Pharmacodynamic Characterization of Nitric Oxide-Mediated Vasodilatory Activity in Isolated Perfused Rat Mesenteric Artery Bed

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Vasodilatory profiles following a short-term infusion of nitric oxide (NO), acetylcholine (ACh), and sodium nitroprusside (SNP) into an isolated perfused mesenteric artery bed were analyzed in rats to examine their vasodilatory efficacy under physiological conditions. These compounds commonly increase the intracellular NO concentration to exert vasodilatory activity. In an experiment with exogenous NO infusion where 100 μl of 1 : 300 diluted NO-saturated solution (approx. 53 pmol of NO) was applied, the infusion caused transient vasodilation in a dose-dependent manner, with the peak vasodilation value being 74.7% of the maximum relaxation value. In experiments with ACh, the peak vasodilation value was 81.5% of the maximum at a dose of 60 pmol. The vasodilation profile of ACh was similar to that of NO infusion, but the ACh-induced vasodilation reduced at a slower rate than that induced by NO infusion. The vasodilatory activity of SNP was less potent than that of ACh, and its peak value was 62.8% of the maximum at a dose of 2000 pmol. However, SNP activity was augmented by removing the vascular endothelium of the mesenteric artery bed, and the peak value reached 67.3% of the maximum at a dose of 60 pmol. Pharmacodynamic analysis indicated that NO and ACh are equivalent regarding their vasodilatory efficacy, while the efficacy of SNP was less than 1% of theirs, as the arterial vascular endothelium impeded intracellular SNP-related NO generation, by which 95% of SNP's vasodilatory efficacy was negated. These findings will be helpful to understand factors influencing the therapeutic efficacy of vasodilators.

Key words nitric oxide; acetylcholine; sodium nitroprusside; vasodilation; pharmacodynamics; vascular endothelium

It is well-known that resistance blood vessels play an important role in maintaining the blood pressure and regulating tissue blood flow.1–3 Therefore, an elevated blood pressure in patients with hypertension is frequently managed by decreasing the peripheral vascular resistance in combination with reducing the cardiac output and/or decreasing the blood volume.2,3 Hypertension requires life-long treatment, and it is a major risk factor for various, serious diseases, such as stroke, heart failure, and renal dysfunction.2,3 It is therefore of importance to understand various therapeutic compounds from the viewpoint of their vasodilatory activity in consideration of efficacy and persistency. Such information contributes to an improvement in the appropriateness and effectiveness of hypertension treatment.

As for the mechanism accounting for vasodilatation, many investigators demonstrated that a cGMP-related process is largely involved in arteriolar smooth muscle relaxation.4–6 That is, following an increase in the intracellular cGMP concentration, protein kinase G is stimulated, and, subsequently, the Ca2+ pump function is activated. This causes the promotion of intracellular Ca2+ efflux, and the resultant decrease in the intracellular Ca2+ concentration leads to arteriolar smooth muscle relaxation. In addition, it is widely known that the arteriolar vascular endothelium plays an important role in vascular tension control by releasing relaxing and contracting factors to promote muscle relaxation and contraction, respectively.7,8 Compared to this detailed knowledge of the mechanism underlying vasodilatation, the differences in the vasodilatory efficacies of various compounds and in their time-dependent profiles are not fully understood.

To clarify the basic aspects of the vasodilatory efficacy and persistency of various therapeutic compounds, we investigated the vasodynamics activities of model vasodilators in an isolated perfused mesenteric artery bed of rats. Nitric oxide (NO), acetylcholine (ACh), and sodium nitroprusside (SNP) were selected as model vasodilators due to their well-known pharmacological characteristics.6,7,9,10 In addition, they involve a common process to exert vasodilatory activity. NO acts as an endothelium-derived relaxing factor,6,11 stimulating soluble guanylate cyclase to promote cGMP production from GTP. ACh interacts with muscarinic acetylcholine receptors on the endothelium, and, subsequently, it stimulates the endothelial nitric oxide synthase to generate NO, resulting in an increase in the cGMP concentration.7,9 SNP, known as an NO donor compound, diffuses into the arteriolar smooth muscle cells.12 SNP decomposes there to release NO, causing an increased cGMP concentration and vasodilation. Considering the common process of vasodilatation, we characterized their vasodilatory profiles in a model-dependent manner to clarify the mechanism accounting for the difference in their vasodilatory efficacies.

MATERIALS AND METHODS

Materials A nitric oxide/nitrogen gas mixture (1 : 9) and nitrogen gas were purchased from Air Water Inc. (Osaka, Japan). Acetylcholine chloride was kindly donated by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Sodium nitroprusside, sodium deoxycholate, and guanethidine sulfate were obtained from Sigma Aldrich Co. (St. Louis, MO, U.S.A.). M ethoxamine hydrochloride and papaverine hydrochloride were supplied by Nippon Shinyaku Co., Ltd. and Dainippon Sumitomo Pharma Co., Ltd., respectively. Other chemicals used were of the finest grade available. Krebs–Ringer bicarbonate solution (pH 7.4) used in this study contained EDTA at a concentration of 27 μM.10,12

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Animals  Male Wistar rats (250—350 g) were purchased from Japan SLIC Inc. (Hamamatsu, Japan), and they were housed at 20—25 °C and 40—50% humidity under a 12-h light/dark cycle. They were allowed free access to a standard laboratory diet and water. All animal experiments were performed in accordance with the guidelines for animal experimentation of Okayama University.

Preparation of the Isolated Perfused Mesenteric Artery Bed  The isolated perfused mesenteric artery bed was prepared according to the reported methods.10,12 After being anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally (i.p.)), each rat was fixed on its back, and a middle incision was gently made in the abdomen, exposing the jejunum and superior mesenteric artery. The exposed tissues were continually moistened by a superfusion with Krebs–Ringer solution at a constant rate of 5 ml/min, while it was bubbled with nitrogen gas for 15 min to purge dissolved oxygen. 

Vasodilation experiments were performed using the same preparation of the mesenteric artery bed with a 20-min interval between experiments. One preparation was used for one model compound. The vasodilatory activity was evaluated based on the decrease in perfusion pressure from the baseline, and it was expressed defining the maximum relaxation value as 100%13. The maximum relaxation value was given as the difference between the baseline and perfusion pressure measured when the artery bed was maximally relaxed, which was induced by introducing 100 μM papaverine into the artery bed after the series of experiments was completed. The perfusion pressure was recorded every second with a digital recording system (PowerLab; Bio Research Center Co., Nagoya, Japan) via a pressure transducer equipped with a signal amplifier (model TP-400T/AP-641G; Nihon Kohden, Tokyo, Japan).

In some experiments, the vascular endothelia of the mesenteric artery bed were removed as described previously.13 Briefly, prior to the perfusion with Krebs–Ringer solution containing 5 μM guanethidine and 2 μM methoxamine, the artery bed was perfused for 30 s with 0.9% NaCl containing deoxycholate at a concentration of 1.8 mg/ml. The artery bed was then perfused with Krebs–Ringer solution for 60 min to stabilize the perfusion pressure. The removal of endothelia was confirmed with the observation that transient vasodilatation was not induced even on the introduction of ACh at a dose of 1 nmol.9,12

Preparation of the NO Solution  The NO solution was prepared using a previously reported method.14 Ten milliliters of isotonic phosphate buffer (pH 7.4) in a test tube was bubbled with nitrogen gas for 15 min to purge dissolved oxygen.15 It was then bubbled with a nitric oxide/nitrogen gas mixture for 15 min to obtain NO-saturated solution. Impurities in the gas mixture were removed before use by it being bubbled through 1 n NaOH solution. The NO-saturated solution was then diluted by 1:1000, 1:700, 1:500, and 1:300 with the phosphate buffer. According to the literature, the NO-saturated solution contains NO at a concentration of 0.16 mM.16 The uses of 100 μl of those diluted solutions therefore correspond to NO at doses of 16, 23, 32, and 53 pmol, respectively. The NO-saturation and dilution processes were carried out every time a series of experiments was performed.

Model-Dependent Analysis of the Vasodilatory Activity  The vasodilation profiles of NO, ACh, and SNP were analyzed in a model-dependent manner with the consideration that these compounds commonly increase the intracellular NO concentration in vascular smooth muscle cells in their vasodilation processes (Fig. 1). Designating the intracellular NO concentration as C, the vasodilatory activity (E) as a percentage of the maximum relaxation value (E_{max}), can be described as follows:

$$\frac{E}{E_{max}} = \frac{C}{C_{S0} + C} = \frac{C}{1 + C_{S0}}$$

(1)

$$C_{N} = \frac{C}{E_{S0}}$$

(2)

where $E_{S0}$ is the intracellular NO concentration causing vasodilation at a strength of 50% of the maximum relaxation. $C_{N}$ is the normalized value for C. $C_{N}$ is described as a function of time, t, as follows:
where \( R_{\text{app}} \) is the normalized value reflecting the apparent rate at which the intracellular NO concentration in the vascular smooth muscle cells increases. The increase in the NO concentration is assumed to last for \( T_e \). As shown in Eq. 4, \( R_{\text{app}} \) is related to the infusion rate of the model compound (\( \dot{R}_{\text{inf}} \)) and the efficacy index of the compound (\( F \)). \( F \) is set at 1 when the vasodilation profiles in the NO experiments are analyzed. \( K \) and \( V \) are the first-order rate constant for the NO elimination process and the volume of distribution of NO in the smooth muscle cells, respectively. In the analysis, the parameters \( R_{\text{app}} \) and \( K \) were determined based on the vasodilation profiles by performing the curve-fitting calculation using the nonlinear least square method.

\[
\begin{align*}
C_N(t) &= \frac{R_{\text{app}}}{K} \cdot (1 - e^{-Kt}) & 0 \leq t < T_e \\
C_N(t) &= C_N(T_e) \cdot e^{-Kt} & T_e \leq t \\
R_{\text{app}} &= \frac{F}{EC_{50} V} \cdot \dot{R}_{\text{inf}}
\end{align*}
\]

RESULTS

Characterization of the Vasodilatory Activity of NO and ACh

As shown in Fig. 2A, immediate vasodilation was observed following an infusion of the NO solution into the mesenteric artery bed, in which the peak value of vasodilation reached 74.7% of the maximum relaxation value when the NO solution was used at a dose of 53 pmol. The peak value decreased as the infused NO dose decreased (Fig. 2A). As for the vasodilation profiles following ACh infusion, the peak value of vasodilation was 81.5% of the maximum relaxation at a dose of 60 pmol, and the vasodilatory activity decreased as the infused ACh dose decreased (Fig. 2B). In addition, after reaching the peak value, the vasodilatory activity of ACh seemed to diminish at a slower rate as compared to that of the NO solution (Fig. 2B).

These vasodilation profiles were then characterized in a model-dependent manner. With the vasodilation profiles of NO solution, it was shown that the rate of increase in the intracellular NO concentration proportionally rises as the infusion rate of NO increases (Fig. 2C). When the linear relationship is assumed, the overall correlation coefficient of the regression line is calculated to be 0.804, being large enough to reject the null hypothesis that there is no linear relationship between the rate of increase in the intracellular NO concentration and the infusion rate of NO (\( p<0.05 \)) (Fig. 2C).
Based on this result, the value of the $EC_{50} \cdot V$ product in Eq. 4 is calculated as 8.2 pmol with the efficacy index, $F$, being set at 1. As for the experiments with ACh, the intracellular NO concentration was similarly shown to increase as the infusion rate of ACh rose (Fig. 2C), in which the overall correlation coefficient of the regression line is given to be 0.697 ($p < 0.05$). Using the determined value of the $EC_{50} \cdot V$ product and the slope of the regression line in the ACh experiments, the efficacy index for ACh was calculated as 1.06, meaning that 1 mol of ACh acts as if it were roughly 1 mol of NO in terms of the vasodilatory activity.

Characterization of the Vasodilatory Activity of SNP
As shown in Fig. 3A, the vasodilation caused by SNP seemed to gradually occur as compared to that caused by NO and ACh. The peak value of the vasodilatory activity of SNP was 62.8% of the maximum relaxation value at a dose of 2000 pmol, and it decreased as the SNP dose decreased (Fig. 3A). The vasodilatory activity of SNP appeared to reduce at a much slower rate than those of NO and ACh (Figs. 2, 3A). Figure 2B shows the vasodilation profile of SNP observed in the mesenteric artery bed treated with deoxycholate to remove the endothelium. In this case, the vasodilatory activity of SNP augmented with its peak value being 67.3% of the maximum relaxation at a dose of 60 pmol (Fig. 3B). The reduction of the vasodilatory activity of SNP appeared to slow when the vascular endothelium was removed (Figs. 3A, B).

In the analysis of the vasodilation profiles, the intracellular NO concentration was shown to slowly increase as the infusion rate of SNP rose, and the efficacy index of SNP was calculated as 0.007 (Fig. 3C). Compared to this, when the mesenteric artery bed was treated with deoxycholate, the intracellular NO concentration increased at a greater rate with an efficacy index of 0.121, indicating that the vasodilatory efficacy of SNP augmented by 17.3 times when the vascular endothelium was removed (Figs. 3A, B).

Characterization of the Intracellular NO Elimination Process
Since the NO elimination process in the arteriolar smooth muscle cells influences the intracellular NO concentration and, thereby, affects the vasodilatory activity, the NO elimination rate constants were then estimated in a model-dependent manner. As shown in Fig. 4, the NO elimination rate constants were almost the same for a given vasodilator, though the rate constants in the NO experiments largely fluctuate. On the other hand, the elimination rate constants vary depending on the vasodilator employed (Table 1). This is
clearly exemplified by the observation that the vasodilatory activity of NO at a dose of 53 pmol is similar to that of ACh at a dose of 60 pmol, but the elimination rate constant given in the NO experiments is larger than that given in the ACh experiments (p<0.05) (Figs. 2, 4). In this case, the elimination half-time in the NO experiments was calculated as 5.3 s, while that in the ACh experiments was 9.8 s. As for the elimination rate constant in the SNP experiments, it was a smaller value than that obtained in the ACh experiments (Table 1). In addition, when the mesenteric artery bed was treated with deoxycholate and the vascular endothelium was removed, the elimination rate constant became significantly smaller than that obtained in experiments without deoxycholate treatment (Table 1).

DISCUSSION

Considering that the increase in the intracellular NO concentration is a common pathway of NO, ACh, and SNP through which they exert vasodilatory activity,\textsuperscript{2,6,7,9} we analyzed the vasodilation profiles of these compounds to clarify the mechanism responsible for the difference in their vasodilatory efficacies.

Firstly, we analyzed the vasodilation profiles of NO (Fig. 2), in which we infused NO solution into the mesenteric artery bed to exogenously increase the intracellular NO concentration in the vascular smooth muscle cells. In this analysis, the value of the EC\textsubscript{50} V product was determined to be 8.2 pmol, indicating that if this amount of NO is generated in the vascular smooth muscle cells, vasodilation occurs at 50% of the strength of the maximum relaxation in the mesenteric artery bed in this study. With this determined value, we then analyzed the vasodilation profiles of ACh. The vasodilation profiles of ACh seem to be similar to those of the NO solution (Fig. 2B). Based on these profiles, the efficacy index of ACh is calculated as close to unity. ACh is therefore considered to be equivalent to NO with regard to the efficacy to induce vasodilation. It was demonstrated that ACh-induced vasodilation is abolished by inhibiting soluble guanylate cyclase and by the combined use of NO synthase inhibitor and NO inactivator, indicating that the vasodilatory activity of ACh is mainly exerted \textit{via} the NO/cGMP pathway.\textsuperscript{7} ACh interacts with muscarinic acetylcholine receptors on the endothelium, initiating its vasodilatory effect. In this reaction, ACh seems to produce NO in the stoichiometric ratio of 1:1, although the NO/cGMP pathway may involve more complex reactions than those reported. The current analysis also indicated that there is a difference in the intracellular NO elimination processes between ACh and NO. That is, as shown in Fig. 4, the intracellular NO concentration in the NO experiments appeared to decrease at a faster rate than that in the ACh experiments. The precise mechanism underlying this difference is currently unclear, but it may be explained by assuming that NO molecules endogenously generated by ACh in the NO/cGMP pathway somehow elude the elimination process until they accomplish their role in the vasodilating reaction, and/or by assuming that NO molecules exogenously introduced into the cell are more quickly eradicated as compared to those endogenously generated.\textsuperscript{15} In addition, it may be rational to think that the elimination phase observed in the vasodilation profiles is a composite phase reflecting at least two processes: one is the NO elimination process in the vascular smooth muscle cells, and the other is the endothelium-independent vasodilation process which is mediated by perivascular nerves and counteracts vascular contraction.\textsuperscript{10,12,17} It has recently been revealed that non-adrenergic non-cholinergic nerves containing calcitonin gene-related peptide (CGRP) are involved in vascular relaxation,\textsuperscript{18} and that ACh indirectly stimulates CGRP-containing nerves,\textsuperscript{12,19} causing endothelium-independent vasodilation. It is likely that ACh-induced nerve-mediated vasodilation lasts for a longer duration than ACh-induced NO-mediated vasodilation, resulting in an underestimation of the NO elimination rate constant.

It is known that SNP releases NO in an equinomolar manner to exert vasodilatory activity. Although this brought about the expectation that the vasodilatory activity of SNP is as potent as that of the NO solution, it is clearly less potent (Figs. 2A, 3A). The calculated efficacy index of SNP indicates that less than 1% of the released NO by SNP contributes to the SNP-induced vasodilation, with a large part of SNP and/or its NO-releasing activity being wasted. On the other hand, it was demonstrated that the SNP-induced vasodilatory activity augments and the efficacy index increases when the vascular endothelium is removed\textsuperscript{20} (Figs. 3B, C). SNP infused in the mesenteric artery bed is thought to diffuse through the vascular endothelium into the vascular smooth muscle cells where it releases NO to exert a vasodilatory effect. Therefore, it is quite likely that the endothelium is a barrier preventing SNP from reaching the smooth muscle cells. In addition, it has also been reported that the vascular endothelium regulates the vascular tone in various ways,\textsuperscript{13} releasing an endothelium-derived contracting factor (EDCF) for counteraction when the vascular smooth muscle enters a relaxed state.\textsuperscript{9} Our observation seems to be consistent with the reported findings. That is, the vascular endothelium suppresses the SNP-induced vasodilatory activity, and since the suppressive factor is removed by treating the mesenteric artery bed with deoxycholate, SNP can fully exert its vasodilatory effect. It has also been shown that the NO elimination rate constant in the SNP experiments clearly becomes smaller when the vascular endothelium is removed (Fig. 4, Table 1), reflecting the fact that the vascular endothelium counteracts the vasodilatory activity induced by SNP.

Regarding the endothelium-mediated contraction, although both ACh and SNP exert vasodilatory activity by generating/releasing NO, there appears to be a difference in their NO elimination rate constants (Fig. 4, Table 1). That is, the intracellular NO concentration in ACh experiments decreases at a faster rate than that in SNP experiments. This suggests

<table>
<thead>
<tr>
<th>Experiment group</th>
<th>p\textsuperscript{a}</th>
<th>Elimination rate constant (s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>22</td>
<td>0.107±0.011</td>
</tr>
<tr>
<td>ACh</td>
<td>24</td>
<td>0.073±0.004\textsuperscript{b}</td>
</tr>
<tr>
<td>SNP</td>
<td>23</td>
<td>0.034±0.002\textsuperscript{c}</td>
</tr>
<tr>
<td>SNP without endothelia</td>
<td>19</td>
<td>0.008±0.001\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data are shown as the means±S.E. \textsuperscript{b} Number of experiments. \textsuperscript{c} Significantly different from the NO group (p<0.05). \textsuperscript{d} Significantly different from the ACh group (p<0.05). \textsuperscript{e} Significantly different from the SNP group (p<0.05).
that the vascular endothelium’s counteraction of the vasodilatory activity is more intensely performed in ACh than in SNP experiments. ACh generates NO in the vascular endothelium, and the generated NO diffuses into the vascular smooth muscle cells to cause muscle relaxation, while SNP diffuses into the vascular smooth muscle cells and releases NO there for vasodilation. On considering these factors in conjunction with the reported findings, it can be thought that the counteraction of the vascular endothelium, probably by releasing EDCF, is associated with the intracellular NO generation process.8)

In summary, we analyzed the vasodilation profiles of NO, ACh, and SNP in a model-dependent manner, and indicated that ACh and NO are equivalent to each other regarding their vasodilatory activity. In addition, it was shown that the vasodilatory activity of SNP is markedly suppressed due to the interference of the vascular endothelium. Pharmacodynamic analysis seems to be useful to characterize the pharmacological activity of various compounds, and to recognize mechanisms underlying the difference in their therapeutic efficacies.

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REFERENCES