Thromboxane A₂ Stimulates Vascular Smooth Muscle Hypertrophy by Up-regulating the Synthesis and Release of Endogenous Basic Fibroblast Growth Factor*

(Received for publication, January 14, 1993, and in revised form, April 24, 1993)

Safdar Aliň, Michael G. Davisť, Michael W. Beckerį, and Gerald W. Dorn II§§

From the Departments $\S$Medicine/Cardiology and $\S$Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

We have shown previously that thromboxane A₂ stimulates hypertrophy of cultured rat aortic smooth muscle cells defined as proteocnogene expression and protein synthesis without DNA synthesis or cellular proliferation (Dorn, G. W., II, Becker, M. W., Davis, M. G. (1992) J. Biol. Chem. 267, 24897-24905). Since endogenous growth modulators could possibly regulate vascular smooth muscle growth to this vasoconstrictor, we tested the hypothesis that thromboxane-stimulated vascular smooth muscle hypertrophy was due to increased expression of endogenously produced basic fibroblast growth factor (bFGF). The thromboxane mimetic (15S)-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid (U46619) (1 µM) increased cultured rat aorta derived smooth muscle cell immunoreactive bFGF content by 351 ± 40% over untreated controls after 24 h. Co-incubation of vascular smooth muscle cells with a specific antisense oligodeoxynucleotide (AS) against codon 60 of bFGF coding sequence reduced thromboxane-stimulated bFGF expression by 72 ± 5% and prevented thromboxane-stimulated hypertrophy (nonsense oligonucleotide had no effects). Addition of exogenous bFGF (20 ng/ml) restored growth to AS-treated/thromboxane-stimulated vascular smooth muscle cells. Furthermore, addition to the culture medium of neutralizing antibody against bFGF inhibited U46619-stimulated vascular smooth muscle hypertrophy by 68 ± 17%, whereas nonimmune IgG had no effect.

Since protein tyrosine phosphorylation is a cell signal associated with growth, thromboxane-stimulated tyrosine phosphorylation was also examined. Exposure to 1 µM U46619 for 10 min increased vascular smooth muscle immunoreactive phosphotyrosine content of 130-144-, 86-, 80-, 75-, and 58-kDa proteins. The tyrosine kinase inhibitor herbimycin A (5 µM) prevented thromboxane-stimulated tyrosine phosphorylation, but not thromboxane-stimulated hypertrophy, suggesting that tyrosine phosphorylation was not required for thromboxane-stimulated vascular smooth muscle growth. These results indicate that increased expression and release of endogenous bFGF, but not direct tyrosine phosphorylation, mediates the hypertrophic vascular smooth muscle response to thromboxane.

Thromboxane A₂ is a potent vasoconstrictor released from activated platelets during intravascular thrombosis (1). Like other contractile agonists, including angiotensin II (2-4), thrombin (5, 6), and serotonin (7), thromboxane A₂ possesses growth-promoting activity in vascular smooth muscle cells (8, 9). Recently, Dorn et al. (10) demonstrated that the thromboxane A₂ analog (15S)-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid (U46619)'-stimulated cellular hypertrophy of cultured rat aortic smooth muscle cells which was characterized by proteocnogene expression and accelerated protein synthesis without DNA synthesis or cell proliferation. The intracellular signals which transduce thromboxane-stimulated vascular smooth muscle hypertrophy have not been defined.

One potentially important signaling system for cellular growth is tyrosine phosphorylation (11). After exposure to platelet-derived growth factor (PDGF) and other growth factors, a protein kinase cascade is activated which culminates in phosphorylation of ribosomal protein S6, thought to play a key regulatory role for protein translation during cell cycle progression (11-13). Vasoconstricting growth promoters such as angiotensin II, endothelin, and vasopressin also enhance tyrosine phosphorylation of a number of proteins via protein kinase C-dependent and independent pathways (14).

The mechanisms by which most vasoconstrictor growth promoters increase cellular phosphorytrosine content are unclear. It has been suggested that thrombin directly activates growth factor receptor tyrosine kinase activity via a proteolytic action (15). A mechanism by which other vasoconstrictors could activate tyrosine kinases is by causing the autocrine or paracrine release of endogenous growth factors. For example, angiotensin II has been shown to induce PDGF A-chain expression in cultured rat aortic smooth muscle cells (16, 17).

The current studies were undertaken to test the hypothesis that thromboxane-stimulated vascular smooth muscle hypertrophy requires endogenous growth factor production. We report that exposure to the thromboxane A₂ mimetic U46619 is associated with rapidly increased cellular phosphorytrosine content and a delayed increase in endogenous bFGF content. Our results indicate that increased expression and release of...
Thromboxane Stimulates Basic Fibroblast Growth Factor

bFGF is required for thromboxane-stimulated hypertrophy but that the initial increase in tyrosine phosphorylated proteins is not necessary for a growth response.

**Experimental Procedures**

**Materials**—Human recombinant bFGF, herbasmin A, trypsin, and antibiotics for tissue culture were obtained from Life Technologies, Inc. Monoclonal antibodies against bFGF and phosphotyrosine were from Upstate Biotechnology Inc. A polyclonal antibody to phosphotyrosine containing proteins was from Life Technologies, Inc. The amphotericin B and heparinase anticoagulant and chemiluminescence immuno blot developing systems were purchased from Bio-Rad. An anti-phosphotyrosine antibody against oligo(ethylene glycol) directed against bFGF was synthesized and purified at the University of Cincinnati Core DNA Facility under the direction of Dr. Jerry Lingrel. Radiochemicals were from Du Pont-New England Nuclear. U46619 and all other reagents and chemicals were obtained from Sigma.

**Measurement of Protein and DNA Synthesis and Cell Culture**—The methods for culture of rat aortic vascular smooth muscle cells and measurement of protein and DNA synthesis by incorporation of [3H]leucine and [3H]thymidine have been described previously in detail (8). Cell cultures were from passages 3 to 6. The smooth muscle nature of the cultured cells was confirmed by positive staining with antibody smooth muscle actin (Sigma) at passages 3 and 5.

**Protein Electrophoresis and Western Blot Analysis**—For measurement of protein phosphorysine content, vascular smooth muscle lysates were centrifuged at 100,000 g for 30 min at 4 °C and the supernatants saved, and protein concentration was determined spectrophotometrically by the method of Bradford (18). Identical amounts of protein were loaded onto 10% discontinuous SDS-polyacrylamide gels and electrophoresed in a minigel apparatus at 75 mV for 2.5–5 h at 10 °C according to Laemmli’s method (19). Proteins were electrophoretically transferred onto nitrocellulose membranes and tyrosine phosphorylated proteins were identified by immunoblot analysis. In some experiments vascular smooth muscle cells stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 15 min or 20 ng/ml purified human bFGF for 30 min were also assayed for phosphorysine accumulation. The effects of the protein kinase inhibitor staurosporine (100 nM) or the specific tyrosine kinase inhibitor herbimycin A (5 μM) (15), added 30 min prior to stimulation with U46619, were examined in some experiments as indicated.

**Measurement of Thromboxane-stimulated Protein and DNA Synthesis**—In our prior study, thromboxane exposure increased vascular smooth muscle cell protein synthesis as measured by [3H]leucine incorporation, but did not increase DNA synthesis or stimulate cell proliferation (10). As these results were at variance with those of Hanasaki et al. (8) who observed thromboxane A2-stimulated DNA synthesis in cultured rat aorta vascular smooth muscle cells, we repeated these studies. Treatment with 1 μM U46619 for 48 h increased [3H]leucine incorporation into growth-arrested vascular smooth muscle cells by 44% compared with controls (vehicle, 9465 ± 770 cpm/well; U46619, 13,589 ± 616 cpm/well, n = 4 pairs, *p* = 0.002). In contrast, but consistent with our prior findings, U46619 failed to stimulate DNA synthesis as measured by the incorporation of [3H]thymidine. Therefore, the thromboxane A2 mimetic U46619 stimulates cultured rat aortic smooth muscle cell protein synthesis but not DNA synthesis, i.e., it stimulates hypertrophy.

**Measurement of Thromboxane-stimulated Tyrosine Phosphorylation**—Since growth factor receptors (13), some vasoconstricting growth promoters (14), and a number of retroviral oncogene products (21) increase tyrosine-specific protein kinase activity, we examined whether protein tyrosine phosphorylation was also stimulated by U46619. The immunoblot analysis, the time course for U46619-stimulated tyrosine phosphorylation of the 75–85-kDa proteins in three additional experiments. Tyrosine phosphorylation was apparent 5 min after addition of 1 μM U46619, peaked at 10 min, and subsequently declined, but remained elevated over unstimulated levels. Similar time courses of U46619-stimulated tyrosine phosphorylation were observed for the higher and lower molecular weight phosphoproteins (data not shown).

**Effects of Tyrosine Kinase Inhibitors on Thromboxane A2-induced Tyrosine Phosphorylation**—The immunoblot in Fig. 1 shows the effects of the specific tyrosine kinase inhibitor herbiasmin A and the less specific kinase inhibitor staurosporine (22) on U46619-stimulated vascular smooth muscle phosphotyrosine accumulation. This figure also shows that tyrosine phosphorylation was stimulated by addition of PMA was by laser densitometry using a LKB Ulro Scan densitometer (LKB Produkter, Gaithersburg, MD).

**Inhibition of bFGF with Antisense Oligodeoxynucleotide or Neutralizing Antibodies**—Rat aortic smooth muscle cells were grown to confluence by serum deprivation as described above and a specific antisense oligonucleotide (5′-GGC-TGC-CAT-GGT-CCG-3′) (20) was added, in the concentrations indicated, at the time of addition of serum-free medium. After 48 h of incubation, the growth-arrested cells were stimulated with 1 μM U46619 for an additional 24 h. Antisense primers were added again at the time of U46619 stimulation. A nonsense oligonucleotide was added in an identical manner as a control to parallel cell cultures. Basic fibroblast growth factor content and [3H]leucine incorporation were measured as described above.

To determine the role of secreted bFGF on U46619-induced growth response, growth-arrested rat aortic vascular muscle cells were stimulated with 1 μM U46619 in the presence of 1.5 μg/ml of a neutralizing antibody against bFGF (Upstate Biotechnology Inc., Type II). Non-specific IgG (1.5 μg/ml) was added in an identical manner to parallel cell cultures as a control. [3H]Leucine incorporation was determined as described above.

**Statistical Analysis**—The results were analyzed by either two-tailed Student’s *t* test or one-way analysis of variance and Neuman Keul’s test as appropriate. All values are expressed as mean ± standard error. A *p* value of <0.05 was considered significant.
Thromboxane Stimulates Basic Fibroblast Growth Factor

**Fig. 1. Thromboxane-stimulated protein tyrosine phosphorylation in vascular smooth muscle cells.** a, confluent quiescent rat aortic smooth muscle cells were exposed to ethanolic vehicle (control (CON)) or U46619 (1 μM) for time points as indicated. Cell monolayers were lysed in situ as described under “Experimental Procedures.” Cell lysates were separated by 10% discontinuous SDS-polyacrylamide gel electrophoresis electrophoresis, transferred to nitrocellulose, and probed with rabbit polyclonal antiphosphotyrosine antibody (top) or mouse monoclonal antiphosphotyrosine antibody (bottom). Relative positions of U46619-stimulated tyrosine phosphorylated proteins in the top blot are indicated by arrows and are from the top: 130-150, 75-85, 60, and 17 kDa. Molecular masses of protein standards are indicated in kDa on the left border. Both monoclonal and polyclonal antibodies showed increased phosphorylation of 130-150- and 75-85-kDa proteins, but polyclonal antibody also resolved other phosphotyrosine-containing proteins. b, time-dependent tyrosine phosphorylation of 75-85-kDa protein (probed with polyclonal antibody) in U46619-stimulated vascular smooth muscle cells as determined by laser densitometry. Each point represents the mean ± standard error of three experiments. Tyrosine content was maximal after 10 min of stimulation and then fell to a plateau level approximately 170% of baseline.

**Fig. 2. Herbimycin A inhibits thromboxane-stimulated tyrosine phosphorylation.** Growth-arrested vascular smooth muscle cells were exposed to vehicle or U46619 (1 μM) for 10 min or preincubated for 30 min with 100 nM staurosporine (U46+S) or 5 μM herbimycin (U46+H) and then stimulated with U46619 (1 μM) for 10 min. Basic fibroblast growth factor (20 ng/ml × 30 min) and PMA (100 nM × 15 min) stimulated cells were run as positive controls. Immunoblots were prepared and probed with polyclonal anti-phosphotyrosine antibody as described in the legend to Fig. 1. Herbimycin, but not staurosporine, inhibited U46619-stimulated vascular smooth muscle tyrosine phosphorylation.

(100 nM) or bFGF (20 ng/ml) to cultured vascular smooth muscle cells. The electrophoresis for these experiments was performed for prolonged periods in order to provide greater resolution of the higher molecular mass tyrosine-phosphorylated proteins. Examination of this immunoblot shows that U46619 phosphorylates tyrosine residues on several individual proteins having molecular masses of 132–144 kDa and distinct 86-, 80-, 75-, and 58.5-kDa proteins. U46619-stimulated tyrosine phosphorylation was inhibited by treatment with herbimycin A. In contrast, staurosporine, a kinase inhibitor which is more specific for protein kinase C, but which also has tyrosine kinase inhibiting activity (22), appeared to attenuate U46619-stimulated tyrosine phosphorylation of only the 58.5-kDa protein.

Different patterns of vascular smooth muscle cell tyrosine phosphorylation after stimulation by bFGF, PMA, and thromboxane A2 are also demonstrated in Fig. 2. Compared with U46619, both bFGF and PMA caused tyrosine phosphorylation of an approximately 200-kDa protein. In contrast, U46619, but not bFGF or PMA, stimulated tyrosine phosphorylation of a 75-kDa protein. All three agonists increased the phosphotyrosine contents of 86-, 80-, and 58.5-kDa proteins.

**Effects of Protein Kinase Inhibitors on Thromboxane-stimulated Hyperplasia**—In order to determine whether tyrosine phosphorylation was a necessary cell signal for U46619-stimulated vascular smooth muscle hypertrophy, the effects of tyrosine kinase inhibition with herbimycin A on U46619-stimulated vascular smooth muscle cell [3H]leucine accumulation were examined. The results of these studies, shown in Table I, demonstrate that preincubation with herbimycin A under conditions which were effective in preventing U46619-stimulated tyrosine phosphorylation (see above) did not inhibit U46619-stimulated [3H]leucine incorporation into vas-
Thromboxane Stimulates Basic Fibroblast Growth Factor

Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[H]Leucine</th>
<th>n</th>
<th>Difference vehicle</th>
<th>Difference U46619</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>8,336 ± 340</td>
<td>29</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum</td>
<td>22,743 ± 2,186</td>
<td>14</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>U46619</td>
<td>13,065 ± 689</td>
<td>30</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>U46 + H</td>
<td>13,356 ± 330</td>
<td>3</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>U46 + S</td>
<td>5,759 ± 2,001</td>
<td>4</td>
<td>0.014</td>
<td>0.001</td>
</tr>
<tr>
<td>U46 + AS</td>
<td>5,159 ± 736</td>
<td>7</td>
<td>NS</td>
<td>0.014</td>
</tr>
<tr>
<td>U46 + AS + bFGF</td>
<td>25,864 ± 8,094</td>
<td>4</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Vehicle</td>
<td>8,872 ± 877</td>
<td>6</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>U46 + anti-bFGF</td>
<td>11,438 ± 764</td>
<td>6</td>
<td>0.052</td>
<td>0.013</td>
</tr>
<tr>
<td>U46 + IgG</td>
<td>16,065 ± 2,101</td>
<td>6</td>
<td>0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

cellular smooth muscle cells.

Since treatment with the protein kinase C activator PMA had increased the phosphotyrosine content of vascular smooth muscle cell proteins, and since staurosporine, a protein kinase inhibitor which exhibits some selectivity for protein kinase C, appeared to inhibit U46619-stimulated tyrosine phosphorylation of only the 58.5-kDa protein, we also examined the effects of staurosporine treatment on thromboxane-stimulated hypertrophy. Results in the table indicate that U46619-stimulated [H]leucine incorporation was dramatically reduced by staurosporine treatment. To ensure that the observed decrease in [H]leucine incorporation was not the result of cell injury or death, we determined that cell viability assayed both by trypan blue exclusion and release of lactate dehydrogenase was identical after 48 h in staurosporine- and vehicle-treated cells (greater than 97% viability by trypan blue, no lactate dehydrogenase release compared with controls). Although the antiproliferative effects of staurosporine are not necessarily related to inhibition of protein kinase C and may represent inhibition of growth factor receptor-mediated tyrosine phosphorylation (23), the results of the staurosporine and herbimycin experiments suggest that tyrosine phosphorylation by U46619 is not necessary for the hypertrophic response to thromboxane A2 in vascular smooth muscle cells.

Endogenous Expression of bFGF in Response to Thromboxane A2 Stimulation—To examine the possibility that thromboxane A2 was stimulating vascular smooth muscle hypertrophy by modulating the expression of an endogenous biological response modifier, we measured bFGF in U46619-stimulated growth-arrested vascular smooth muscle cells. The immunoblot in Fig. 3 illustrates that the 22-kDa isoform of bFGF increased by 331 ± 40% (n = 7, p < 0.001) over unstimulated controls after 24 h of exposure to U46619 and remained elevated by 182 ± 17% (n = 4, p < 0.01) after 48 h.

To examine the significance of bFGF expression in thromboxane A2-stimulated vascular smooth muscle hypertrophy, bFGF translation and expression were inhibited with a specific antisense (AS) oligodeoxynucleotide (20). Fig. 4 shows the effect of pre-addition of 30 μM bFGF AS on U46619-stimulated bFGF expression. In six separate experiments, bFGF levels in U46619-treated vascular smooth muscle were reduced by 72 ± 5% or approximately to the levels observed in untreated cells. In contrast, addition of 30 μM nonsense oligonucleotide as a control had a slight nonspecific inhibitory effect on U46619-stimulated bFGF expression which did not achieve statistical significance (Fig. 4).

To determine whether endogenous bFGF was important for the hypertrophic response to U46619, the effects of bFGF AS on U46619-stimulated vascular smooth muscle [H]leucine accumulation were assessed. Inhibition of bFGF expression

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46619}}{\text{Control}} \]

\[ \frac{\text{U46 + AS}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]

\[ \frac{\text{Vehicle}}{\text{Control}} \]

\[ \frac{\text{U46 + anti-bFGF}}{\text{Control}} \]

\[ \frac{\text{U46 + IgG}}{\text{Control}} \]

\[ \frac{\text{U46 + AS + bFGF}}{\text{Control}} \]
with 30 μM bFGF AS abolished thromboxane-stimulated hypertrophy (Table 1), whereas treatment with identical concentrations of nonsense oligonucleotide had no effect (n = 2; data not shown). Neither significant inhibition of bFGF nor attenuation of [3H]leucine accumulation were observed when bFGF AS was added at a concentration of 7.5 μM (not shown). However, to further insure that antisense inhibition of bFGF was not having a nonspecific effect on vascular smooth muscle cell proliferation, we added exogenous bFGF (20 ng/ml) to cells previously treated with U46619 in the presence of 30 μM bFGF AS and found that the cellular ability to accumulate [3H]leucine was restored (Table 1).

Finally, we determined whether endogenous bFGF was functioning as an autocrine factor, i.e. a factor which is released and has modulating effects on the same cell type, or as an intracrine factor, i.e. a factor which exerts its modulating effects on the cell in which it is produced, without being released. In six experiments, addition to the culture medium of an anti-bFGF antibody which neutralizes the biological activity of bFGF inhibited U46619-stimulated [3H]leucine accumulation by 69 ± 17%, whereas nonimmune immunoglobulin (control) was without inhibitory activity (Table 1).

Taken together, the above studies suggest that thromboxane-stimulated vascular smooth muscle hypertrophy is critically dependent upon increased endogenous synthesis and release of bFGF.

DISCUSSION

The current studies were undertaken to identify and determine the relative importance of cellular messengers which transduce the vascular smooth muscle hypertrophic response to a synthetic thromboxane A2 analog, U46619. The most significant finding is that endogenous vascular smooth muscle synthesis and release of bFGF is necessary for thromboxane-mediated hypertrophy. This conclusion is based on the following results: 1) U46619 exposure caused quiescent vascular smooth muscle cells to hypertrophy and up-regulate endogenous bFGF; 2) specific inhibition of endogenous bFGF with an antisense oligodeoxynucleotide prevented U46619-stimulated vascular smooth muscle hypertrophy; and 3) neutralization of the biological activity of extracellular bFGF with a specific monoclonal antibody inhibited U46619-stimulated vascular smooth muscle hypertrophy. In contrast, U46619-stimulated tyrosine phosphorylation was not necessary for vascular smooth muscle hypertrophy, since inhibition of phosphotyrosine accumulation with herbimycin A did not prevent [3H]leucine incorporation. To our knowledge, this is the first demonstration that a vasoconstricting agonist transduces vascular smooth muscle cell growth by stimulating the formation of basic fibroblast growth factor.

Thromboxane A2, a cyclooxygenase metabolite of arachidonic acid, is one of the most potent known vasoconstrictors. In vascular smooth muscle, thromboxane-stimulated second messengers include activation of phospholipase C, production of 1,4,5-inositol trisphosphate with release of calcium from intracellular stores, activation of protein kinase C, and phosphorylation of myosin light chains (24). Although these cell signals are also stimulated by other vasoconstrictors which promote vascular smooth muscle growth (25), studies by Berk et al. (3) indicate that neither the concentration of intracellular calcium nor protein kinase C activity are critical mediators for vascular smooth muscle growth induced by vasoconstrictors.

Phosphorylation of tyrosine and serine/threonine residues on specific cellular proteins constitutes a cell signaling cascade initiated by classical growth factors including PDGF, epidermal growth factor (EGF), and bFGF (13), but its role in signal transduction of G-protein linked receptors is not clearly understood. Tyrosine phosphorylation of phospholipase C-γ1 appears to be necessary for thrombin-induced mitogenesis of cultured vascular smooth muscle cells (15). Herein we have shown that U46619 increased the phosphotyrosine content of proteins with molecular masses of 132-144, 86, and 75 kDa. The identity of these phosphotyrosine-containing proteins is not known, but 135-kDa phospholipase C, 85-kDa phosphatidylinositol 3-kinase, and 74-kDa p74src are possible candidates, as they are similar in molecular mass.

Herbimycin A was used to determine the importance of tyrosine phosphorylation in thromboxane-stimulated vascular smooth muscle hypertrophy. In contrast to its reported effects on thrombin-stimulated vascular smooth muscle growth (15), herbimycin failed to significantly inhibit U46619-stimulated vascular smooth muscle hypertrophy. Thus, although thromboxane A2 activates one or more tyrosine kinases (or inhibits tyrosine phosphatases), thromboxane-stimulated tyrosine phosphorylation is apparently not critical for transducing vascular smooth muscle hypertrophy.

Our results showing inhibition of U46619-stimulated vascular smooth muscle [3H]leucine incorporation with 100 nM staurosporine are consistent with a role for protein kinase C in thromboxane-stimulated hypertrophy. Staurosporine inhibits protein kinase C with an IC50 of 2.7 nM, but it inhibits the tyrosine kinase activity of epidermal growth factor receptors with an IC50 of 630 nM (22). Thus, at the concentration employed herein, only protein kinase C should have been significantly inhibited. The Western blot in Fig. 2 shows that staurosporine did not substantially inhibit U46619-stimulated tyrosine phosphorylation. Yet, staurosporine had a profound inhibitory effect on vascular smooth muscle cellular accumulation of [3H]leucine, suppressing it to levels below even the vehicle-treated controls (Table 1). This inhibitory effect was apparently not due to cytotoxicity, since staurosporine did not increase vascular smooth muscle lactate dehydrogenase release or trypan blue staining. In preliminary studies, we have found that calphostin C, the most specific inhibitor of protein kinase C currently available (22), has identical effects on thromboxane-stimulated vascular smooth muscle hypertrophy. Therefore, it can be inferred that activation of protein kinase C is necessary for thromboxane-stimulated hypertrophy.

The most remarkable finding of the present study is that vascular smooth muscle bFGF expression increased greater than 3-fold after 24 h of exposure to a stable thromboxane analog and that bFGF was required for thromboxane-stimulated hypertrophy. Basic fibroblast growth factor is a 16-25-kDa multifunctional protein which exerts mitogenic effects on a wide range of cell types derived from mesoderm and ectoderm (26). Basic fibroblast growth factor has been identified in both normal and malignant tissues and may be classified as an angiogenic factor in that it is expressed primarily in vascular-rich tissues and promotes the growth of both vascular endothelium and smooth muscle. Cassell's group has compared the expression of bFGF in freshly isolated bovine aorta smooth muscle cells and in cultured cells from the same tissue (27). Using radioimmunoassay, immunoblotting, and 3T3 mitogenesis assays, these investigators found that freshly isolated cells contain measurable, but small, amounts of bFGF compared with the cultured cells. The recent report by Lindner and Reidy (28) that neutralization of bFGF with specific antibodies inhibits neointimal proliferation after balloon catheter injury of rat carotid arteries

3 G. W. Dorn II, unpublished results.
shows that bFGF plays a significant role in modulating the growth of vascular smooth muscle following in vivo vascular injury. Their follow-up study further suggested that the source of the bFGF responsible for neointimal proliferation in balloon-damaged vessels was endogenous release from injured vascular smooth muscle cells (29). Our results in thromboxane-stimulated cultured cells confirm that the ability of vascular smooth muscle cells to synthesize and release bFGF can be a critical event regulating vascular smooth muscle cell growth.

Although bFGF appears to lack a signal sequence which would direct its release along conventional secretory pathways (30, 31), it is deposited in large amounts in the extracellular matrix synthesized by vascular smooth muscle cells in tissue culture (27). Furthermore, 25% of cell-associated bFGF is located at the nucleus, suggesting that it may function intracellularly as a transacting regulator of gene transcription (27).

Thus, it was possible that vascular smooth muscle cell hypertrophy was regulated by bFGF in either an intracellular manner, i.e. intracellular bFGF interacting with its cell membrane or nuclear receptors without being released, or an autocrine manner, i.e. released and acting externally. We found that neutralization of extracellular bFGF with a specific antibody inhibited thromboxane-stimulated [3H]leucine incorporation into cultured vascular smooth muscle cells by approximately 70%. This suggests that thromboxane-stimulated vascular smooth muscle cells can secrete bFGF, perhaps into the extracellular matrix, thereby allowing bFGF to act as an autocrine factor. Incomplete inhibition of U46619-stimulated [3H]leucine incorporation by anti-bFGF, but complete inhibition by AS bFGF (see Table I), further suggests that endogenous bFGF has additional intracellular effects.

Endogenous expression of bFGF can be modified by a variety of biological stimuli. Platelet-derived growth factor increases the expression of bFGF mRNA in cultured vascular smooth muscle cells (32). Several studies have demonstrated that the bioactivity of bFGF can also be modulated by transforming growth factor β (TGFβ) which amplifies the mitogenic effect of bFGF on corneal endothelial cells and osteoblasts (33, 34), but inhibits bFGF-induced proliferation of vascular endothelial cells (35, 36). Although TGFβ3 was not measured in the current study, an inhibitory effect of endogenously produced TGFβ3 may explain why we observed hyperplasia, not hyperproliferation, associated with increased bFGF synthesis and release in thromboxane-stimulated cells. Dzau and associates (37) reported that vascular smooth muscle hypertrophy stimulated by angiotensin II was associated with delayed increase in TGFβ3 and that inhibition of TGFβ3 with anti-TGFβ3 antibody changed the vascular smooth muscle response to angiotensin II from hypertrophy to hyperplasia.

Together with the current study, these results raise the possibility that the growth state of vascular smooth muscle cells is homeostatically regulated by external factors which modulate levels of endogenous growth promoters (i.e. bFGF or PDGF) and inhibitors (i.e. TGFβ).

That thromboxane A2 regulates the expression of bFGF in vascular smooth muscle cells is particularly intriguing in light of our prior findings that growth factors such as bFGF and PDGF can regulate the expression of receptors for thromboxane A2 in cultured vascular smooth muscle cells (38). Using radioligand binding to thromboxane A2 receptors, we found that bFGF and PDGF down-regulated thromboxane receptors at concentrations of the growth factors, which were below their respective thresholds for mitogenesis. We postulated that down-regulation of this vasoconstrictor receptor by growth factors might have been related to transformation of the vascular smooth muscle cells from a quiescent/contractile state to a proliferative/synthetic state. Together with our previous study describing the hypertrophic effects of contractile prostanooids (10), and the current study showing that thromboxane can increase bFGF expression, these observations provide further evidence for regulatory interactions between vasoconstrictors and growth factors in vascular smooth muscle cells.

The results of any study examining the growth response of cells in culture must be cautiously interpreted with respect to in vivo tissues. Vascular smooth muscle cells in their native state are quiescent and express low levels of bFGF. Tissue culture causes changes in the functional status of vascular smooth muscle cells from quiescent to proliferative, and this alteration in itself can increase the basal levels of bFGF (27).

Therefore, the conditions of tissue culture, i.e. exposure to serum containing mitogens and the change to a proliferative phenotype, may partially simulate the environment of smooth muscle cells in an injured artery where disruption of the intima and associated basement membrane permits growth factors derived from blood elements to directly stimulate smooth muscle cell migration and proliferation. In vivo, these events result in formation of a smooth muscle rich neointima which can cause luminal compromise. Immunohistochemical examination and in situ hybridization studies of neointima from balloon-injured animal arteries and of endarterectomy specimens from atherosclerotic human arteries have shown increased levels of growth factors and growth factor receptors in these lesions (39-43). Therefore, the currently observed up-regulation of endogenous smooth muscle bFGF may be most relevant to a clinical analog of experimental vascular injury, i.e. restenosis following balloon angioplasty. If endogenous bFGF up-regulation also modulates smooth muscle growth to other vasoconstrictors or to mechanical injury, then selective targeting of this protein, using antisense or other techniques, may be effective in preventing neointimal proliferation after angioplasty.

REFERENCES

Thromboxane Stimulates Basic Fibroblast Growth Factor