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**Title: Impaired vascular mechanotransduction in a transgenic mouse model of CADASIL arteriopathy**

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**ABSTRACT**

**Background and purpose** - CADASIL is an inherited small-vessel disease responsible for lacunar strokes and cognitive impairment. The disease is caused by highly stereotyped mutations in *Notch3*, expression of which is highly restricted to vascular smooth muscle cells (vSMC). The underlying vasculopathy is characterized by degeneration of vSMC and the accumulation of granular osmiophilic material (GOM) and Notch3 protein within the cell surface of these cells. In this study we assessed early functional changes related to the expression of mutant *Notch3* in resistance arteries.

**Methods** - Vasomotor function was examined *in vitro* in arteries from transgenic mice that express a mutant *Notch3* in vSMC. Tail artery segments from transgenic and normal wild-type male mice were mounted on small-vessel arteriographs and reactivity to mechanical (flow and pressure) forces and pharmacological stimuli were determined. Mice were studied at 10-11 months of age when vSMC degeneration, GOM deposits and Notch3 accumulation were not yet present.

**Results** - Passive arterial diameter, contraction to phenylephrine and endothelium-dependent relaxation to acetylcholine were unaffected in transgenic mice. By contrast, flow-induced dilation was significantly decreased and pressure-induced myogenic tone significantly increased in arteries from transgenic mice compared with wild-type mice.

**Conclusions** - This is the first study providing evidence that mutant *Notch3* impairs selectively the response of resistance arteries to flow and pressure. The data suggest an early role of vascular dysfunction in the pathogenic process of the disease.

## INTRODUCTION

CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) is an inherited small vessel disease characterized by recurrent ischemic strokes, cognitive impairment and premature death<sup>1-3</sup>. On neuropathological examination, brains show a diffuse myelin loss and multiple small deep infarcts located within the white matter and basal ganglia. These changes are caused by a distinctive arteriopathy characterized by a progressive degeneration of vascular smooth muscle cells (vSMC) and the accumulation of granular osmiophilic material (GOM) within the basement membrane of these cells. Arterial changes are systemic although symptoms are restricted to the central nervous system<sup>4-5</sup>.

CADASIL is caused by highly stereotyped mutations in the Notch3 receptor, which lead to an odd number of cysteine residues within an Epidermal Growth Factor Repeat (EGFR) of the extracellular domain<sup>6-9</sup>. Recent works demonstrated that vSMC are the primary targets of the pathogenic process. In human adults, expression of Notch3 is highly restricted to vSMC. In CADASIL, there is an abnormal accumulation of Notch3 at the plasma membrane of vSMC because of an impaired clearance of the receptor<sup>10</sup>. Consistent with these data, transgenic mice that express a mutant *Notch3* in vSMC develop arterial lesions similar to that seen in CADASIL patients<sup>11</sup>.

Functional consequences of vessel changes in the CADASIL pathogenesis process remain to be elucidated. In CADASIL patients, two types of lesions affecting resistance arteries have been described that might affect hemodynamic. First, narrowing of the arterial lumen may reduce the baseline blood flow. Second, degeneration of vSMC may impair the vasomotor function. Analysis of transgenic mice that express a mutant *Notch3* revealed a third type of lesions that precede vSMC degeneration, GOM deposits and Notch3 accumulation. These changes are characterized the disruption of anchorage and adhesion of vSMC to neighboring cells as well as cytoskeleton abnormalities of vSMC<sup>11</sup>. Flow (shear stress) is the main mechanical stimulus activating vascular endothelial cells whereas pressure is the main mechanical stimulus responsible for a sustained vasoconstrictor (myogenic) tone in resistance arteries<sup>12-13</sup>. We and others have previously shown that vascular mechanotransduction to shear stress and tensile stress relies on the integrity of cytoskeletal proteins and intercellular communications<sup>12-17</sup>. On the basis of the aforementioned observations, we hypothesized that these early vascular changes may impair vasomotor function. In the present work, we assessed the vascular reactivity in response to vasoactive agents and mechanical forces in isolated arteries from transgenic mice expressing the Arg90Cys mutant Notch3 protein and wild-type littermates mice. We studied mice at 10 months of age when vSMC cytoskeleton and adhesion defects were present but GOM deposits, Notch3 accumulation and vSMC degeneration were absent. We focused on the tail caudal arteries because alterations have been especially well characterized in these vessels<sup>11</sup>.

## METHODS

### Animals

The transgenic mouse model of CADASIL arteriopathy used in this study has been described previously<sup>11</sup>. In brief, transgenic mice express a mutant human Notch3, carrying the arginine-to-cysteine mis-sense mutation at amino acid position 90 in the EGFR n°2, with the expression driven by the *SM22a* promoter. Two independent transgenic founder lines (TgMa and TgVe) that express a low level of mutant *Notch3* (< 50% of murine *Notch3*) in the tail arteries have been established, with TgVe expressing a higher level than TgMa. Male transgenic mice heterozygous for the transgene and their wild-type littermates were used. All mice were studied at 10-11 months when vSMC degeneration, GOM deposits and Notch3 accumulation were not yet detected.

The procedure followed in the care and euthanasia of the study animals was in accordance with the European Community Standards on the Care and Use of Laboratory Animals (Ministère de l'Agriculture and Préfecture de Paris, authorizations 00577 and 75-071).

### Determination of blood pressure

Blood pressure was measured by direct intra-arterial recording. Tygon catheter was inserted into the femoral artery on anesthetized mice with inhaled halothane. Mouse was allowed to recover from anesthesia and held under minimal restraint. Rectal temperature was maintained at 37 °-37.7C. Heart rate and blood pressure were monitored using a 4-Multichannel recorder (Gould RS 3400).

### Vascular Function

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (75 to 100 mg/kg). Tail caudal arteries were removed and cannulated at both ends in a video-monitored perfusion system (LSI, Burlington, VT) as previously described<sup>15,18</sup>. Briefly, arteries were bathed in a 5 ml organ bath containing physiological salt solution of the following composition (in mM) : 135.0, NaCl, 15.0, NaHCO<sub>3</sub>, 4.6 KCl, 1.5, CaCl<sub>2</sub>, 1.2, MgSO<sub>4</sub>, 11.0, glucose, 5.0, N-2-hydroxy-ethylpiperazine-N-2-ethylsulfonic acid. The pH was 7.4. The pO<sub>2</sub> was maintained at a value of 160 mmHg and the pCO<sub>2</sub> at a value of 37 mmHg. The bath solution was changed continuously at a rate of 4 ml min<sup>-1</sup>. (pH 7.4, pO<sub>2</sub> 160 mmHg, pCO<sub>2</sub> 37 mmHg). Perfusion of the artery was achieved with a similar physiological salt solution. Pressure was controlled by a servo-perfusion system and flow was generated by a peristaltic pump. Flow-induced dilation was studied by increasing flow rate by steps from 0 to 50  $\mu$ l. min<sup>-1</sup>. Data were obtained from arterial segments precontracted with phenylephrine to approximately 50% to 60% of maximum response and with intraluminal pressure set at 75 mmHg as described<sup>14</sup>. Myogenic tone was determined by increasing intraluminal pressure by steps from 10 to 150 mmHg without intraluminal flow<sup>15</sup>.

Diameters measured in normal physiological salt solution were considered as diameter under active tone or "active diameter" At the end of each experiment arterial segments were superfused with a

Ca<sup>2+</sup>-free physiological salt solution containing EGTA (2 mM) and sodium nitroprusside (10 μM) and pressure steps were repeated in order to determine the arteries' passive diameter<sup>14</sup>.

Results are given in micrometers for artery diameters. Myogenic tone was expressed as active tone (passive diameter - active diameter, in μm) or as the percentage of passive diameter (measured diameter/passive diameter X 100). Flow-induced relaxation was expressed as increase in diameter (μm) as a function of shear stress due to flow in each vessel. Shear stress was calculated for each individual segment of artery as previously described<sup>13-15</sup>  $\{\tau = 4 \eta \cdot Q / \pi \cdot r^3\}$ , where  $\eta$  is viscosity (poise = dyn . s . cm<sup>-2</sup>), Q is flow (ml/s), and r is radius (cm)}.

In a separate series of arterial segments, we examined dose-dependent contraction of tail arterial segments in response to phenylephrine (10 nmol/L to 1 mmol/L) and relaxation in response to acetylcholine (10 nmol/L to 100 μmol/L) after precontraction with phenylephrine (50% of the maximal contraction) as previously described<sup>15</sup>. Contraction in response to phenylephrine was expressed as change in diameter (μm) due to phenylephrine. Relaxation in response to acetylcholine was expressed as the percentage of dilation of the active tone (precontraction)<sup>15,16</sup>.

## Drugs

N-2-hydroxy-ethylpiperazine-N-2-ethylsulfonic acid (HEPES), ethylenbis-(oxyethyleninitrolo) tetraacetic acid (EGTA), phenylephrine, acetylcholine, were purchased from Sigma (St. Louis, MO). Other reagents were purchased from Prolabo (Paris, France). Drugs were dissolved and diluted in physiological salt solution (HEPES buffer). Concentrations are expressed as final concentration of each drug in the organ bath.

## Statistical analysis

Data are expressed as mean ± SEM. Significance of the differences between groups was determined by analysis of variance (ANOVA for consecutive measurements: responses to pressure, flow, phenylephrine and acetylcholine). Means were compared by unpaired t test or Bonferroni's test. *P* <0.05 was considered to be statistically significant.

## RESULTS

Body weight was not affected by the expression of the mutant protein (41±2 versus 39±2 g, transgenic mice (line TgMa or TgVe) versus wild-type littermate, n= 8 to 12 per group). Mean arterial blood pressure, measured in femoral artery, was not significantly different between transgenic and wild-type mice (108.3 ±4.9, n= 6 in TgMa mice; 105.6±1.8, n=5 in TgVe mice and 111.6±2.7, n=5 in wild-type mice).

### Pressure-induced arterial tone

In isolated tail caudal arteries stepwise increases in intraluminal pressure from 0 to 50 mmHg induced

an increase in artery diameter, whereas further step increases in pressure induced no more increase or a decrease in diameter reflecting the development of myogenic tone. The difference between passive diameter and the diameter measured under physiological conditions was considered as myogenic tone (Figure 1A). Myogenic tone was significantly increased in arteries from transgenic mice compared with their wild-type littermates (Figure 1A). Myogenic tone, when normalized to the corresponding passive diameters, was also significantly increased in arteries from transgenic mice compared with their wild-type littermates (Figure 1B).

Isolated tail caudal arteries were also submitted to stepwise increases in intraluminal pressure, in a  $\text{Ca}^{2+}$ -free physiological salt solution containing EGTA (2 mM) and sodium nitroprusside (10  $\mu\text{M}$ ). Under these conditions, arteries responded by a progressive rise in diameter. Internal diameter ranged from  $289.6 \pm 16.4 \mu\text{m}$  (pressure, 25 mmHg) to  $346.3 \pm 20.1 \mu\text{m}$  (pressure, 150 mmHg) in control mice. Passive arterial diameter curves were unaffected in transgenic mice as compared with their wild-type littermates (Figure 1C).

### **Flow-induced responses**

In arteries submitted to a pressure of 75 mmHg, increasing flow by step induced a progressive arterial dilation. Arterial dilations were quantified as increases in diameter. Flow (shear stress)-induced dilation was significantly reduced in caudal arteries from transgenic mice as compared with arteries from wild-type mice (Figure 2A).

From the flow and diameter data obtained, wall shear stress was calculated and plotted against the changes in arterial diameter. Figure 2B shows that a given step increase in wall shear stress elicits a significantly attenuated increase in diameter of caudal arteries of transgenic mice compared with those of wild-type mice.

### **Responses to vasoactive agents**

We examined contraction of tail caudal arteries from transgenic and wild-type mice in response to phenylephrine. All arteries contracted in a dose-dependent manner in response to phenylephrine. Vasoconstriction in response to phenylephrine was similar in transgenic and wild type mice (Figure 3A).

To study vasorelaxation, precontraction was matched in arteries from transgenic and wildtype mice. Acetylcholine induces an endothelium-dependent relaxation. Tail caudal arteries relaxed in a dose-dependent fashion in response to acetylcholine. Responses to acetylcholine were not impaired in transgenic mice as compared with their wild-type littermates (Figure 3B).

## **DISCUSSION**

In this study we showed that transgenic mice expressing a CADASIL mutant *Notch3* in vSMC exhibited a significant increase in pressure-induced contraction and a significant decrease in flow-

induced dilation in isolated caudal arteries. Vascular dysfunction was detected in 10 month-old transgenic mice, when mutant arteries exhibit disruption of adhesion of vSMC to neighboring endothelial cells and vSMC as well as cytoskeleton abnormalities of vSMC characterized by an increased number of dense plaques and dense bands<sup>11</sup>. In contrast phenylephrine-induced contraction and acetylcholine -induced dilation were unaffected in these transgenic mice, indicating that the defective transduction of mechanical forces did not arise from a global dysfunction of vSMC. Together the data indicate that transgenic mice exhibit a specific defect in the transduction of shear and tensile stress into dilation and constriction respectively. Impaired mechanotransduction with preserved agonist or receptor-induced tone has been previously reported in mice lacking *vimentin* or *dystrophin*<sup>14,15</sup>. Thus our findings further support the notion that mechanical forces (flow, pressure) and chemical stimuli act, at least in a great part, through different pathways<sup>19-22</sup>.

The specific molecular basis for the mechanotransduction defect in *Notch3* mutant arteries remains to be elucidated. Both myogenic tone and flow-mediated dilation depend on cytoskeleton integrity and cell to cell force transmission through the cytoskeleton, integrins and the extracellular matrix<sup>12-17</sup>. Previous works showed that pressure induced arterial tone involved an initial calcium entry into arteriolar SMC, subsequent activation of signaling pathways (PKC, PLC, Rho-A..) that sensitized the contractile apparatus to calcium and further resulted in the stimulation of actin polymerization in vSMC<sup>17,19,20,22</sup>. Increased myogenic tone likely arises from a direct deleterious effect of mutant *Notch3*, expression of which was specifically targeted in vSMC. Ultrastructural analyses are consistent with an increased actin polymerization in SMC of mutant arteries<sup>11</sup>. In contrast impaired flow-induced dilation, which primarily involves endothelial cells, likely results from an indirect effect of mutant *Notch3*. Ultrastructural changes of endothelial cells have been detected in mutant arteries<sup>11</sup>. Additionally, one might speculate that defective myoendothelial communications or even a chronic change in smooth muscle tone could account for this specific endothelial cell dysfunction<sup>23</sup>. Additional studies are required to address this issue.

Previous studies reported in CADASIL patients an impaired cerebral vasoreactivity, as determined by attenuated vasodilation to inhaled carbon dioxide or acetazolamide and, abnormal vasoconstrictor reactivity in isolated small gluteal arteries<sup>24-26</sup>. Of note, all three studies included essentially symptomatic patients with a mean age above 45 years, an age where the pathological hallmarks of the CADASIL arteriopathy, including GOM deposits, *Notch3* accumulation and vSMC degeneration, are already apparent. Thus these human studies have been difficult to interpret because it was unclear whether impaired vasoreactivity had or not a primary responsibility in the pathogenic process and whether it arose from vascular cells dysfunction or degeneration. In the present study, we demonstrate that vascular mechanotransduction is very early impaired, before the appearance of the pathological hallmarks of CADASIL. Thus, our findings suggest an early role of vascular dysfunction in the pathogenic process of the disease.

Reduced cerebral blood flow has been demonstrated in CADASIL patients using positron emission tomography, magnetic resonance imaging bolus tracking or phase contrast<sup>24,27-30</sup>. Pressure and flow

are two mechanical stimuli that determine the basal vascular tone in resistance arteries and allow for a rapid adaptation to changes in blood flow and pressure. Attenuation in flow-induced dilation might lead to a lesser adaptation to increases in blood flow in organs when a metabolic need requires a higher blood supply. Similarly, increased myogenic tone might lead to a lesser adaptation to decreases in blood pressure. Because there is no organ in the body as dependent as the brain on a continuous blood supply, defective mechanotransduction to shear and tensile stress might reduce cerebral blood flow sufficiently to cause ischemic cell injury.

In conclusion, the present study provides the first evidence that vascular reactivity to the mechanical factors, flow and pressure, is early and selectively impaired in arteries expressing a CADASIL mutant *Notch3*. Treatments that improve vascular mechanotransduction could be beneficial in *Notch3* mutation carriers at risk of developing the CADASIL disease.

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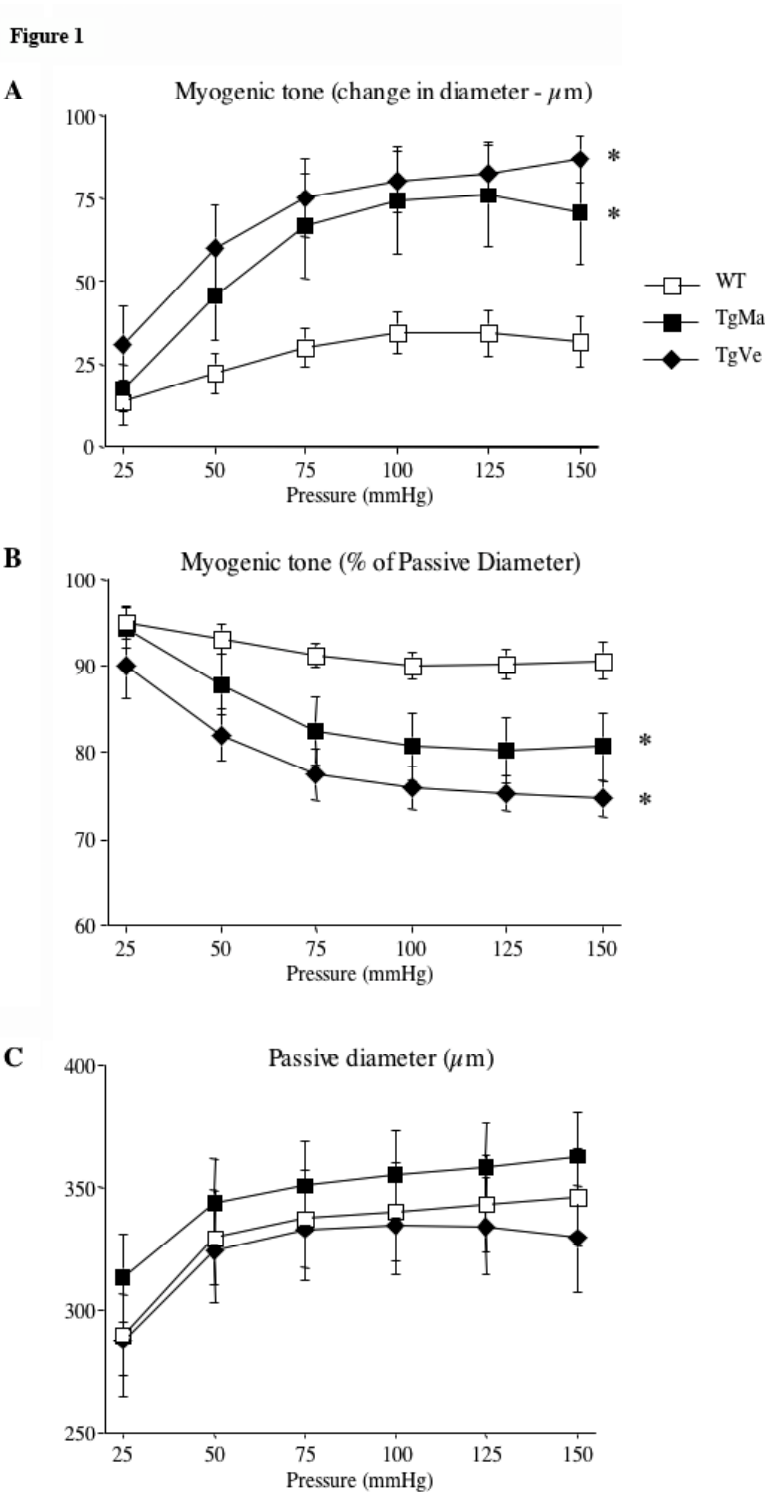
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**Figure 1:** Diameter changes measured in response to pressure in isolated tail caudal arteries.

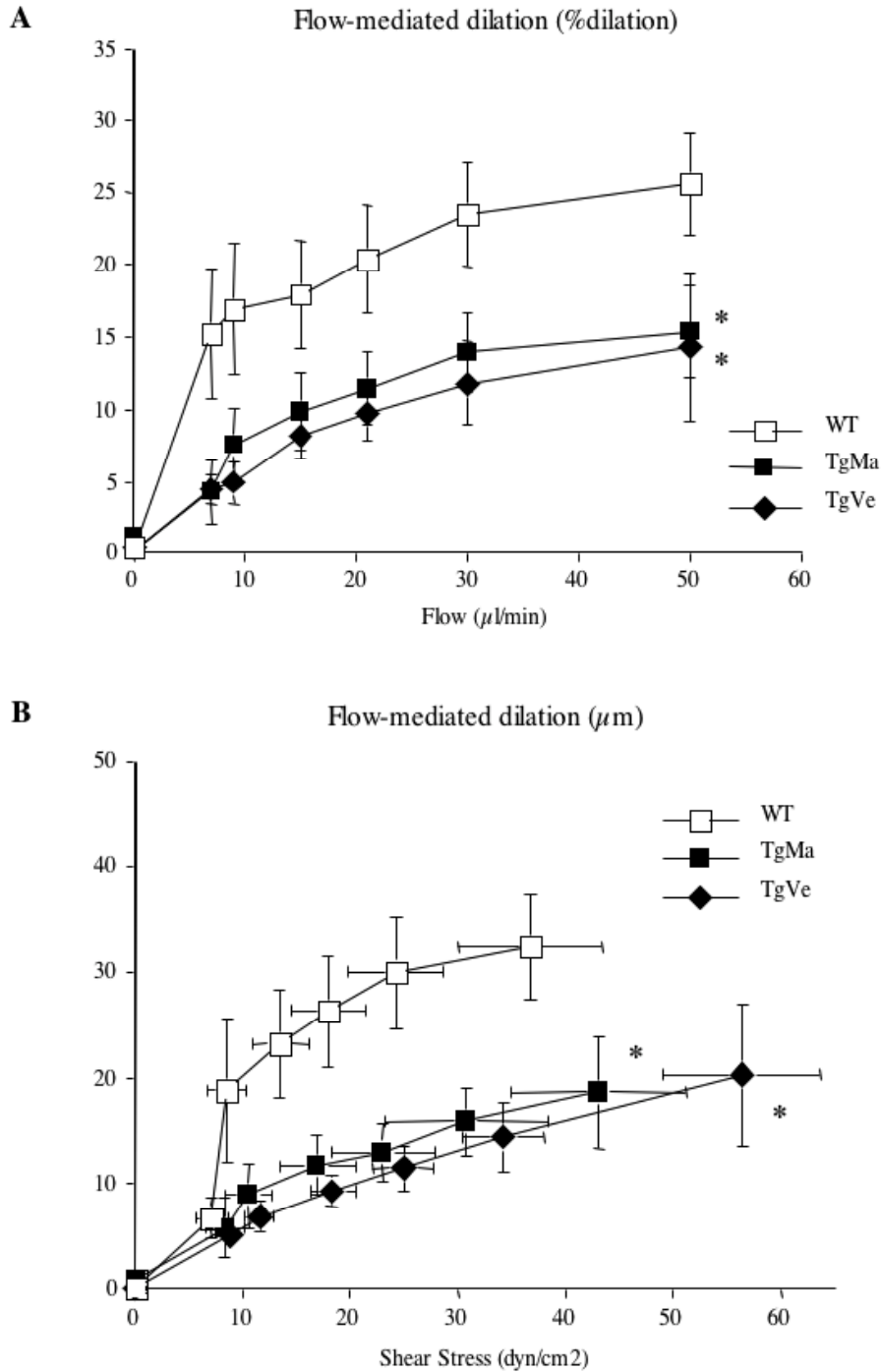
A-Changes in diameter in response to step increases in pressure (myogenic tone) in caudal arteries. Myogenic tone is expressed as active tone ( $\mu\text{m}$ ).

B- Myogenic tone expressed as percentage of passive diameter.

C- Passive diameter measurements were determined in a  $\text{Ca}^{2+}$ -free isotonic salt solution containing EGTA (2mmol/L) and in the presence of sodium nitroprusside ( $10\mu\text{mol/L}$ ) in caudal arteries. Caudal arteries were isolated from wild-type ( $n=12$ ) and transgenic mice ( $n=9$  and  $n=10$ , lines TgMa and TgVe respectively).

\*  $P < 0.001$ ; two-factor ANOVA, transgenic versus wild-type mice.

Figure 2

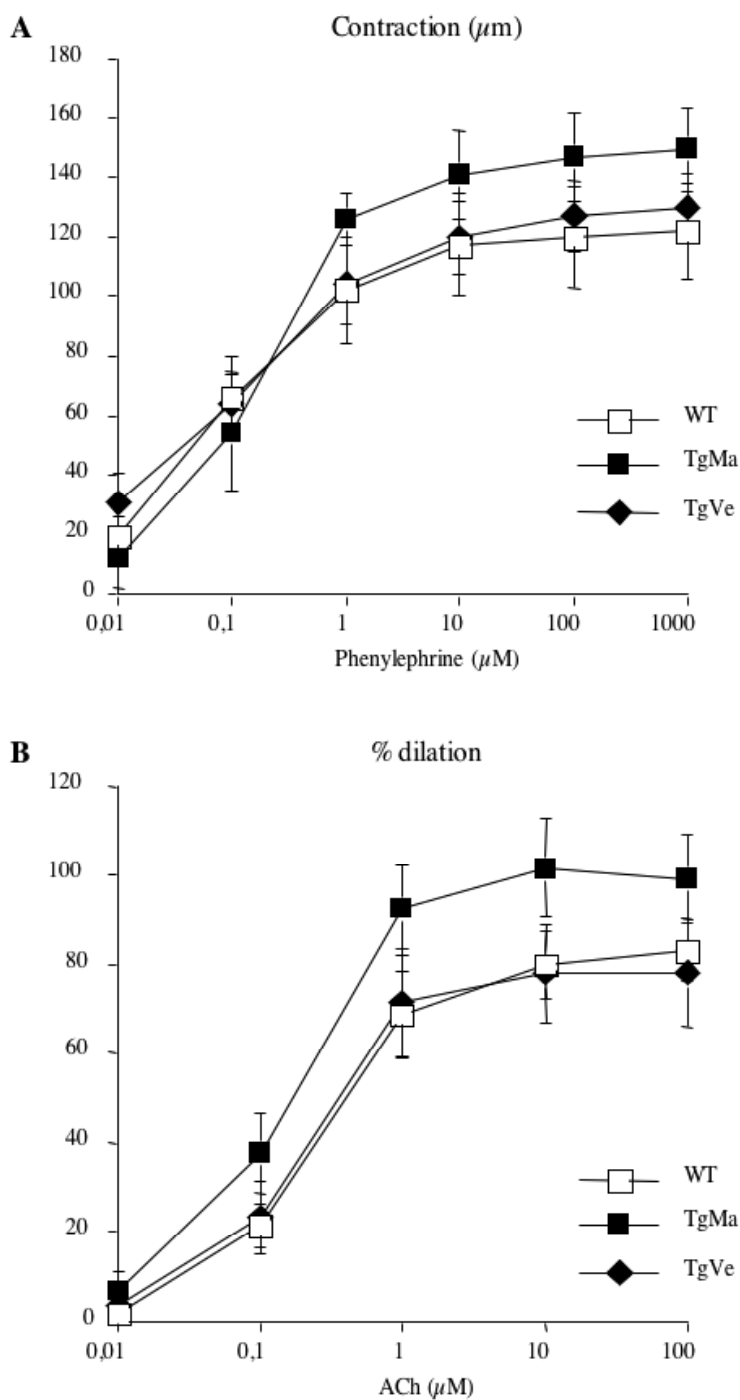


**Figure 2:** Diameter changes measured in response to flow (shear stress) in isolated tail caudal arteries. A- Changes in diameter in response to stepwise increases in intraluminal flow were determined in caudal arteries submitted to a pressure of 75mmHg and precontracted with phenylephrine. Flow-mediated dilation is expressed as percentage dilation of active tone by flow.

B- Flow-induced dilation (in  $\mu\text{m}$  change in diameter) is expressed as a function of shear stress. Caudal arteries were isolated from wild-type (n=12) and transgenic mice (n=9 and n=10, lines TgMa and TgVe respectively).

\* P < 0.001; two-factor ANOVA, transgenic versus wild-type mice.

Figure 3



**Figure 3:** Concentration-dependent-contraction to phenylephrine (A) and concentration-dependent-dilation to acetylcholine (B) in tail caudal arteries isolated from wild-type (n=12) and transgenic mice (n=8 and n=9, lines TgMa and TgVe respectively). No significant difference; 2-factor ANOVA, transgenic versus wild-type mice.