Changes of Brain Endothelin Levels and Peripheral Endothelin Receptors by Chronic Cigarette Smoke in Spontaneously Hypertensive Rats

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Abstract. The present study was conducted to evaluate the contribution of endothelin (ET) to the pharmacodynamic response to chronic cigarette smoke in spontaneously hypertensive rats (SHR). The contribution of ET was studied consequent to the hemodynamic response following 8 weeks of cigarette smoke by determining the changes in tissue ET-1 content and ET receptors. The blood pressure (BP) at the early phase of smoking and the heart rate (HR) 24 h later were apparently reduced in SHR, while the HR at the early phase was transiently elevated in normotensive Wistar Kyoto (WKY) rats. Tissue ET-1 levels in the hypothalamus, striatum, and cortex of SHR were higher than those in WKY rats, and these higher levels in SHR were reduced by exposure to chronic cigarette smoke. The ET-1 contents in the medulla oblongata and midbrain of both strains were clearly increased by smoke exposure, although the levels of SHR and WKY rats were not different. In addition, the immunoreactivity of the ET type A receptor in the adrenal glands and type B receptor in the kidneys of SHR showed a different response to smoke exposure as compared to WKY rats. Our present findings suggest that the changes of ETs may relate to the pharmacodynamic effects of chronic cigarette smoke.

Keywords: endothelin, spontaneously hypertensive rat, cigarette smoking, hypertension, endothelin receptor

Introduction

Although there have been numerous clinical studies on the deleterious effects of chronic cigarette smoke, the influence of habitual cigarette smoke exposure on patients with diseases brought about through their lifestyle (such as hypertension and diabetic mellitus) has not been thoroughly investigated (1 – 3). The effects of chronic smoke exposure or exposure to the major psychoactive constituent of the smoke, nicotine, have been evaluated using experimental animals; however, no evidence could be found on the effects of these agents on animals with hypertension (4). Therefore, we initially evaluated the effects of chronic cigarette smoke on

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the pharmacodynamic response of spontaneously hypertensive rats (SHR) (5). In these studies, a characteristic effect, being not always unfavorable for the development of hypertension in animals, was observed on the hemodynamic response of SHR, compared with that of normotensive Wistar Kyoto (WKY) rats used as the control. The underlying basis of this pharmacodynamic response to the cigarette smoke has been generally been examined in the context of the action of nicotine and/or catecholamines. On the other hand, recent reports have linked various autacoids to cardiovascular disease; in particular, the endothelins (ETs) have received much attention in this regard, but no evidence has been documented on the contribution of ETs to the effects of chronic cigarette smoke in SHR.

Accordingly, we evaluated the contribution of central and peripheral endothelins in mediating the hemo-
dynamic response induced by chronic cigarette smoke in SHR. The levels of ET-1 and/or its receptors were assessed in both central and peripheral regions of SHR and WKY rats. The observations indicate a role for ETs in mediating the effects of chronic cigarette smoke in SHR.

Materials and Methods

Experimental animals

All animal experiments were performed in accordance with The Japanese Pharmacological Society “Guiding Principles for the Care and Use of Laboratory Animals” and were approved by the Animal Care Committee of Nara Medical University.

Ten-week-old, male, young adult SHR (SHR/Ncrj) and age-matched male WKY rats were purchased from Charles River Japan, Inc. (Yokohama). These animals were used for the smoking experiment after preconditioning for 2 weeks. The animals were fed with commercial solid diet and water, ad libitum, throughout the preconditioning and experimental periods in the animal room maintained at a room temperature of 22 ± 1°C, with the humidity at 55 ± 10%, and the light cycle with a 12-h interval from 8:00 A.M. to 8:00 P.M. The animals received occasional heart rate (HR) and blood pressure (BP) measurements by tail-cuff during the preconditioning; this process reduced the measurement-induced stress during the study and allowed for animal selection for group assignments.

Method for smoking

Animals were compulsively given cigarette smoke using a Hamburg II apparatus by the method of Suemaru et al. (6). The Long Peace cigarette with filter (Japan Tobacco Industry Co., Ltd., Tokyo), with nicotine and tar contents of 1.9 and 2.1 mg, respectively, was used. The daily compulsive cigarette smoke exposure in the apparatus was conducted with 10 animals, simultaneously, being exposed to 30 pieces cigarettes over 20-min periods between 9:00 and 11:00 A.M. Such smoking was repeated for 5 days from Monday to Friday each week. The animals in the non-smoking group were kept for 20 min in the holder without cigarette smoke.

Method for estimating HR and BP

The HR and BP of animals were measured using a tail-cuff apparatus (Model MK-1100; Muromachi Kikai Co., Ltd., Tokyo). Baseline levels were measured 10 times, each after stabilizing for 15 min in a hot box maintained at 35°C; in order to avoid any misjudgment brought about by a single estimate, the average value for each animal was accepted as its baseline value.

Study design

The SHR and WKY rats, respectively, were matched on the basis of their body weight, HR, and BP as assessed during the preconditioning; and then they were divided into two experimental groups, smoking and non-smoking, respectively. An experiment was performed using 5 SHR and WKY rats each for the smoking and non-smoking groups, respectively, and then the experiment was repeated twice. There were no statistical differences among the mean group values with respect to body weight, HR, and BP before the start of the smoking experiments. The weekly smoking exposure continued for 8 weeks. The pharmacodynamic changes of these animals were assessed at three different phases after the daily smoking. The early phase after daily smoking was defined as the time of 15 – 55 min after smoking, while the late phase was 125 – 165 min (according to the time required for estimating the HR and BP). The evaluation of pharmacodynamic responses at the early phase was performed every Thursday, while that at the late phase was performed every Friday. The response 24 h later was evaluated on Saturday after the weekly smoking. The pharmacodynamic response measurements at the different phases were not carried out on the same day in order to avoid the effects of stress in measuring by the tail-cuff method. On the Saturday of the 8th week, the pharmacodynamic responses 24 h later were not assessed in both experiments. Animals of an experimental group were used to prepare their tissue samples 24 h after the final smoking, while those of another group received the smoking in order to prepare the tissue samples at the late phase. For preparing the tissue samples, animals were sacrificed under pentobarbital sodium anesthesia (Nembutal, 50 mg/kg, i.p.; Dainippon Pharmaceutical Co., Ltd., Osaka). After collecting the arterial blood from the abdominal artery, rats were decapitated, and brain, heart, adrenal glands, and kidneys collected. Brain tissue was divided into 6 parts: hypothalamus, striatum, cortex, medulla oblongata, midbrain, and hippocampus. The tissue samples were immediately frozen with liquid nitrogen and stored at −80°C for determining the content of ET-1, while those for evaluating the immunoreactivities of ET receptors were fixed with 10% para-formaldehyde and embedded in paraffin. The tissue for determining the change of ET-1 and ET receptors in the present studies were decided by pilot studies using the tissue samples obtained from the animals described in our previous report (5).

Method for determining ET-1 content in brain regions

A commercial kit (Endothelin ELISA Kit; Biomedica, Vienna, Austria) was used for determining ET-1 content of brain regions. The frozen materials prepared as above
were homogenized with phosphate-buffered saline (PBS) (pH 7.4, 0.9% NaCl, 2 mM KCl, 8 mM Na₂HPO₄, and 15 mM KH₂PO₄ including pepstatin (5 μg/ml). Samples were centrifuged at 12,000 × g for 15 min at 4°C, and then the supernatant was again ultracentrifuged by 100,000 × g for 1 h at 4°C. Two hundred microliters of the supernatant was applied into each well on the plate and then incubated for 18 h at room temperature. After the incubation, the plate was washed with PBS three times, and then the secondary antibody was applied into the each well and incubated for 1 h at 37°C. Again, the plate was washed three times with PBS following the incubation for 30 min at room temperature and after the addition of coloring reagent. After the incubation, a stop solution was added and the absorbance of each well was measured at 450 nm using the plate reader, and then the ET-1 content was assessed from the standard curve.

The protein concentration in the samples was determined using the BCA Protein Assay Kit (Pierce, Boston, MA, USA). Bovine serum albumin solution was used as the standard material at concentrations of 0, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/ml, respectively, together with 5 μg/ml of pepstatin. Fifty microliters of the standard solution was mixed with 1 ml of A solution and 50 μl of B solution and then incubated for 1 h in the chamber at 37°C. After incubation, the absorbance at 562 nm was measured, and then the standard curve was made.

Analysis of ET receptor expression by immunohistochemistry

After decapitation, the tissue samples of brain, heart, adrenal gland, or kidney were fixed with 10% paraformaldehyde. Endogenous peroxidase activity was quenched by incubation in methanol containing 0.3% H₂O₂ for 30 min. After blocking with 10% goat serum for 20 min, sections were incubated with primary antibody (anti ET type A receptor antibody, anti ET type B receptor antibody; IBL, Fujioka) diluted in PBS (pH 7.4, 0.9% NaCl, 10 mM Na₂HPO₄·7H₂O, and 10 mM NaH₂PO₄) for 16 h (brain, 0.75 μg/ml; heart, 0.75 μg/ml; adrenal gland, 0.5 μg/ml; kidney, 4 μg/ml) followed by incubations with secondary antibody, peroxidase labeled biotinated streptavidine for 20 min each, and 3,3-diaminobenzidine for 5 min. Each incubation step was carried out at room temperature except for the primary antibody treatment at 4°C. Slides were counterstained with hematoxylin and mounted. The negative controls were incubated with human ET receptors peptides in place of primary antibody. The intensity was designated as positive when more than 30% of cells stained. The immunoreactivities was analyzed by scores for the staining. Scores were given as follows: no staining, 0; weak, 1; moderate, 2; and strong, 3.

Statistical analyses of data

Data are shown as the mean ± S.E.M. Data of the systolic BP and HR were analyzed over time by two-way, repeated measures ANOVA followed by the Sheffé modified test; those of the brain ET-1 content and the scores on immunoreactivities were analyzed by the Mann-Whitney U-test.

Results

Determining tissue ET-1 and ET receptors

Changes in ET-1 content due to chronic smoking were detected primarily in brain regions 24 h after the final day of smoke exposure, while changes in the ET receptors were observed in adrenal glands and kidneys at the late phase and 24 h after the daily smoking. Slight changes in the hypothalamic ET receptors were not consistently observed. These preliminary observations lead to the design of the present study.

Pharmacodynamic responses during chronic cigarette smoke

The pharmacodynamic responses in SHR and WKY rats during the chronic cigarette smoke exposure for 8 weeks are represented in Figs. 1 and 2. The daily smoking produced a marked increase in HR during the early phase in WKY rats (Fig. 1A), but not in SHR (Fig. 1B). The HR 24 h after the smoke exposure was reduced in the SHR as compared to the non-smoking group. Thus, it was more probable for the relative rise in HR detected in the WKY rats during the early phase to reach statistical significance because of the reduction observed in SHR. On the other hand, SHR showed a clear reduction in systolic BP during the early phase of daily smoking (Fig. 2B), an effect not seen in WKY rats (Fig. 2A). These trends in the pharmacodynamic responses during the chronic cigarette smoke are consistent with previous reports.

The changes of ET-1 content in brain regions

Differences in ET-1 concentrations between the tissue prepared from SHR and WKY rats, and moreover the smoking and non-smoking groups, were detected in a number of brain regions. In the non-smoking groups, ET-1 levels of the hypothalamus, striatum, and cortex were higher in the SHR than those in WKY rats (Fig. 3); the levels in other regions were not different between SHR and WKY rats (Figs. 4 and 5). The chronic cigarette smoke clearly reduced the increased concentrations of ET-1 in the hypothalamus, striatum, and cortex in
SHR 24 h after the final day of smoking (Fig. 3) and markedly increased the ET-1 contents in the medulla oblongata and midbrain in both strains (Fig. 4). In contrast, the ET-1 levels of the hippocampus were not affected by the chronic smoking in either strain (Fig. 5).

**Changes of ET receptors in adrenal glands and kidneys**

The changes in ET receptor immunoreactivity following chronic cigarette smoke were consistent with those of adrenal glands and kidneys. The expressions of both ET type A and B receptors were detected in the adrenal
Contribution of Endothelins in Smoking

The expression of type A receptor was specific in the medulla cells, while that of type B receptor was non-specific. Moreover, the immunoreactivity of type A receptor was minimally expressed in the non-smoking group of SHR, but quite apparent in the medulla during the late phase of the daily smoking in SHR, and still more increased 24 h later (Fig. 6). The intensities of type A receptor staining in WKY rats showed no difference among the preparations from the non-smoking and smoking groups (Table 1). The reduced intensities in the non-smoking group of SHR were similar during the late phase and 24 h later. However, the intensities in the smoking group of SHR became apparently higher than those in the non-smoking group during the late phase of daily smoking and were similar to the levels

Fig. 2. Changes of systolic blood pressure during the chronic cigarette smoke for 8 weeks in WKY rats (A) and SHR (B). According to the legends in Fig. 1.
observed in WKY rats 24 h later. On the other hand, the nonspecific expressions of ET type B receptor exhibited a similar intensity in all preparations obtained from the adrenal glands of both SHR and WKY rats independently of the cigarette smoke.

In the kidneys, the expression of ET type A receptor was not specific across regions and not different between the preparations from SHR and WKY rats or cigarette smoke exposure. However, the type B receptor was specifically expressed in the distal tubules of both SHR and WKY rats, and the immunoreactivities were highest during the late phase of daily smoking in both strains. Moreover, high levels were observed even in the non-smoking groups of both strains (Fig. 7 and Table 2).
Fig. 6. Representative patterns of the immunoreactivity indicating endothelin type A receptor in the preparations obtained from the adrenal glands of SHR. Photo A is a sample from the late phase of the non-smoking group, photo B is one from the late phase of the smoking group, and photo C is the sample from the smoking group 24 h later. Scale bar: 50 μm.

Fig. 7. Representative patterns of the immunoreactivity indicating endothelin type B receptor in the preparations obtained from the kidneys of SHR. According to the legends in Fig. 6.
However, the intensities of staining were markedly lower in the non-smoking SHR and WKY rats and also in the smoking group of WKY rats 24 h later.

**Studies for evaluating ET-1 and ET receptors in heart**

The changes of ET-1 content and receptor immunoreactivity were also studied in the heart, but none were detected.

**Discussion**

Although a number of studies have been performed evaluating the influence of habitual cigarette smoke on patients with various diseases relating to their lifestyle, none have used animal models of hypertension (7, 8). We previously reported the pharmacodynamic responses in the SHR receiving chronic cigarette smoke compared with those in WKY rats (5). In that study, various tissue samples were stored for further investigating the causes of the observed pharmacodynamic responses. In regard to the relationship between the cigarette smoke and the pharmacodynamic responses, there are many possible mechanisms including nicotine and its metabolites, catecholamines, various autacoids, and so on. In the present study, we chose to evaluate the contribution of ETs. Various documents have already been offered on the role of ETs in the pharmacodynamic responses, but their role in smoking has not been clarified (9). Moreover, there are some reports not supporting the conclusion that ETs contribute to the development of hypertension in SHR (10, 11). In the present study, differences in ET-1 content in brain regions and in ET receptor expression in the adrenal glands and kidneys between SHR and WKY rats were observed. As for the ET-1 content in the brain regions, three different regions were classified on the basis of differences in between SHR and WKY rats as well as changes due to the chronic cigarette smoke. The first included the hypothalamus, striatum, and cortex. Their ET-1 content in the non-smoking groups were higher in SHR than WKY rats, suggesting that the higher levels may relate to the characteristics of SHR. Moreover, these increased levels were reduced to the level of WKY rats 24 h after the final day of chronic smoking. The second class includes the medulla oblongata and midbrain, with an increase in ET-1 content through the cigarette smoke exposure with no difference between SHR and WKY rats. The third class is the hippocampus in which there was no difference in the content of ET-1 between SHR and WKY rats without regard to cigarette smoke exposure.

Various studies have been already been performed to determine the content of tissue ET-1 in order to evaluate its contribution to hypertension in SHR. For example, Wong and Jeng reported the highest levels were detected

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### Table 1. Quantitative evaluation on the endothelin type A receptors in the adrenal glands of SHR and WKY rats receiving the chronic cigarette smoke

<table>
<thead>
<tr>
<th>Phase after smoking</th>
<th>SHR Non-smoking group</th>
<th>SHR Smoking group</th>
<th>WKY rats Non-smoking group</th>
<th>WKY rats Smoking group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late phase</td>
<td>0.5 ± 0.0</td>
<td>2.0 ± 0.1**</td>
<td>3.0 ± 0.0</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>24 h later</td>
<td>0.4 ± 0.1</td>
<td>3.0 ± 0.0**</td>
<td>3.0 ± 0.0</td>
<td>3.0 ± 0.0</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.E.M. of 5 animals. Scores of the staining for the immunoreactivities are as follows: no staining, 0; weak, 1; moderate, 2; and strong, 3. The difference of mean values between the non-smoking and smoking group was analyzed by the Mann-Whitney U-test, and the statistical significance is indicated as **P<0.01.

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### Table 2. Quantitative evaluation on the endothelin type B receptors in the kidney of SHR and WKY rats receiving the chronic cigarette smoke

<table>
<thead>
<tr>
<th>Phase after smoking</th>
<th>SHR Non-smoking group</th>
<th>SHR Smoking group</th>
<th>WKY rats Non-smoking group</th>
<th>WKY rats Smoking group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late phase</td>
<td>2.7 ± 0.3</td>
<td>2.7 ± 0.2</td>
<td>2.7 ± 0.3</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>24 h later</td>
<td>0.1 ± 0.1</td>
<td>0.8 ± 0.4**</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.E.M. of 5 animals. Scores of the staining for the immunoreactivities are as follows: no staining, 0; weak, 1; moderate, 2; and strong, 3. The difference of mean values between the non-smoking and smoking group was analyzed by the Mann-Whitney U-test, and the statistical significance is indicated as **P<0.01.
in the kidneys of SHR and about half of that found in the brain (12). In addition, Iyer et al. documented changes in ET-1 content in brain regions of SHR and WKY rats across various ages, indicating a lower level in SHR than in WKY rats (13). However, there have been no studies comparable to our present results on the ET-1 content in the brain regions following chronic smoke exposure.

An important brain function is the regulation of the hemodynamic response via the baroreflex function of the nucleus tractus solitarius (NTS). Earlier workers have reported accelerated HR regulation using SHR, relating this to an $\alpha_2$-adrenergic receptor (14 – 16). George et al. (17) and Hauger et al. (18) documented a neuronal connection between the hypothalamus and NTS, and Hauger et al. (18) showed the hypothalamus-pituitary-adrenal gland axis as the center of stress regulation. Gulati et al. reported an elevation in BP following centrally administered ET-1 (19). This evidence may suggest that the increased HR and BP in SHR are consequent to the roles of catecholamines and ETs in the brain. Although the details should be further investigated, the initial effect of ET-1 in the brain regions of SHR exposed to chronic smoking may relate to the baroreflex.

The changes of ET levels to smoke exposure in the second class of brain regions was apparent in both strains 24 h after the daily smoking, a time not associated with the hemodynamic response. Accordingly, although the cardiovascular response is mediated by the brain, the changes of ET-1 content may be somewhat independent of the hemodynamic responses. However, as the enhanced content of ET-1 is assessed 24 h after the daily smoking, it is presumed that the ET-1 levels in the regions may be always be maintained at a higher level during the chronic smoking. Therefore, in order to understand a transient normalization of various vital responses associated with daily smoking, the time course should be studied in more detail to determine whether such an elevated content returns transiently after the daily smoking. As for the hemodynamic responses, a transient rise of HR at the early phase of daily smoking is common to both strains; thus, the relationship between this event and the central regulation of HR through the baroreflex also needs to be clarified.

On the other hand, regarding the role of peripheral ETs, it has been documented that ETs promote the secretion of catecholamines from the adrenal glands via the type A receptors (20 – 22). Ozaki et al. reported a higher content of adrenal catecholamines in SHR compared with WKY rats (23).

Our experimental results indicated a remarkable decrease of the type A receptor in the adrenal medulla cells of SHR. Thus, it seems likely that SHR over-secrete catecholamines from adrenal glands consequent to the low levels of ET-1. However, the over-secretion is reduced as the receptor expression is increased by the chronic cigarette smoke, and chronic smoking produces a down regulation, transiently. Although we could not assess the changes of ET-1 content in the adrenal medulla in the present experiments, it is presumed that the secretion of catecholamines from adrenal glands should occur via an ET-mediated process even in SHR receiving chronic smoking. In contrast, such a catecholamine-secretion in WKY rats occurs without regard to the smoking, and therefore, the hemodynamic response by the catecholamines secreted owing to smoking should be seen only in SHR. The characteristic effect in SHR is the reduction of BP during the early and late phases of the daily smoking. Of course, the rise of HR in both strains during the early phase of daily smoking may relate to the catecholamine secretion. To complete the story, it needs to be further investigated that the local content of ETs in adrenal medulla cells rises directly or indirectly via the action of smoke components such as nicotine during the early phase of daily smoking.

Finally, Zeidel et al. has reported that ET-1 blocks the sodium reabsorption through inhibiting the renal tubular Na$^+$-K$^+$ ATPase activity via the type B receptor (24). As seen in the present study (Table 2), the expression of type B receptor was markedly promoted during the late phase of daily smoking in both strains regardless of the smoking. Although the receptor immunoreactivity in non-treated animals was not shown in the table, those of the non-smoking group 24 h later corresponded to their values. Accordingly, the expression of type B receptor in the distal tubules is minimal in both strains, but it seems likely that the handling stress through the smoking experiments easily alters expression of the receptor during the late phase. In this regard, animals do excrete urine when kept in the holder. However, the expression of type B receptor was maintained at a low level up to 24 h later only in SHR exposed to the smoke. Although it is not clear whether such a Na$^+$ diuretic property contributes to the reduction of BP, this event may be helpful for the reduction of BP in SHR.

We think these data do not offer a clear explanation about the mechanism why the change of brain ET contents and immunoreactivity of ET receptor affects the change of the BP and HR in SHR and WKY by chronic cigarette smoke.

As further experiments, we should evaluate the effect of ET antagonists on systemic hemodynamic change during chronic cigarette smoking. Furthermore, it is also very important to measure the blood catecholamine
concentration and urinary catecholamine excretion. However, although we tried to measure the blood catecholamine concentration in rats by acute challenge of nicotine, which was the same dose as in the chronic cigarette smoke used in this experiment, we could not detect any significant changes (data are not shown). So, we think that it may be difficult to evaluate precisely the change of the blood catecholamine concentration and urinary catecholamine excretion under our experimental situation.

In conclusion, there is evidence that central and peripheral systems ETs play a role in the hemodynamic response to chronic cigarette smoke exposure in SHR and WKY rats.

Acknowledgments

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