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Chronic sensory denervation reduces thrombin-stimulated endothelin release from aortic endothelial cells

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Abstract. The long-term (trophic) influence of perivascular nerves on the endothelium was investigated by measuring changes in thrombin-stimulated release of the potent vasoconstrictor, endothelin, after selective chronic denervation. Rat pups were treated with either guanethidine or capsaicin to destroy sympathetic or sensory nerves, respectively. The abdominal aortas from the rats at three months of age (5 pooled per experiment) were incubated with 4U thrombin/ml in medium for 24 h at 37 °C, and the amount of endothelin released from the preparation determined by immunoassay. After neonatal sensory denervation there was a significant reduction in the thrombin-stimulated release of endothelin compared to the controls (0.012 ± 0.012 (4) compared to 0.063 ± 0.012 (6), pmol/cm^2/24 h , $p < 0.02$). There was no change in endothelin release after sympathetic denervation. In summary, sensory nerves play a trophic role in the expression of endothelin in endothelial cells of the intima.
Key words. Sympathectomy; sensory denervation; endothelin; endothelium; plasticity.

There are several naturally occurring and disease-related conditions in which there are chronic or long-term changes in the autonomic innervation of vascular smooth muscle. During ageing, for example, there are changes in the innervation profile of vessels reflecting differential expression of coexisting neurotransmitters[1,2]. Diseases, such as diabetes, characterised by autonomic failure, often display cardiovascular abnormalities and altered perivascular innervation patterns[3]. In addition, perivascular sympathetic hyperinnervation has been implicated in the trophic changes associated with the progression of hypertension[4,5]. There is now increasing evidence for an ongoing long-term interaction between nerves at the adventitial-medial border and endothelial cells of the intima, which also regulate vascular tone by release of vasoactive substances[6–10].
Endothelin (ET) is a potent vasoconstrictor which is synthesized and released by endothelial cells[11,12] and is thought to play an important role in the regulation of vascular tone and smooth muscle proliferation[13]. It is not stored in vesicles but is produced on demand at the level of mRNA transcription[11]. Thrombin is known to enhance the mRNA expression and release of this peptide by a mechanism involving mobilisation of intracellular Ca^{2+} and protein kinase C activation[14–17].
In the present study, we have investigated the effects of selective experimental long-term denervation on endothelial synthesis of endothelin, using thrombin to stimulate its release from intact endothelial cells. For this purpose, abdominal aorta was taken from adult rats that had been either sympathectomised or had undergone sensory denervation shortly after birth.

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Materials and methods

Animal treatments. To destroy primary afferent (sensory) nerves, male Wistar rat pups were treated with either capsaicin (50 mg/kg in saline containing 10% ethanol and 10% Tween 80) or the dissolving medium (vehicle-treated controls). Subcutaneous injections were given on days 2, 3, 4 and 7 after birth. On days 2 and 3 injections were carried out under ice anaesthesia to reduce discomfort due to the immediate capsaicin-induced release of sensory neurotransmitters from primary afferent nerve fibres; subsequently, the animals experience loss of sensation due to the selective destruction of these nerves. This regime for capsaicin treatment has been shown to lead to depletion of substance P (SP)-immunoreactive nerves from the vasculature[18].
Long-lasting sympathectomy was performed by treatment of male Wistar rat pups with subcutaneous injections of 50 mg/kg guanethidine sulphate (Ismelin, Ciba-Geigy) in saline from day 8 after birth for 3 weeks, 5 days per week[19,20]. The control group of rats received saline injections in the same way. Rats were killed at 3 months of age by an overdose of CO_2 gas.
Tissue handling. The abdominal aortas were carefully dissected and immediately placed in M199-20mM HEPES buffer pH 7.4, containing 100 U/ml penicillin (medium). Five aortas were pooled for each experiment, to provide a sufficient endothelial area for detectable ET

release. They were opened longitudinally, any remaining blood gently washed off, and then placed in 2 ml fresh medium containing 4 U/ml thrombin and incubated at 37 °C in an atmosphere of 97.5% O_2/2.5% CO_2 in a humidified chamber for 24 h. In some experiments the endothelium was mechanically removed from all 5 vessels by intimal scraping before incubation in thrombin. The medium was decanted, taking care not to damage the abdominal aorta samples, and frozen in liquid nitrogen, together with medium containing thrombin which had been incubated in the absence of the aorta samples, until subsequent extraction and assay. Random vessels were stained with silver nitrate to check that the endothelium remained. The intimal area of the abdominal aorta samples was measured.

Immunoassay. Thawed conditioned media samples were passed through Sep-Pak cartridges (Waters, Milford, USA) which had been primed with methanol, water and 0.1% trifluoroacetic acid (TFA, chromatography grade), washed with 0.1% TFA and eluted with 50:50 acetonitrile:0.1% TFA. After lyophilisation, the samples were reconstituted in phosphate buffered saline containing 0.1% gelatine and the ET, SP and arginine vasopressin (AVP) levels were determined using enzyme-linked immunosorbent assays, as previously described[21]. Data were expressed as the mean ± S.E. pmol/cm²/24 h (n = number of experiments) and compared using the two-tailed Student's t-test. A p value of < 0.05 was taken as significant.

Results

In the present study, thrombin was used at an optimal concentration to stimulate ET release from aortic strips[15]. A 24 h incubation period allowed sufficient accumulation of ET in the medium for immunodetection, whereas after only 4 h the level was low (control rats, 0.018 pmol/cm²/4 h).

The incubated media from vessels from which the endothelium had been removed contained no detectable ET (n = 2). The amount of ET released into the medium from abdominal aortas from denervated and non-denervated rats are given in figure 1. Abdominal aorta with intact endothelium from capsaicin-treated rats released significantly less ET upon incubation with thrombin than the vehicle-treated controls, $p < 0.02$ (fig. 1a). There were no significant differences in thrombin-stimulated ET release between guanethidine-treated rats and their appropriate vehicle-treated controls (fig. 1b). SP and AVP concentrations in the media were below the level of detection after 24 h incubation with thrombin.

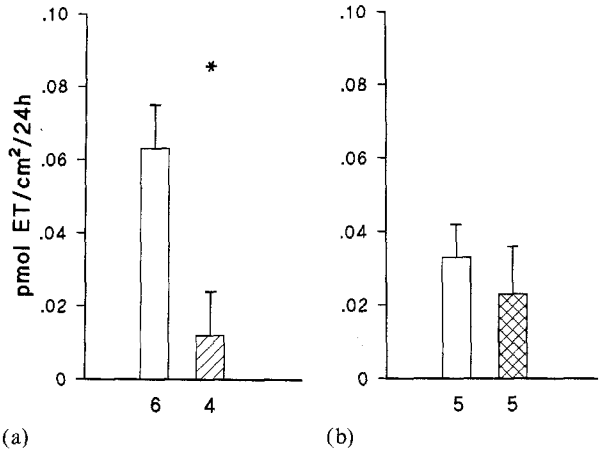

Figure 1. Levels of ET released from abdominal aorta incubated with 4 U/ml thrombin, from *a*) capsaicin-treated (hatched bar) and *b*) guanethidine-treated (cross-hatched bar) rats, compared to their respective vehicle-treated controls (blank bar closest to treatment bar; the vehicle was different for the two drugs). Data expressed as mean ± S. E. pmol/cm²/24 h. The n values are given on the x axis, *$p < 0.02$.

Discussion

The results of the present study, showing decreased thrombin-stimulated endothelin release following experimental sensory denervation, are pertinent to the overall effect of autonomic perivascular plasticity on vascular tone. The endothelial responses to denervation may be more pronounced in selected vessels: for example, whilst sympathectomy did not appear to influence the thrombin-stimulated release of endothelin in the abdominal aorta, increased shear stress-induced release of ET was noted from the mesenteric bed[9]. On the other hand, the normal high flow-stimulated release of ET from endothelial cells isolated from the thoracic aorta was suppressed in sympathectomised rats[22]. In contrast to the abdominal aorta there was no evidence for an effect of chronic sensory denervation on endothelial ET production from the mesenteric arterial bed (unpublished observations) or from isolated brain microvessels[10].

The physiological importance of ET is indicated by the finding that its endothelial expression and release is regulated by several chemical and mechanical factors in addition to thrombin and shear stress[11,17]. The endothelium is a source of several other vasoactive substances, including ATP, SP, angiotensin II and AVP[23–27], which act via specific endothelial receptors to stimulate nitric oxide production and bring about vasodilatation[28]. AVP and angiotensin II also stimulate ET release[29]. Results from the present study indicate that thrombin does not stimulate the release of detectable levels of SP or AVP at concentrations which have been shown to induce ET release[15]. Shear stress is a stimulus for SP release from endothelial cells[12,18]. There is evidence that the shear stress-induced release of SP and ATP from endothelial cells is also influenced by long-term selective denervation[10,22]. Thus, during long-term changes in perivascular

innervation, the resultant effect of changes in the release of ET, which is purely a constrictor in the aorta[30], will be balanced by concomitant changes in the release of vasodilator substances from endothelial cells of the intima.

The mechanism by which events at the adventitial-medial border are communicated to endothelial cells of the intima of vessels may require the intervention of a substance(s), produced either by nerves or the medial smooth muscle, which acts at the level of transcription. Altered endothelial expression of peptides in response to chronic electrical stimulation of perivascular nerves indicates that the level of smooth muscle activity may be related to the presence of such a substance(s)[7]. Our present results support the suggestion that changes in smooth muscle tone which accompany sensory denervation may trigger changes in the levels of such trophic molecules affecting the transcription of mRNA encoding ET in the endothelium. Chronic sensory stimulation would thus be expected to lead to increased endothelial expression of ET; however, these studies have not yet been performed. Another means of communication may be via a sensory-afferent mechanism that promotes the production of a circulating molecule which regulates ET synthesis. If this is the case, however, inter-vessel differences in response would not be expected.

In summary, the presence or absence of sensory nerves in some way influences the expression of endothelin from endothelial cells of the intima of the rat abdominal aorta. It is proposed that changes in expression of sensory nerves in ageing and disease may lead to changes in endothelial-mediated vascular control.

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