Protease-Activated Receptor-2 Stimulates Angiogenesis and Accelerates Hemodynamic Recovery in a Mouse Model of Hindlimb Ischemia

Anna F. Milia,* Maria B. Salis,* Tiziana Stacca, Alessandra Pinna, Paolo Madeddu, Marcello Trevisani, Pierangelo Geppetti, Costanza Emanueli

Abstract—Proteinase-activated receptors (PAR-2) are expressed by the cardiovascular system and mediate vasodilation, plasma protein extravasation, and endothelial cell proliferation, all regarded as essential steps for neovascularization. We investigated the angiogenic action of PAR-2 signaling in vivo. The effect of the PAR-2 activating peptide (PAR-2AP, SLIGRL-NH$_2$) was assessed in the absence of ischemia, and the therapeutic potential of PAR-2AP and the PAR-2 agonist trypsin (at 300 and 1.5 nmol IM daily for 21 days, respectively) was also tested in mice subjected to unilateral limb ischemia. PAR-2AP increased capillarity in normoperfused adductor skeletal muscles, whereas neither the vehicle of the PAR2-AP nor the PAR-2 reverse peptide (PAR-2RP, LRGILS-NH$_2$) did produce any effect. In addition, both PAR-2AP and trypsin enhanced reparative angiogenic response to limb ischemia, an effect that was not produced by PAR-2RP or the vehicle of PAR-2 agonists. Potentiation of reparative angiogenesis by PAR-2AP or trypsin resulted in an accelerated hemodynamic recovery and enhanced limb salvage. In conclusions, our study is the first to demonstrate the angiogenic potential of PAR-2 stimulation in vivo. If similar effects occur in humans, PAR-2AP agonists could have some therapeutic potential for the treatment of tissue ischemia. (Circ Res. 2002;91:346-352.)

Key Words: proteinase-activated receptor-2 ■ angiogenesis ■ skeletal muscle ■ ischemia

The proteinase-activated receptor-2 (PAR-2) belongs to a recently described family of 7-transmembrane G protein–coupled receptors (PARs). PARs, rather then being stimulated through ligand receptor occupancy, are activated by serine protease–induced proteolytic cleavage of their N terminus, resulting in the generation of a new tethered ligand that interacts with the receptor within extracellular loop-2.1–3 The endogenous activator of the first discovered PAR-1 is thrombin,1,2 although PAR-2 is not responsive to this protease. Instead, PAR-2 is reportedly activated by trypsin,1,2 mast cell tryptase,4 and complement factor Xa,5 but the identity of the endogenous ligand remains uncertain. The short synthetic activating peptide (PAR-2AP, SLIGRL-NH$_2$, in the rat and mouse), corresponding to the new amino terminus exposed after enzymatic cleavage, but not the reverse peptide (PAR-2RP, LRGILS-NH$_2$), activates the receptor and for this reason has been used to explore the pathophysiological role of PAR-2.6 Several clues suggest that PAR-2 is implicated in neovascularization. First, PAR-2 is widely expressed in the cardiovascular system.6–12 Second, PAR-2 mediates endothelial cell (EC) mitogenesis in vitro,10 and promotes vasodilation11–15 and microvascular permeability in vivo,16 all regarded as essential steps of the angiogenesis program. Third, PAR-2 expression is upregulated by cytokines, including tumor necrosis factor-α, interleukin-1β, and lipopolysaccharide, that are implicated in inflammatory angiogenesis.17 To the best of our knowledge, however, no information is available regarding direct stimulation of angiogenesis by PAR-2. Furthermore, it is not known if activation of PAR-2 may positively interfere with posts ischemic reparative processes at the microvascular level.

To address these issues, we first evaluated if locally administered PAR-2AP increases the capillarity of normoperfused adductor muscles. Then, we studied the effect of PAR-2 activation by PAR-2AP or trypsin in a mouse model of unilateral hindlimb ischemia.18,19 Modulation of PAR-2 gene expression by ischemia was also assessed.

The present study provides experimental evidence for a role of PAR-2 in neovascularization. Specifically, we documented that PAR-2 agonists increase capillary density in skeletal muscles either in the presence or the absence of an ischemic environment. The posts ischemic healing and limb salvage produced by PAR-2 agonists may be the consequence
of these microvascular effects. Finally, a role of PAR-2 in spontaneous reparative process is suggested by upregulation of the receptor by ischemia.

Materials and Methods

All procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md). Experiments were performed in male CD1 mice (27 to 33 g body wt; Charles River, Comerio, Italy).

Microvascular Effects of PAR-2AP in Normoperfused Muscles

Mouse PAR-2AP and PAR-2RP were synthesized at the Experimental and Clinical Medicine Department of the University of Ferrara (Italy). Left adductor muscles of unanesthetized mice were daily injected with PAR-2AP or PAR-2RP at the dose of 100 nmol (PAR-2AP: n = 5; PAR-2RP: n = 5), 300 nmol (PAR-2AP: n = 9; PAR-2RP: n = 8), or 900 nmol (PAR-2AP: n = 5; PAR-2RP: n = 4) in 20 μL sterile saline, or vehicle (n = 11) for 14 days. Animals were then euthanized to allow histological analysis of muscular capillary density of injected and contralateral noninjected adductors (vide infra). The neovascularization effects of PAR-2AP were compared with those evoked by activation of kinin B1 receptor, a recognized proangiogenic and proinflammatory mechanism.19 For this purpose, an additional group of CD1 male mice (n = 14) was treated with the selective B1 agonist Sar[D-Phe9]des-Arg9-BK (R916, kindly provided by Prof Regoli, University of Ferrara, Italy) at the daily dose of 100 nmol (in 20 μL saline) intramuscularly. This dose of the B1 receptor agonist was previously found to produce angiogenesis and to be effective in a mouse model of unilateral hindlimb ischemia.19

Modulation of PAR-2 Expression by Ischemia

Mice were anesthetized with 2,2,2-tribromoethanol anesthesia (Sigma-Aldrich, 880 nmol/kg body wt, IP), and the left femoral artery was exposed, dissected free, and excised.18,19 Adductor muscles were harvested at 0, 1, 3, 7, and 14 days after induction of ischemia (n = 5 each time point) for RT-PCR analysis of PAR-2 gene expression. For this purpose, total RNA was isolated from liquid nitrogen frozen muscles. cDNA was made from total RNA using iScript Kit (Sigma-Aldrich, 880 mmol/kg body wt, IP), and the left femoral arterioles were sequentially measured by tail-cuff plethysmography before and after induction of unilateral limb ischemia. The ischemic to contralateral foot perfusion ratio was then calculated.18,19 In order to avoid any confounding effect related to drug delivery, measurements were always performed 24 hours apart from the last injection of the compounds or their vehicles.

The acute action of PAR-2AP injection on limb blood flow was assessed in a supplementary group of mice. Limb blood flow was measured under basal conditions and then sequentially for 15 minutes after the injection of PAR-2AP at 300 or 900 nmol into normoperfused or ischemic adductors (n = 6 each group).

Histological Assessment of Neovascularization

Capillary and myofiber density of both adductor muscles were determined at necropsy. Anesthetized mice were perfused with phosphate buffer saline (PBS, 1 minute), and then with 10% buffered formalin (10 minutes) at 100 mm Hg via the abdominal aorta. After paraffin embedding, 3-μm-thick sections were cut from each sample with muscle fibers transversely oriented, and stained with hematoxylin and eosin. Sections were analyzed in a blinded fashion using an ocular reticule (960-μm2 area) at 1000× magnification. Twenty-five fields were randomly counted and averaged. The number of capillary (n_cap) and myofiber (n_fiber) profiles per field was used to compute capillary or myofiber numerical density per square millimeter of muscle, according to the following formulas: n_cap/mm2 = n_cap in total fields per total field area and n_fiber/mm2 = n_fiber in total fields per total field area. In addition, capillary density was normalized by myofiber density (n_cap/n_fiber).19

Limb Blood Flow Measurement

Hindlimb blood flow of anesthetized mice was measured by laser Doppler flowmetry (Lica, Sweden) under basal conditions and then weekly after induction of unilateral limb ischemia. The ischemic to contralateral foot perfusion ratio was then calculated.18,19 In order to avoid any confounding effect related to drug delivery, measurements were always performed 24 hours apart from the last injection of the compounds or their vehicles.

Statistical Analysis

All results are expressed as mean±SEM. Multivariate repeated-measures ANOVA was performed to test for interaction between time and grouping factor. In multiple comparisons among independent groups in which ANOVA and F test indicated significant differences, the statistical value was determined according to the Bonferroni’s method. Differences within and between groups were determined using paired or unpaired Student’s t test, respectively. The comparative incidence of limb salvage was evaluated by χ2 analysis. A value of P<0.05 was interpreted to denote statistical significance.

Results

Microvascular Effects of PAR-2 Activation in Normoperfused Muscles

Long-term local administration of PAR-2AP increased muscular capillary density in a dose-dependent manner (P<0.05) (Figure 1A). In contrast, neither PAR-2RP (Figure 1A) nor the PAR-2AP vehicle (data not shown) affected capillarity (P=NS versus contralateral noninjected muscles). Myofiber density was similar among different experimental groups (P=NS for all comparisons, Figure 1B). Consequently, the increase in capillarity induced by PAR-2AP was confirmed after normalization by myofiber number (Figure 1C).

The typical microvascular pattern observed in adductor muscles treated with PAR-2AP as compared with those treated with its vehicle is reported in Figure 2. No morpho-
logical sign of inflammation was detected in those muscles injected with PAR-2AP, with the exception of a modest leukocyte infiltration observed in those muscles receiving the highest dose of PAR-2AP. The magnitude of the angiogenic response produced by the PAR-2AP was similar to that induced by the kinin B$_1$ agonist R916 (100 nmol: 886±102 in PAR-2AP versus 1058±60 cap/mm$^2$ in R916; $P=NS$).

**Modulation of Muscular PAR-2 Gene Expression by Ischemia**

As shown in Figure 3, expression of mRNA of PAR-2 increased transiently in adductor muscles after induction of ischemia (1.9-fold at 1 day; $P<0.05$). Expression of mRNA of PAR-2 returned to baseline values after approximately 2 weeks.

**Effects of PAR-2AP on Reparative Angiogenesis**

Spontaneous angiogenesis in response to ischemia, documented by increased capillary density, occurred in adductor muscles of control mice injected with either PAR-2RP (Figure 4A) or saline (Figure 5A), with no difference between the two treatments ($P=NS$). The native capillary response was strongly potentiated by PAR-2AP (1213±97 versus 868±22 cap/mm$^2$ in PAR-2RP; $P<0.01$) or trypsin (1107±62 versus 755±39 cap/mm$^2$ in saline; $P<0.01$). The results were confirmed after normalization by myofiber density (Figure 4C and 5C).

**Effect of PAR-2AP on Postischemic Perfusion Recovery**

As shown in Figure 6, postischemic hemodynamic recovery was accelerated in mice in which PAR-2 was activated by PAR-2AP or trypsin. By day 21, limb salvage was achieved in 65% and 78% of PAR-2AP– and trypsin-treated mice, respectively. The effect produced by these PAR-2 agonists was significantly higher ($P<0.05$) than that obtained in PAR-2RP– or saline-treated mice (13% and 10%, respectively). Systemic hemodynamics were not altered by treatments or surgery (data not shown). No acute vasodilatory response was observed after intramuscular injection of PAR-2AP ($P=NS$ versus basal).

**Discussion**

Although the murine gene encoding the PAR-2 was cloned in 1994, the pathophysiological importance of PAR-2 remains poorly understood. PAR-2 signaling has been implicated in pain transmission, inflammatory responses, and tissue injury, as well as in the regulation of cardiovascular function. The present study provides the first evidence of...
a proangiogenic role for PAR-2 and points to a therapeutic potential of PAR-2 agonists for the treatment of conditions of peripheral ischemia.

The angiogenic action of exogenous PAR-2AP does not appear to be restricted to an ischemic milieu, as the PAR-2 activator promotes capillarization in normoperfused muscles. This observation supports the view that an ischemic environment is not strictly required for neovascularization to develop; however, this concept is not universally accepted. Comparative studies with R916 indicated that the magnitude of the angiogenic response produced by the PAR-2AP and the kinin B1 agonist was similar, thus indicating that PAR-2 activation results in a robust effect to increase muscular microvasculature. We found no evidence of inflammatory reactions in PAR-2AP–injected muscles, except for a modest leukocyte infiltration observed in those receiving the highest dose of the activating peptide. Thus, in normoperfused tissues, PAR-2 signaling may induce capillary prolif-

Figure 3. A, Representative PCR bands of PAR-2 expression in adductor muscles harvested at 0, 1, 3, 7, and 14 days after induction of limb ischemia. Levels of GAPDH mRNA are shown for comparison. B, Time course of the ratio between PAR-2 and GAPDH mRNA level, expressed as PCR band optical density (O.D.). Values are mean±SEM. *P<0.05 vs time 0.

Figure 4. Bar graph shows the effects of the mouse PAR-2AP (filled columns) and control PAR-2RP (hatched columns) on capillary density (A), myoфибber density (B), and capillary to myoфибber ratio (C) of ischemic adductors. Contralateral normoperfused adductors (open columns) are shown for reference. Unilateral limb ischemia was induced by left femoral artery resection. Starting on the same day of surgery, mice received daily injections of 300 nmol PAR-2AP or PAR-2RP into the ischemic adductor. Twenty-one days from surgery, ischemic (I) and contralateral muscles (C) were harvested for histology. Values are mean±SEM. *P<0.05 vs contralateral; §P<0.05 vs PAR-2RP.
eration by directly targeting endothelial cells rather than through a proinflammatory mechanism. This is in agreement with previous in vitro studies showing a proliferative response in cultured human umbilical vein ECs (HUVECs) exposed to PAR-2AP or trypsin. However, it is likely that, under ischemic conditions, PAR-2 agonists can mount a more robust reparative response by amplifying proinflammatory mechanisms implicated in angiogenesis. For instance, PAR-2 activation induces HUVECs to release the proangiogenic interleukin-6. Furthermore, leukocytes express PAR-2 receptors and participate in the release of angiogenic substances after homing at sites of active neovascularization. Finally, the proangiogenic, proinflammatory substance P (SP) is released from perivascular endings of dorsal root ganglia neurons after PAR-2 stimulation.

To assess the possible contribution of vasodilation to the angiogenic effect of PAR-2 stimulation, limb blood flow was measured before and after local PAR-2AP injection. The observation that short-term PAR-2 activation does not affect local or systemic hemodynamics seems to exclude this hypothesis.

The possible role of PAR-2 in the reparative processes that result in tissue recovery is suggested by increased mRNA levels in ischemic adductor muscles. Unfortunately, a definite conclusion on this issue is hindered by the lack of information
regarding the role of ischemia in PAR-2 protein expression and the precise localization of the receptor within different vascular cells. Further limitations for the comprehension of the physiological role of PAR-2 in angiogenesis derive from the absence of high-affinity receptor antagonists and by the fact that PAR-2 knockouts are currently unavailable in our laboratory. Irrespective of the physiological role of the putative endogenous PAR-2 agonist(s), it is of importance that stimulation of PAR-2 with exogenous activators potentiates reparative angiogenesis and accelerates hemodynamic recovery, thereby improving limb salvage. Comparisons with previous results obtained in our laboratory using the human serine protease tissue kallikrein19,20 or with previous data with other known stimulators of angiogenesis indicate that PAR-2 agonists have beneficial effects quantitatively comparable with many of these compounds in a murine model of limb ischemia.36

Present data clearly showing that PAR-2 activation results in angiogenesis may provide possible interpretation for the finding that receptor expression is upregulated in the tumor environment.37,38 Trypsin, secreted by malignant cells into the extracellular space, may activate vascular PAR-2 in the vicinity of the tumor, thus promoting tumoral angiogenesis. This mechanism may contribute in the well-known ability of trypsin to facilitate cancer growth and diffusion.39

In conclusion, we have demonstrated that potentiation of PAR-2 activation induces neovascularization in skeletal muscles. The present study provides new insights into the vascular action of PAR-2. If PAR-2 activation exerts similar effects in humans, PAR-2 agonists may have a therapeutic potential in the treatment of clinical conditions characterized by deregulated neovascularization.

Acknowledgment
Research costs were covered by I.N.B.B. intramural funding to C.E.

References


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Circ Res. 2002;91:346-352; originally published online August 1, 2002; doi: 10.1161/01.RES.0000031958.92781.9E

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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