end of the treatment using the tail-cuff-method (Phymep, Paris, France).

Ultrasound study
Transthoracic echocardiography was performed as previously described1 with a Toshiba Powervision 6000, SSA 370A device equipped with an 8- to 14-MHz linear transducer under isoflurane anaesthesia (0.75% to 1.0% in oxygen) with spontaneous ventilation. The body temperature was maintained with a heating pad. Data were transferred online to a computer for offline analysis (Ultrasound Image Workstation-300A, Toshiba). Heart rate was measured from EKG tracing. Systolic and diastolic diameters of ascending aorta were measured from time mode imaging as gated on R wave from simultaneous EKG tracing. The left ventricle (LV) was imaged in time-motion mode from parasternal long-axis view to obtain measurements of the left atrium (LA) and LV (LV end-diastolic and end-systolic diameters, ejection fraction, LV mass). Pulsed-wave Doppler tissue was obtained from the posterior wall for the measurement of maximal systolic and diastolic wall velocities.

Pulse Wave Velocity (PWV)
Measurement of thoraco-abdominal pulse wave velocity (PWV) was carried out as previously described2 using a low-volume pressure transducer (P23XL, Becton Dickinson).

2-Sample harvesting
Body weight and body length (nose to rump) were measured at the end of each experiment while the animals were anesthetized for sacrifice. The descending thoracic aortas were removed for the determination of aortic elastin, collagen, and cell protein contents. The heart was removed and weighed. The infrarenal abdominal aortas were perfused at a pressure of 100mmHg for 30min. with buffered formalin for histological staining or for 15min with buffered 3% glutaraldehyde for electron microscopy.

3- Histology and electron microscopy
Formalin-fixed samples of infrarenal abdominal aorta were embedded in paraffin to obtain 5µm-thick sections and stained with orcein, hematoxylin-eosin or Gallego’s stain. For ultrastructural analysis, samples of glutaraldehyde-fixed abdominal aorta were counterstained with lead citrate and examined using a Zeiss 109 transmission electron microscope.

Morphometry
Eight images (at magnification x400) from one abdominal aortic section stained with orcein for each animal of experiment 2 were analysed with the software ImageJ 1.34s (Rasband W,
NIH, USA). On each image, we performed respectively 10, 20, 40 and 20 measures of media, internal elastic lamina, elastic lamellae and external elastic lamina thickness. The number of vSMC nuclei in the media was counted on sections of abdominal aorta stained with hematoxylin-eosin.

4- Purification and quantification of aortic elastin and collagen
Biochemical analysis of the descending thoracic aorta was performed as previously described\(^3\)\(^4\). Length and width of thoracic aortas were measured under a dissecting microscope prior to this procedure. Elastin and collagen were both expressed as a percentage of thoracic aortic dry weight or as mg per cm of thoracic aorta.

5- mRNA quantification
Total RNAs were extracted with the E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, Inc.) and genomic DNA was digested with DNase-I (Rnase-Free DNase I Set, Omega Bio-Tek, Inc.) following supplied protocol. Total RNA were then quantified with Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen Cergy Pontoise, France). Two hundred nanograms of total RNAs were used for reverse transcription and relative quantification of TE, fibrillin-1, fibulin-5, LOX and LOXL1 mRNAs and TE pre-messenger RNA was performed. Tropoelastin (TE), fibrillin-1, LOX, LOXL1 mRNAs (for primer sequences see\(^5\)) were evaluated by real-time PCR using the CFX96 device with the iQ SYBR Green supermix Kit (Biorad, Marnes-la-Coquette, France) after reverse transcription of mRNAs performed with M-MLV reverse transcriptase and in the presence of Oligo(dT)\(^{12-18}\) Primer (Invitrogen, Cergy Pontoise, France). Free genomic-DNA RNAs were reverse transcribed with random primer hexamers (Invitrogen, Cergy Pontoise, France) prior to the TE pre-messenger RNA quantification amplifying the intron 35 (TE\(^{35}\))\(^6\). Results were normalized to the quantity of total RNA and expressed as arbitrary units.

6 - Statistical Analysis
Data are expressed as means ± SD. For all data analysis, the non-parametric Mann-Whitney U test was applied.

References


