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Abstract

The present studies investigated behavioral and neurochemical aspects of the noradrenergic and serotonergic nervous systems in streptozotocin-induced diabetic mice. We previously reported that intrathecal (i.t.) injection of norepinephrine significantly potentiated antinociception in diabetic mice compared to that in non-diabetic mice, and that antinociception due to norepinephrine injection was completely abolished by pretreatment with yohimbine, an $\alpha_2$-adrenoceptor antagonist. The present studies demonstrated that i.t. injection of clonidine also showed more potent antinociceptive activity in diabetic mice than in non-diabetic mice, but that i.t. methoxamine injection did not affect diabetic or non-diabetic mice. The antinociceptive potency due to i.t. injection of 5-HT was significantly lower in diabetic than in non-diabetic mice. In a neurochemical study, we found that the density of $[^3]H$-rauwolscine binding sites in spinal $\alpha_2$-adrenoceptors was significantly higher in diabetic than in non-diabetic mice, but that the binding affinity was unchanged. Spinal norepinephrine turnover was determined by measuring the decline in tissue norepinephrine concentration at 3 h after injection of the tyrosine hydroxylase inhibitor $\alpha$-methyl-p-tyrosine. The spinal norepinephrine concentration decreased to 43.7% from the baseline in non-diabetic mice, while it was 21.0% in diabetic mice. These results suggest that, based on the decrease of norepinephrine release in the spinal cord, up-regulation of spinal $\alpha_2$-adrenoceptors caused the increase of antinociception due to i.t. injection of an $\alpha_2$-adrenoceptor agonist in streptozotocin-induced diabetic mice, and it seemed that the stimulation of $\alpha_2$-adrenoceptors potentiated the antinociceptive effect. Thus, the spinal noradrenergic systems play an important moderating role in diabetes-induced neuropathic pain.
Key words: Antinociception; diabetes; descending pain inhibitory systems; spinal cord; α2-adrenoceptors
1. Introduction

Diabetes is commonly associated with neuropathic pain, which is often considerable. However, the mechanism underlying neuropathic pain has not been elucidated. It is known that the pathophysiology of neuropathic pain in the diabetic is accompanied by neurochemical alterations in the nervous system. A number of neurotransmitters likely modulate the ascending and descending pain pathways and norepinephrine and 5-hydroxytriptamine (serotonin, 5-HT) have been implicated as mediators of endogenous analgesia via the descending pain inhibitory pathways (Basbaum and Fields, 1984; Yaksh, 1985). Wei et al. (1999) reported that an imbalance in these inhibitory mechanisms contributes to central sensitization and hyperexcitability of spinal and supraspinal pain-transmitting pathways, leading to persistent pain. Our previous investigation (Omiya et al., 2005) demonstrated that intrathecal (i.t.) injection of norepinephrine produced more-potent antinociception in streptozotocin-induced diabetic mice than in non-diabetic mice and that the antinociceptive effect of norepinephrine was antagonized by pretreatment with yohimbine, an $\alpha_2$-adrenoceptor antagonist. These results suggested that the spinal $\alpha_2$-adrenoceptors play an important role in analgesic effects in diabetic animals. Moreover, duloxetine, a selective and potent dual 5-HT- and norepinephrine-reuptake inhibitor, showed significantly more efficacy than a placebo on most outcome measures in the management of diabetic peripheral neuropathic pain (Goldstein et al., 2005). This effect is due to the ability of duloxetine to enhance both 5-HT and norepinephrine functions in descending pain modulatory pathways. These results suggested that 5-HT and norepinephrine inhibit pain via the descending pain pathway in diabetic neuropathy. However, the participation of noradrenergic and serotonergic nervous systems in the
occurrence of neuropathic pain in the diabetic is not clear. In the present studies, we investigated behavioral and neurochemical aspects of noradrenergic and serotonergic systems in streptozotocin-induced diabetic mice. Noradrenergic and serotonergic neuronal activity was determined by measuring the decline in tissue norepinephrine concentration after injection of the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine, measurement of the density and affinity of spinal α2-adrenoceptors using [3H]-rauwolscine, and evaluation of antinociceptive effects by a tail-pressure test.

2. Materials and methods

2.1. Animals

Male ddY mice (Japan SLC, Hamamatsu, Japan) initially weighing 20-25 g were used. The animals were housed five to a cage under a 12-h light and dark cycle (light between 7:00-19:00) and had free access to food and water ad libitum throughout the experiments. All experimental procedures were performed according to the “Guidelines for the Care and Use of Laboratory Animals” approved by the Laboratory Animal Committee of Tsumura & Co.

2.2. Induction of diabetes

Diabetes was induced by an intravenous injection of streptozotocin (150 mg/kg) dissolved in 33.3 mM citrate buffer solution at pH 4.5. According to a previous report (Kamei et al., 1993), the serum glucose level in mice that had been injected was significantly elevated compared to that in age-matched control mice in 1 to 2 weeks. The experiments were conducted 2 weeks after injection of streptozotocin.
In the study of mice with diabetes of long duration, it was conducted 4 weeks after injection of streptozocin. Mice with glucose levels above 400 mg/dl were considered diabetic.

2.3. Drugs

5-Hydroxytryptamine hydrochloride, methoxamine hydrochloride, clonidine hydrochloride, and α-methyl-DL-p-tyrosine methyl ester hydrochloride (α-MT, a tyrosine hydroxylase inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and these drugs were dissolved in 0.9% physiological saline. They were injected intrathecally (i.t.) in a volume of 5 µl according to the method described by Hylden and Wilcox (1980). α-MT was injected subcutaneously at a concentration of 200 mg/kg.

2.4. Nociception test

Antinociceptive responses were evaluated by a tail-pressure test (Pressure analgesimeter; Ugo Basile, Milan, Italy) in mice as described previously (Ohara et al., 1991; Omiya et al., 2000). The animals were adapted to the test procedure by prior exposure to the test apparatus. Mice were subjected to pressure on the tail at a point 1 cm distant from the root. The force applied to the tail was increased at a constant rate of 16 g/s, and the threshold for struggling behavior was measured. The average threshold of three trials constituted the baseline nociceptive threshold at 0 h. Changes in the threshold induced by drugs were monitored at 10-min intervals for 1 h after their administration. The antinociceptive effects over time were calculated as the area under the curve (AUC) by plotting the increase in the nociceptive threshold (Δ g) from
the value at 0 h on the ordinate and the time interval (h) on the abscissa. The AUC is the area of the nociceptive threshold between 0 and 1 h.

2.5. α2-Adrenoceptor binding assay

Crude synaptic membranes were prepared according to the method of Neylon and Summers (1985). Mice were decapitated 2 weeks after injection of streptozotocin. The spinal cord (L1-L4) was removed and weighed immediately. All procedures were carried out on ice. Tissues were homogenized in 10 volumes of 50 mM Tris-HCl buffer (pH 7.4) using a microhomogenizer (NS-310E, Niti-on, Tokyo, Japan) at setting 8 for 30 sec. The homogenate was centrifuged at 30,000 x g for 15 min at 4°C. The resulting supernatant was discarded, and pellet was washed three times by re-homogenization and centrifugation in 50 mM Tris-HCl buffer. Finally, the concentration of the membrane suspension was adjusted to 100 ml/wet weight with Tris-HCl buffer containing 5 µM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 0.1% ascorbic acid for the binding assay. The protein concentration was determined by the method of Lowry et al. (1951). [3H]-rauwolscine binding was determined using the method of Neylon and Summers (1985). About 300 µg protein of the membrane preparation was incubated for 60 min at 25°C with 0.5 to 20 nM concentrations of [3H]-rauwolscine (PerkinElmer, Inc., Waltham, MA, USA; specific activity: 82.0 Ci/mmol). Non-specific binding was determined by 10 µM phentolamine (Sigma-Aldrich). At the end of the incubation period, the assay samples were rapidly filtered under vacuum through Whatman GF/B glass filters and washed three times with ice-cold Tris-HCl buffer. The filters were then placed in 5 ml Cleasol I scintillation cocktail (Nakalai Tesque, Kyoto, Japan) for 12 h, and the radioactivity retained was
counted in a Packard liquid scintillation counter (PerkinElmer Inc.). The number of binding sites ($B_{\text{max}}$) and the dissociation constant ($K_d$) were calculated from the Scatchard analysis by a linear regression program.

2.6. Concentration of norepinephrine in spinal cord of diabetic mice

Non-diabetic and diabetic mice were decapitated before or 3 h after subcutaneous administration of α-MT at a dose of 200 mg/kg. According to the method of Sidman et al. (1971), the L1-L4 segment of the spinal cord was rapidly removed. The dissected samples were weighed, homogenized with an ultrasonicator (SMT Co., Ltd, Tokyo Japan) in 0.2 ml of 0.2 M perchloric acid including 0.1 mM Na$_2$EDTA and 50 ng/ml isoproterenol (Sigma-Aldrich) as an internal standard, and centrifuged at 15,000 x g for 15 min. The supernatants were collected in microtubes and analyzed by HPLC with electrochemical detection. The mobile phase was composed of 83% acetate/citrate buffer containing 5 mg/l Na$_2$EDTA, 500 mg/l sodium octanesulfonic acid, and 17% methanol. The flow rate was maintained at 0.5 ml/min, and the samples were injected into the column (3.0 $\phi \times$ 150 mm; EICOMPAK SC-50DS, Eicom, Kyoto, Japan). The substances were oxidized with a graphite electrode at a potential of $+750$ mV relative to an Ag/AgCl reference electrode, and the electrochemical detector (ECD-100; Eicom) was set at a gain of 2.0 nA full scale. The peaks generated were recorded, and their heights were measured.

2.7. Statistical analysis

The results are expressed as means ± S.E.M. and analyzed by one- or two-way ANOVA followed by Student’s $t$-test or Dunnett’s test. Values of $P<0.05$
were considered significant.

3. Results

3.1. Changes in mechanical nociceptive threshold in diabetic mice

In diabetic mice 2 weeks after injection of streptozotocin, the baseline mechanical nociceptive threshold decreased 27.2% as compared with the threshold in age-matched non-diabetic mice, whereas the baseline nociceptive threshold in diabetic mice 4 weeks after injection was not different than the threshold in non-diabetic mice (Table 1). Intrathecal injection of the vehicle (saline) did not change the nociceptive threshold in diabetic mice between 0 h and 1 h (2 weeks: 74.8±1.0 g – 82.2±3.3 g, 4 weeks: 125.0±4.0 g – 130.9±4.0 g).

3.2. Antinociceptive effect of i.t. injection of noradrenergic or serotonergic agonist in diabetic mice

Intrathecal injection of clonidine, an $\alpha_2$-adrenoceptor agonist, at a dose of 0.3-3 µg increased antinociception in diabetic and non-diabetic mice [two-way ANOVA: treatment, $F(1, 1)=41.86$, $P<0.0001$; dose, $F(1, 2)=98.93$, $P<0.0001$; treatment×dose, $F(1, 56)=3.48$, $P=0.0378$] (Fig. 1). The clonidine-induced antinociceptive effects were significantly higher in diabetic than in non-diabetic mice. However, methoxamine, an $\alpha_1$-adrenoceptor agonist, (30-300 µg, i.t.) did not affect antinociception in diabetic or non-diabetic mice (Fig. 2). On the other hand, i.t. injection of 5-HT at doses of 3-100 µg also increased the antinociception, but the antinociceptive potency of 5-HT was significantly lower in diabetic mice than in
non-diabetic mice [two-way ANOVA: treatment, $F(1, 1)=14.95, P=0.0003$; dose, $F(1, 3)=22.08, P<0.0001$; treatment×dose, $F(1, 56)=2.03, P=0.1203$] (Fig. 3).

3.3. $\alpha_2$-Adrenoceptor binding in diabetic mice

The specific binding of various concentrations of $[^3]$H-rauwolscine (0.5 to 20 nM) to spinal cord synaptosomal preparations is shown in Fig. 4. The maximum binding ($B_{\text{max}}$) was significantly higher in diabetic mice (165.9 ± 4.5 fmol/mg protein) than in non-diabetic mice (137.3 ± 5.4 fmol/mg protein) ($P<0.01$). However, the binding affinity ($K_d$) was not affected in either group (non-diabetic mice: 20.0 ± 0.8 nM vs. diabetic mice: 21.0 ± 0.8 nM).

3.4. Concentration of norepinephrine in spinal cord of diabetic mice

Spinal norepinephrine turnover was determined by measuring the decrease in the tissue norepinephrine concentration at 3 hr after injection of $\alpha$-MT, a tyrosine hydroxylase inhibitor. The results are summarized in Table 2. The basal levels of norepinephrine in the spinal cord did not differ between diabetic and non-diabetic mice. After the injection of $\alpha$-MT, the spinal cord norepinephrine concentration decreased 43.7% from baseline in non-diabetic mice, while the spinal norepinephrine concentration in diabetic mice decreased 21.0%, a statistically significant difference ($P<0.01$). On the other hand, there was no statistically significant difference between the basal concentrations of 5-HT in diabetic and non-diabetic mice (non-diabetic mice: $1077.2 \pm 114.4$ ng/g tissue, n=10 vs. diabetic mice: $1043.4 \pm 128.6$ ng/g tissue, n=12).
4. Discussion

Several studies reported that chronic hyperalgesia produced a functional change in the descending noradrenergic and serotonergic systems (Miura et al., 2005; Mochizuki, 2004; Tanabe et al., 2005). In diabetic neuropathy, the analgesic activity of relatively selective or selective serotonin reuptake inhibitors is less potent than that of relatively selective norepinephrine reuptake inhibitors (Courteix et al., 1994; Max et al., 1992). Our previous investigation (Omiya et al., 2005) demonstrated that i.t. injection of norepinephrine produced more-potent antinociception in streptozotocin-induced diabetic mice than in non-diabetic mice, and that the effect of norepinephrine was antagonized by pretreatment with yohimbine. In this study, the antinociceptive potency of intrathecally injected 5-HT was significantly lower in diabetic mice than in non-diabetic mice. These observations suggest that the spinal noradrenergic system is a stronger modulator of analgesic effects in diabetes than the serotonergic system. We focused on the spinal α-adrenoceptors in diabetic mice. Intrathecal injection of clonidine, an α₂-adrenoceptor agonist, produced a potent analgesic activity in non-diabetic and diabetic mice. However, i.t. injection of methoxamine, an α₁-adrenoceptor agonist, did not produce any analgesic activity in either non-diabetic or diabetic mice. These findings suggest that spinal cord α₂-adrenoceptors play an important role in the analgesic effects due to stimulation by an α₂-adrenoceptor agonist, whereas the α₁-adrenoceptor is not involved. In addition, the dose-response curves for the analgesic activity of intrathecally injected clonidine or norepinephrine (Omiya et al., 2005) were lower in the diabetic mice, confirming that the analgesic action mechanism mediated by α₂-adrenoceptors was enhanced in diabetic mice in comparison with that in non-diabetic mice. To clarify the mechanism involved
in norepinephrine/clonidine-induced enhancement of analgesic action, we conducted a binding assay to investigate changes in spinal cord $\alpha_2$-adrenoceptors in diabetic mice. The binding density ($B_{\text{max}}$) of $\alpha_2$-adrenoceptors in the diabetic mice was significantly higher than that in the non-diabetic mice; furthermore, there was no difference between the $K_d$ values of the non-diabetic and diabetic mice. These results suggest that the enhancement of the antinociceptive potency in diabetic mice is associated with an increase in the density of spinal cord $\alpha_2$-adrenoceptors. Bitar et al. (1999) reported that an increase in the tail-flick latency in streptozotocin-induced diabetic rats was related to the down-regulation of $\alpha_2$-adrenoceptors in the spinal cord. The difference between our results and theirs may depend on the duration of diabetes or variations in species. Indeed, 2 weeks after injection of streptozotocin, diabetic mice exhibited a lower nociceptive threshold than non-diabetic mice, but the baseline threshold in diabetic mice 4 weeks after injection of streptozotocin was not lower, indicating that the latter diabetic state no longer decreased in response to mechanical stimulation.

Moreover, intrathecal injection of clonidine did not produce antinociception in diabetic mice 4 weeks after injection of streptozotocin (data not shown), whereas i.t. injection of clonidine produced a prominent antinociception in diabetic mice 2 weeks after injection of streptozotocin. These findings are similar to the behavioral observations of Bitar and Pilcher (1997). The functional changes in the spinal cord $\alpha_2$-adrenoceptors are related to the duration of diabetes. To elucidate the etiology of the up-regulation of $\alpha_2$-adrenoceptors in diabetic mice, we determined spinal norepinephrine turnover by measuring a decline of the tissue norepinephrine concentration 3 h after injection of $\alpha$-MT in diabetic mice. $\alpha$-MT treatment markedly decreased the spinal norepinephrine concentration in non-diabetic mice, whereas 3 h after $\alpha$-MT treatment it still remained
in diabetic mice. Ohtani et al. (1997) reported that the extracellular concentration of norepinephrine in the hypothalamus was remarkably decreased in streptozotocin-treated rats even 2 weeks after induction of diabetes. It was also reported that the extracellular norepinephrine concentration in the locus coeruleus nucleus depended on neuronal amine synthesis (Fernandez-Pastor et al., 2005). Raiteri et al. (1983) suggested that the sensitivity of postsynaptic $\alpha_2$-adrenoceptors is up-regulated after noradrenergic denervation. Moreover, functional supersensitivity appears to be due to an increase in the density of $\alpha_2$-adrenoceptors in the spinal cord of rats in which the descending norepinephrine pathway is chemically affected (Dooley et al., 1983; Roudet et al., 1994). These reports indicate that a reduction of the extracellular norepinephrine concentration in the noradrenergic terminals is strongly involved in the up-regulation of spinal $\alpha_2$-adrenoceptors in diabetic mice. The up-regulation of $\alpha_2$-adrenoceptors should lead to the increase of antinociceptive potency induced by norepinephrine or clonidine in diabetic mice.

In this study, an analgesic effect mediated by $\alpha_1$-adrenoceptors was not shown in non-diabetic or diabetic mice. It has been reported that the contractile responses to $\alpha_1$-adrenoceptors were enhanced in diabetic mesenteric arteries (Dresner et al., 1997; Ishikawa et al., 2004). The peripheral $\alpha_1$-adrenoceptors play an important role in vasoconstriction, but the spinal cord $\alpha_1$-adrenoceptors apparently do not contribute to antinociceptive activity in diabetic animals.

We concluded that a decrease in norepinephrine release in the spinal cord of diabetic animals induced the up-regulation of $\alpha_2$-adrenoceptors, suggesting that the stimulation of $\alpha_2$-adrenoceptors potentiated the antinociceptive effect. Furthermore, the supersensitivity was mediated by $\alpha_2$-adrenoceptors. Thus, the spinal noradrenergic
systems play an important moderating role in diabetes-induced neuropathic pain. The $\alpha_2$-adrenergic agonist-related stimulation of $\alpha_2$-adrenoceptors and selective norepinephrine reuptake inhibitors may be useful for relieving neuropathic pain in diabetics.

References


Figure Legends

Fig. 1. Dose-response curves obtained with intrathecally injected clonidine in non-diabetic and diabetic mice. The nociceptive threshold was determined by the tail-pressure test every 10 min after clonidine treatment for 1 h, and the antinociceptive activities were expressed as the AUC. Each point represents the mean ± S.E.M. of 10-11 animals. **P<0.01 compared with the non-diabetic mice (Student’s t-test).

Fig. 2. Dose-response curves obtained with intrathecally injected methoxamine in non-diabetic and diabetic mice. The nociceptive threshold was determined by the tail-pressure test every 10 min after methoxamine treatment for 1 h, and the antinociceptive activities were expressed as the AUC. Each point represents the mean ± S.E.M. of 10-11 animals.

Fig. 3. Dose-response curves obtained with intrathecally injected serotonin in non-diabetic and diabetic mice. The nociceptive threshold was determined by the tail-pressure test every 10 min after serotonin treatment for 1 h, and the antinociceptive activities were expressed as the AUC. Each point represents the mean ± S.E.M. of 8 animals. **P<0.01 compared with the non-diabetic mice (Student’s t-test).

Fig. 4. Specific binding of $[^3]$H-rauwolscine to mouse spinal cord membranes. The inset is a Scatchard plot of specific $[^3]$H-rauwolscine binding. B/F is the ratio of specifically bound to free $[^3]$H-rauwolscine. Each value plotted represents the mean of duplicate determinations of 6 experiments. Binding parameters ($K_d$ in nM and $B_{max}$ in...
fmol/mg protein) for non-diabetic mice were: $K_d = 20.0\pm0.8$, $B_{\text{max}} = 137.3\pm5.4$; for diabetic mice: $K_d = 21.0\pm0.8$, $B_{\text{max}} = 165.9\pm4.5^{**}$. **$P<0.01$ compared with the non-diabetic mice (Student’s $t$-test).
Clonidine (μg, i.t.)

Diabetic mice
Non-diabetic mice

**Fig. 1**
Fig. 2
**Fig. 3**

- **AUC** subscripts **$0-1$ h** (g $\cdot$ h)

- **5-HT** subscripts (**μg, i.t.**)
Fig. 4

Bound (fmol/mg protein)

[\textsuperscript{3}H]-rauwolscine (nM)

- O Non-diabetic mice
- ● Diabetic mice
Table 1  Nociceptive responses to pressure stimulation after streptozotocin treatment

<table>
<thead>
<tr>
<th></th>
<th>Baseline threshold (g)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>2 weeks</td>
<td>4 weeks</td>
</tr>
<tr>
<td>Non-diabetic mice</td>
<td>102.8 ± 1.3</td>
<td>117.8 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Diabetic mice</td>
<td>74.8 ± 1.0</td>
<td>126.4 ± 5.2</td>
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</table>

Values are expressed as the mean ± S.E.M. of 6-11 animals.

*P < 0.01 compared with the 2 weeks non-diabetic mice (Student's *t*-test).
Table 2  Concentrations of NE in spinal cord of non-diabetic and diabetic mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NE concentration (ng/ g wet tissue)</th>
<th>% change</th>
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<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>3 h</td>
</tr>
<tr>
<td>Non-diabetic—saline</td>
<td>474.2 ± 24.0</td>
<td>419.0 ± 15.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-diabetic—α-MT</td>
<td>474.2 ± 24.0</td>
<td>267.1 ± 13.4</td>
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<tr>
<td>Diabetic—α-MT</td>
<td>456.5 ± 18.6</td>
<td>360.6 ± 16.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.E.M. of 9-13 animals.
<sup>a</sup> P<0.01 compared with the non-diabetic α-MT group (Dunnett's test).