Substance P Is Involved in the Cutaneous Blood Flow Increase Response to Sympathetic Nerve Stimulation in Persistently Inflamed Rats

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Abstract: Changed vascular functions have been reported in several pathological conditions, such as chronic regional pain syndrome, obstructive vascular disease, and inflammation. Our previous experiments also showed that electrical stimulation of the lumbar sympathetic trunk (sympathetic stimulation: SS), which normally induces a decrease in blood flow (BF), caused a BF increase in about half of the measured sites in rats persistently inflamed with complete Freund’s Adjuvant (Al rats). We also showed that the BF-increase response was only partially suppressed by the α₁ antagonist at a higher dosage, suggesting the involvement of nonadrenergic mechanisms. We hypothesize that nonadrenergic mechanisms mediating vasodilatation might involve a vasodilating neuropeptide such as substance P (SP) that is released from sympathetic nerve terminals. In this experiment, we conducted an examination using an NK-1 receptor antagonist to determine whether SP plays any role in changed response to SS in Al rats, and also an immunohistochemical examination of whether SP is expressed in the lumbar sympathetic nerve ganglia (SG) of Al rats. The administration of an NK-1 receptor antagonist, CP-96,345, significantly reduced the BF-increase response to SS in Al rats, but its inactive enantiomer, CP-96,344, had no effect. Immunohistochemistry for SP revealed that SP-ir positive SG neurons (mean 13 neurons/rat) were found in 5 of 8 Al rats, whereas only one neuron was stained in 8 control rats. These results suggest that NK-1 receptor activation is involved in the BF-increase response to SS, and that this activation is in part mediated by SP from lumbar SG that was synthesized de novo in inflamed animals.

Key words: sympathetic nerve stimulation, substance P, blood flow, immunohistochemistry, adjuvant-arthritis rats.

Abnormal skin temperature, coloring of affected areas, and sweating have been observed in association with complex regional pain syndrome (CRPS) [1]. These clinical findings suggest that sympathetic nerve activity is altered in this condition, and in fact some researchers have reported changed sympathetic nerve activities, including abnormal sympathetic vasomotor activities and denervation-induced supersensitivity of adrenoceptors [2–4]. Altered vascular responses have also been reported in other pathological conditions. For example, Lundberg et al. reported that electrical stimulation of the sympathetic nerve caused cutaneous vasodilatation in patients with obstructive vascular disease [5]. McDugall et al. reported that the vasoconstriction response to electrical stimulation of sympathetic or saphenous nerves was diminished in rats with complete Freund’s adjuvant (CFA)-induced knee joint inflammation [6, 7]. Reduced vasoconstriction response to sympathetic nerve stimulation was also observed in rats with carrageenan inflammation [8]. Our previous experiments showed that electrical stimulation of the lumbar sympathetic trunk (sympathetic stimulation: SS), which normally induces a decrease in blood flow (BF), induced a BF increase in 50–67% of measured sites in rats persistently inflamed with CFA (AI rats) [9]. BF was also increased by a small dosage of exogenous noradrenaline (NA) at the sites where BF increased in response to SS. Because this response to NA was blocked by both α₁- and α₂-adrenoceptor antagonists, the involvement of α-adrenoceptors in the BF-increase response to NA was suggested. However, BF-increase response to SS was reduced...
only by α1-adrenoceptor antagonist, and only at a higher dosage. β-adrenoceptor antagonist did not influence the BF-increase response to SS. This result and the different effects of α-adrenoceptor antagonists on SS- and NA-induced blood flow changes suggest that the BF-increase response to SS involves, besides NA, nonadrenergic mechanisms. We hypothesize that nonadrenergic mechanisms mediating vasodilatation might involve a vasodilating neuropeptide such as substance P (SP) [10] that is released from sympathetic nerve terminals. SP signaling reportedly contributes to vascular abnormalities in a rat model of CRPS [11].

SP is normally expressed in small dorsal root ganglion (DRG) neurons and their fibers, and its expression is up-regulated in inflamed conditions [12]. The nerve growth factor (NGF) that is produced in inflamed tissues plays an important role in this up-regulation by interacting with TrkA receptor, a high-affinity NGF receptor [13]. In contrast to DRG neurons, sympathetic ganglion neurons rarely express SP, though SP-containing nerve fibers are commonly observed running through the sympathetic ganglia [14]. Therefore these nerve fibers are considered to be collaterals of primary afferent neurons [15]. Sympathetic postganglionic neurons also express TrkA receptors, and NGF plays important roles in the development and maintenance of sympathetic neurons [16]. Thus the possibility remains that SP is up-regulated in sympathetic ganglia and released from peripheral nerve terminals to induce vasodilatation.

The aim of the present study, therefore, was to examine whether SP plays any role in the altered vascular response, i.e., the vasodilating response, to SS. We also examined whether SP expression is up-regulated in the lumbar sympathetic nerve ganglia of AI rats.

METHODS

Induction of adjuvant inflammation. Twenty-three male Lewis rats (LEW/Cj, Charles River Co., Yokohama, Japan) were used. They were 9 weeks old at the time of CFA injection without anesthesia. Adjuvant inflammation was induced in 15 rats (AI rats) by subcutaneously inoculating 0.1 ml of CFA, a suspension of heat-killed Mycobacterium butyricum (Difco Laboratories, Detroit, MI, USA) in paraffin oil (6 mg/ml), into the distal third of the tail. Eight untreated rats served as controls. All rats were kept on a 12-h light/dark cycle at 23°C with free access to food and water. Seven AI rats were used in the electrophysiological experiment, and another 8 AI and 8 control rats were used for the immunohistochemistry. Rats that survived more than 2 weeks (maximum 7 weeks) after inoculation of the CFA were used for the electrophysiological experiment. The Journal of Physiological Sciences Vol. 57, No. 6, 2007

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received saline (saline group). The lumbar sympa
thetic chain was exposed under sodium pentobarbital anesthesia (45
mg/kg, i.p.). Either 5 µl of saline as a control (saline group)
or 1 mg colchicine in 5 µl saline (colchicine group) was in-
jected just beneath the lumbar sympathetic chain, and a
piece of cotton soaked with colchicine solution was placed
above this chain in the colchicine group. Two days later, the
deply anesthetized rats were perfused intracardially with
PBS followed by fixative solution consisting of 4%
paraformaldehyde and 0.5% picric acid in PBS. The lumbar
sympathetic ganglia (SG) L4 and L5 were removed, post-
fixed in the same fixative for 1 night, and then placed in
30% sucrose in PBS for 4 days at 4°C. The SG were cut lon-
gitudinally in 10 µm sections on a cryostat, mounted on
slides and air-dried, and stored at −80°C until used.

The sections were hydrated in PBS, and the endogenous
peroxidase was then eliminated. Afterward, the sections
were preincubated in a solution containing 10% normal
goat serum (NGS) in PBS for 1 night at 4°C. They were
then placed in rabbit anti-SP antiserum (1:10,000 or 20,000,
diluted with PBS containing 2% NGS; Dia Solin, Stillwa-
ter, USA) and stored for 3–4 days at 4°C. Following washes
in PBS, the sections were incubated for 1 night with goat
antirabbit IgG (1:200) at 4°C. They were rinsed in PBS and
subsequently placed in ABC reagents (Vector Lab, Inc.,
Burlingame, USA) for 30 min. After further rinsing, the
peroxidase label was developed in 0.03% 3,3′-
diaminobenzidine tetrahydrochloride (DAB; Dojindo Lab., Kumamo-
to, Japan) containing Ni and activated with 0.01% H2O2 in
Tris Buffer (pH 7.6). As a staining reference, we stained
DRGs, which normally contain SP positive neurons, simul-
taneously with SG. After the time that was required for
stained reaction products to appear in the DRG sections, we
examined for the presence of reactive substances to DAB in
the SG sections. Excess reaction product was washed out
with PBS, and the sections were counterstained with meth-
yl green (Waldeck-Gmbh & Co. KG Muenster, Germany).
The sections were dehydrated and placed under coverslips
for light microscopy. The specificity of immunostaining
was tested by omitting the primary antiserum or by preab-
sorbing the antibody with 100 µg/ml of SP (Sigma, St. Lou-
is, USA) overnight at 4°C.

We selected every third or more section of the whole
SG in each rat. The number of DAB-labeled cells in L4 or
L5 of the SG was counted under a light microscope with
the aid of an image analysis system, Leica Qwin (Leica
Co., Ltd., Wetzlar, Germany). The density of the immu-
noreaction product was quantified using a 256-level grey
scale. The threshold for the SP-ir positive neuron profiles
was determined to be 178, and all neurons whose mean
cytoplasmic density was below this threshold were count-
ded. The ratios of rats having SP-ir positive neuron(s) in
SG were compared between groups using a Fisher’s exact
probability test.

All experimental procedures were approved by the An-
imal Care Committee, Nagoya University.

RESULTS

Two of the laser Doppler flowmeter probes were placed
on the plantar skin, and the BF responses to SS at these
two sites were recorded simultaneously. We usually se-
lected one site first where the BF clearly increased in re-
response to SS, and then for a site with a BF decrease. SS
induced either an increase or a decrease of BF in AI rats.
Typical BF responses to SS in the AI rats are shown in
Fig. 1 (leftmost recording).

**Fig. 1.** Changes in blood flow (BF) and mean
arterial pressure (MAP) responses to the
electrical stimulation of sympathetic nerve
(SS) by the injection of CP-96,345 and CP-
96,344 in an inflamed rat. Ordinate: BF (in
arbitrary units) and MAP (in mm Hg). BF re-
sponses 1 and 2 (BF1 and BF2) were simul-
taneously measured at two points indicated
by the corresponding labels in the schematic
drawing of the hind paw. The bar under the
MAP recording indicates the period of SS, 5
s. SS induced BF-increase and -decrease
responses in the same adjuvant-inflamed
rat. Arrows show that the drug was injected
shortly after the previous SS. The number
under the MAP recording indicates the time
elapsed after the first SS. The BF-increase
response was suppressed by CP-96,345,
but it was unaffected by CP-96,344. In con-
trast, the BF-decrease response was unaf-
fected by either drug.
Effects of NK-1 receptor antagonist in SS-induced BF increase response

CP-96,345 (1 mg/kg) tended to cause a decrease and a subsequent increase in the baseline BF and MAP, but BF at both sites and MAP had returned to preinjection levels twenty min after drug administration (data not shown). CP-96,344 also had some effect on baseline BF and MAP, but these values had also returned to the preinjection levels by 20 min after injection. Therefore the BF response to SS was examined 20 min after drug injection.

When SS was applied 20 min after CP-96,345, the BF-increase response was almost completely suppressed (Fig. 1, top recording) in 5 of 7 cases examined. In 2 cases, the BF-increase response was not influenced by CP-96,345. It recovered at 60 min after injection. It must be noted that CP-96,345 did not affect the BF-decrease response induced by SS (Fig. 1, middle recording). MAP was slightly increased by SS, but this was not changed by CP-96,345 application. CP-96,344 also affected neither the BF-increase nor the BF-decrease response to SS (Fig. 1).

Figure 2 shows the summary of the effects of CP-96,345 and CP-96,344. The ∆BF (BF-increase response) to SS was significantly changed by CP-96,345 (one-way ANOVA, †p < 0.05), and ∆BF at 25 min after the first SS (that means, 20 min after CP-96,345 injection) was significantly suppressed when compared with ∆BF before the CP-96,345 injection (Bonferroni’s test, **p < 0.01).

Fig. 2. Effects of CP-96,345 and CP-96,344 on BF-increase and BF-decrease responses to the electrical stimulation of sympathetic nerve (SS). Ordinate: changes in the BF from baseline value (∆ blood flow, in arbitrary units). Either the maximum (BF-increase response) or the minimum (BF-decrease response) values to SS before and after injections of CP-96,345 (solid lines) and CP-96,344 (dashed lines) were measured as shown in the insets, and mean values with SE were plotted (n = 7). Abscissa: time after the SS before the CP-96,345 or CP-96,344 injection. CP-96,345 or CP-96,344 was injected at 5 min (marked with an underline and "inj"). Note that there was a significant change in the blood flow increase response (∆BF) to SS in the CP-96,345 series (one-way ANOVA with repeated measures, p < 0.05), and ∆BF at 25 min after the first SS (that means, 20 min after CP-96,345 injection) was significantly suppressed when compared with ∆BF before the CP-96,345 injection (Bonferroni’s test, **p < 0.01).

Fig. 3. Immunohistochemistry of substance P (SP) expression in the L4 sympathetic ganglion of an adjuvant-treated and a control rat. A: CFA rat; B: control rat. SP-ir positive cells are labeled in a CFA rat (rat No. 15 in Table 1). White arrowheads show labeled SP containing fibers in sympathetic ganglia in A and B. SP-ir positive fibers (stained dark) were seen running parallel to the long axis of the SG. There were no SP-ir positive cells in the sympathetic ganglia of control rats (B). Scale: 30 µm.
Immunohistochemistry of substance P

We examined 4 to 11 sections from each animal. Small numbers of SP-ir positive SG neurons (sample photos in Fig. 3A) were observed in sections obtained from AI rats, irrespective of whether they received colchicine. SP-ir positive cell somas were observed scattered over the SG, and a few labeled cells were occasionally localized side by side in SG, as in Fig. 3A. SP-containing fibers were also observed, and some were traced to the soma (leftmost open arrowhead in Fig. 3A). In contrast, only one of the eight control animals had SP-ir positive neurons in SG. The exceptional control animal that had one SP-ir neuron in its SG was one that had received colchicine (Table 1). SP-containing fibers were also observed in almost every section from the control rats (open arrowhead, Fig. 3B). In summary, SP-ir neurons were found in 5 of the 8 AI rats (Table 1), and the ratio of rats with SP-ir positive neurons was significantly higher in AI rats than in the control group ($p < 0.05$). In 3 of the SP-ir positive AI rats, SP-ir was found in both the L4 and L5 SG, and in another 2 AI rats SP-ir was in only the L4 or L5 SG. The number of neurons having SP-ir varied from 0 to 17 in a section, and from 0 to 43 (mean 13) in a rat.

DISCUSSION

(1) SP was involved in the BF increase response to SS

In this experiment, the BF-increase response to SS was reduced by CP-96,345, but not by CP-96,344. The dosage used, 1 mg/kg (injected into the branch of the femoral artery), is considered to be sufficient to completely block NK-1 receptors, based on our preliminary experiments: the effective results were obtained by an intravenous injection of 2–5 mg/kg in AI rats. Furthermore, enough dosage determined by the provider of the drug (Pfizer) was 0.3 mg/kg, i.v. These results suggest that the SS-induced BF-increase response is mediated by SP through the NK-1 receptor and by NA (see below). In our previous study [9], we found in the present study that SP was expressed in the lumbar sympathetic ganglia in some of the AI rats, but not in intact animals. After a blockade of axonal transport by colchicine [17], more AI rats had SP-ir, but only one control rat had one SP-ir neuron, though SP-ir positive fibers were always found. The absence of SP-ir positive neurons and the presence of SP-ir positive nerve fibers in lumbar SG in the control rats are in agreement with the findings of previous reports [14]. It is reported that SP is up-regulated in DRG neurons in CFA-inflamed rats [12], and that the concentration of SP increases in the serum of patients with long-standing rheumatoid arthritis [18]. In contrast, there has been no direct evidence that SP is up-regulated in the lumbar SG of inflamed animals and transported and released from sympathetic terminals [19]. Our present observation is, to our knowledge, the first to show how SP might be explained by the involvement of SP-induced active vasodilatation. It should be mentioned that in this study the BF-increase response to SS was incompletely suppressed after an injection of CP-96,345, with half of the response remaining. Because the dosage of CP-96,345 was large enough to completely suppress NK-1 receptor, this remaining response could not have been mediated by NK-1 receptor; thus, it might have been mediated by adrenoceptors, as we have already shown [9]. Another reason for this residual BF response after treatment with CP-96,345 might be that the contribution of substance P is variable among animals, as suggested by the absence of SP-ir positive SG neurons in some AI rats.

(2) Where does SP come from?

We found in the present study that SP was expressed in the lumbar sympathetic ganglia in some of the AI rats, but not in intact animals. After a blockade of axonal transport by colchicine [17], more AI rats had SP-ir, but only one control rat had one SP-ir neuron, though SP-ir positive fibers were always found. The absence of SP-ir positive neurons and the presence of SP-ir positive nerve fibers in lumbar SG in the control rats are in agreement with the findings of previous reports [14]. It is reported that SP is up-regulated in DRG neurons in CFA-inflamed rats [12], and that the concentration of SP increases in the serum of patients with long-standing rheumatoid arthritis [18]. In contrast, there has been no direct evidence that SP is up-regulated in the lumbar SG of inflamed animals and transported and released from sympathetic terminals [19]. Our present observation is, to our knowledge, the first to show

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Table 1. Number of SP-ir positive cells in L4 or L5 sympathetic ganglia. An injection of adjuvant and/or colchicine is indicated as +. The number of sections counted in each animal is also presented.
an increased expression of SP in SG as a result of inflammation. An inflammatory cytokine, IL-1, is reported to stimulate the up-regulation of SP through the induction of a leukemia inhibitory factor in SG [19]. The effect of immunocytokines on SP expression has been studied in relation to nerve injury [20], and it is possible that IL-1 is also increased in SG with peripheral inflammation. NFG is another candidate substance for the stimulation of SP up-regulation. It is known that NFG transported from inflamed tissues plays an important role in the up-regulation of SP in DRG [21]. Because SG neurons also express TrkA receptors [16], NFG that was transported from inflamed peripheral target tissues might play some role in the up-regulation of SP in SG neurons, the same as in DRG neurons in adjuvant inflammation. SP involved in the BF-increase response to SS might originate from SP-containing neurons in SG. There is, however, a problem with this conclusion because the number of SP-ir cells was small, and some AI rats had no SP-ir positive cells in SG at all. This might be why the SS-induced BF-increase response was not suppressed at all by SP antagonist in 2 of the 7 AI rats in the present experiment. Because all SG had SP-ir positive fibers, many of which are considered to be afferents, SS might also have stimulated these afferent fibers to release SP from their terminals. The possibility that somatic afferents innervating the hind limbs run in the SG has previously been neglected for indirect reasons [22], but our present results may call for a reconsideration of this possibility. Another possibility is that the SS might have stimulated sensory neurons through possible sympathetic-sensory coupling. C-polymodal receptors were reported to be activated by NA in CFA rats [23], and this might result in a substance P release from peripheral sensory terminals. This possibility is open for future study.

In conclusion, the involvement of both adrenergic-passive and nonadrenergic-active mechanisms through NK-1 receptors has been suggested in the BF-increase response to SS. Furthermore, NK-1 activation involved in the BF-increase response might be in part mediated by SP from lumbar SG that was synthesized de novo in inflamed animals.

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In conclusion, the involvement of both adrenergic-passive and nonadrenergic-active mechanisms through NK-1 receptors has been suggested in the BF-increase response to SS. Furthermore, NK-1 activation involved in the BF-increase response might be in part mediated by SP from lumbar SG that was synthesized de novo in inflamed animals.