THE ADAPTIVE RESPONSES to repeated exercise are many. A key component is proliferation of the microcirculation in the exercised skeletal muscles (see Ref. 10 for review) that allows greater blood flow, O2 transport, and metabolic clearance to support higher workloads. Patients with chronic diseases such as chronic obstructive pulmonary disease, heart failure, and renal failure uniformly show decreased exercise capacity that is not greatly improved even by transplantation to replace the faulty organ (18, 19, 26, 27). We hypothesize that, in part, this may reflect skeletal muscle abnormalities, including decreased capillarity.

A variety of vasodilator substances have been identified in recent years, and some, such as adenosine, nitric oxide (NO), and prostaglandins, appear to be released acutely in the muscle microvasculature during exercise (7, 8, 11, 16, 20). These molecules have been shown to contribute to exercise-induced vasodilatation (13, 14), and NO has also been implicated in gene expression and regulation of gene products for angiogenic growth factors (29). Thus NO may play a role in the regulation of vascular endothelial growth factor (VEGF) (2), as may prostacyclin (9, 21) and adenosine (22). It is easy to hypothesize a role for such molecules, secreted by microvascular endothelium during exercise, perhaps via shear stress (15), as signaling the need for increased capillary formation to accommodate greater exercise capacity to the myocytes.

We have shown that a single exercise bout (1) and others have found that several days of chronic stimulation (5) in a naive rat increases VEGF gene expression severalfold as well as that for basic fibroblast growth factor (bFGF) and transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)) to a lesser degree, presumably initiating angiogenesis.

The above constellation of observations led to the purpose of the work described herein: to determine whether NO, various dilator prostaglandins (PGE1, PGE2, and PGJ2), or adenosine individually have the capability of affecting angiogenic growth factor gene expression in resting skeletal muscle when infused in doses sufficient to produce substantial physiological changes in blood flow and blood pressure. We fully realize that such an approach does not prove an essential or contributing role for any such vasodilator in natural exercise-induced angiogenesis. Rather, it points the way to future work by identifying dilators with a potential angiogenic role.

METHODS

Animals, Anesthesia, and Surgery

This study was approved by the University of California, San Diego, Animal Subjects Committee. Female Wistar rats, aged 8–12 wk and weighing 250 ± 10 g, were used throughout the study in groups of 5–7. One group was used as a control group and received only normal saline. Six additional groups were studied. Table 1 shows the vasodilators used in each group and the dose of each one. Only one dilator was used in any one rat, and each was infused for 60 min at a constant concentration and rate of infusion into the left femoral artery (3 ml/h). Rats were all anesthetized with pentobarbital sodium (40–60 mg/kg ip) and mechanically ventilated (Harvard rodent ventilator, model 683) to maintain arterial Po2, Paco2, and pH in the normal range. This was considered important, since it is well known that VEGF in particular is hypoxia inducible. Maintenance doses of pentobarbital sodium were given to maintain a steady level of...
Table 1. Vasodilators used and their infusion rates

<table>
<thead>
<tr>
<th>Vasodilator</th>
<th>Dose, µg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>200</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>4.2</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>100</td>
</tr>
<tr>
<td>PGE₁</td>
<td>1.9</td>
</tr>
<tr>
<td>PGE₂</td>
<td>1.7</td>
</tr>
<tr>
<td>PGI₂</td>
<td>1.7</td>
</tr>
</tbody>
</table>

anesthesia, and temperature was held constant by using a heating pad. An electromagnetic flow probe (1RB693 Transonic Systems, Ithaca, NY) was placed around the left femoral artery. A carotid artery was cannulated to measure systemic blood pressure.

**Protocol**

After surgical preparation, carotid artery pressure and femoral blood flow were recorded. The vasodilator infusion was then begun, and 10 min were allowed to reach new stable values. Invariably, femoral flow was higher than baseline, and a partial ligature was tightened around the femoral artery to restore flow to baseline levels. The purpose of this tactic was to eliminate the possibility of increased muscle blood flow per se affecting gene expression.

After 1 h of dilator infusion, the left gastrocnemius muscle was removed in toto, weighed, and frozen in liquid nitrogen. No further processing was done until all animals in a dilator group had been studied, so that all molecular biological measurements could be done together. While awaiting this processing, samples were stored at −80°C. Note that no exercise or electrical stimulation of the muscles occurred at any time in this study.

**Molecular Biological Analyses**

We limited the question at hand to gene expression, that is, to Northern analysis. The reason for this is that our prior work (1) demonstrated significant increases in VEGF, bFGF, and TGF-β1 mRNA abundance after exercise, and our present quest was to determine whether any of the above-named vasodilators could be, even in part, responsible for this.

Protocol

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**Table 2. Hemodynamic data**

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Peak</th>
<th>Postligature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.4 ± 0.7</td>
<td>2.5 ± 0.7</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>Flow, ml/min</td>
<td>136 ± 11</td>
<td>126 ± 8</td>
<td>126 ± 8</td>
</tr>
<tr>
<td>Pressure, mmHg</td>
<td>80 ± 18</td>
<td>79 ± 24</td>
<td>79 ± 24</td>
</tr>
<tr>
<td>Adenosine</td>
<td>3 ± 0.9</td>
<td>7.4 ± 1.7</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>Flow, ml/min</td>
<td>124 ± 9</td>
<td>85 ± 8</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>Pressure, mmHg</td>
<td>55 ± 15</td>
<td>17 ± 5</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>2 ± 0.6</td>
<td>5.2 ± 0.5</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Flow, ml/min</td>
<td>132 ± 8</td>
<td>82 ± 8</td>
<td>98 ± 6</td>
</tr>
<tr>
<td>Pressure, mmHg</td>
<td>54 ± 6</td>
<td>17 ± 3</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>2 ± 0.4</td>
<td>6.4 ± 1.3</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Flow, ml/min</td>
<td>135 ± 6</td>
<td>69 ± 6</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>Pressure, mmHg</td>
<td>62 ± 9</td>
<td>13 ± 2</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>PGE₁</td>
<td>3 ± 0.6</td>
<td>7.5 ± 1.4</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>Flow, ml/min</td>
<td>131 ± 8</td>
<td>114 ± 10</td>
<td>99 ± 3</td>
</tr>
<tr>
<td>Pressure, mmHg</td>
<td>46 ± 10</td>
<td>14 ± 3</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>PGE₂</td>
<td>1 ± 0.1</td>
<td>4.5 ± 0.6</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Flow, ml/min</td>
<td>120 ± 11</td>
<td>98 ± 10</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>Pressure, mmHg</td>
<td>70 ± 6</td>
<td>24 ± 4</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>PGI₂</td>
<td>2 ± 0.3</td>
<td>4.4 ± 0.4</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Flow, ml/min</td>
<td>132 ± 5</td>
<td>112 ± 8</td>
<td>106 ± 6</td>
</tr>
<tr>
<td>Pressure, mmHg</td>
<td>52 ± 7</td>
<td>28 ± 3</td>
<td>28 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE.
the gastrocnemius muscle. Each has six items, one for each of the six vasodilators used, which are identified. The horizontal dashed line in Figs. 1–4 shows the result expected if the agent had no effect on gene expression. Significance of changes is given with each item. The increases are for 18S-normalized in RNA levels for the indicated growth factor, compared with saline control values.

VEGF: Both nitroprusside and acetylcholine increased VEGF mRNA abundance by similar amounts that approximated 50% (Fig. 2). Both agents had effects of similar statistical significance, *P* < 0.05. Adenosine, PGE1, and PGE2 had no significant effect on VEGF expression. Nitroprusside and acetylcholine separately increased VEGF mRNA abundance by about the same amount. Adenosine, PGE1, and PGE2 had no significant effect.

**Fig. 1.** Northern blot for vascular endothelial growth factor (VEGF) mRNA in all control rats and in all rats given nitroprusside, acetylcholine, and prostacyclin. Nitroprusside and acetylcholine increased, whereas prostacyclin decreased mRNA levels.

**Fig. 2.** Gene expression for VEGF: mRNA values (normalized by 18S RNA to correct for lane loading) are shown for each of the 6 vasodilators. In each case, values are normalized by saline control data such that lack of effect of any one agent would result in a numerical value of 1.0. Nitroprusside and acetylcholine, which both act via nitric oxide, separately increased VEGF mRNA abundance by ~50%, whereas PGI2 reduced VEGF mRNA abundance by about the same amount. Adenosine, PGE1, and PGE2 had no significant effect. NS, not significant.

**Fig. 3.** Data presented and formatted similarly to those of Fig. 2 but for basic fibroblast growth factor (bFGF). Effects on bFGF gene expression were minimal and confined to a small reduction of mRNA levels associated with PGI2.

**Fig. 4.** Data in similar format to Figs. 2 and 3, showing effects of vasodilators on gene expression for transforming growth factor-β1 (TGF-β1). Adenosine, nitroprusside, and PGE1 led to significant if modest reductions in gene expression, whereas PGE2 led to a small increase in TGF-β1 mRNA.
mRNA levels, whereas PGI₂ produced an ~40% decrease that was highly significant (Fig. 2).

bFGF. None of the vasodilators led to significant increases in bFGF mRNA (Fig. 3), but one, PGI₂, slightly but significantly reduced gene expression for bFGF. Thus, as previously seen in the treadmill running in the rat, bFGF responded less than VEGF to the vasodilator stimuli (1).

TGF-β1. Effects of the vasodilators on TGF-β1 gene expression were somewhat different than for the relatively consistent changes for the above two growth factors. PGE₂ slightly enhanced mRNA levels (Fig. 4) by some 20%, whereas adenosine, nitroprusside, and PGE₁ all led to modest reductions in mRNA levels. The most consistent and also the largest effect was a 50% decrease produced by PGE₁ (Fig. 4).

DISCUSSION

This study has shown that in resting, normally perfused rat gastrocnemius muscle vasodilators of various kinds given for 1 h in doses that reduce total systemic vascular resistance to about one-third of normal do have effects on mRNA abundance of the potentially angiogenic growth factors VEGF, bFGF, and TGF-β1. In some cases, mRNA levels increased, in some decreased, as Figs. 2–4 show for each growth factor.

Choice of Growth Factors for Investigation

We focus the discussion on VEGF as the likely major growth factor based on the work of others showing that, in other systems, inhibition of angiogenesis occurs when VEGF is inhibited (3, 12). However, bFGF is also angiogenic (28), although in our system (1) 1 h of exercise in the rat had considerably greater effects on VEGF than on bFGF mRNA levels. The importance of TGF-β1 to exercise-induced angiogenesis is less certain, but, given its major role in regulation of the extracellular matrix, it appears reasonable to examine (1). Thus all three factors may be relevant to muscle angiogenesis, although definitive roles for each in this context remain to be established.

Choice of Vasodilators Tested

The six vasodilators all represent normally active endogenous vasodilator molecules or processes in vascular biology. Thus adenosine is normally produced in exercising muscle (16a), as are NO (8) and PGI₂ (16). We chose two NO “donors,” i.e., nitroprusside and acetylcholine, as two readily available but separate ways to enhance NO levels. That they provided quite concordant results for VEGF (increased mRNA) and bFGF (lack of effect) gives greater confidence in the role of NO. Moreover, it suggests that for VEGF it is NO per se that is important, since while acetylcholine acts with endothelial NO synthase (NOS) activation, nitroprusside bypasses this pathway and acts as a direct NO donor. That NO may play a role at all in angiogenesis is suggested by the work of Ziche et al. (29). Those investigators showed that NOS inhibition by Α°-nitro-L-arginine methyl ester blocked VEGF- but not bFGF-induced angiogenesis in the rabbit corneal implant preparation. Our results are quite consistent with those of Ziche et al. (29): VEGF but not bFGF mRNA abundance was increased by NO donors in vivo in normal muscle in our study.

That adenosine had no effect on VEGF gene expression in skeletal muscle (despite substantial and prolonged vasodilatation) contrasts with reports in the literature linking adenosine to VEGF gene activation in vascular endothelial and smooth muscle cell cultures (22, 25). The lack of effect of PGE₁ and PGE₂ contrasts with the work of Harada et al. (6) in osteoblasts, but in all of these studies it is difficult to compare cell culture studies with in vivo experiments, especially when different tissues are involved. Similarly, the downregulation we saw with PGI₂ contrasts with data by Höper et al. (9) in cell culture and in isolated lungs.

Opposing Effects of NO and PGI₂ on VEGF mRNA

It is of particular interest that, despite similar vasodilator effect in the present work, NO enrichment enhanced VEGF mRNA, whereas PGI₂ reduced it to a roughly similar degree (Fig. 2). These two dilators may both be secreted acutely during natural exercise (7, 8, 11, 16, 20), and our results suggest the possibility that NO and PGI₂ could be modulators of the VEGF response, controlling gene expression by effects in opposite directions. Whether this hypothesis has merit will have to await specific NO- and prostaglandin-blocking studies that examine not only gene expression for VEGF but also the ultimate degree of angiogenesis (or lack of) in response to exercise training. Thus we would expect that NOS inhibition would impair and PGI₂ inhibition would enhance the angiogenic response to exercise training.

The present study showed rather small (yet statistically significant) changes in mRNA abundance (in response to vasodilator infusion), changes much smaller than those seen in natural exercise. We do not think that these different degrees of gene expression should be overinterpreted, but they suggest a modulatory rather than controlling role for NO and PGI₂ in stimulating VEGF. We did not want to give such high doses of vasodilators that cardiovascular instability might have resulted, yet a threefold fall in vascular resistance was produced (Table 2). This is comparable to the roughly twofold reduction in overall resistance in intact exercise in the rat where substantial increases in cardiac output are accompanied by very little increase in mean arterial blood pressure. Thus data from Gonzalez et al. (4) show total blood flow increasing from 214 to 456 ml·min⁻¹·kg⁻¹ and mean arterial pressure increasing from 116 to 120 mmHg.

Our tactic of returning femoral flow to normal (in the face of continuing vasodilatation) by the partial ligation deserves discussion. We did this because of the possibility that increased muscle blood flow per se, via increases in shear stress, might alter VEGF gene expression. In the meantime, we have found in the canine gastrocnemius that even fivefold increases in...
blood flow for 60 min without active muscle contraction fail to alter VEGF gene expression (23). Thus 10 min of twofold elevation in flow should not be confusing.

Further evidence that any physical factors accompanying the vasodilator effects of these agents are not the primary mechanisms of their effects on angiogenic growth factor gene expression comes from the similar degrees of vasodilatation from all six agents (Table 2) yet different effects on gene expression, i.e., increased, decreased, or none. If, for example, NO has an effect on VEGF directly by virtue of hemodynamic effects (changes in pressure or flow), this should have resulted in all six vasodilators increasing VEGF gene expression.

In summary, administration of several normally occurring vasodilators or their activators into the arterial circulation of resting rat skeletal muscle does, variably, alter angiogenic growth factor gene expression as measured by mRNA responses to doses sufficient to lower systemic vascular resistance to one-third of normal for a period of 1 h. Specifically, VEGF mRNA levels are increased by NO (nitroprusside or acetylcholine) and decreased by PGI2, whereas adenosine, PGE1, and PGE2 have no effect. For bFGF, PGI2 also reduces its mRNA level, but no other dilators tested affected gene expression. For TGF-β1, adenosine, nitroprusside, and PGE1 reduced its mRNA, whereas PGE2 slightly increased it. Although these results suggest possible roles for those dilators in signaling initiation of exercise-induced skeletal muscle angiogenesis, their biological significance cannot be established from the present work.

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