Substance P Is Released From the Endothelium of Normal and Capsaicin-Treated Rat Hind-Limb Vasculature, In Vivo, by Increased Flow

Vera Ralevic, Pamela Milner, Olga Hudlická, Frantisek Kristek, and Geoffrey Burnstock

The rat hind-limb vasculature releases substance P when subjected to a rapid increase in flow through the vascular bed. This release also occurs during high flow after rats have been capsaicinized, when loss of substance P-containing nerve fibers was verified by immunohistochemistry. Air treatment, a procedure shown by transmission electron microscopy to have removed endothelial cells from the arteries but not arterioles or capillaries of the hind-limb preparations, eliminated this release. Thus, the substance P released is unlikely to arise from perivascular nerves but rather from arterial endothelial cells. (Circulation Research 1990;66:1178-1183)

A number of naturally occurring vasodilators have been shown to evoke their responses by stimulation of production of endothelium-derived relaxing factor (EDRF) in endothelial cells. These substances include acetylcholine (ACh), substance P (SP), ADP, ATP, bradykinin, thrombin, and histamine (for reviews see Furchgott1 and Vanhoutte et al2). Removal of the endothelium and the subsequent abolition of a relaxant response to these substances played a crucial part in early investigations, although these studies were largely confined to isolated vessels or vascular beds. More recently, gossypol, a selective and irreversible inhibitor of the EDRF-mediated vasodilation in isolated arteries, has been used for demonstration of endothelium-dependent relaxations to ACh, SP, and ATP in the rabbit hind limb in vivo.3-5 Of the naturally occurring vasodilators acting via the intimal surface, SP is one of the most potent studied,1,6 and receptors for this peptide have been demonstrated, by autoradiographic analysis, on the endothelium of the dog carotid artery7 and renal artery.8 In view of these studies, there is strong evidence that SP may have a physiological role in the regulation of vascular tone, and hence of blood flow, by an endothelium-dependent mechanism.

A local endothelial source for vasoactive agents was first suggested by the electron microscopic immunocytochemical localization of choline acetyltransferase (the ACh-synthesizing enzyme) in vascular endothelial cells in the rat brain.9 Since then, further electron microscopic studies have shown that endothelial cells may also be a source of SP and 5-hydroxytryptamine (5-HT) in the rat femoral and mesenteric arteries,10 and of 5-HT in the rat coronary artery.11 Insight into the physiological role of endothelial cells has come from studies demonstrating that increments in flow through large conduit arteries in vitro or in situ12,13 and at the microvascular level14 induce active vasodilatation of arterial smooth muscle that is abolished on removal of the endothelium. Further, it has been shown that an increase in flow induces the release of EDRF from the canine femoral artery15,16 and the rabbit hind limb.3 This finding suggests that endothelial cells play a role in blood flow regulation by releasing EDRF.

In the present study, we sought to determine whether SP is released from endothelial cells of the perfused rat hind-limb preparation in response to an increase in flow through the vascular bed.

Materials and Methods

Perfusion of Rat Hind-Limb Vasculature In Vivo

Male Wistar rats (300–420 g) were anesthetized by injection with sodium pentobarbitone (60 mg/kg i.p.,
diluted 1:1 with saline). The right jugular vein was cannulated for administration of heparin and additional anesthetic if necessary, and the animal’s blood pressure was monitored by a pressure transducer (model P23, Gould, Cleveland, Ohio) from a cannula to the right carotid artery. The trachea was also cannulated. The abdomen was opened, and the left renal artery and vein and the vessels supplying the body wall, the scrotum, and all visible arterial and venous side-branches in the pelvic and abdominal region were ligated. After intravenous administration of heparin (1,000 units), the aorta was cannulated to allow perfusion of the hindquarters. The vena cava was cannulated and the outflow directed away from the animal via a fraction collector (model 201-202, Gilson, Worthington, Ohio). The hindquarters were initially flushed with 0.9% saline containing heparin (100 units/ml) and then perfused with a regulated pulsatile flow, by use of a Watson-Marlow pump, with a Krebs-Ringer bicarbonate solution (gassed with 95% O₂-5% CO₂ and maintained at 37° C) containing (mM) NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, NaHCO₃ 25.0, D-glucose 5.5, CaCl₂·2H₂O 2.5, with added bovine serum albumin (10 g/l) and insulin (10 units/l). Perfusion pressure was measured by a pressure transducer (model P23, Gould) from a side arm of the inflow cannula and was matched so as not to exceed the animal’s blood pressure. Flow was measured by timed collection in a graduated cylinder.

Flow of the Krebs-Ringer bicarbonate solution through the hindquarters was allowed to stabilize for 15–20 minutes before collection. High flow was achieved by rapidly increasing the output of the pump to approximately three times that of the basal flow for 2 minutes. Fractions (0.12 min/fraction) were collected at basal flow immediately before high flow and over the period of high flow. This procedure was repeated to produce a second period of high flow after 15–20 minutes of recovery at basal flow. Fractions were collected in polypropylene tubes, which were immediately placed on ice.

Endothelium Removal

After the first period of high flow, the endothelium was removed, according to a protocol developed in this laboratory, from the vasculature of five rats by introduction of air into the perfusion line as a bubble over a period of 5 seconds. This procedure was repeated at 10-second intervals for a total duration of 5 minutes. Transmission and scanning electron microscopic examination of the perfused hind-limb vasculature, the artery supplying the extensor hallucis propius muscle, and the extensor hallucis proprius muscle were immersed in 3% glutaraldehyde in 0.1 M phosphate buffer. During fixation, arteries were cleaned and cut into blocks. After washing in several changes of 0.1 M phosphate buffer, the blocks were postfixed in 2% OsO₄, dehydrated in ascending grades of alcohol, and embedded in Spurr resin for electron microscopy. The ultrathin sections were stained with lead citrate and subsequently examined by use of an electron microscope (model 301, Philips Electronic Instruments, Mahwah, New Jersey).

Results

The basal flow through the hind-limb vasculature was 9.5±0.8 ml/min (n=15), with a perfusion pressure of 36.7±1.3 mm Hg (n=15). High flow was approximately three times that of basal flow; the two consecutive periods of high flow were at rates of 28.4±0.7 and 28.8±1.1 ml/min (n=15), with perfusion pressures of 69.1±1.8 and 75.4±5.8 mm Hg, respectively. In untreated rats (n=5) there was a significant release of SP during both the first and second periods of high flow, from 4.4±3.4 to...
FIGURE 1. Levels of substance P (SP), expressed as picomoles per minute, released from rat hind-limb vasculature during two periods of low and high flow in untreated and capsaicinized rats with no treatment between the two periods of increased flow, and in untreated rats with air treatment between the two periods of increased flow. *p<0.05, **p<0.02, ***p<0.001.

30.7±9.5 pmol/min (p<0.05) and from 1.6±0.5 to 37.8±6.7 pmol/min (p<0.001), respectively (Figure 1). Release of SP peaked rapidly within the first half minute from onset of high flow, reached a maximum at approximately 1 minute from onset, and then fell off gradually to return to the basal release level before the 2-minute period of high flow was terminated.

Capsaicin-treated rats (n=5) showed a similar, significant evoked release of SP during both the first and second periods of high flow, from 2.7±1.5 to 44.9±13.6 pmol/min (p<0.02), and from 4.4±1.8 to 41.8±11.9 pmol/min (p<0.02), respectively (Figure 1). Depletion of SP from the primary sensory neurons of these animals was confirmed by immunohistochemical analysis of the femoral artery and the artery supplying the hallucis proprius muscle. In both these arteries, there was almost total depletion of SP-immunofluorescent fibers compared with those from control animals, while NPY-containing fibers were unaffected (Figure 2).

The introduction of air into the rat hind limb was shown by transmission and scanning electron microscopic examination to selectively remove the endothelium from the arteries of the hind limb (Figures 3a and 3b), leaving the endothelium of arterioles and capillaries intact (Figure 3c). There was no significant difference in flow rate or perfusion pressure of the vascular bed after introduction of air; in these five animals the flow rate before air was 9.0±0.5 ml/min, with perfusion pressure of 34.0±2.5 mm Hg, while after air the flow was 8.8±0.7 ml/min and perfusion pressure was 52.0±14.5 mm Hg. In air-treated animals (n=5) the first increment of flow, that prior to air treatment, evoked a significant release of SP from 1.5±0.3 to 21.4±6.4 pmol/min (p<0.02). After air treatment, however, there was no significant release of SP, although the level (7.2 pmol/min) was slightly higher than basal values (2.2 pmol/min) (Figure 1).

Discussion

The present results demonstrate that SP is released from the rat hind limb with increased flow only when endothelial cells are present and that the source of this SP is not periarterial nerves.

SP, one of the most potent of the naturally occurring vasodilators, has been shown to elicit relaxation of vascular smooth muscle by acting on receptors on the luminal surface of endothelial cells. Its effects have been demonstrated in isolated vessels such as the rabbit and dog renal and celiac arteries, in vessels in situ such as the canine femoral artery, and, more recently, in vivo in the rabbit hindquarter. Together with the localization of SP receptors on endothelial cells, there is overwhelming evidence that SP can act as a regulator of vascular smooth muscle tone, exerting its effects via the endothelium.

Although pharmacological studies favor SP as a mediator of vascular tone, it would be more likely to have a physiological role if there was a local source of this agent. A supply of SP from within the endothelial cells themselves has already been proposed based on electron microscopic immunocytochemical localization of SP in endothelial cells of the rat femoral artery. Thus, the endothelial cells are a likely source of the SP released from the rat hind limb on increased flow. However, release from alternative sources has to be considered. SP is not present in platelets, and its concentration in plasma is very low. Although a neural origin of the SP released on perfusion is unlikely in view of the fact that it would have to traverse the media, muscle coat, and elastic lamina to reach the lumen, SP has been shown to relax the canine femoral artery on adventitial appli-
cation, albeit at a potency some 50–100 times less than on luminal application. For elimination of SP from periarterial nerves as a contributor to the release observed in high flow, animals were treated with capsaicin, a neurotoxin that depletes up to 91% of primary sensory neurones (for reviews see Fitzgerald and Nagy). In these animals, the quantity of SP released during high flow was similar to that observed in untreated animals, which suggests that the origin of this SP was independent of SP contained within the primary sensory neurones innervating the hind limb.

To ensure that the released SP was indeed of an endothelial origin, we used air treatment, together with the periods of high flow, to selectively remove the endothelium from the arteries of the rat hind limb, as demonstrated by transmission electron microscopy. Before air treatment, high flow evoked a significant release of SP. When these same animals were subjected to a second period of high flow after air treatment, there was no significant release of SP. The small amount of SP detected after air treatment may arise from the remaining intact endothelium of arterioles and capillaries. Since there was no change in peripheral resistance of the vascular bed after air treatment, it is unlikely that microembolization played a significant part in the experiments. However, since microembolization cannot be ruled out and could result in parts of the microcirculation being prevented from exposure to the perfusion medium after air treatment, we cannot entirely exclude nonneuronal cells of the microcirculation as a source of SP.

This study, together with the evidence for the localization of SP within endothelial cells of the rat coronary artery and rat mesenteric and femoral arteries, strongly suggests an endothelial origin for SP. Our recent demonstration of release of SP from cultures of human umbilical vein endothelial cells grown on microcarrier beads during increased flow (unpublished observation) is consistent with this hypothesis. The technique of increasing flow in this study was employed as a stimulus to demonstrate that SP may be released from endothelial cells in vivo; it is possible that a similar mechanism may occur physiologically. It should be noted, however, that concomitant with an increase in flow rate there are often changes in pressure and edema formation that could be related to the release of SP. In this study, bovine serum albumin was incorporated into the

**Figure 2.** Immunofluorescence micrographs showing SP-like immunoreactive nerve fibers in rat femoral artery (panel a) and artery supplying extensor hallucis proprius muscle (panel b). These fibers are almost completely absent in femoral artery (panel c) and in artery supplying extensor hallucis proprius muscle (panel d) after neonatal capsaicin treatment. (The faint SP immunoreactive fiber in panel c was a rare example.) Calibration bar=50 μm.
perfusate to minimize edema formation. The requirement of the endothelium for flow-dependent dilatation and the flow-induced release of EDRF have been well documented.  

In pharmacological studies, the production of EDRF occurs secondary to an initial stimulus. Increased flow alone may be a sufficient initial stimulus to cause direct release of EDRF. However, in the light of this and
other studies, it is possible that EDRF release may occur secondary to the interaction of a substance such as SP with its intimal receptor after the release of this substance from endothelial cells.

In conclusion, this study demonstrates that SP is released from the perfused rat hind limb with high flow. The source of release is likely to be the endothelial cells in arteries since a) capsaicin treatment destroyed SP-containing C fibers but did not affect release and b) removal of the endothelium from the arteries abolished release.

References

Key Words: vascular endothelial cells • substance P • capsaicin • increased flow
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