Inhibition of ERK1/2 Phosphorylation: A New Strategy to Stimulate Elastogenesis in the Aorta

Morgane Lannoy, Séverin Slove, Liliane Louedec, Christine Choqueux, Clément Journé, Jean-Baptiste Michel, Marie-Paule Jacob

Abstract—Haploinsufficiency of elastin leads, in more than half of patients with Williams–Beuren syndrome, to development of supravalvular aortic stenosis and hypertension. Determining mechanisms implicated in elastin synthesis would be of interest to find new elastogenic molecules to treat such a pathology. Here, we analyzed the signaling pathway linking intracellular calcium concentration to elastin regulation to find new molecules able to increase elastin synthesis. Their elastogenic ability was then investigated, in vitro and in vivo, using inhibitors of the highlighted pathway. The Brown Norway rat strain was used here as an arterial elastin-deficient model. Our data indicated that A23187, a calcium ionophore, decreases elastin expression in cultured vascular smooth muscle cells, both transcriptionally and post-transcriptionally. Addition of A23187 induced transient activation of extracellular signal-regulated kinases 1/2, leading to an upregulation of activator protein-1 transcription factors, which correlated with the inhibition of elastin gene transcription. Pretreatment with U0126, an inhibitor of extracellular signal-regulated kinases 1/2 phosphorylation, abolished the inhibition of elastin gene transcription by A23187. In vitro, U0126 increased elastin synthesis and in vivo, 24 hours after an intravenous administration, elastin gene transcription and elastin mRNA levels were increased in the rat aorta. A chronic treatment, diffusing U0126 for 10 weeks, increased aortic elastin content without changing cell number and collagen content. In conclusion, calcium ionophore represses elastin gene transcription via activation of extracellular signal-regulated kinases 1/2 pathway and activator protein-1 transcription factors. Moreover, we provide strong evidence that inhibition of extracellular signal–regulated kinases 1/2 increases elastin synthesis and could thus be suitable for treating vascular pathologies characterized by diminished arterial elastin content. (Hypertension. 2014;64:00-00.) ● Online Data Supplement

Key Words: activator protein-1 ■ calcium ■ elastin ■ ERK1/2 pathway

Among extracellular matrix components, elastin is the main protein present in elastic fibers, conferring elasticity to tissues submitted to mechanical distortion, such as the aorta, and maintaining vascular smooth muscle cell (vSMC) phenotype in a nonproliferative state. A deficit in elastin synthesis or the degradation of elastic fibers could lead to vascular pathologies like supravalvular aortic stenosis (SVAS) and its simultaneous hypertension. Elastin deficiency in elastin-null mice also leads to stenosis. Reestablishing a normal elastin level in this mouse model prevents perinatal lethality because of aortic occlusion. Thus, finding new molecules capable of stimulating elastin synthesis would be of interest to treat human SVAS.

Elastin synthesis is modulated by several factors including basic fibroblast growth factor (bFGF), tumor necrosis factor-α, epidermal growth factor (EGF), heparin-binding EGF, or transforming growth factor-α. The mechanism most studied is probably the increased elastin gene transcription by bFGF in human vSMCs and rat pulmonary fibroblasts. EGF and bFGF were shown to decrease elastin gene transcription by activating extracellular signal-regulated kinases 1/2 (ERK1/2), resulting in its translocation to the nucleus and subsequent induction of c-fos and fra1. Heterodimers c-fos/c-jun and fra1/c-jun form the activator protein-1 (AP1) complex that represses elastin gene transcription through its binding to an AP1/CAMP response element–like sequence located on the human elastin gene. However, these cytokines and growth factors have limitations for clinical use in patients with abnormal elastin metabolism.

To find new molecules that enhance elastin synthesis, our laboratory has been engaged, over the past decade, in deciphering the mechanisms involved in elastin regulation. By comparing 7 inbred rat strains, we showed that the Brown Norway (BN) rat has the lowest aortic elastin content. Furthermore, we reported that the difference in aortic elastin content between BN and LOU rats is explained by a decrease in the synthesis of tropoelastin, the soluble precursor of elastin, and that elastin polymorphism only accounted for 3.9% of the variance of the aortic elastin content in F2 BN×LOU rats. A
genome-wide search for quantitative trait loci influencing the aortic elastin content was thus undertaken using this F2 cohort and many genes encoding for potassium (K+) channels were present in these quantitative trait loci, suggesting that K+ could regulate aortic elastin content.10 Indeed, we recently reported that in vitro several K+ channel openers (minoxidil, diazoxide, and pinacidil) increased elastin synthesis in vSMCs, whereas glibenclamide, a K+ channel blocker, decreased its transcription.20 Furthermore, in the in vivo administration, to the BN rat, of minoxidil or diazoxide increased aortic elastin content.20

The inhibition of elastin synthesis in the presence of high concentrations of K+ was blocked by addition of nifedipine, a calcium (Ca2+) antagonist, indicating that the regulation of elastin gene transcription by K+ implicates Ca2+ influx.21 Thus, in the present study, we used the Ca2+ ionophore A23187 to analyze the effect of Ca2+ on elastin synthesis and to describe the pathway(s) implicated in the regulation of elastin synthesis by Ca2+ to find new molecules capable of inducing elastin synthesis.

Methods

See the online-only Data Supplement for description of the methods used in this study.

Results

An Increase in vSMC Intracellular Calcium Concentration Decreases Elastin Synthesis, Transcriptionally and Post-Transcriptionally

To increase the intracellular calcium concentration ([Ca2+]i), vSMCs were incubated with different concentrations of A23187, a Ca2+ ionophore. Several mRNAs encoding for proteins composing elastic fibers or catalyzing the cross-linking of elastin monomers were quantified after 24 hours of treatment. Significant decreases in the steady-state levels of elastin mRNA were detected for concentrations equal or higher than 10 μmol/L (Figure 1A). The same concentration-dependent expression pattern was obtained for mRNA encoding fibrillin-1, fibrillin-5, LOX, and LOXL1 (Figure S1A–S1D). A second increase in ERK1/2 phosphorylation appeared from 2 to 12 hours after addition of the drug. A concomitant and significant increase in ERK2 proteins was observed (Figure S3E and S3F). The first increase in ERK1/2 phosphorylation was closely followed by an increase in mRNA and proteins encoding AP1 transcription factors, the transcriptional level of the elastin gene, and the variation in elastin mRNA. After the addition of A23187, phosphorylation of ERK1/2 significantly increased after 10 minutes (Figure 2A and Figure S3A–S3D). A second increase in ERK1/2 phosphorylation appeared from 2 to 12 hours after addition of the drug. A concomitant and significant increase in ERK2 proteins was observed (Figure S3E and S3F). The first increase in ERK1/2 phosphorylation was closely followed by an increase in mRNA and proteins encoding AP1 transcription factors. Indeed, after addition of the drug, c-fos expression increased and decreased with the same time-dependent expression pattern as the phosphorylation of ERK1/2. Both c-fos mRNA (Figure 2B) and protein (Figure 2A and Figure S3G) were

Figure 1. The calcium ionophore A23187 decreases elastin synthesis, transcriptionally and post-transcriptionally. Steady-state levels of elastin mRNA (A), elastin pre-mRNA (B), c-fos mRNA (C), and c-jun mRNA (D) were measured in vascular smooth muscle cells (vSMCs) incubated for 24 hours with different concentrations of A23187 (◼) or in controls (▲). E, Elastin mRNA level was quantified in vSMCs as a function of time after the addition of 60 μmol/L 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside (DRB), in absence (●) or in presence (◼) of 10 μmol/L A23187. Elastin mRNA levels are expressed as relative expression, 1 being the level at t0. n=6 for all experiments. *P<0.05; **P<0.01 compared with the control condition.
ERK1/2 Pathway

Downregulation of AP1 Transcription Factors Upregulates Elastin Synthesis

Because the activation of ERK1/2 is required to inhibit elastin synthesis by Ca^{2+}, the inhibition of ERK1/2 phosphorylation and its downstream targets, the AP1 transcription factors, seems to be a good strategy to increase elastin synthesis. So, we analyzed the effect of c-fos and c-jun downregulation on elastin expression. After 72 hours of vSMC transfection with 100 μmol/L c-fos small interfering RNA, c-fos protein expression was analyzed by immunoblot (Figure S4A) and was decreased 2-fold relative to β-tubulin (Figure S4B), whereas its mRNA was decreased 4-fold (Figure S4D). The downregulation of c-fos led to a significant increase in the steady-state level of elastin mRNA relative to β-actin (Figure 3A) or total RNA (Figure 4B). The downregulation of c-jun had the same effect on elastin expression. Indeed, after 72 hours of c-jun small interfering RNA transfection, c-jun was downregulated at both the protein (Figure S4A and S4C) and mRNA levels (Figure S4D). The treatment of cells with 100 μmol/L U0126 before the addition of A23187 abolished the increase in ERK1/2 phosphorylation and c-fos and c-jun mRNA level induced by A23187 only (Figure 3A–3C). The ability of A23187 to inhibit elastin gene transcription was also completely abolished by U0126 (Figure 3D). This result demonstrates that the inhibition of elastin gene transcription initiated by A23187 requires the activation of ERK1/2. Inhibition of ERK1/2 phosphorylation thus seems to be a good strategy to increase elastin synthesis.
Downregulation of activator protein-1 transcription factors upregulates elastin synthesis. Elastin mRNA level was measured in vascular smooth muscle cells transfected for 72 hours with 100 nmol/L c-fos small interfering RNA (siRNA), 50 nmol/L c-jun siRNA (NT,  ), or with the nontargeting siRNA (NT,  ) normalized to β-actin mRNA levels (A) or to total RNA concentrations (B). n=3 or 4 for all experiments. Statistical significance was determined using an unpaired 2-tailed Student t test. \( *P<0.05; **P<0.01 \) compared with the control condition.

Figure 4. Downregulation of activator protein-1 transcription factors upregulates elastin synthesis. Elastin mRNA level was measured in vascular smooth muscle cells transfected for 72 hours with 100 nmol/L c-fos small interfering RNA (siRNA), 50 nmol/L c-jun siRNA ( ), or with the nontargeting siRNA (NT, ). normalized to β-actin mRNA levels (A) or to total RNA concentrations (B). n=3 or 4 for all experiments. Statistical significance was determined using an unpaired 2-tailed Student t test. \( *P<0.05; **P<0.001 \) compared with the control condition.

Inhibition of ERK1/2 Phosphorylation Increases Elastin Synthesis in vSMCs

Because inhibition of ERK1/2 phosphorylation seems to be a potential strategy to stimulate elastin synthesis, vSMCs were treated with 10 μmol/L U0126. After 24 hours of treatment, phosphorylation of ERK1/2 relative to β-tubulin or total ERK1/2 was significantly decreased (Figure 5A and Figure 5SA–SSD). This inhibition led to an increase in elastin gene transcription (Figure 5B and Figure 5E) and in the steady-state level of elastin mRNA (Figure 5C). In the same experimental conditions, mRNA encoding for fibrillin-1, fibrillin-5, LOX, LOXL1, and collagen-I (Figure S6) or β-actin and GAPDH (Figure S6) were not significantly modified. AP1 transcription factors were also analyzed. After 24 hours of treatment, c-fos expression, a well-characterized downstream target of activated ERK1/2, was decreased at both mRNA (Figure 5D) and protein (Figure 5A and Figure 5SE) levels. On the contrary, fra1 mRNA (Figure S6), c-jun mRNA (Figure 5E), and c-jun protein (Figure 5A and Figure S5F) showed no significant variations. The effect of ST638, an inhibitor of all tyrosine kinases (100 μmol/L, 1 hour), which also induced an increase in elastin gene transcription (Figure S7) additionally supports the conclusion that inhibition of ERK1/2 phosphorylation increases elastin synthesis.

Figure 5. U0126, an inhibitor of MEK1/2, increases in vitro elastin synthesis. A. Phosphorylated extracellular signal-regulated kinases 1/2 (p-ERK1/2), ERK1/2, c-fos, c-jun, and β-tubulin proteins were extracted from control vascular smooth muscle cells (vSMCs) or vSMCs incubated for 24 hours with 10 μmol/L U0126 and analyzed by immunoblot. Steady-state levels of elastin pre-mRNA (B) and elastin (C), c-fos (D), c-jun (E) mRNAs were measured in the corresponding conditions. n=6, controls ( ), U0126 ( ), \( *P<0.05; **P<0.01 \) compared with the control condition.

In Vivo Inhibition of ERK1/2 Phosphorylation Increases Elastin Synthesis in Young BN Rat Aorta

Two concentrations of U0126, 1 or 5 mg/kg, were administered intravenously to young BN rats to analyze their effects after 1, 24, or 48 hours on mRNA and proteins of the aortic arch. One hour after injection of 5 mg/kg U0126, ERK1/2 phosphorylation was significantly decreased, as shown by immunoblot (Figure 6A and Figure S8). After 24 hours, phosphorylation of ERK1/2 had returned to its normal level. With the same dose of inhibitor (5 mg/kg), elastin pre-mRNA was significantly increased 1 and 24 hours after the injection and elastin mRNA, after 24 and 48 hours (Figure 6B and 6C). When a lower concentration of inhibitor was used (1 mg/kg), the same variations in elastin pre-mRNA and mRNA levels were observed, but only the increases in elastin pre-mRNA after 1 hour and in elastin mRNA after 48 hours were significant.

Then, a chronic treatment was performed in young BN rats, using micro-osmotic pumps that diffused U0126 for 10 weeks, to analyze its effect on the quantity of polymerized elastin within the aortic extracellular matrix. After 10 weeks of treatment, body weight and body length of control and treated rats were similar (Table S1), but the composition of the aortic extracellular matrix was modified. U0126 increased insoluble elastin content by 3.7% when it was expressed as a percentage of the thoracic aortic dry weight (Figure 7A) and by 5.9% when expressed as mg/cm aorta/cm nose-to-rump length (Figure 7B) without affecting the percentage of collagen (Figure 7C) or the percentage of cell proteins (control: 15.21±3.8; U0126: 16.27±1.0). Histological studies were performed on infrarenal abdominal sections to analyze the effect of U0126 on elastic fiber network organization and vSMC proliferation. Hematoxylin–eosin staining showed that there was no difference in vSMC density in the media of the U0126 group compared with controls (control: 7129±1150 nuclei/mm2; U0126: 7052±1067 nuclei/mm2). Orcein staining showed that elastic lamellae in the U0126 group were well organized and thicker than in controls (Figure 7D). Quantification of the thickness of the internal elastic lamina (IEL) and medial elastic lamellae (EL) confirmed this result. Indeed, EL thickness of the U0126 group was significantly increased by 11% compared with controls (P<0.05), whereas IEL thickness was increased by 6.8% (P=0.281). The effect of
the treatment on the thickness of IEL and EL using a 2-way ANOVA analysis followed by the Scheffé test was also significant \((P<0.01)\). Investigation of the cardiovascular parameters using echocardiography and blood pressure measurements, performed at the end of the experiment, did not reveal any differences between controls and the U0126 group (Table S1).

In summary, treatment with U0126, which inhibits phosphorylation of ERK1/2, increased elastin gene expression in vSMCs and elastin content and thickness of elastic lamellae in the thoracic aorta of BN rats, without functional or morphological cardiovascular alterations.

**Discussion**

The present study is the first proof-of-concept of using U0126, an inhibitor of ERK1/2 phosphorylation, to increase elastin synthesis in a rat model deficient in aortic elastin content. Using vSMCs isolated from the aorta of the BN rat, we have shown that the marked increase in intracellular \(\text{Ca}^{2+}\) mediated by A23187 decreases the steady-state levels of several mRNAs encoding for proteins implicated in elastic fiber formation. We also showed that A23187 decreases both the transcription of the elastin gene and the stability of elastin mRNA. Furthermore, we provide evidence that the decrease in elastin gene transcription mediated by an increase in \([\text{Ca}^{2+}]_i\) requires the phosphorylation of ERK1/2 and the recruitment of AP1 transcription factors. Our study specifically demonstrates for the first time that inhibition of ERK1/2 phosphorylation increases elastin synthesis both in vitro, in vSMCs, and in vivo, in the BN aorta, suggesting that inhibition of ERK1/2 phosphorylation might be a potential therapeutic strategy in vascular pathologies due to a deficit in elastin, such as SVAS.

Among molecules described to regulate elastin synthesis, we note that the downregulation of the elastin gene in rat

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**Figure 6.** Inhibition of extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylation by a single injection of U0126 stimulates elastin gene transcription in the aorta of Brown Norway (BN) rats. **A**, Phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, and \(\beta\)-tubulin proteins were extracted from the aortic arch of BN rats 1 or 24 hours after the injection of U0126 (1 or 5 mg/kg) and analyzed by immunoblot \((n=6)\). **B**, Steady-state levels of elastin pre-mRNA were measured in aortic arch of controls (◼) and rats treated with 1 or 5 mg/kg U0126 (▄) for 1 and 24 hours \((n=6)\). **C**, Steady-state levels of elastin mRNA were measured in aortic arch of controls (◼, \(n=10\)) and rats treated with 1 or 5 mg/kg U0126 (▄) for 1 hour \((n=6)\), 24 hours \((n=10)\), and 48 hours \((n=4)\). *P<0.05; **P<0.01 compared with the control condition.

**Figure 7.** A chronic treatment of Brown Norway rats with the inhibitor of MEK1/2, U0126, increases aortic elastin content. Elastin (A and B) and collagen (C) contents are expressed as the percentage of aortic dry weight (A and C) or weight/cm aorta/cm nose-to-rump length (B) in control rats (◼) and rats treated with U0126 (▄). \(n=9\) in each group. *P<0.05 compared with the control group. **D**, Hematoxylin–eosin or orcein staining of transverse sections of the abdominal aorta from control rats and U0126-treated rats. Scale bar, 10 \(\mu\)m. **E**, The thickness of internal elastic lamina (IEL) and elastic lamellae (EL) was measured on orcein-stained sections of abdominal aorta from control rats (◼, \(n=7\)) and rats treated with U0126 (▄, \(n=8\)). ns, not significant; *P<0.05 compared with controls.
fibroblasts by both bFGF and EGF is mediated by activation of the ERK1/2 cascade.\textsuperscript{13-15} ERK1/2 activated by bFGF and EGF translocates to the nucleus where it accumulates, resulting in phosphorylation of Elk-1, which then induces c-fos expression that upregulates fra1.\textsuperscript{14,15} Both fra1 and c-fos can be heterodimerized with c-jun to form the complex AP1. In the human elastin gene promoter, an AP1-like site located at -564 to -558 bp has been described to repress elastin gene transcription mediated by recruitment of API transcription factors.\textsuperscript{5,10,13,16} This AP1-like site (TGTTCTCT) is conserved in the rat elastin gene promoter at -568 to -574 from the ATG translation initiation site (NC_005111). So, we made the hypothesis that API transcription factors could downregulate the rat elastin gene in the same way as for the human elastin gene via fixation of API on the promoter. Here, we have shown that an inhibition of elastin gene transcription occurs after activation of the ERK1/2 pathway and is correlated with the increase in API transcription factor expression.

Inhibition of ERK1/2 phosphorylation abolished the effect of A23187 on elastin gene transcription and so convincingly demonstrated that A23187-initiated elastin gene transcription inhibition requires the activation of ERK1/2. Silencing of the API transcription factors c-fos and c-jun increased the steady-state level of elastin mRNA, demonstrating the implication of API in elastin gene regulation. Taken together, these results provide strong evidence that a mobilization of Ca\textsuperscript{2+} in vSMCs results in activation of ERK1/2 and API expression which repress elastin gene transcription, as described in bFGF- and EGF-stimulated rat fibroblasts.

Variations in [Ca\textsuperscript{2+}] in vSMCs could occur when the free K\textsuperscript{+} concentration is modified. Previously, it was demonstrated that induction of K\textsuperscript{+} efflux by minoxidil, a K\textsubscript{ATP} channel opener, increased elastin synthesis in vSMCs and skin fibroblasts at a pretranslational level.\textsuperscript{23-25} On the contrary, high extracellular K\textsuperscript{+} concentrations decrease elastin synthesis in vSMCs.\textsuperscript{26} Inhibition of the effect of K\textsuperscript{+}-mediated elastin reduction by nifedipine, an inhibitor of Ca\textsuperscript{2+} influx, suggested that K\textsuperscript{+} regulates elastin synthesis via an influx of Ca\textsuperscript{2+}.\textsuperscript{21} A decrease in the intracellular concentration of potassium ([K\textsuperscript{+}]) thus leads to an increase in elastin synthesis via hyperpolarization of the membrane and the closing of voltage-dependant Ca\textsuperscript{2+} channels.\textsuperscript{23-27} Whereas an increase in [K\textsuperscript{+}], depolarizes the membrane and induces the opening of voltage-dependant Ca\textsuperscript{2+} channels, which leads to an increase in [Ca\textsuperscript{2+}], and consequently a decrease in elastin synthesis.\textsuperscript{21} Using vSMCs isolated from the BN rat aorta, we demonstrate in this study that A23187, a Ca\textsuperscript{2+} ionophore, decreases the steady-state levels of several mRNAs encoding for proteins composing elastic fibers (elastin, fibrillin-1, and fibrulin-5) or catalyzing the cross-linking of elastin monomers (LOX and LOXL1). Previously, we reported that glibenclamide, a K\textsuperscript{+} channel blocker, acts as A23187 by decreasing the expression of elastin, fibrillin-1, fibrulin-5, LOX, and LOXL1 mRNAs in vSMCs, whereas different K\textsuperscript{+} channel openers such as minoxidil sulfate, diazoxide, and pinacidil increase their steady-state levels.\textsuperscript{20} These results support the previous report that K\textsuperscript{+} regulates elastin expression via a Ca\textsuperscript{2+} influx.\textsuperscript{21} This hypothesis was further strengthened by analysis of the expression of API transcription factors; A23187 and glibenclamide increase their expression, whereas K\textsuperscript{+} channel openers decrease their expression (Figure S9). Both K\textsuperscript{+} and Ca\textsuperscript{2+} could regulate elastin gene transcription via the ERK1/2-API signaling pathway.

ERK1/2 is a common signaling pathway, which is activated by several factors, some of which (bFGF, EGF; HB-EGF) have already been implicated in the downregulation of elastin synthesis.\textsuperscript{6,14,15} Thus, inhibition of its phosphorylation seems to be a promising strategy to regulate elastin synthesis in models deficient in elastin content. Using aortic vSMCs isolated from the BN rat, an inbred strain deficient in elastin synthesis, we demonstrated that inhibition of ERK1/2 phosphorylation by U0126, an inhibitor of MEK1/2 activity, increases elastin gene transcription. Inhibition of factors upstream to ERK1/2 using ST638, an inhibitor of all tyrosine kinases, also increased elastin gene transcription, further supporting the ability of ERK1/2 inhibitors to increase elastin expression. Moreover, U0126 treatment had no effect on the expression of several mRNAs encoding for other proteins implicated in elastic fiber formation (fibrillin-1, fibrulin-5, LOX, and LOXL1), strengthening the interest of using U0126 in models deficient in elastin content because of its specificity. Using U0126 to increase elastin synthesis should not alter the expression of the other genes implicated in the formation of elastic fibers and thus will not lead to defective elastic fiber formation.\textsuperscript{26-31} On the contrary, this specific effect is not deleterious to the formation of mature elastic lamellae and completely cross-linked elastin. Furthermore, because we have shown in this study, and previously,\textsuperscript{20} that [Ca\textsuperscript{2+}] and [K\textsuperscript{+}] can regulate the expression of fibrillin-1, fibrulin-5, LOX, and LOXL1, it is probable that the expression of these other elastic components is regulated via a signaling pathway distinct from ERK1/2.

In vitro, U0126 had no effect on the expression of several mRNAs encoding for proteins implicated in elastic fiber formation. In vivo, we demonstrated that U0126 increased insoluble elastin content, expressed both as a percentage of the dry weight of the thoracic aorta and as mg/cm aorta/cm nose-to-rump length. The significant increase in insoluble elastin content in U0126-treated animals demonstrated that the newly formed elastin was adequately cross-linked, as it was resistant to the chemical treatment used for its purification. This was further supported by the homogeneous increase in elastic fiber thickness observed at the histological level. The greater increase in EL thickness compared with that of the IEL could be explained by the presence of a VSMC layer that synthesizes elastin on each side of the EL, whereas the IEL has a layer of vSMCs on one side and endothelial cells on the other side. However, this treatment induced no changes in the relative content (%) of collagen. Moreover, U0126 did not change the number of vSMCs, which was confirmed by the measurement of cell protein concentration in the 0.3% SDS extract of each aorta. So, taking together the in vitro and the in vivo results, U0126 increases elastin synthesis without affecting the quantity of the other aortic components.

SVAS, isolated or in patients with Williams–Beuren syndrome (WBS), is an autosomal dominant disease characterized by a hyperproliferation of vSMCs and their migration to the intima that can lead to an occlusion of the aorta.\textsuperscript{2} A correlation has been found in SVAS and WBS between the low
level of elastin deposition and the increase in vSMC proliferation. Moreover, in vitro, this abnormal vSMC proliferation in SVAS and WBS could be reversed by addition of exogenous insoluble elastin, demonstrating that the reduced elastin content in SVAS and WBS leads to the increase in vSMC proliferation. Recently, SVAS induced pluripotent stem cell–derived SMCs were produced. As described in SVAS and WBS SMCs, SVAS induced pluripotent stem cell–derived SMCs had fewer organized networks of smooth muscle alpha actin, a hallmark of mature contractile SMCs, and showed an abnormal proliferation, respectively reversible with addition of insoluble elastin or U0126. The combined effects of U0126 on abnormal proliferation of SVAS induced pluripotent stem cell–derived SMCs and elastin synthesis that we describe in this study might provide the basis for a potential therapeutic strategy for SVAS patients.

The most common form of stenosis in WBS patients is SVAS, but stenoses can also occur in other arteries, such as the descending aorta and the pulmonary, coronary, renal, mesenteric, and intracranial arteries. Thus, it is important to increase elastin synthesis within all arteries to prevent the development of stenoses. In our study, whatever the part of the aorta analyzed and whatever the methodology used, we observed an increase in elastin synthesis. Moreover, the increase in elastin mRNA from rats euthanized 1, 24, or 48 hours after an injection of U0126 at 1 or 5 mg/kg is the same within the aortic arch and the thoracic aorta (data not shown). Thus, U0126 seems to have a beneficial effect on elastin synthesis throughout the aorta, whatever the embryological origin of the vSMCs.

**Perspectives**

Haploinsufficiency of elastin in patients with WBS leads, in more than half of cases, to development of SVAS and hypertension. The discovery of molecules able to enhance elastin synthesis would be of interest to treat this condition. In the present study, we demonstrated that an increase in [Ca²⁺], decreases (1) the steady-state level of mRNAs encoding for proteins composing elastic fibers in vitro, (2) both elastin gene transcription and its mRNA stability, and (3) elastin gene transcription via activation of the ERK1/2-AP1 signaling pathway. Moreover, we have shown that inhibition of ERK1/2 phosphorylation increases (1) elastin gene transcription in vitro without affecting the steady-state level of mRNAs encoding for other proteins composing elastic fibers and (2) elastic fiber thickness and aortic insoluble elastin content in the BN rat in vivo, without functional or morphological cardiovascular alterations. Silencing of c-fos and c-jun, two AP1 transcription factors that are downstream to ERK1/2, increases elastin synthesis in vitro; thus, it would be of interest to further study the effect of silencing AP1 transcription factors on elastogenesis in vivo. It would be also of interest to treat elastin-deficient or haploinsufficient mice with elastogemec molecules, such as U0126, to analyze the effect of elastin neosynthesis on the development of aortic stenosis and hypertension. Inhibitors of ERK1/2 phosphorylation could be used in a clinical trial for treating young patients with WBS, as currently underway with the elastogenic molecule, minoxidil, that is a potassium channel opener (NCT00876200).

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**Disclosures**

None.

**References**


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INHIBITION OF ERK1/2 PHOSPHORYLATION, A NEW STRATEGY TO STIMULATE ELASTOGENESIS IN THE AORTA

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Short title: ERK1/2 inhibition induces elastic fiber formation

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Detailed Methods

Animals

Male inbred Brown Norway (BN/Orl Rj) rats were supplied by Elevage Janvier (Le Genest-St-Isle, France). The studies were carried out under authorization N° C 75-18-03 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 N° 2012-15/698-0082.

Primary cultures and in vitro experiments

Thoracic aorta of 6 week-old BN rats were dissected out and media isolated from adventitia. Media were fragmented and digested with 0.14% (w/v) type I collagenase (220U/mg, Life Technologies) and 0.04% (w/v) porcine pancreatic elastase (4.65U/mgP, Worthington Biochemical Corporation) solution for 30min. at 37°C. Then, to release vSMCs, digested fragments were vigorously agitated in fresh medium and cultured in Dulbecco’s modified Eagle’s low glucose medium (DMEM, Life Technologies), supplemented with 20% fetal bovine serum (FBS, PAA Laboratories) and antibiotic cocktail (PAA Laboratories): penicillin (100U/mL), streptomycin sulphate (100µg/ml) and amphotericin B (250ng/ml) at 37°C in a 5% CO2/95% air incubator. In all pharmacological experiments described here, confluent cultures at passage 5 in 9,6cm² culture wells were used. vSMCs were deprived of FBS for 24 hours and then drugs dissolved in DMSO and diluted in FBS-free DMEM were added to wells. In control wells, a similar volume of DMSO was added. Cells were incubated for different times and different concentrations with A23187 - a calcium ionophore - (Sigma-Aldrich) or U0126 - an inhibitor of MEK1/2 kinase activity - (Cell Signaling Technology). For the study of mRNA degradation, vSMCs were deprived for 24 hours and then preincubated with A23187 (10 µmol/L) in FBS-free DMEM for 4 hours. A23187 (10 µmol/L), diluted in FBS-free DMEM containing 60 µmol/L of 5,6-Dichlorobenzimidazole-1-β-D-ribofuranoside (DRB, Sigma-Aldrich), an inhibitor of RNA synthesis, was then added to wells and vSMCs studied 6 and 20 hours later.
**siRNA transfection**

Subconfluent vSMCs at passage 5 in 9.6cm² culture wells were deprived of FBS for 24 hours. ON-TARGETplus SMARTpool c-fos (L-099915-02) and c-jun (L-089158-01) siRNA were respectively used at 100 and 50 nmol/L to transfect vSMCs using DharmaFECT 2 Transfection Reagent (Thermo Fisher Scientific). A similar concentration of ON-TARGETplus Non-targeting Pool (D-001810-10-05) was added to control wells. Cells were analyzed 72 hours after siRNA transfection.

**RNA and protein purification**

To purify RNA and proteins from a same sample, cultured vSMCs or aortic arch were lysed in 1ml of Qiazol® Lysis Reagent (Qiagen). Addition of 200µL of chloroform (Sigma-Aldrich) followed by a centrifugation (15min., 12,000g), separated the solution into an aqueous phase, containing RNA, and an organic phase with proteins. mRNA, pre-mRNA and miRNA present in the aqueous phase were then purified with the miRNeasy Mini kit (Qiagen) and genomic DNA was digested with DNase-I (Rnase-Free DNase Set, Qiagen), according to the manufacturer's directions. Proteins of the organic phase were precipitated by adding 1.2 ml of isopropanol (Sigma-Aldrich). Protein pellets were washed with a 0.3mol/L guanidine hydrochloride, 95% ethanol solution, vacuum dried and resuspended in 100µL of 62.5mmol/L Tris-HCl, 10% glycerol and 2% SDS solution.

**Reverse transcription-PCR**

Total RNA was quantified with Quant-iT™ RiboGreen® RNA Assay Kit (Life Technologies). Two hundred nanograms of total RNA were used to perform Reverse transcription with miScript Reverse Transcription Kit (Qiagen) to reverse transcript mRNA, pre-mRNA and miRNA. Real-time PCR were performed in the CFX96 device with the iQ SYBR Green supermix Kit Detection (Bio-Rad) using specific primers (Table S2). Results were normalized to the total RNA concentration and expressed as relative expression.
**Immunoblotting**

Protein concentrations were determined with BC Assay Protein Quantitation Kit (Interchim). Samples were separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes (GE Healthcare). Membranes were then blocked with PBS-T [Phosphate-buffered saline - 0.1% Tween 20] containing 5% (w/v) BSA for 1 h at RT, and incubated overnight at 4°C with primary antibodies: p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), c-fos, c-jun or β-tubulin (Cell Signaling Technologies). After washings with PBS-T, membranes were incubated with peroxidase-conjugated affiniPure goat anti-(rabbit IgG) (Jackson ImmunoResearch Laboratories) for 1 h. Immune complexes were revealed using ECL+ chemiluminescence kit (GE Healthcare) and visualized by exposure to films (GE Healthcare). Densitometric analysis was performed using the PC version of NIH Image software (Scion Image) after photography with a computer-assisted camera (GS-800 Calibrated Densitometer, Bio-Rad). Specific protein levels were normalized to β-tubulin protein levels.

**In vivo experiments**

First, we performed two short-term *in vivo* experiments where 8 week-old BN rats were injected intravenously with 1 or 5mg/kg of U0126 (Cell Signaling Technology) solubilized in DMSO and diluted in physiologic serum, as previously described \(^1,^2\). A similar volume of DMSO was injected into control rats. In the first experiment, rats were sacrificed 24 and 48hrs after the injection whereas, in the second one, they were sacrificed 1 and 24hrs after the injection. For the long-term treatment, a protocol from a previous publication \(^3\) were adapted to treat 3 week-old BN rats for 10 weeks with U0126 (Cell Signaling Technology). U0126 solubilized in DMSO and then diluted in physiologic serum at 10 µmol/L were continuously delivered using an intraperitoneally-implanted micro-osmotic pump (Alzet Osmotic Pumps). The Model 1004 was used to diffuse the drug for 4 weeks and the model 2006 for 6 additional weeks. Control rats were also implanted intraperitoneally with micro-osmotic pumps that diffused the same concentration of DMSO as in treated rats. The experiment included 9 control rats and 9 treated rats. Body weights were measured twice a week from the beginning to the end of the experiments. Body length (nose-to-rump) was measured at the end of each experiment, on anesthetized animals before sacrifice.
Blood pressure measurements

Non-invasive measurements of systolic arterial pressure and heart rate were performed four weeks after the beginning, and at the end of the treatment using the tail-cuff-method (Phymep).

Ultrasound study

Transthoracic echocardiography was performed as previously described with a Vevo2100 (Visualsonics, Toronto, Canada) equipped with a 15-22MHz linear transducer held by the Vevo Imaging Station (Integrated Rail System), allowing precise probe positioning with a bench mounted adjustable rail system, under isoflurane anaesthesia (0.75% to 1.0% in oxygen) with spontaneous ventilation. The body temperature was maintained with a heating pad. Heart rate was measured from EKG tracing. Systolic and diastolic diameters of ascending aorta (AA) were measured from time-mouvement mode imaging, as gated from simultaneous EKG recording. Time-motion mode from parasternal long-axis view was obtain for measurements of the left atrium diameter (LA), left ventricle (LV) end-diastolic and end-systolic diameters, and septum and posterior wall thickness. LV ejection fraction, and LV mass were computed. Pulse wave Tissue Doppler was used to assess posterior wall systolic (Spw) and diastolic (Epw) velocities.

Sample harvesting

At the end of all in vivo experiments, the aortic arch was removed and lysed in 1ml of Qiazol to purify RNA and proteins. At the end of the long-term in vivo experiment, 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 30 min. with buffered formalin for histological analysis.

Histology

Formalin-fixed infrarenal abdominal aortas were embedded in paraffin to obtain 6µm-thick sections and stained with orcein or hematoxylin-eosin. For each animal, four images (at magnification x400) from abdominal aortic sections stained with orcein or hematoxylin-eosin were scanned with a computer-assisted camera (Nanozoomer 2.ORS, Hamamatsu Photonics) and analysed with the software Nanozoomer Digital Pathology (Hamamatsu Photonics). On each image of abdominal aortic section stained with orcein, we performed, blindly, 10, 10 and
20 measurements of media, internal elastic lamina and elastic lamellae thickness, respectively. The number of vSMCs nuclei in the media was counted on sections of abdominal aorta stained with hematoxylin-eosin.

Purification and quantification of aortic elastin and collagen

Biochemical analysis of the descending thoracic aorta was performed as previously described\textsuperscript{5, 6}. Length and width of thoracic aortas were measured under a dissecting microscope. Elastin and collagen were both expressed as a percentage of thoracic aortic dry weight.

Statistical Analysis

Values are expressed as means ± SD. The significance of differences between groups was tested using the non-parametric Mann-Whitney U test unless otherwise stated. Statistical significance was defined as [*], p<0.05; [**], p<0.01. Statistical analysis was performed using GraphPad Prism version 5.00 software.
Reference List


<table>
<thead>
<tr>
<th>Morphological or Functional Parameters</th>
<th>Control (n=6)</th>
<th>U0126 (n=6)</th>
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<tr>
<td><strong>Animal data</strong></td>
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<tr>
<td>Body weight (g)</td>
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<td>Left atrial diameter (mm)</td>
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<td>AA diastolic diameter (mm)</td>
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Table S1: A chronic treatment of BN rats with U0126 does not modify morphological and functional parameters of the heart.

Body weight, body length and systolic blood pressure were measured after treatment of BN rats with U0126 for 10 weeks. Morphologic parameters of the heart and ascending aorta (AA), and functional cardiac parameters of control rats and rats treated with U0126 were recorded by echography. No significance variations were observed compared to the control group.
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Table S2: Primer sequences used to perform real-time PCR.
Figure S1: The calcium ionophore A23187 decreases the steady-state levels of several mRNAs encoding for proteins composing elastic fibers (fibrillin-1 and fibulin-5) or catalyzing the cross-linking of elastin (LOX and LOXL1) whereas fra1 mRNA level is increased.

Steady-state levels of fibrillin-1 (A), fibulin-5 (B), LOX (C), LOXL1 (D) and fra1 (E) mRNAs were measured in vSMCs incubated for 24 hours with different concentrations of A23187 (□) or in controls (■) (n=6). [*], p<0.05; [**], p<0.01 compared to the control condition.
Figure S2: The increase in miR-29b induced by the calcium ionophore A23187 occurs too late to explain the decreased elastin mRNA stability.

Steady-state levels of elastin mRNA (A), miR-29a (B), miR-29b (C) and miR-29c (D) were measured in vSMCs incubated for different times with 0.5 μmol/L A23187. n=6, controls (■), A23187 (□), [*], p<0.05; [**], p<0.01 compared to the control condition.
Figure S3: The calcium ionophore A23187 stimulates ERK1/2 phosphorylation and AP1 transcription factors expression before decreasing elastin synthesis.

Quantification of phosphorylated ERK1/2 (p-ERK1/2, A, B, C, D), ERK1/2 (E, F), c-fos (G) and c-jun (H) proteins extracted from control vSMCs (■) and vSMCs incubated for different times with 0.5μmol/L A23187 (□) on immunoblots, normalized to β-tubulin (A, B, E, F, G, H) or ERK 1/2 (C, D) proteins (n=6). [*], p<0.05; [**], p<0.01 compared to the control condition.
Figure S4: Specific siRNAs down-regulate AP1 transcription factors.

(A) C-fos, c-jun and β-tubulin proteins were extracted from vSMCs transfected for 72 hours with 100 nmol/L c-fos siRNA, 50 nmol/L c-jun siRNA (●) or with the non-targeting siRNA (NT, □) and analyzed by immunoblot. C-fos (B) and c-jun (C) immunoblots were quantified and normalized to β-tubulin. C-fos (D) and c-jun (E) mRNAs were quantified for the different conditions, normalized to total RNA concentrations. n=3 or 4 for all experiments. Statistical significance was determined using an unpaired two-tailed Student’s t-test. [*], p<0.05; [**], p<0.01 compared to the control condition.
Figure S5: U0126, an inhibitor of MEK1/2, increases in vitro elastin synthesis.
Quantification of phosphorylated ERK1/2 (p-ERK1/2, A, B, C, D), c-fos (E) and c-jun (F) proteins extracted from control vSMCs (■) and vSMCs incubated for 24 hours with 10μmol/L U0126 (□) on immunoblots, normalized to β-tubulin (A, B, E, F) or ERK 1/2 (C, D) proteins (n=6). [**], p<0.01 compared to the control condition.
Figure S6: U0126, an inhibitor of MEK1/2, increases *in vitro* elastin gene transcription without affecting mRNA encoding for several other proteins composing elastic fibers or catalyzing the elastin cross-linking.

Steady-state levels of elastin pre-mRNA 2, and fibrillin-1, fibulin-5, LOX, LOXL1, collagen-1, β-actin, GAPDH and Fra1 mRNAs were measured in control vSMCs (■) or in vSMCs incubated for 24 hours with 10μmol/L U0126 (□) (n=6). [**] p<0.01 compared to the control condition.
Figure S7: ST638 increases in vitro elastin synthesis

Elastin pre-mRNA was measured in vSMCs incubated for 1 hour with 100 µmol/L ST638 (■) or in controls (■) (n=6). [**], p<0.01 compared to the control condition.
Figure S8: Inhibition of ERK1/2 phosphorylation with a single injection of U0126 stimulates elastin gene transcription in the aorta of BN rats.

Quantification of phosphorylated ERK1/2 proteins (p-ERK1/2) extracted from aortic arch of BN rats treated for 1 or 24 hours with 1 or 5 mg/kg (▲) or in controls (■) on immunoblots, relative to β-tubulin (A, B) or ERK1/2 (C, D) proteins (n=6). [*], p<0.05 compared to the control condition.
Figure S9: Potassium channel openers decrease AP1 transcription factors whereas a potassium channel blocker increases their expression.

Steady-state levels of c-fos (A) and fra1 (B) were measured in vSMC incubated for 24 hours at different concentrations of an ATP-dependent potassium channel blocker (glibenclamide, □), three ATP-dependant potassium channel openers (minoxidil-sulfate and diazoxide, pinacidil, ■) or in controls (■) (n=6). [*], p<0.05; [**], p<0.01; [***], p<0.001 compared to the control condition.