VSM growth is stimulated in sympathetic neuron/VSM cocultures: role of TGF-β2 and endothelin

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During development, blood vessels must clearly grow to match the vascular supply to tissue metabolic demands. In the healthy adult animal, there is little vascular growth but many cardiovascular diseases, including hypertension (17) and atherosclerosis (12, 22), are characterized by aberrant vascular growth that may compromise cardiovascular function. The mechanisms that regulate physiological and/or pathological vascular growth are not well understood.

In vivo studies indicate that sympathetic innervation promotes vascular growth (4, 12, 17, 23, 31). The mechanism underlying this action has not been identified. In vitro studies suggest multiple potential mechanisms that may mediate sympathetic stimulation of vascular growth. Activation of sympathetic neurons causes the release of neurotransmitters and cotransmitters that have been shown to stimulate the growth of vascular smooth muscle (VSM) and endothelial cells (EC) (5, 23, 28, 32). In addition, many neurons, including sympathetic neurons, produce growth factors, the release of which is not dependent on neuronal activity (6).

In the present study, a sympathetic neuron/VSM coculture model is used to determine if and how postganglionic sympathetic neurons stimulate the growth of VSM.

MATERIALS AND METHODS

Materials. Dulbecco's modified essential medium (DMEM), penicillin-streptomycin, and glutamine were purchased from Gibco Life Technologies. Fetal bovine serum (FBS) was purchased from Summit Biotechnology. Collagenase (type 2), hyaluronidase, and trypsin were purchased from Worthington Biochemicals. Mitomycin C and smooth muscle α-actin antibody were purchased from Sigma. Rhodamine-labeled secondary antibody was purchased from Boehringer Mannheim Chemicals. Tyrosine hydroxylase (TH) primary antibody and fluorescein-labeled secondary antibody were purchased from Chemicon International.

Vascular cell culture. EC isolated from adult (>90 days) male Sprague-Dawley rats were a generous gift from Dr. Paula Grammas (University of Oklahoma). These cells exhibited the distinct cobblestone morphology characteristic of EC and they took up acetylated low-density lipoprotein (27). EC were used from passages 11–20. VSM was isolated from explants of adult male Sprague-Dawley rat aortas (21). These cells exhibited characteristic "hill and valley" growth patterns and immunohistochemical labeling with a monoclonal antibody for smooth muscle-specific α-actin. VSM was used from passages 3–10. Vascular cells were grown in low glucose DMEM supplemented with 10% FBS, 1 mM glutamine, 100 units penicillin, and 100 units streptomycin. Cells were maintained at 37°C in a humidified 5% CO2 environment.

Superior cervical ganglion cultures. Rat pups (3–4 days of age, male and female, Sprague-Dawley, Harlan) were anesthetized with metofane and euthanized by removing their hearts. Sympathetic superior cervical ganglia (SCG) were collected and enzymatically dissociated for 20 min at 37°C in a collagenase-hyaluronidase solution (10 mg/ml bovine serum albumin, 4 mg/ml collagenase, 1 mg/ml hyaluronidase) and then for 3 min in trypsin (3 mg/ml). Dissociated cells were applied to collagen-coated dishes. Neuronal cultures (SCG) were grown in DMEM supplemented with 10% FBS, 50 ng/ml NGF, penicillin-streptomycin, and glutamine. Nonneuronal cultures [SCG(−)] were grown in DMEM supplemented with 10% FBS, penicillin-streptomycin, and glutamine without NGF. NGF is absolutely required for the survival of sympathetic neurons, and these cultures contained nonneuronal SCG cells but not neurons. SCG and SCG(−) cultures were maintained at 37°C in a humidified 5% CO2 environment.
SCG/vascular and SCG(−) VSM cocultures. One day after plating, SCG and SCG(−) cultures were treated with an antimitotic agent (mitomycin C, 10 µg/ml for 1 h) to prevent the growth of nonneuronal cells. After removal of the mitomycin C, VSM or EC was added to SCG cultures and VSM was added to SCG(−) cultures and allowed to attach overnight. Transwell SCG/VSM cocultures. SCG were plated in a 24-well tissue culture dish. One day after plating, the SCG were growth arrested (10 µg/ml mitomycin C for 60 min). After removal of the growth-arresting agent, VSM (1 × 10^4 cells/well) was plated on a transwell insert (0.4 µm pores), which sits 1 mm above the well containing the SCG. Thus the SCG and VSM are cultured in the same medium and can reciprocally exchange soluble effectors, but they are not in close proximity and cannot make physical contact. As a control, VSM was also grown on the transwell insert but in the absence of SCG.

Immunohistochemistry. Four-day SCG/VSM cocultures were rinsed with 0.1 M phosphate-buffered saline (PBS, 19 mM sodium phosphate monobasic, 81 mM sodium phosphate dibasic, 0.05 sodium chloride, pH 7.4) and fixed for 2 h in 4% paraformaldehyde in 0.1 M PBS. The cells were then permeabilized (1 h in 0.1 M PBS, 0.2% Triton X-100, 0.9% hydrogen peroxide) and blocked (30 min in 5% normal goat serum). To label sympathetic neurons, cells were incubated with TH (rate-limiting enzyme in catecholamine synthesis) primary antibody (rabbit, 1:4,000) overnight at room temperature, washed with 0.1 M PBS, and incubated with a fluorescein-labeled secondary antibody (donkey anti-rabbit IgG fluorescein, 1:200) overnight at 4°C. To label VSM, cocultures were then incubated with smooth muscle α-actin primary antibody (mouse, 1:400) for 1 h at 37°C, washed, and then incubated with a rhodamine-labeled secondary antibody (goat antimouse IgG rhodamine, 1:10) for 1 h at 37°C. Immunofluorescence was visualized with a Nikon diaphot microscope with appropriate fluorescent filters.

Fluorescence imaging of intracellular calcium. For these experiments the SCG, VSM, and SCG/VSM cultures were placed in HEPES-buffered saline, pH 7.4, and loaded with 1 µM of the membrane permeant Ca2+ indicator dye, fura 2 (the acetoxymethyl ester form) for 30 min at room temperature. Excess dye was removed by washing the cells twice. The cells were then incubated for 15 min at room temperature to allow cytosolic esterases to cleave the ester rendering the dye impermeant. Calcium in the cells was visualized with a Nikon Diaphot microscope equipped for epifluorescence coupled to a Hamamatsu silicon-intensified target camera. The images were recorded and analyzed with an IMAGE-1/FL quantitative fluorescent measuring program (Universal Imaging). Fluorescence ratio images of 510 nm emissions resulting from 340 and 380 nm excitation were acquired. This fluorescence ratio was measured as an index of free cytosolic calcium.

Proliferation assays. For cell growth assays, all cells were plated in 24-well collagen-coated dishes. Cell growth was assayed in VSM and EC cultures and in SCG/VSM, SCG/EC, and SCG(−)/VSM cocultures. Cell growth was assessed as increases in cell number. Cell numbers were counted electronically (Coulter Electronics). For growth-arrested cells, the effectiveness of growth arrest was verified by counting cell number before and 5 days after growth arrest. For all cultures and cocultures, approximately 1 × 10^4 cells/well were plated. For all cultures and cocultures, the day after all cells were plated, three wells were counted to determine the starting cell number (day 0 cell count). The remaining wells of cells were then placed in DMEM supplemented with 5% FBS, penicillin-streptomycin, glutamine, and 50 ng/ml NGF and allowed to grow for 4 days (5% FBS submaximally stimulated the growth of VSM and EC and thus inhibitory and/or stimulatory effects could be assessed). The cells were then counted (day 4 cell count), and the percent increases in VSM or EC number were determined. (For the VSM or EC cultures, the percent increase in cell number = [(day 4 VSM or EC count − day 0 VSM or EC count)/day 0 VSM or EC count] × 100. For the cocultures, the percent increase in VSM or EC number = [(day 4 coculture count − day 0 coculture count)/day 0 VSM or EC count] × 100. In the cocultures, because the SCG or SCG(−) cells were growth arrested, any increase in cell number is attributable to increases in VSM or EC number. Sympathetic stimulation of vascular cell growth was calculated as percentage increases in number in the presence of SCG minus percent increases in cell number in the absence of SCG.

Northern analysis. RNA was isolated as described by Chirgwin et al. (7). Briefly, cells that had been grown in culture for 4 days were lysed with 4 M guanidium isothiocyanate centrifuged through a gradient of cesium chloride. Pelleted RNA was purified by chloroform-butanol extraction and ethanol precipitation, separated by electrophoresis through an agarose (1.2%)-formaldehyde gel, and transferred to nitrocellulose. The RNA was immobilized on the nitrocellulose by baking for 2 h at 80°C. After prehybridization, RNA was labeled by hybridization to radioactively labeled cDNAs encoding for transforming growth factor-β1 (TGF-β1) (obtained from American Type Culture Collection (ATCC); 3), TGF-β2 (obtained from ATCC; 18), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (13). After hybridization, nitrocellulose membranes were washed under stringent conditions and exposed to X-ray film.

Statistical analysis. All data are expressed as means ± SE. All growth experiments were performed in triplicate; the triplicate data were averaged for each number. Unpaired t-tests were used to determine statistically significant differences (P < 0.05).

RESULTS

SCG/VSM cocultures. The goal of the present studies was to determine if and how sympathetic neurons modulate the growth of VSM. An in vitro coculture model was used to achieve this goal. These cultures contained postganglionic sympathetic neurons and a small percentage (10–20%) of nonneuronal cells. The SCG cells were growth arrested to prevent the growth of nonneuronal cells, and then VSM was added to the cultures. The initial ratio of neurons to VSM in the cocultures was approximately 1:1. Figure 1 shows a representative coculture immunohistochemically labeled with TH (green fluorescence), the rate-limiting enzyme in catecholamine synthesis and thus a marker for postganglionic sympathetic neurons, and smooth muscle α-actin (red fluorescence), a marker for VSM. Note that in Fig. 1 no VSM exhibited green fluorescence and no sympathetic neurons exhibited red fluorescence, indicating that the immunohistochemical labeling with both TH and smooth muscle α-actin was specific.

In vivo, sympathetic neurons form synaptic connections with VSM. Figure 1 shows that processes emanating from the sympathetic neurons appear to make contact with the VSM, suggesting that the neurons could make "synapses" with the VSM in the SCG/VSM cocultures. In vivo, acetylcholine (ACh) released from preganglionic sympathetic nerve terminals stimulates...
norepinephrine (NE) release from postganglionic nerve terminals that then act on VSM. The ability of SCG neurons to make synapses on VSM in culture was assessed in three experiments. Fluorescence ratios of fura 2-loaded SCG, VSM, and SCG/VSM cultures were measured to monitor changes in intracellular calcium. In these experiments both the SCG and VSM were mitomycin treated. In VSM cultured in the absence of SCG, 7 of 9 cells sampled demonstrated an increase in intracellular calcium in response to the addition of $10^{-4}$ M NE. Fluorescent ratios before the addition of NE were $0.4 \pm 0.006$ (means $\pm$ SE). The addition of NE increased the fluorescent ratio to $2.09 \pm 0.19$ (peak ratio). This indicates that the sympathetic neurotransmitter NE could act on these cells. The addition of ACh ($10^{-4}$ M) did not increase intracellular calcium in any VSM cultured in the absence of SCG neurons (data not shown), suggesting that ACh does not directly act on VSM to regulate intracellular calcium. The addition of ACh to SCG grown in the absence and presence of VSM increased calcium in SCG neurons (Fig. 2, solid line), suggesting that ACh would promote the release of neurotransmitter from these neurons. Fluorescent ratios measured in five neurons were $0.43 \pm 0.04$ before and $1.77 \pm 0.24$ after the addition of ACh. In three independent cocultures, ACh increased intracellular calcium in VSM (Fig. 2, dotted line), suggesting that ACh can indirectly modulate intracellular calcium in VSM by activating the SCG neurons. For these VSM ($n = 3$), the fluorescent ratios were $0.45 \pm 0.07$ before and $1.25 \pm 0.18$ after the addition of ACh. Thus the morphological data in Fig. 1 and the functional data in Fig. 2 suggest that in the coculture model used in the present study, postganglionic sympathetic neurons make functional synapses with VSM.

VSM growth is stimulated in SCG/VSM cocultures. VSM growth (% increase in VSM number) in the presence of sympathetic neurons (presence of SCG, $856 \pm 81\%$) was greater than that in the absence of sympathetic neurons (absence of SCG, $626 \pm 66\%$; $P < 0.05$; Fig. 3A), suggesting that sympathetic neurons stimulate the growth of VSM in SCG/VSM cocultures. The difference between growth in the presence of SCG and that in the absence of SCG ($231 \pm 54\%$) is the stimulation attributable to the SCG neurons. Sympathetic

![Fig. 1. Superior cervical ganglia (SCG)/vascular smooth muscle (VSM) coculture. Sympathetic neurons in representative coculture were labeled with tyrosine hydroxylase primary antibody and fluorescein-labeled secondary antibody. VSM was labeled with α-smooth muscle primary antibody and rhodamine-labeled secondary antibody.](image1)

![Fig. 2. Acetylcholine (ACh) stimulates an increase in cytosolic free calcium in VSM in SCG/VSM coculture. SCG and VSM in coculture were loaded with calcium indicator dye fura 2 before treatment with ACh as described in MATERIALS AND METHODS. Fluorescence ratio is index of cytosolic free calcium. Addition of ACh induces rapid increase in intracellular calcium in SCG and VSM in coculture. This trace is representative of traces recorded from 3 VSM from 3 independent cocultures.](image2)
neurons did not stimulate the growth of EC in SCG/EC cocultures (Fig. 3B). Growth of EC in the presence of SCG (943 ± 159% increase) was not significantly different from that in the absence of SCG (849 ± 70% increase; P > 0.05).

As noted earlier, the SCG cultures contain a small fraction of nonneuronal cells. To verify that the stimulation of VSM in SCG/VSM cocultures was dependent on the sympathetic neurons and not the nonneuronal cells, VSM growth was assessed in SCG(-)/VSM cocultures. The SCG(-) cultures contain nonneuronal superior cervical ganglion cells but no postganglionic sympathetic neurons. VSM growth was not stimulated in SCG(-)/VSM cocultures (Fig. 3C). In fact, VSM growth in the presence of nonneuronal superior cervical ganglion cells [presence of SCG(-)], 95 ± 16% increase] was less than that in the absence of these cells [absence of SCG(-)], 267 ± 60% increase].

The effects of SCG on VSM growth were also assessed in transwell cocultures. In these cocultures SCG and VSM are cultured in the same medium and thus can reciprocally exchange stable soluble mediators, but the cells are separated by 1 mm, which prevents physical contact and/or close proximity. In this coculture system, SCG did not stimulate VSM growth (Fig. 4, solid bar). The lack of stimulation in transwell cocultures is in contrast to the stimulation that was observed in parallel SCG/VSM cocultures that allowed physical contact and/or close proximity (Fig. 4, open bar).

TGF-β2 and endothelin are required for SCG stimulation of VSM growth in SCG/VSM cocultures. Figure 3 indicates that SCG neurons stimulate the growth of VSM in cocultures in which the two cell types are in contact or close proximity. The data in Fig. 4 indicate that close proximity or cell contact is required for this stimulation. TGF-β is produced by neurons (11, 26) and VSM (18, 20), is activated by heterotypic cell interactions that require close proximity or cell contact (2), and stimulates VSM growth (25, 30). Does TGF-β mediate SCG stimulation of VSM growth in SCG/VSM cocultures? Neutralization of the activity of TGF-β2 with an antibody (goat) inhibited SCG stimulation of VSM growth in SCG/VSM cocultures (Fig. 5). Growth of VSM in the absence of SCG was not affected by the TGF-β2 antibody (425 ± 32% increase in the absence vs. 406 ± 34% increase in the presence of the antibody; P > 0.05), but growth of VSM in the presence of SCG was inhibited (589 ± 68% increase in the absence of antibody vs. 445 ± 27% increase in the presence of antibody; P < 0.05). In the presence of the TGF-β2 neutralizing antibody, SCG stimulation of VSM growth was inhibited by 27% (445 ± 27% vs. 325 ± 22%).

Fig. 3. Sympathetic neurons stimulate growth of VSM in SCG/VSM cocultures. A: VSM growth in presence of SCG cells (+ SCG, solid bar) was significantly greater (*) than growth in absence of SCG (- SCG, open bar, n = 8, P < 0.05). B: endothelial cell (EC) growth in presence of SCG cells (+ SCG, solid bar) is not significantly different from EC growth in absence of SCG cells (- SCG, open bar, n = 8, P > 0.05). C: VSM growth in presence of nonneuronal SCG cells (+ SCG(-), solid bar) is not significantly greater than growth in absence of nonneuronal SCG cells [- SCG(-), open bar, n = 4, P > 0.05]. All data are means ± SE.

Fig. 4. Sympathetic neurons do not stimulate growth of VSM in SCG/VSM transwell cocultures. Growth of VSM in the presence of SCG in direct contact and/or close proximity cocultures was significantly greater than growth in absence of SCG (* P < 0.05, unpaired t-test). In parallel transwell cocultures, VSM growth in the presence of SCG was not significantly different from VSM growth in absence of SCG (P > 0.05).
antibody, VSM growth in the presence of SCG (445 ± 27%) was not significantly greater than that in the absence of SCG (406 ± 34%). A nonimmune goat IgG (data not shown) and an antibody that neutralized the activity of TGF-β1 did not alter VSM growth in the presence or absence of SCG, and thus these antibodies did not modulate SCG stimulation of VSM growth (Fig. 5).

The data in Fig. 5 indicate that TGF-β2 is required for sympathetic stimulation of VSM growth in SCG/VSM cocultures. Thus the data in Fig. 5 also suggest that TGF-β2 is produced in SCG/VSM cocultures. Northern analysis of TGF-β expression in SCG, VSM, and SCG/VSM cultures supports this suggestion. TGF-β2 mRNAs (4.0 and 5.0 kb) were expressed in SCG, VSM, and SCG/VSM cultures (Fig. 6A). Under equivalent experimental conditions, expression of TGF-β1 mRNA (2.5 kb) was undetectable, although this mRNA was easily detected in cultures of bovine EC (Fig. 6B). GAPDH mRNA was detectable in all samples.

TGF-β2 can stimulate the growth of VSM by increasing the production of VSM mitogens (14, 25). Endothelin is a VSM mitogen (1, 29) that is produced by postganglionic sympathetic neurons (9) and VSM (15), and TGF-β increases VSM production of endothelin-1 (15). Thus the sympathetic stimulation of VSM growth observed in SCG/VSM cocultures could be mediated by TGF-β2 and endothelin-1. To test this hypothesis, VSM growth was assessed in the presence and absence of endothelin receptor A antagonists BQ-123 and BQ-610. Similar results were obtained with these two agents, and thus the data were combined. The addition of 1 µM BQ-123 (or BQ-610) inhibited sympathetic stimulation of VSM growth in SCG/VSM cocultures (Fig. 7).

The data in Figs. 5 and 7 suggest that sympathetic stimulation of VSM growth in SCG/VSM cocultures is dependent on the activity of TGF-β2 and that the effects of TGF-β2 are mediated by endothelin. If this is the case, then TGF-β2 should stimulate the growth of VSM in the absence of SCG, and endothelin antagonism should prevent this stimulation. The effects of TGF-β2 and BQ-123 on VSM growth in the absence of SCG are shown in Fig. 8. In the absence of BQ-123, 10 ng/ml TGF-β2 stimulated growth (44.6 ± 24.9% increase in cell number, open bar). In the presence of 10 µM BQ-123, TGF-β2 no longer stimulated and in fact inhibited VSM growth (41.0 ± 22.4% decrease in cell number, solid bar).

DISCUSSION

The majority of blood vessels in animals and humans are innervated by the sympathetic nervous system. It is well known that sympathetic innervation modulates the contractile activity of the vasculature and is thus an important determinant of blood pressure (17). Sympathetic innervation also has been reported to modulate the growth of blood vessels (4, 12, 17, 24, 31), but much
that in absence of BQ-123 (BQ-123. *Growth in presence of BQ-123 was significantly less than (BQ-123, open bar) and presence (2) growth was measured in presence of 10 ng/ml TGF-
BQ-123 or BQ-610, BQ-123, open bar) of endothelin-A receptor antagonists (1 µM receptor antagonists was significantly (*) less than that in absence (−BQ-123, open bar) of endothelin-A receptor antagonists (1 µM BQ-123 or BQ-610, n = 6, P < 0.05).

less is known about this action or the mechanism of this action. The data presented demonstrate that postganglionic sympathetic neurons stimulate the growth of VSM in an in vitro coculture system and identify novel mechanisms whereby sympathetic neurons may act on VSM.

An in vitro coculture model was used to study sympathetic stimulation of VSM growth. The cocultures contained postganglionic sympathetic neurons and a small percentage of nonneuronal cells isolated from SCG of neonatal rats and vascular cells from adult rat aortas. The sympathetic and vascular cells were cultured in the same dish in close proximity or direct contact. In these cultures, the sympathetic neurons could modulate vascular cell growth by releasing growth factors from their cell bodies and/or nerve terminals.

Figure 1 suggests that in the cocultures used in the present studies, processes from the neurons make contact with the VSM. Do they actually make synapses? If the neurons were making functional synapses with the smooth muscle cells, selective activation of the neurons should cause the release of neurotransmitter, which would then act on the smooth muscle. Fluorescence imaging of intracellular calcium was used to test this hypothesis. ACh was used to selectively activate the neurons. ACh increased intracellular calcium in SCG neurons cultured in the presence and absence of VSM, suggesting that addition of ACh to the cultures would promote the release of NE from the neurons. ACh did not alter intracellular calcium in VSM cultured in the absence of SCG, indicating that ACh did not directly modulate intracellular calcium in these cells. NE increased intracellular calcium in VSM cultured in the absence of SCG, indicating that VSM could respond to this neurotransmitter if it was released from SCG neurons in the SCG/VSM cocultures. Addition of ACh to SCG/VSM cocultures increased intracellular calcium in VSM as well as SCG neurons (Fig. 2), suggesting that functional synapses were present. The frequency of SCG/VSM synapse formation was not assessed in the present study but is currently under investigation.

VSM growth was stimulated in SCG/VSM cocultures (Fig. 3A). SCG/VSM coculture produced a 230 ± 54% increase in VSM growth. This increase is comparable to that induced by maximally active concentrations of other VSM mitogens (10, 29). EC growth was not stimulated in SCG/EC cocultures (Fig. 3B), indicating that SCG produce a factor or factors that stimulate the growth of VSM but not EC.

SCG cultures used in the present study contained postganglionic sympathetic neurons as well as a small number of nonneuronal cells. SCG stimulation of VSM growth could be mediated by either or both of these cell types. To determine whether nonneuronal SCG could stimulate VSM growth, VSM was cocultured with SCG cultures that had been grown in the absence of NGF [SCG(−)]. These SCG(−) cultures contained nonneuronal cells but no neurons. VSM growth was not stimulated in SCG(−)/VSM cocultures (Fig. 3C). In fact, VSM growth was inhibited, indicating that SCG stimulation of growth in SCG/VSM cocultures requires postganglionic sympathetic neurons and that the presence of the neurons overrides any inhibitory effects associated with the nonneuronal cells.

VSM growth was not stimulated in SCG/VSM transwell cocultures (Fig. 4). In these cocultures, the cells are cultured in the same medium and thus can reciprocally exchange stable soluble mediators, but the VSM and the SCG are separated by 1 mm and thus are not in close proximity or contact. Thus SCG stimulation of VSM growth in coculture is dependent on proximity or contact between SCG and VSM.

TGF-β has been reported to stimulate VSM growth (25, 30), is produced by vascular cells (16, 18, 20) and many neurons (11, 26), and is activated when heterotypic cells are in close proximity or contact (2). Thus TGF-β was a likely mediator of the SCG stimulation of VSM growth observed in the present studies. When SCG and VSM were cocultured in the presence of an antibody that neutralized the activity of TGF-β, SCG no longer stimulated the growth of VSM (Fig. 5). SCG did not stimulate VSM growth when cocultures were grown
in the presence of a nonimmune goat IgG (data not shown), indicating that the effect of the TGF-β2 antibody was attributable to the neutralization of the activity of TGF-β2. The ability of SCG to stimulate the growth of VSM in coculture was also unaffected by antibodies that neutralized the activity of TGF-β1 (Fig. 5).

The present studies using neutralizing antibodies suggest that active TGF-β2 but not TGF-β1 is produced in VSM, SCG, and SCG/VSM cultures. Consistent with previous observations (18, 20), mRNA encoding for TGF-β2 was detected in VSM (Fig. 6A). TGF-β2 mRNA was also detected in SCG cultures and in SCG/VSM cocultures (Fig. 6A). This isoform of TGF-β is known to be expressed by many other neurons (11, 26), but this is the first report that it is expressed by postganglionic sympathetic neurons. The amount of TGF-β1 mRNA in VSM, SCG, and SCG/VSM cultures was considerably less than that of TGF-β2 mRNA (Fig. 6B). In the present studies, Northern analysis was performed on cultures in which the VSM was confluent. These cultures would be comparable to the high density VSM cultures used by Hamet et al. (16), in which TGF-β1 mRNA expression was also very low. The preferential expression of TGF-β2 and not TGF-β1 by sympathetic neurons is consistent with previous reports of TGF-β isoform expression in other neurons (11, 26).

The data in Fig. 7 indicate that endothelin A receptor antagonism also inhibits stimulation of VSM growth in SCG/VSM cocultures. Endothelin, acting at endothelin A receptors, is a mitogen for VSM (1, 29) that is produced by postganglionic sympathetic neurons (9) and rat VSM (15). TGF-β is known to increase VSM endothelin-1 mRNA and peptide expression (15). Thus the data in Figs. 5–7 support the hypothesis that TGF-β2 is produced in SCG/VSM cocultures and stimulates the production of endothelin, which stimulates the growth of VSM. Further support for this hypothesis is provided by the data shown in Fig. 8. VSM growth in the absence of SCG was slightly stimulated by TGF-β2. This stimulation was inhibited by BQ-123, an endothelin A receptor antagonist. In fact, in the presence of BQ-123, TGF-β2 inhibited rather than stimulated VSM growth.

In vivo studies clearly indicate that sympathetic innervation modulates the physiological growth of VSM that occurs during development (4, 31) and the pathological growth that occurs in hypertension (17) and in response to vascular injury (12). The goal of the present studies was to determine the mechanism whereby sympathetic innervation modulates VSM growth. An in vitro coculture model was used to achieve this goal. Several lines of evidence suggest that this model is appropriate for studying sympathetic regulation of VSM growth. SCG neurons grown in culture are biochemically and electrophysiologically similar to those in vivo (8). The VSM used in the present studies, which was isolated from adult rat aortas, have growth properties similar to those of VSM in developing arteries and in injured arteries (22). Two differences between in vivo and the in vitro model should be noted. In vivo, only postganglionic sympathetic nerve terminals (not cell bodies) would be in close proximity to VSM; in vitro, VSM has access to both cell bodies and nerve terminals. Also, in the presently used in vitro model, the neurons were not stimulated; in vivo, the neurons would be intermittently stimulated. Studies are in progress to extend the current in vitro observations, and determine the role of TGF-β2 and endothelin in sympathetic regulation of VSM growth in vivo.

In summary, the present studies demonstrate that postganglionic sympathetic neurons stimulate the growth of VSM in cocultures that allow close proximity or direct contact between the neurons and VSM. Data are also presented demonstrating that the VSM, SCG, and SCG/VSM cultures express mRNA encoding for TGF-β2, and that if the activity of TGF-β2 is inhibited, the SCG no longer stimulate VSM growth. Finally, inhibition of endothelin binding to endothelin A receptors is also shown to prevent SCG stimulation of VSM growth in SCG/VSM cocultures, suggesting that endothelin and TGF-β2 are required for SCG stimulation of VSM growth.

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