Sympathetic innervation promotes vascular smooth muscle differentiation

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The sympathetic nervous system (SNS) is an important modulator of vascular smooth muscle (VSM) growth and function. Several lines of evidence suggest that the SNS also promotes VSM differentiation. The present study tests this hypothesis. Expression of smooth muscle myosin (SM2) and α-actin were assessed by Western analysis as indexes of VSM differentiation. SM2 expression (normalized to α-actin) in adult innervated rat femoral and tail arteries was 479 ± 115% of that in noninnervated carotid arteries. Expression of α-actin (normalized to GAPDH or total protein) in 30-day-innervated rat femoral arteries was greater than in corresponding noninnervated femoral arteries from guanethidine-sympathectomized rats. SM2 expression (normalized to α-actin) in neonatal femoral arteries grown in vitro for 7 days in the presence of sympathetic ganglia was greater than SM2 expression in corresponding arteries grown in the absence of sympathetic ganglia. In VSM-endothelial cell cultures grown in the presence of dissociated sympathetic neurons, α-actin (normalized to GAPDH) was 300 ± 66% of that in corresponding cultures grown in the absence of neurons. This effect was inhibited by an antibody that neutralized the activity of transforming growth factor-β2. All of these data indicate that sympathetic innervation increased VSM contractile protein expression and thereby suggest that the SNS promotes and/or maintains VSM differentiation.

MATERIALS AND METHODS

Vascular smooth muscle (VSM) differentiation is critical for the normal development of blood vessels. VSMs must differentiate from the synthetic phenotype required for growth of blood vessels to the contractile phenotype required for appropriate regulation of blood pressure and blood flow. VSM differentiation is associated with increased expression of contractile proteins such as smooth muscle actin and myosin (12, 14, 15, 17, 20, 21, 24, 28). In many cardiovascular diseases including hypertension, atherosclerosis, and ischemic heart disease, differentiated contractile VSM redifferentiates to a more synthetic phenotype (1, 13, 20, 23, 28). This "developmental regression" can compromise cardiovascular function. The physiological and pathological mechanisms that modulate the differentiation of VSM are not well understood.

The sympathetic nervous system is an important determinant of vascular growth and function (5, 6, 8, 27, 33). The effects of the sympathetic nervous system on VSM differentiation have not been studied extensively, but there is evidence to suggest that sympathetic neurons (SNs) promote or maintain VSM differentiation. Dimitriadou et al. (10) observed that sympathetic denervation produced morphological changes in VSM and suggested that these changes were characteristic of VSM dedifferentiation. Kacem et al. (19) found that VSMs in denervated arteries expressed more vimentin than corresponding innervated arteries. These authors suggested that vimentin was a marker for dedifferentiated VSM. The mechanisms of these effects were not investigated.

The present studies tested the hypothesis that SNs promote the differentiation of VSM. Smooth muscle myosin heavy chain [200-kDa isoform, SM2 (20, 28)] and/or α-actin (12, 14, 28) expression were assessed as indexes of VSM differentiation in arteries and VSM grown in vivo and in vitro in the absence and presence of sympathetic innervation. The present studies also tested the hypothesis that sympathetic modulation of VSM differentiation was mediated by transforming growth factor (TGF)-β.

Materials

The use of animals in these studies was in accordance with the National Institutes of Health guidelines for the humane care and use of animals in research and was approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Materials. Dulbecco’s modified essential media (DMEM), penicillin-streptomycin, and glutamine were purchased from Gibco Life Technologies. Fetal bovine serum (FBS) was purchased from Serum Technologies or GIBCO Life Technologies. Nerve growth factor (NGF) was purchased from Collaborative Biomedical Products. Collagenase (type 2), hyaluronidase, and trypsin were purchased from Worthington Biochemical. Mitomycin C and smooth muscle α-actin antibody were purchased from Sigma. Collagen was generously provided by Dr. Carson Cornbrook (Department of Anatomy and Neurobiology, University of Vermont). SM2 antibody was generously provided by Art Rovner (Department of Molecular Physiology and Biophysics, University of Vermont or was purchased from Biomedical Technologies. GAPDH antibody was purchased from Biodesign.

Cell and organ culture. Endothelial cells (ECs) isolated from adult (>90 days of age) male Sprague-Dawley rats were a generous gift from Dr. Paula Grammas (University of Oklahoma; Ref. 9). These cells exhibited the distinct cobblestone morphology characteristic of ECs and took up acetylated LDL. ECs were used from passages 11-20. VSMs were isolated from explants of aorta of adult male and female Sprague-Dawley rats (29). These cells exhibited characteristic "hill-and-valley" growth patterns and immunohistochemical labeling with a monoclonal antibody for smooth muscle-specific α-actin. VSMs were used from passages 2-6. Vascular cells were grown in low-glucose DMEM supplemented with 10% FBS, 1 mM glutamine, and 100 units each of penicillin and streptomycin. Cells were maintained at 37°C in a humidified 5% CO2 environment.

Postganglionic SNs were isolated from superior cervical ganglia (SCG) of neonatal rats. SCG were collected and enzymatically dissociated for 20 min at 37°C in a collagenase-hyaluronidase solution [that contained (in mg/ml) 10 bovine serum albumin, 4 collagenase, and 1 hyaluronidase] and then for 10 min in trypsin (3 mg/ml).

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Equivalent numbers of dissociated cells were applied to collagen-coated tissue culture dishes. SN cultures were grown in DMEM supplemented with 10% FBS, 50 ng/ml NGF, penicillin-streptomycin, and glutamine at 37°C in a humidified 5% CO₂ environment. For SN-vascular cocultures, vascular cells were added to the SNs the day after plating. Parallel cultures that contained equal numbers of vascular cells without neurons were also plated. Mitomycin C (10 μg/ml for 1 h) was then added to all cultures to arrest growth of non-neuronal cells (2).

The effects of SNs on VSM differentiation were also studied in organ cultures. Femoral arteries from 10-day-old rats were grown for 7 days in the presence of absence of SCG obtained from the same rats. The arteries and/or ganglia were grown on collagen in DMEM-F-12 supplemented with 50 ng/ml NGF.

**Sympathectomy.** Rat pups were sympathectomized with guanethidine starting 2 days after birth. Pups were injected 5 days/wk for 3 wk (a total of 15 injections) with 50 mg/kg guanethidine. This protocol irreversibly inhibits the development of innervation to the vasculature (18). The effectiveness of sympathectomy was verified by staining of femoral arteries with glyoxylic acid as described by Lindvall and Bjorklund (22).

**Western analysis.** Cells were lysed with three cycles of rapid freeze thawing and were sonicated. Arteries were homogenized in phosphate-buffered saline (PBS) that contained protease inhibitors. Protein concentrations of samples were determined using the Bio-Rad Bradford protein assay. Equal amounts of protein were diluted with an equal volume of electrophoresis running buffer, boiled for 5 min, and electrophoresed on 10% or 12% acrylamide gels. Gels were transferred to nitrocellulose membranes. After transfer, the gels were stained (GelCode Blue stain reagent; Pierce) to verify that equal amounts of protein were loaded for each sample. The membranes were blocked with PBS that contained 3% nonfat dry milk. Membranes were briefly rinsed and then incubated while shaking for 60 min at room temperature in PBS that contained 3% nonfat dry milk and a 1:5,000 dilution of mouse α-actin primary antibody (Sigma) or a 1:1,000 (Roivner antibody) or 1:150 (Biomedical Technologies antibody) dilution of SM2, or a 1:1,000 dilution of GAPDH antibody. (For some but not all experiments, GAPDH expression was also assessed to confirm equal protein loading.) Membranes were again briefly rinsed and were incubated while shaking in PBS that contained 3% nonfat dry milk and a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse α-actin (Bio-Rad) for 60 min at room temperature. The horseradish peroxidase was detected with enhanced chemiluminescence (Pierce) and documented on autoradiographic film. Signals were quantitated densitometrically.

**Histology.** Histology was performed at the University of Vermont Cell Imaging Facility. Arteries were fixed in 4% paraformaldehyde and embedded in epoxy resin. Sections were cut and stained with toluidine blue. Images were viewed on an Olympus microscope, captured with an Olympus camera and Magnafire software, and analyzed using MetaMorph software.

**Statistical analysis.** All data are expressed as means ± SE. Unpaired t-tests were used to determine statistically significant differences (P < 0.05).

**RESULTS**

During development, VSMs must differentiate from undifferentiated mesenchyme to mature contractile VSMs that are present in adult arteries. VSM differentiation is associated with increases in smooth muscle α-actin and myosin. Smooth muscle α-actin levels increase early in development (12, 28); SM2 levels increase later (21). Vascular injury results in VSM dedifferentiation (1, 13, 20, 23, 28). VSM dedifferentiation is the reverse of differentiation and is associated with decreases in smooth muscle α-actin and SM2 levels; SM2 levels decrease early in dedifferentiation and α-actin levels decrease later (1, 20). In the present studies, α-actin and/or SM2 were measured as markers of early and late VSM differentiation.

If sympathetic innervation promotes the expression of smooth muscle α-actin and/or SM2, blood vessels that are innervated should express more of these proteins than blood vessels that are not innervated. To test this hypothesis, contractile protein expression in adult innervated arteries was compared with adult noninnervated arteries. It is well known that rat tail arteries are innervated (31). Glyoxylic acid staining indicated that rat femoral arteries are innervated and rat carotid arteries are not (Fig. 1A). Western analyses indicated that α-actin expression in innervated tail and femoral arteries was similar to that in noninnervated arteries. Western analyses indicated, however, that SM2 expression was greater in the innervated arteries (Fig. 1B). Quantitative analysis of Western analysis data from five carotid, five tail, and two femoral arteries indicated that SM2 normalized to α-actin was significantly greater in innervated (tail and femoral) arteries.

The data in Fig. 1 indicate that adult fully differentiated innervated arteries expressed more SM2 than adult fully differentiated carotid arteries. These data suggest that innervation promotes the expression of SM2 but do not provide insight into the effects of sympathetic innervation on the VSM differentiation that occurs during development. To test the hypothesis that sympathetic innervation promotes VSM differentiation during development, expression of smooth muscle α-actin was assessed in femoral arteries that developed in the presence and absence (guanethidine sympathectomy) of sympathetic innervation. Sympathetic innervation to rat femoral arteries develops postnatally. The animals were sympathectomized postnatally with repeated injections of guanethidine between postnatal days 2 and 23. Arteries were taken from animals at 30 days of age to determine the effects of sympathectomy on developing arteries and at 90 days of age to determine the effects of sympathectomy on fully developed arteries. In 30-day-old rats, α-actin (normalized to GAPDH or total protein) in arteries that developed in the absence of innervation was 35 ± 27% of that in arteries that developed in the presence of innervation (P < 0.05; one-tailed paired t-test; n = 4). Guanethidine sympathectomy did not affect α-actin expression in femoral arteries of 90-day-old animals. Representative Western analyses of 30- and 90-day-old arteries (n = 4 and 3, respectively) are shown in Fig. 2A.

Guanethidine produces systemic sympathectomy that could result in innervation-independent systemic cardiovascular effects and could alter VSM contractile protein expression. To determine whether this was the case, α-actin expression was also assessed in carotid arteries from 30-day-old control and sympathectomized animals. As shown in Fig. 1, carotid arteries are not innervated in control or sympathectomized animals. Three independent analyses indicated that guanethidine sympathectomy did not decrease carotid α-actin expression (Fig. 2B; P > 0.05), which suggests that the decrease in α-actin expression in femoral arteries from sympathectomized rats was innervation dependent.

Sympathetic innervation promotes the growth as well as the differentiation of VSMs. It was possible that the decrease in α-actin observed in the femoral arteries of 30-day-old sympathectomized animals was due to decreased VSM. Morphological analyses suggest this was not the case (Fig. 2C). Sympa-
The effects of sympathetic innervation on dedifferentiation of VSM also occurs when arteries and VSMs are placed in culture (13). The effects of sympathetic innervation on dedifferentiation, the arteries were analyzed immediately after harvest (0 days in culture) and after 7 days in culture is shown. To assess the effects of sympathetic innervation on dedifferentiation, the arteries were grown in culture for 7 days in the absence and presence of SCG. These ganglia contain postganglionic SNs representative of those that would innervate blood vessels. The artery and artery-ganglion cocultures contained NGF (50 ng/ml), which promoted extensive axon outgrowth from the ganglia as shown in Fig. 3A. A representative Western analysis of SM2 expression in femoral arteries analyzed immediately after harvest (0 days in culture) and after 7 days in culture is shown. To assess the effects of sympathetic innervation on dedifferentiation, the arteries were grown in culture for 7 days in the absence and presence of SCG. These ganglia contain postganglionic SNs representative of those that would innervate blood vessels. The artery and artery-ganglion cocultures contained NGF (50 ng/ml), which promoted extensive axon outgrowth from the ganglia as shown in Fig. 3A.

As a first step toward determining how SNs modulate VSM differentiation, the roles of potential mediators were studied in VSM-SN cocultures. The data in Figs. 1–3 suggest that postganglionic SNs would increase α-actin and/or SM2 expression in VSMs. To confirm this in the coculture model, VSMs and dissociated postganglionic SNs were grown for 8 days either alone or in coculture. Expression of α-actin and SM2 was then assessed in the VSMs grown in the presence and in the absence of SNs. In the cocultures used in the present study, the VSMs could not reliably be separated from the SNs. Thus the cell extracts from the VSM-SN cocultures contained both VSM and neuronal material. To control for any effects associated with the presence of neuronal materials, VSMs grown in the absence of SNs were analyzed in the presence of an equal amount of SN material. To control for any effects associated with the presence of neuronal materials, VSMs grown in the absence of SNs were analyzed in the presence of an equal amount of SN material. This was achieved by combining parallel cultures of VSMs and SNs grown alone immediately before Western analysis. Protein assays confirmed that the total amount of material in the cocultures was not different from that in VSMs grown alone (combined +). In all three analyses, myosin expression was 0% (undetectable) of α-actin expression in arteries grown in the absence of sympathetic ganglia and 55 ± 16% in the presence of sympathetic ganglia. Expression of α-actin (as a percentage of total protein and/or GAPDH) increased in one experiment but did not change in the other two experiments. SM2 expression in the artery cultured in the presence of the ganglia was less than that in the artery that was not grown in culture (data not shown).
not modulate VSM α-actin expression. However, SNs in the presence of ECs increased smooth muscle α-actin expression (Fig. 4). Expression of α-actin in VSMs grown in the presence of ECs and SNs (SN-EC-VSM coculture) was greater than that in VSMs grown only in the presence of ECs (combined EC-VSM and SN cultures). SM2 was not detectable in any of the cultures.

TGF-β is produced by ECs, VSMs, SNs, and EC-VSM and SN-VSM cocultures (2, 8). TGF-β is known to promote the differentiation of VSMs (15–17) and induce the expression of smooth muscle α-actin (16, 17) and myosin (17). This suggests that TGF-β may play a role in sympathetic induction of VSM α-actin and/or myosin. This hypothesis was tested. TGF-β2 is an isoform of TGF-β that is produced by rat vascular cells and SNs (8). The role of this TGF-β isoform was considered (Fig. 5). Figure 5A shows that TGF-β2 (5 ng/ml) increased α-actin expression in EC-VSM cultures grown in the absence but not the presence of an antibody that neutralized the activity of TGF-β2. As noted in Fig. 4, in the absence of an antibody that neutralized the activity of TGF-β2 antibody or in the presence of a control antibody, SNs increased α-actin expression in EC-VSM cultures. In the presence of the TGF-β2 antibody, SNs no longer increased α-actin expression.

DISCUSSION

The present study demonstrates for the first time that sympathetic innervation promotes VSM differentiation as determined by induction or maintenance of contractile protein expression. Several lines of evidence in support of this conclusion are presented, as follows: 1) SM2 expression in innervated rat tail and femoral arteries is greater than that in noninnervated carotid arteries, 2) α-actin expression in femoral arteries that developed in the presence of sympathetic innervation was greater than that in femoral arteries that developed in the absence of sympathetic innervation, 3) SM2 expression in arteries cultured in the presence of sympathetic ganglia was greater than that in arteries cultured in the absence of sympathetic ganglia, and 4) α-actin expression in EC-VSM cocultures that were cultured in the presence of postganglionic SNs was greater than that in EC-VSM cocultures cultured in the absence of SNs.
The overall hypothesis for the present study is that sympathetic innervation promotes or maintains the differentiation of VSMs. To test this hypothesis, the effects of sympathetic innervation on VSM differentiation were studied. Expression of α-actin and/or SM2 was studied as an index of VSM differentiation. Are these relevant markers of differentiation? Smooth muscle α-actin increases during early postnatal differentiation of VSMs (12, 14) and decreases when VSM dedifferentiates in response to vascular injury (1, 13). α-Actin is routinely used to study the mechanisms underlying VSM differentiation both in vivo and in vitro (12–17, 20, 24, 28, 32). SM2 is considered the most stringent marker of VSM differentiation (20, 26). Unlike α-actin, SM2 expression is limited to smooth muscle cells (26). SM2 is undetectable in embryonic and early postnatal VSMs but is easily detectable in young and adult animals (20). These later stages of VSM are associated with increases in SM2 but no change in smooth muscle α-actin (12).

The data in Fig. 1 indicate that in adult rats, innervated tail and femoral arteries express more SM2 than noninnervated carotid arteries. These data are consistent with the overall hypothesis of the present studies that sympathetic innervation promotes VSM differentiation by inducing or maintaining the expression of α-actin and/or SM2. This is the first study to compare SM2 expression in these arteries, and it is unclear whether innervation is the only factor that contributes to the difference in SM2. Carotid arteries are larger than femoral and tail arteries; the structure of the elastic carotid artery differs from that of the muscular femoral and tail arteries; and the embryonic origin of VSM in the two arteries is different (32). Studies by Topouzis and Majesky (32) suggest that the difference in embryonic origin would not affect SM2 expression. These investigations cultured VSMs derived from the neural crest (carotid artery) and mesodermal mesenchyme (tail and femoral arteries) and found no difference in expression of seven markers of VSM differentiation.

The functional significance of the difference in SM2 expression between innervated and noninnervated arteries is unclear. All arteries were obtained from adults, and thus both innervated and noninnervated arteries are fully differentiated. SM2 is not the only myosin isoform expressed in arteries, and studies by Sherwood and Eddinger (30) suggest that differences in SM2 expression do not correlate with changes in contractile function. Recent evidence (4, 30) suggests that innervated arteries express more of another isoform of myosin, SM-B, and this isoform does affect contractile function (3, 4). These data are consistent with innervated arteries being more differentiated for contractile function.

VSM differentiation was also studied in femoral arteries that differentiated in the presence and absence of innervation. Femoral arteries that differentiated in the absence of innervation were from animals that were sympathectomized immediately after birth with guanethidine. This method of sympathectomy irreversibly prevents sympathetic innervation of blood vessels and other sympathetic targets (18). Glyoxylic acid staining for the presence of catecholamines (22) confirmed that femoral arteries from control animals were heavily innervated and arteries from sympathectomized animals lacked detectable innervation (data not shown). Figure 2 indicates that sympathectomy decreased α-actin in femoral arteries of 30-day-old rats, which suggests that innervation promoted or maintained α-actin expression in arteries of young rats. Data from adult animals (90 days of age) showed no difference, which suggests that innervation delays rather than prevents the increase in α-actin expression. These data indicate that sympathetic innervation promotes postnatal differentiation of VSM characterized by increases in α-actin (12).

The method of sympathectomy used in the present studies was systemic and thus could have resulted in innervation-independent systemic effects that decreased α-actin in femoral arteries. This is unlikely, because sympathectomy did not decrease α-actin expression in carotid arteries, which are not innervated (Fig. 2B).

When blood vessels are injured, VSMs undergo dedifferentiation characterized by decreases in SM2 followed by de-
creases in smooth muscle α-actin (see Fig. 3A; Refs. 1, 13, 20, 23, 28). To assess the effects of innervation on dedifferentiation, neonatal femoral arteries were grown in culture in the absence and presence of sympathetic ganglia. The data from these experiments indicate that sympathetic ganglia did not affect the expression of α-actin (see Fig. 3C). However, arteries grown in the presence of ganglia expressed more SM2 than those grown in the absence of ganglia, which suggests that innervation inhibited VSM dedifferentiation.

Neurovascular cocultures were also used to study the effects of sympathetic innervation on VSM differentiation. These cell culture models are routinely used to study many aspects of VSM (2, 6, 8, 16, 17, 27–29), SN (7, 8, 25), and EC (2, 9, 17) function. The VSM cultures used in the present study are representative of dedifferentiated VSMs found in injured and neonatal rat arteries and thus are representative of VSMs whose differentiation would be affected by sympathetic innervation after vascular injury or during vascular development. The SNs used in this study were isolated from SCG of neonatal rats. These neurons are representative of neurons that would innervate blood vessels and potentially modulate VSM differentiation during postnatal development and in response to vascular injury. The ECs used in the present study were isolated from the aortas of adult rats (9). These ECs display many of the characteristics of ECs in neonatal and adult arteries. Neurovascular cocultures were used to study how interactions between SNs, ECs, and VSMs affect VSM differentiation. Neurovascular cocultures were also used to identify potential mechanisms that underlie sympathetic effects on VSM differentiation. Several lines of evidence suggest that cell-to-cell interactions in the cocultures used in this study are representative of cell-to-cell interactions in blood vessels. In the cocultures, the different cell types were in contact or close apposition with one another just as they are in vivo. EC-VSM interactions in cocultures and in intact arteries modulate EC (2) and VSM growth (6) and TGF-β activity (2). SN-VSM interactions in cocultures and in vivo allow for the formation of synapses, stimulate VSM growth (8), and promote neuron survival (7).

Figures 1–3 suggest that SNs would increase α-actin and/or SM2 expression in VSM cultures. SM2 was undetectable in the cultures used in the present study, and thus the effects of innervation on α-actin were considered. SNs did not increase α-actin expression (see Fig. 4). Previous studies from my laboratory (8) indicate that SNs promote the growth of VSMs. Growth and differentiation of VSMs have in many cases been inversely correlated (28). SNs thus appear to promote the growth but not the differentiation of VSMs in culture.

Sympathetic innervation to blood vessels develops late in gestation or shortly after birth (31). SNs interact with VSMs that are already incorporated into blood vessels and thus are associated with vascular ECs. ECs are known to modulate VSM function, and ECs and sympathetic mediators are known to interact to modulate VSM growth (6). Therefore, it was likely that ECs and SNs would interact to modulate VSM α-actin expression. To test this hypothesis, the effects of SNs on VSM differentiation were assessed in the presence of ECs. SNs did increase α-actin expression when VSMs were grown in the presence of ECs (see Fig. 4). Several lines of evidence suggest that this increase in α-actin expression was not due to changes in VSM cell number or protein, as follows: 1) equal numbers of SNs, ECs, and VSMs were plated in combined cultures and cocultures, and both cultures were irreversibly growth arrested with mitomycin C, which prevented any cell growth; 2) total protein quantity in combined cultures was not different than in cocultures; and 3) the observed increase in α-actin expression is supported by the data in Figs. 1–3, which were obtained using alternate models and approaches.

In vitro and in vivo studies indicate that TGF-β promotes the differentiation of VSM and increases α-actin expression (15–17). TGF-β is produced by SNs (8), VSMs (2, 8), and ECs (2), and active TGF-β is produced in SN-VSM (8) and EC-VSM (2) cocultures. The data in Fig. 5 indicate that TGF-β contributed to the SN-induced increase in α-actin expression.

The present study identifies a novel action of the sympathetic nervous system. The data indicate that sympathetic innervation promotes and/or maintains the differentiation of VSMs. Differentiation of VSMs that occurs during postnatal vascular development is a determinant of vascular contractile function. Dedifferentiation of VSMs that occurs in response to vascular injury (1, 13, 20) is thought to contribute to hypertension, atherosclerosis, and postangioplasty restenosis. SNs are likely to modulate postnatal developmental differentiation and injury-induced dedifferentiation.

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