Nestin upregulation characterizes vascular remodeling secondary to hypertension in the rat

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1Program in Biomedical Sciences, Université de Montréal, Montréal, Québec, Canada; 2Department of Physiology, Université de Montréal, Montréal, Québec, Canada; and 3Research Center, Montreal Heart Institute and Université de Montréal, Montréal, Québec, Canada

Submitted 5 November 2014; accepted in final form 3 February 2015

Tardif K, Hertig V, Duquette N, Villeneuve L, El-Hamamsy I, Tanguay J, Calderone A. Nestin upregulation characterizes vascular remodeling secondary to hypertension in the rat. Am J Physiol Heart Circ Physiol 308: H1265–H1274, 2015. First published March 13, 2015; doi:10.1152/ajpheart.00804.2014.—Proliferation and hypertrophy of vascular smooth muscle cells represent hallmark features of vessel remodeling secondary to hypertension. The intermediate filament protein nestin was recently identified in vascular smooth muscle cells and in other cell types directly participated in proliferation. The present study tested the hypothesis that vessel remodeling secondary to hypertension was characterized by nestin upregulation in vascular smooth muscle cells. Two weeks after suprarenal abdominal aorta constriction of adult male Sprague-Dawley rats, elevated mean arterial pressure increased the media area and thickness of the carotid artery and aorta and concomitantly upregulated nestin protein levels. In the normal adult rat carotid artery, nestin immunoreactivity was observed in a subpopulation of vascular smooth muscle cells, and the density significantly increased following suprarenal abdominal aorta constriction. filamentous nestin was detected in cultured rat carotid artery- and aorta-derived vascular smooth muscle cells and an analogous paradigm observed in human aorta-derived vascular smooth muscle cells. ANG II and EGF treatment of vascular smooth muscle cells stimulated DNA and protein synthesis and increased nestin protein levels. Lentiviral short-hairpin RNA-mediated nestin depletion of carotid artery-derived vascular smooth muscle cells inhibited peptide growth factor-stimulated DNA synthesis, whereas protein synthesis remained intact. These data have demonstrated that vessel remodeling secondary to hypertension was characterized in part by nestin upregulation in vascular smooth muscle cells. The selective role of nestin in peptide growth factor-stimulated DNA synthesis has revealed that the proliferative and hypertrophic responses of vascular smooth muscle cells were mediated by divergent signaling events.

Nestin; carotid artery; aorta; hypertension; vascular remodeling

DURING THE DEVELOPMENT of the central nervous system (CNS), a population of neuroepithelial stem cells was initially identified via expression of the intermediate filament protein nestin (8, 18). Nestin is a 240-kDa protein and a member of the class VI family of intermediate filament proteins, and, in contrast to other classes, it is unable to self-assemble and form homodimers because of a short NH2 terminus (37, 39). Therefore, nestin will form heterodimers with other intermediate filament

proteins, including vimentin and desmin (37). The promoter region upstream of exon 1 of the nestin gene does not contain any identifiable elements regulating expression. However, the nestin gene does contain regulatory elements in the various intron regions that drive expression in a cell-specific manner (37). In neural progenitor/stem cells, nestin expression is independently regulated by restricted enhancer elements identified in the second intron (43). In humans, a highly conserved region that directed expression was also identified in the second intron of the nestin gene (21). However, nestin expression driven by the second intron was not limited to CNS-resident stem cells, since a transgenic mouse containing the 5.8-kb fragment of the promoter region and the 1.8-kb fragment of the second intron of the rat nestin gene linked to the reporter green fluorescent protein (GFP) identified progenitor/stem cell populations in the skin, skeletal muscle, and heart (2, 5, 11, 12, 14, 23). Despite the plethora of data supporting the premise that nestin expression identified a stem cell phenotype, recent studies have detected the intermediate filament protein in numerous non-stem cell populations during physiological growth and pathological remodeling. Nestin was highly expressed in developing skeletal myofibers and downregulated following maturation, upregulated in endothelial cells during reparative angiogenesis, and induced in mesangial cells following injury and in scar myofibroblasts and cardiac myocyte-like cells bordering the peri-infarct/infarct region of the ischemically damaged rodent and human heart (1, 3, 4, 7, 9, 11, 24, 25, 31, 32). However, in contrast to neural progenitor/stem cells, expression of the intermediate filament protein in skeletal myofibers and endothelial cells was driven by the first intron of the nestin gene (1, 42, 43). Biologically, nestin expression was reported to play a seminal role in cell proliferation (4, 9, 37, 38). Vascular remodeling is a hallmark feature of hypertension, characterized in part by the proliferation and hypertrophy of vascular smooth muscle cells (13). A recent study reported that a subpopulation of vascular smooth muscle cells residing in the normal rat aorta expressed the intermediate filament protein nestin and was actively engaged in the cell cycle (27). Moreover, nestin upregulation was identified in newly formed blood vessels during physiological and pathological remodeling and increased expression observed during neointimal growth following balloon injury of the rat carotid artery (1, 11, 12, 24, 27). In vitro studies have further demonstrated that peptide growth factors implicated in vascular remodeling induced

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tin expression in rat aortic vascular smooth muscle cells (15a, 16). Based on these observations, nestin upregulation in vascular smooth muscle cells may represent an early event of vessel remodeling secondary to hypertension. Thus the present study tested the hypothesis that nestin expression was increased during vessel remodeling following suprarenal abdominal aorta constriction of adult male Sprague-Dawley rats and that the intermediate filament protein directly contributed to peptide growth factor-stimulated vascular smooth muscle cell proliferation.

METHODS

Animal models. Suprarenal abdominal aorta constriction was performed on adult male Sprague-Dawley rats (9–11 wk old; Charles River Canada), employing a 21-gauge needle as previously described (26). Two weeks after surgery, mean arterial pressure and left ventricular function were determined (12). The heart, left common carotid artery, and aorta were subsequently removed for analysis. The use and care of laboratory rats was according to the Canadian Council for Animal Care and approved by the Animal Care Committee of the Montreal Heart Institute. 

Vessel morphology and collagen content. Formalin-fixed 6- to 8-μm-thick sections of the left common carotid artery and aorta (aortic arch and thoracic region) were stained with hematoxylin-phloxine-saffron (HPS) and images captured with the Olympus QICAM color video camera interfaced with an Olympus CXX41 microscope. Vessel wall morphology (media thickness, mm; media area, mm²) was measured with Image-Pro software (version 7; Media Cybernetics, Rockville, MD). HPS staining was also used to determine the number of vascular smooth muscle cell nuclei and density normalized to media area. Masson’s trichrome staining measured intraluminal collagen accumulation in the carotid artery. Image-Pro was used to calculate collagen content, and the data were normalized to the media area.

Rat vascular smooth muscle cells. The left common carotid artery or aortic arch of adult male Sprague-Dawley rats (9–11 wk old) was cut longitudinally and the lumen gently rubbed with a cotton swab to remove the endothelium. Vessel segments (3–5 mm in length) were digested in Dulbecco’s modified Eagle’s medium (DMEM; low glucose; HyClone Laboratories, Logan, UT) containing collagenase (type II; 1 mg/ml) at 37°C for 5 h. Cells were filtered (40-μm nylon mesh; Corning, Corning, NY), cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Grand Island, NY), 2% penicillin-streptomycin, 1% fungizone, epidermal growth factor (EGF; 25 ng/ml), and basic fibroblast growth factor (10 ng/ml), and grown until confluent. Experiments were performed on first/second-passage vascular smooth muscle cells plated at a density of 125–150 cells/mm² in DMEM containing 10% FBS for 24 h. Cells were subsequently washed and the media replaced with DMEM supplemented with insulin-transferrin-selenium (BD Bioscience, Bedford, MA) for 48 h. Vascular smooth muscle cells were treated for 24 h with angiotensin II (ANG II; 10⁻⁶ M; Sigma), EGF (10 ng/ml; R&D Systems), transforming growth factor-β1 (TGF-β1; 5 ng/ml; R&D Systems), or 10% FBS to assess nestin expression, DNA, and protein synthesis. Briefly, cells were treated for 24 h with [3H]leucine (2 μCi/ml; MP Biomedicals, Santa Ana, CA) or 4–6 h before the end of stimulation with [3H]thymidine (1 μCi/ml; PerkinElmer, Waltham, MA) to assess protein and DNA synthesis, respectively. Thereafter, cells were washed twice with cold PBS, and then 1 ml of cold 5% trichloroacetic acid was added for 30–60 min to precipitate protein or DNA. The precipitates were subsequently washed twice with cold water and resuspended in 500 μl of 0.4 M NaOH. Aliquots were counted in a scintillation counter. In each experiment, data were the average of three to six independent wells.

Human aortic vascular smooth muscle cells. The aorta was obtained from two non-diabetic, non-hypertensive male patients (47 and 55 yr old) undergoing the Ross procedure at the Montreal Heart Institute. The tissue was collected in Medium 199 (M199; Gibco, Life Technologies, Burlington, ON, Canada) supplemented with penicillin-streptomycin (50 units of penicillin and 50 μg of streptomycin; Gibco), fungizone (102.5 μg/ml; Gibco), and 10% FBS (Fisher Scientific, Nepean, ON, Canada). Under sterile conditions, tissue was washed with phosphate-buffered saline (PBS; Gibco), the adventitia removed, and the tunica media placed in a 50-ml tube containing 10 ml of a type II collagenase (220 U/mg; Gibco). After collagenase treatment at 37°C for 2 h, the nondigestible tissue was removed, 5 ml of M199 supplemented with penicillin-streptomycin, fungizone, and 10% FBS were added, and the tube was centrifuged (3,500 rpm, 5 min, 4°C). The pellet was resuspended in 2 ml of M199 supplemented with penicillin-streptomycin, fungizone, and 10% FBS, and the cells were plated in a T25 flask coated with 2% gelatin derived from bovine skin (Sigma, St. Louis, MO) and placed in an incubator at 37°C with 5% CO₂. After reaching confluence, the cells were passaged and plated in a T75 flask coated with 2% gelatin and grown until confluent. Thereafter, cells were trypsinized and subsequently passaged in T75 flasks or plated in P6 plates coated with 2% gelatin and grown until 80% confluence in 10% FBS. Immunofluorescence was performed on second/third-passage cells, and vascular smooth muscle cell lineage was confirmed for each preparation.

Western blot analysis. Lysates (30–50 μg) prepared from the left common carotid artery, aortic arch, or cultured vascular smooth muscle cells were subjected to SDS-polyacrylamide gel (10%) electrophoresis and transferred to a polyvinylidene difluoride membrane (Perkin Elmer Life Sciences, Boston, MA) (12). Antibodies used include mouse monoclonal anti-nestin (~240 kDa; 1:500; Chemicon, Temecula, CA), mouse monoclonal anti-endothelial nitric oxide synthase (eNOS; ~130 kDa; 1:500; BD Bioscience, Mississauga, ON, Canada), goat monoclonal anti-CD31 (~130 kDa; 1:500; Santa Cruz Biotechs, Santa Cruz, CA), rabbit polyclonal anti-caldesmon (~70 kDa; 1:2,500; Abcam, Cambridge, MA), rabbit polyclonal anti-smooth muscle-22α (~230 kDa; 1:5,000; Abcam), rabbit polyclonal anti-smooth muscle α-actin (~43 kDa; 1:5,000; Abcam), rabbit polyclonal anti-vimentin (~57 kDa 1:500; Abcam), and mouse monoclonal anti-GAPDH (1:5,000; Ambion, Austin TX). After overnight incubation at 4°C, the appropriate secondary antibody conjugated to horseradish peroxidase (1:20,000; Jackson ImmunoResearch, West Grove, PA) was added and bands were visualized utilizing the ECL detection kit (Perkin Elmer). Films were scanned with ImageJ software, and the target protein signal was depicted as arbitrary light units normalized to GAPDH protein.

Immunofluorescence. Formalin-fixed 6- to 8-μm-thick sections of the left common carotid artery were subjected to the antigen retrieval method and stained with the mouse monoclonal anti-nestin (1:150; Chemicon), rabbit polyclonal anti-smooth muscle α-actin (1:100; Abcam), or goat monoclonal anti-CD31 (1:100; Santa Cruz Biotechnologies) (12). Rat (primary and 1st/2nd passage) and human vascular smooth muscle cells were plated on glass coverslips, fixed with 4% paraformaldehyde, and stained with mouse monoclonal anti-nestin (1:500; Chemicon), mouse monoclonal anti-human nestin (1:500; Santa Cruz Biotechnologies), rabbit polyclonal anti-smooth muscle α-actin (1:200; Abcam), rabbit monoclonal anti-CD31 (1:100; Santa Cruz Biotechnologies), rabbit polyclonal anti-smooth muscle-22α (1:5,000; Abcam), rabbit polyclonal anti-smooth muscle-22α (1:5,000; Abcam), and mouse monoclonal anti-CD31 (1:500; Abcam). The nucleus was identified with To-PRO-3 (1.5 μM; Invitrogen) or 4',6'-diamidino-2-phenylindole (1.5 μM; Sigma). Secondary antibodies used were goat anti-mouse IgG conjugated to Alexa Fluor 555 (1:800; Invitrogen) or goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:800; Invitrogen). Immunofluorescence was visualized using a confocal LSM710 Zeiss microscope with the Zeiss LSM Image Browser. The density of vascular smooth muscle cells expressing nestin was determined with maximum projections derived from a z stack (voxel size of 143 × 143 × 250 nm in zyx).
Table 1. **Hemodynamics of sham and 2-wk SAC rats**

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
<th>LVSP, mmHg</th>
<th>LVEDP, mmHg</th>
<th>+dP/dt, mmHg/s</th>
<th>−dP/dt, mmHg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>10</td>
<td>112 ± 6</td>
<td>272 ± 8</td>
<td>145 ± 8</td>
<td>10 ± 2</td>
<td>6,176 ± 216</td>
</tr>
<tr>
<td>SAC</td>
<td>8</td>
<td>132 ± 6*</td>
<td>269 ± 15</td>
<td>185 ± 9*</td>
<td>8 ± 1</td>
<td>7,850 ± 533*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of sham or suprarenal abdominal aorta-constricted (SAC) rats examined. MAP, mean arterial pressure; HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of contraction; −dP/dt, rate of relaxation. *P < 0.05 vs. sham (unpaired t-test).

RESULTS

**Left ventricular contractility and cardiovascular remodeling of suprarenal abdominal aorta-constricted rats.** Two weeks after suprarenal abdominal aorta constriction of adult male Sprague-Dawley rats, mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), and the rates of left ventricular contraction (+dP/dt) and relaxation (−dP/dt) were significantly elevated compared with values in sham rats (Table 1). Absolute left ventricular weight and left ventricular weight normalized to body weight were increased after a greater afterload was imposed on the heart, whereas right ventricular weight remained unchanged (Table 2).

In the carotid artery of suprarenal abdominal aorta-constricted rats, a hypertrophic response was evident, because media area and thickness were significantly greater compared with values in sham rats (Table 3). Remodeling of the carotid artery suprarenal abdominal aorta-constricted rats was also attributed in part to vascular smooth muscle cell proliferation, because the number of nuclei alone and after normalization to the media area were significantly increased compared with values in sham rats (Table 3). A reactive fibrotic response was also prevalent, since intraluminal collagen content (%collagen; plane) and normalized to the vessel media area (μm²; average of at least 3–4 distinct fields). Non-specific staining was determined after the addition of the conjugated secondary antibody alone.

**Lentiviral construct.** The lentiviral construct containing the short hairpin RNA (shRNA) directed against nestin was prepared as previously described (4). The biological impact of the empty lentivirus and the lentivirus containing the shRNA directed against nestin was determined by measuring DNA and protein synthesis of infected first/second-passage carotid artery-derived vascular smooth muscle cells (4).

**Statistics.** Data are means ± SE, and n represents the number of rats or individual preparations of vascular smooth muscle cells used per experiment. Data were evaluated using Student's unpaired t-test or one-way ANOVA (GraphPad InStat), and a significant difference was determined using the Student-Newman-Keuls multiple comparisons post hoc test and a value of P < 0.05 considered statistically significant. The magnitude of nestin and vimentin expression in the aortic arch of normal and suprarenal abdominal aorta-constricted rats was variable and led to a significant difference between the standard deviations. Therefore, a nonparametric two-tailed t-test (GraphPad InStat) was performed and a value of P < 0.05 considered statistically significant.

Table 2. **Body and heart weight of sham and 2-wk SAC rats**

<table>
<thead>
<tr>
<th></th>
<th>BW, g</th>
<th>Heart, g</th>
<th>LV, mg</th>
<th>LV/BW, mg/g</th>
<th>RV, mg</th>
<th>RV/BW, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>7</td>
<td>401 ± 5</td>
<td>1.14 ± 0.07</td>
<td>612 ± 23</td>
<td>1.44 ± 0.08</td>
<td>237 ± 12</td>
</tr>
<tr>
<td>SAC</td>
<td>8</td>
<td>383 ± 13</td>
<td>1.54 ± 0.06*</td>
<td>789 ± 45*</td>
<td>2.09 ± 0.12*</td>
<td>245 ± 13</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of sham or SAC rats examined. BW, body weight; LV, left ventricle; RV, right ventricle. *P < 0.05 vs. sham (unpaired t-test).
banded rats (Fig. 1A). Previous studies have detected the existence of multiple isoforms of vimentin, and phosphorylation of the intermediate filament protein has also been reported (20, 28, 34). It remains unknown if the lower molecular weight band detected in the present study represented an isoform or a dephosphorylated state of vimentin. Aortic remodeling secondary to suprarenal abdominal aorta constriction (n = 8) was also associated with the concomitant upregulation of nestin and vimentin protein levels compared with sham rats (n = 4) (Fig. 1, C and D). However, nestin and vimentin protein expression in the aortic arch of sham and banded rats did not correlate with MAP (nestin, r² = 0.001; vimentin, r² = 0.037) or LVSP (nestin, r² = 0.001; vimentin, r² = 0.045).

An immunofluorescence approach revealed that a subpopulation of vascular smooth muscle cells in the carotid artery of normal adult rats was associated with nestin immunoreactivity (409 ± 105 cells/mm²; n = 4 rats; Fig. 2A), and modest diffused staining of the intermediate filament protein was also detected in endothelial cells. Nestin upregulation in the carotid artery of banded rats was attributed in part to the increased density of nestin-expressing vascular smooth muscle cells (720 ± 73 cells/mm²; n = 6 rats; P < 0.05 vs. sham; Fig. 2, B and C). Nestin immunoreactivity of CD31⁺ endothelial cells persisted in the carotid artery of suprarenal abdominal aorta-constricted rats (Fig. 2, B and C), and in all tissues examined, staining intensity was greater than in sham rats (Fig. 2A).

Filamentous nestin detected in rat and human cultured vascular smooth muscle cells and induced by peptide growth factors. A vascular smooth muscle cell phenotype was confirmed, because carotid artery- and aorta-derived cells (n = 3 independent preparations) coexpressed filamentous caldesmon and smooth muscle-22α (Fig. 2D and Ref. 36). Nestin-immunoreactive filaments were detected in a subpopulation of caldesmon⁺/smooth muscle-22α⁺ vascular smooth muscle cells (Fig. 2D). Caldesmon and smooth muscle-22α immunoreactivity was also detected in second/third-passage vascular smooth muscle cells isolated from the aorta of two patients, and filamentous nestin staining was identified in a subpopulation of cells (Fig. 2E).

ANG II acting directly or indirectly via the paracrine release of peptide growth factors plays a seminal role in vascular remodeling (13, 22, 30, 35, 40). DNA synthesis, as measured by [³H]thymidine uptake, was significantly increased following a 24-h exposure to the panel of peptide growth factors (Fig. 3, B and D). In parallel, a 24-h exposure of carotid artery- and aorta-derived vascular smooth muscle cells to ANG II, EGF, and TGF-β₁ (Fig. 3, E–H) significantly increased nestin protein levels compared with untreated cells (n = 4–6 independent preparations). By contrast, vimentin, smooth muscle α-actin, caldesmon, and smooth muscle-22α expression remained unchanged following exposure of vascular smooth muscle cells to ANG II, EGF, and TGF-β₁ (Fig. 3, E–H).

Role of nestin in DNA and protein synthesis in carotid artery-derived vascular smooth muscle cells. Filamentous nestin was detected in the majority of carotid artery-derived vascular smooth muscle cells infected with the empty lentivirus (Fig. 4A). By contrast, the infection of carotid artery-derived vascular smooth muscle cells with a lentivirus containing a shRNA directed exclusively against nestin (n = 4 independent preparations) significantly reduced expression of the intermediate filament protein compared with cells infected with the empty lentivirus (n = 4 independent preparations) (Fig. 4, A and B). The expression of lineage specific markers in nestin-depleted cells was similar to that of carotid artery-derived vascular smooth muscle cells infected with the empty lentivirus (Fig. 4B). Basal DNA synthesis, as measured by [³H]thymidine uptake, was significantly reduced in nestin-depleted carotid artery-derived vascular smooth muscle cells (12,500 ± 2,310 counts/min; n = 4 independent preparations; P < 0.05 vs. empty lentivirus) compared with cells infected with the empty lentivirus (21,141 ± 1,789 counts/min; n = 4 independent preparations). Furthermore, [³H]thymidine uptake in response to ANG II (10⁻⁴ M) and EGF (10 ng/ml) was significantly attenuated in nestin-depleted cells (Fig. 4C). Basal protein synthesis, as measured by [³H]leucine uptake, was reduced in nestin-depleted carotid artery-derived vascular smooth muscle cells (2,017 ± 422 counts/min; n = 4 independent preparations; P < 0.05 vs. empty lentivirus) compared with cells infected with the empty lentivirus (3,280 ± 210 counts/min; n = 4 independent preparations). However, ANG II- and EGF-stimulated [³H]leucine uptake in nestin-depleted cells was similar to that in carotid artery-derived vascular smooth muscle cells infected with the empty lentivirus (Fig. 4D).

**DISCUSSION**

Vessel remodeling secondary to hypertension was characterized by vascular smooth muscle cell hypertrophy, proliferation, and reactive fibrosis leading to increased arterial wall stiffness and worsening of vascular tone (6, 13). An analogous paradigm of vessel remodeling was observed in suprarenal

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**Table 3. Vessel morphology of sham and 2-wk SAC rats**

<table>
<thead>
<tr>
<th></th>
<th>Carotid artery</th>
<th>Aortic Arch</th>
<th>Thoracic Aorta</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>SAC</td>
<td>Sham</td>
</tr>
<tr>
<td>Wall thickness, mm</td>
<td>0.054 ± 0.002</td>
<td>0.076 ± 0.004*</td>
<td>0.112 ± 0.008</td>
</tr>
<tr>
<td>Media area, mm²</td>
<td>0.125 ± 0.007</td>
<td>0.213 ± 0.018*</td>
<td>0.68 ± 0.05</td>
</tr>
<tr>
<td>Total no. of nuclei</td>
<td>259 ± 18</td>
<td>617 ± 76*</td>
<td>1,830 ± 145</td>
</tr>
<tr>
<td>No. of nuclei per mm²</td>
<td>2,132 ± 193</td>
<td>2,991 ± 345*</td>
<td>2,660 ± 258</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of sham or SAC rats examined. *P < 0.05 vs. sham (unpaired t-test).
Two weeks after banding, carotid artery remodeling was evident as increased media area, thickness, and intraluminal collagen deposition were observed in banded rats. Furthermore, the number of vascular smooth muscle cell nuclei alone as well as the density following normalization to the media area were also elevated in the carotid artery of suprarenal banded rats, supporting the premise that proliferation significantly contributed to vessel remodeling. Analogous to the carotid artery, media area and thickness were increased in the aortic arch and thoracic region of suprarenal abdominal aorta-constricted rats. The number of vascular smooth muscle cell nuclei in the aortic arch and thoracic region was also significantly elevated in banded vs. sham rats. However, following normalization to the media area, the density of vascular smooth muscle cell nuclei in the aortic arch and thoracic region was modestly lower compared with that in sham rats. Therefore, despite the presence of a proliferative response, vascular smooth muscle cell

Fig. 1. Nestin protein expression in the vasculature of suprarenal abdominal aorta-constricted adult male rats. A–D: Nestin protein levels were increased in the carotid artery (A and B) and aortic arch (C and D) of suprarenal abdominal aorta-constricted (SAC) rats, whereas CD31 and endothelial nitric oxide synthase (eNOS) expression remained unchanged. In A, 2 samples of this gel were removed (top, indicated by dashed lines) because nestin expression was not detected. The tissue samples exhibiting a positive nestin signal in A were subsequently reexamined for CD31 and eNOS expression. E and F: nestin protein expression in the carotid artery of sham (filled circles) and SAC rats (open circles) correlated with mean arterial pressure and left ventricular (LV) systolic pressure. Protein expression was normalized to GAPDH; n = 7–8 sham rats, n = 7–8 banded rats. *P < 0.05 vs. sham.
Fig. 2. Nestin expression in the rat vasculature and in isolated rat and human vascular smooth muscle cells. A: in the carotid artery of normal adult rats, nestin immunoreactivity (red fluorescence) was detected in a subpopulation of smooth muscle α-actin (SMA)-immunoreactive (green fluorescence) vascular smooth muscle cells. In addition, weak and diffused nestin staining was observed in endothelial cells lacking SMA immunoreactivity. B: in the carotid artery of SAC rats, nestin (red fluorescence) staining was detected in SMA-immunoreactive (green fluorescence) vascular smooth muscles, and density was greater compared with that in sham rats (see RESULTS). Furthermore, compared with sham rats (shown in A), nestin staining of endothelial cells was greater in banded rats. C: in the carotid artery of SAC rats, nestin (red fluorescence) staining of endothelial cells was confirmed with CD31 (green fluorescence) coimmunoreactivity, and the signal was greater compared with that in sham rats (shown in A). Furthermore, nestin staining was detected in vascular smooth muscle cells lacking CD31 immunoreactivity. In each panel of A–C, the arrow indicates the luminal side and the nucleus identified by 4′,6-diamidino-2-phenylindole (blue fluorescence). D: in rat carotid artery-derived vascular smooth muscle cells, filamentous nestin staining was detected in caldesmon- and smooth muscle-22α (SM-22α)-expressing cells. E: a subpopulation of human aorta-derived vascular smooth muscle cells characterized by caldesmon and SM-22α expression was associated with filamentous nestin. The nucleus was identified by To-PRO-3 (blue fluorescence).
Fig. 3. Peptide growth factor-mediated DNA and protein synthesis and nestin expression in rat carotid artery- and aortic arch-derived vascular smooth muscle cells. A–D: 24-h exposure of carotid artery (A and B)- and aorta-derived vascular smooth muscle cells (C and D) to angiotensin II (ANG II; 10^{-6} M), epidermal growth factor (EGF; 10 ng/ml), or fetal bovine serum (FBS; 10%) significantly increased [{\textsuperscript{3}H}]thymidine and [{\textsuperscript{3}H}]leucine uptake. Transforming growth factor-β1 (TGF-β1; 5 ng/ml) treatment reduced [{\textsuperscript{3}H}]thymidine uptake but concomitantly increased [{\textsuperscript{3}H}]leucine uptake. E and F: 24-h exposure of carotid artery-derived vascular smooth muscle cells to ANG II, EGF, or TGF-β1 upregulated nestin protein levels, whereas SMA, caldesmon, SM-22α, and vimentin expression remained unchanged. The dashed line indicates a sample removed from the gel that was not relevant to the study. G and H: 24-h exposure of aorta-derived vascular smooth muscle cells to ANG II, EGF, or TGF-β1 upregulated nestin protein levels, whereas vimentin expression remained unchanged. Protein expression was normalized to GAPDH; n = 4–6 independent preparations. *P < 0.05 vs. untreated cells.
hypertrophy was the predominant adaptive response of the aorta during the early phase of remodeling secondary to suprarenal abdominal aorta constriction.

Previous studies have reported nestin upregulation in newly formed blood vessels during physiological and pathological remodeling (1, 11, 12, 24). Likewise, neointimal growth following balloon injury of the rat carotid artery has been associated with nestin expression (27). Consistent with its role in vascular remodeling, the present study has demonstrated that nestin protein levels were upregulated in the carotid artery and aortic arch following suprarenal abdominal aorta constriction of adult male Sprague-Dawley rats and attributed in part to the increased density of vascular smooth muscle cells expressing the intermediate filament protein. Furthermore, the magnitude of nestin expression in the carotid artery alone positively correlated with the rise of MAP and LVSP. As previously discussed, nestin is unable to self-assemble and form homodimers because of a short NH2 terminus (37). Thus nestin will form heterodimers with other intermediate filament proteins, including vimentin (37). Consistent with this premise, vimentin protein levels were significantly increased in the carotid artery and aortic arch of suprarenal abdominal aorta-constricted rats in this study, and a positive correlation was observed between the magnitude of expression and MAP and LVSP in the carotid artery alone. Thus, since vimentin expression has been reported in endothelial and vascular smooth muscle cells, both cell types may have contributed to the reported upregulation in the vasculature of banded rats (15, 19).

In experimental models of hypertension, including suprarenal abdominal aorta constriction, ANG II acting directly and/or indirectly via the synthesis of peptide growth factors EGF and TGF-β significantly influenced vascular and cardiac remodeling (10, 22, 29, 30, 33, 35, 40). On the basis of these observations, we performed in vitro experiments to assess the impact of ANG II, EGF, and TGF-β on nestin expression in cultured vascular smooth muscle cells. The intermediate filament protein nestin was detected in a subpopulation of carotid artery-derived vascular smooth muscle cells, peptide growth factor-stimulated [3H]thymidine uptake was attenuated, whereas [3H]leucine uptake was equivalent to that in nestin-expressing vascular smooth muscle cells. Values are means ± SE; n = 4 independent preparations. *P < 0.05 vs. cells infected with the empty lentivirus.
vascular smooth muscle cells to ANG II, EGF, and TGF-β1 significantly increased nestin protein levels, whereas the expression of vimentin and lineage specific markers remained unchanged. The disparate action on DNA synthesis and nestin expression suggested that upregulation of the intermediate filament protein was insufficient to promote entry into the cell cycle in response to TGF-β1. Thus one or more of the myriad signaling events recruited by TGF-β1 may have suppressed a downstream target of nestin required for DNA synthesis (41).

Nonetheless, the in vitro data suggest that nestin upregulation in the vasculature of suprarenal abdominal aorta-constricted rats may have occurred in part via ANG II acting directly and/or in a concerted fashion via the synthesis and release of EGF and TGF-β1. The contrasting pattern of vimentin and nestin expression in cultured vascular smooth muscle cells further suggested that the upregulation of the two intermediate filament proteins in the vasculature of banded rats was mediated in part by distinct biological events. Lastly, the detection of filamentous nestin in human aortic vascular smooth muscle cells supports the premise that increased expression of the intermediate filament protein may also represent a phenotypic event of pathological vascular remodeling in hypertensive patients.

In several distinct cell types, nestin expression correlated with an increased proliferative response (4, 9, 37, 38). Nestin was also detected in a diverse range of cancers, directly implicated in proliferation and expression in several tumors strongly correlated with poor prognosis (17). In the vasculature of the adult rat, two recent studies reported that nestin expression in a subpopulation of vascular smooth muscle cells correlated with cell cycle entry (27, 36). Thus the reported increase in the number of vascular smooth muscle cell nuclei in the carotid artery and aorta of suprarenal abdominal aorta-constricted rats may have been attributed in part to the upregulation of nestin and subsequent reentry into the cell cycle. Unfortunately, an in vivo transgenic approach to selectively deplete nestin in vascular smooth muscles does not presently exist. However, an in vitro approach to directly address the role of nestin in the reentry of vascular smooth muscle cells into the cell cycle was feasible and involved the use of a lentivirus containing a shRNA that selectively targeted the intermediate filament protein. Compared with that in nestin-expressing carotid artery-derived vascular smooth muscle cells, the basal uptake of [3H]thymidine and ANG II- and EGF-stimulated DNA synthesis were significantly attenuated in nestin-depleted cells. Thus the established proliferative role of nestin was consistent with the increased density of vascular smooth muscle cell nuclei and concomitant greater density of nestin-expressing vascular smooth muscle cells in rats subjected to suprarenal abdominal aorta constriction. By contrast, ANG II- and EGF-mediated [3H]leucine uptake in nestin-depleted vascular smooth muscle cells was equivalent to that in nestin-expressing cells. These data suggest that the hypertrophic component of vascular remodeling in suprarenal abdominal aorta-constricted rats was not directly dependent on nestin upregulation. Lastly, the apparent greater staining intensity of nestin in endothelial cells of banded rats may likewise provide a proliferative advantage during vascular remodeling (1, 17, 24).

Vessel remodeling is the end of result of established hypertension. The present study has demonstrated that the increased density of a subpopulation of nestin-expressing vascular smooth muscle cells was associated with significant vessel remodeling (e.g., hypertrophy, proliferation and fibrosis) in the hypertensive rat secondary to suprarenal abdominal aorta constriction. Moreover, the magnitude of nestin expression in the carotid artery of banded rats significantly correlated with the rise of mean arterial pressure. Lastly, the seminal contribution of nestin to vascular smooth muscle cell proliferation in response to a variety of peptide growth factors supports the premise that upregulation of the intermediate filament protein may represent an incipient event of vessel remodeling secondary to hypertension.

ACKNOWLEDGMENTS
We thank Marie-Élaine Clavet and Amelie Bourget for technical assistance and France Thériault for excellent secretarial assistance.

GRANTS
This work was supported by Heart and Stroke Foundation of Canada/Quebec Award G-11-CA-4771 and Canadian Diabetes Association Grant OG-3-11-3267-AC.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
K.T., V.H., N.D., and L.V. performed experiments; K.T., V.H., L.V., LE.-H., J.-F.T., and A.C. analyzed data; K.T. and A.C. interpreted results of experiments; K.T. prepared figures; K.T. drafted manuscript; J.-F.T. and A.C. edited and revised manuscript; A.C. conceived and design of research; A.C. approved final version of manuscript.

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