Antioxidative Action of the Nitrovasodilator Nicorandil: Inhibition of Oxidative Activation of Liver Microsomal Glutathione S-Transferase and Lipid Peroxidation

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ABSTRACT—Antioxidative effects of the nitrovasodilator nicorandil (SG-75) and denitrated SG-75 (SG-86) were examined in vivo and in vitro. When the isolated rat liver was reperfused with Krebs-Henseleit solution after a 90-min ischemia, microsomal GSH S-transferase activity was increased significantly by oxidative modification of the sulfhydryl group of the enzyme. The increase in the transferase activity after ischemia/reperfusion was depressed by SG-75 but not by SG-86. Furthermore, only SG-75 significantly inhibited lipid peroxidation and the activation of microsomal GSH S-transferase induced by hydrogen peroxide treatment of liver microsomes. These data indicate that SG-75 has an antioxidative action and the nitro group of SG-75 may play a critical role for this action.

Keywords: Nicorandil, Antioxidant, Lipid peroxidation, Glutathione S-transferase, Liver microsome

Nicorandil, [SG-75, N-(2-hydroxyethyl)-nicotinamide nitrate; Fig. 1] has been used as a nitrovasodilator for the treatment of angina pectoris. In addition to the vasodilative action of SG-75, recent evidence also indicates that it has anti-free radical and neutrophil-modulating properties in vitro: iron-mediated hydroxyl radical production and superoxide anion formation from neutrophils were inhibited by SG-75 (1, 2). Since reactive oxygen species generated during ischemia/reperfusion of organs cause harmful effects or dysfunction (3–6), the anti-free radical action of SG-75 seems favorable for the treatment of ischemic diseases. In our laboratory, it has been clarified that the liver microsomal glutathione (GSH) S-transferase of rats is activated through oxidative modification by reactive oxygen species in vitro (7–9) and by oxidative stress such as ischemia/reperfusion of isolated rat liver (10). To evaluate the antioxidative effect of SG-75, we determined whether the oxidative activation of liver microsomal GSH S-transferase caused by ischemia/reperfusion of the liver is depressed by this agent. Furthermore, the inhibitory effect of SG-75 on lipid peroxidation was also examined in vitro.

MATERIALS AND METHODS

Chemicals

Reagents used in the experiments were obtained from the following sources: reduced glutathione (GSH), cumene hydroperoxide and 1,1,3,3-tetramethoxypropane (Sigma Chemical Co., St. Louis, MO, USA); NADPH (Oriental Yeast, Tokyo); heparin (Nacalai Tesque, Kyoto); hydrogen peroxide (Santoku Kagaku, Tokyo); and sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA). All other chemicals used were of analytical grade.
Animals and liver perfusion

Male Sprague-Dawley rats (150–300 g) that had been starved overnight were used. After anesthetizing the rat with sodium pentobarbital (50 mg/kg, i.p.), the liver was removed and continuously perfused with Krebs-Henseleit solution (control) or reperfused for 60 min after a 90-min ischemia as previously reported (10). In the case of treatment with agents, the reperfusion buffer contained 1 mM SG-75 or SG-86. Liver cytosol and microsomes were prepared as described previously (8).

Measurement of enzyme activity and lipid peroxidation

GSH S-transferase and GSH peroxidase activities were measured by the methods of Habig et al. (11) and Reddy et al. (12) using 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide as substrates, respectively. Lipid peroxidation in microsomes was measured by the method of Buege and Aust (13) as follows: liver microsomes (0.5 mg/ml) prepared from untreated rats were incubated with 0.75 mM hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.4) at room temperature for 30 min in the presence or absence of agents in 1 ml, and then 2 ml of thiobarbituric acid (0.375% in trichloroacetic acid) was added. After heating at 100°C for 15 min followed by centrifugation of the reaction mixture, the 535 nm absorbance of the supernatant was measured. Thus lipid peroxidation was calculated as thiobarbituric acid reactive substances (TBARS) using 1,1,3,3-tetramethoxypropane as the standard. Denitration from nitrates was measured by the method of Habig et al. (11). Protein concentration was determined by the method of Lowry et al. (14).

Statistical analyses

Data were expressed as the mean ± S.D. Significance of difference was calculated by Student's t-test, where P values < 0.05 were taken as significant.

RESULTS

Effect of SG-75 or SG-86 on GSH S-transferase activation by ischemia/reperfusion of liver

As shown in Table 1, microsomal GSH S-transferase activity, but not cytosolic transferase, was increased significantly after ischemia/reperfusion of the isolated liver, and the increase was depressed when SG-75 was added into the reperfusion buffer. The microsomal transferase activity was not altered by perfusion with SG-75 alone without ischemia. When the ischemic liver was reperfused with SG-86, the microsomal GSH S-transferase activity was slightly increased.

Table 2 shows the activation of microsomal GSH S-transferase by the SH-alkylation with N-ethylmaleimide after ischemia/reperfusion with or without SG-75 and SG-86. The transferase activity was increased by N-ethylmaleimide to 3.3-fold in the control, but increased to 4.0-fold after ischemia/reperfusion. When the ischemic liver was reperfused with SG-75, the activation of the enzyme by the agent was similar to the control, whereas in the case of reperfusion with SG-86, the increase in the transferase activity was 4.0-fold as compared to 4.6-fold in the control. The microsomal GSH S-transferase activity was decreased to about 90% of the initial level by dithiothreitol in the control and SG-75-treated liver, but

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Microsome</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GST</td>
<td>GSH-Px</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(µmol/mg/min)</td>
<td>(µmol/mg/min)</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>0.066±0.011</td>
<td>0.056±0.010</td>
</tr>
<tr>
<td>I/R</td>
<td>7</td>
<td>0.082±0.007*</td>
<td>0.061±0.012</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0.062±0.017</td>
<td>0.046±0.007</td>
</tr>
<tr>
<td>I/R + SG-75</td>
<td>6</td>
<td>0.053±0.007</td>
<td>0.039±0.011</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>0.077±0.018</td>
<td>0.051±0.013</td>
</tr>
<tr>
<td>SG-75</td>
<td>4</td>
<td>0.074±0.023</td>
<td>0.045±0.016</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>0.075±0.006</td>
<td>0.048±0.013</td>
</tr>
<tr>
<td>I/R + SG-86</td>
<td>4</td>
<td>0.080±0.009</td>
<td>0.048±0.011</td>
</tr>
</tbody>
</table>

The isolated liver was continuously perfused (control) or reperfused for 60 min with or without 1 mM of SG-75 or SG-86 after a 90-min ischemia. GSH S-transferase (GST) and GSH peroxidase (GSH-Px) activities in the cytosol and microsomes were measured as described in Materials and Methods. Values are presented as the mean ± S.D. *: The liver was perfused with 1 mM SG-75 for 60 min without ischemia. Control vs. treated, *P < 0.05.
was about 70% after ischemia/reperfusion or after reperfusion with SG-86. These results demonstrated that the SH group in the microsomal transferase was decreased after ischemia/reperfusion or after reperfusion with SG-86, whereas the decrease in the thiol content of the enzyme was recovered in the SG-75-reperfused liver.

**Antioxidant effect of nitrates in vitro**

The effect of SG-75 and SG-86 on hydrogen peroxide-induced lipid peroxidation and activation of microsomal GSH S-transferase in vitro is presented in Table 3. The transferase activity was increased to 1.7-fold by treatment of the microsomes with hydrogen peroxide and to 1.5-fold in the presence of SG-75. The increase in the transferase activity by the oxidant was not altered by SG-86. Hydrogen peroxide-induced lipid peroxidation of liver microsomes was significantly inhibited in the presence of SG-75 but not in the presence of SG-86. As shown in Fig. 2, SG-75 inhibited lipid peroxidation dose-dependently.

Of the nitrates, nipradilol and isosorbide dinitrate caused a 63% and a 21% inhibition of hydrogen peroxide-induced lipid peroxidation, respectively, compared to the 29% inhibition by SG-75. Nitroglycerin did not affect the lipid peroxidation (Table 4).

**Denitration of nitrates in liver microsomes**

The amounts of nitrite anion released from various nitrates in liver microsomes are shown in Table 5. Nitroglycerin and isosorbide dinitrate released nitrite anions, but SG-75 and nipradilol did not.

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### Table 2. Effect of dithiothreitol or N-ethylmaleimide on microsomal GSH S-transferase (GST) activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>GST-activity (μmol/mg/min)</th>
<th>N-ethylmaleimide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>none</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>0.066±0.011</td>
<td>0.057±0.009 (87±9)</td>
</tr>
<tr>
<td>I/R</td>
<td>7</td>
<td>0.082±0.007*</td>
<td>0.059±0.010 (72±7**)</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0.062±0.017</td>
<td>0.055±0.017 (83±10)</td>
</tr>
<tr>
<td>I/R + SG-75</td>
<td>6</td>
<td>0.053±0.007</td>
<td>0.048±0.007 (88±7*)</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>0.075±0.006</td>
<td>0.066±0.008 (87±10)</td>
</tr>
<tr>
<td>I/R + SG-86</td>
<td>4</td>
<td>0.080±0.009</td>
<td>0.059±0.011 (73±8)</td>
</tr>
</tbody>
</table>

Microsomes prepared from control- and ischemia/reperfusion-livers were incubated with 10 mM dithiothreitol for 10 min or with 1 mM N-ethylmaleimide for 2 min at room temperature, and then GST activity was measured as described in Materials and Methods. Values are presented as the mean±S.D. The value in the parentheses shows percent of none-treated GST activity. Control vs. treated, *P<0.05, **P<0.01; I/R vs. SG-75-treated, †P<0.05.

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### Table 3. Effect of SG-75 or SG-86 on hydrogen peroxide-dependent increase in microsomal GSH S-transferase (GST) activity and lipid peroxidation in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H₂O₂</th>
<th>GST activity (nmol/mg/min)</th>
<th>TBARS (nmol MDA/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−</td>
<td>0.127±0.016</td>
<td>0.263±0.028</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.219±0.035**</td>
<td>1.958±0.137*</td>
</tr>
<tr>
<td>SG-75 (1 mM)</td>
<td>+</td>
<td>0.190±0.027**</td>
<td>1.393±0.238***‡‡‡</td>
</tr>
<tr>
<td>SG-86 (1 mM)</td>
<td>+</td>
<td>0.212±0.020*</td>
<td>1.864±0.131**‡‡‡</td>
</tr>
</tbody>
</table>

Liver microsomes prepared from nontreated rats were incubated with 0.75 mM hydrogen peroxide (H₂O₂) in the presence or absence of SG-75 or SG-86 for 30 min at room temperature. GST activity and lipid peroxidation (TBARS) in the incubation mixtures were measured as described in Materials and Methods. Values are presented as the mean±S.D. of three incubations. Control vs. treated, *P<0.01, **P<0.05; H₂O₂ vs. nitrate-treated, †P<0.05, ‡‡‡P<0.01.

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![Fig. 2. Effect of various concentrations of SG-75 on hydrogen peroxide-induced lipid peroxidation of liver microsomes in vitro. Microsomes prepared from nontreated rats were incubated with 0.75 mM hydrogen peroxide in the presence or absence of SG-75 for 30 min at room temperature, and lipid peroxidation was measured as thiobarbituric acid-reactive substance (TBARS). The TBARS value in the control was 1.43±0.10 nmol/mg, and each point shows the mean of duplicate incubations.](image-url)
DISCUSSION

SG-75 is a vasodilator with one nitro group in its molecular structure, and it is used clinically for the treatment of ischemic diseases such as angina pectoris. It is well known that ischemia-reflow of tissue organs can generate reactive oxygen species, leading to tissue damage (3-6). Thus if SG-75 has an antioxidative action, it is favorable for the treatment of ischemic diseases. Indeed, it has been demonstrated that SG-75 scavenges hydroxyl radicals and inhibits superoxide anion production in neutrophils in vitro (1, 2). In the present study, we confirmed the antioxidative action of SG-75 using ischemia/reperfusion of the isolated liver of rats and also microsomal lipid peroxidation. Liver microsomal GSH S-transferase, as reported previously (10), is activated by oxidation of the sulfhydryl group in the enzyme during oxidative stress such as that occurring when the isolated liver is reperfused after ischemia or is perfused with hydrogen peroxide. Since SG-75 itself does not inhibit microsomal GSH S-transferase activity, it is possible to evaluate the antioxidant effect of SG-75 by measuring the transferase activation in ischemia/reperfusion of the liver. As shown in Tables 1 and 2, when the ischemic liver was perfused with the buffer containing SG-75, the increase in the microsomal GSH S-transferase activity seen in the liver after ischemia/reperfusion was prevented. Furthermore, although microsomal GSH S-transferase is activated by N-ethylmaleimide via covalent binding with the SH group, the activation was decreased after ischemia/reperfusion of the liver and was recovered by reperfusion with SG-75. Since reactive oxygen species produced during ischemia/reperfusion of the liver react with the protein thiol resulting in oxidative activation of the transferase (10), it is clear that an oxidation of the SH group in the enzyme during reperfusion of the ischemic liver was inhibited by SG-75. Thus, it was demonstrated that SG-75 acts as an antioxidant in ischemia/reperfusion of the liver.

The antioxidative action of SG-75 was further examined in vitro. When 0.75 mM H₂O₂ was incubated with liver microsomes, lipid peroxidation and the activation of glutathione S-transferase induced by H₂O₂ were inhibited by SG-75 (Fig. 2, Table 3). Since the lipid peroxidation and the activation of glutathione S-transferase in microsomes by H₂O₂ increase linearly with H₂O₂ concentration, the in vitro experiments using 0.75 mM H₂O₂ may reflect an internal condition that occurs during oxidative stress. Thus these data indicate that SG-75 acts as an antioxidant not only in vitro but also in vivo.

In contrast to SG-75, SG-86 that lacks the nitro group in the structure of SG-75 did not show the antioxidative effect. Thus it was suggested that the nitro group in SG-75 is critical for the antioxidative effect of this agent.

To clarify the role of the nitro group in the prevention of lipid peroxidation, other nitrovasodilators were examined. Hydrogen peroxide-induced lipid peroxidation was inhibited markedly by nipradilol, moderately by SG-75 and isosorbide dinitrate, and scarcely by nitroglycerin. These results show that the nitro group may contribute to the antioxidative action of nitrates. Since vasodilation of nitrates is mediated by nitric oxide formed via nitrite anion (15), and nitric oxide can act as an antioxidant in some conditions (16), it is presumed that nitric oxide released from nitrates relates to an antioxidative effect. However, when nitrates were incubated with liver microsomes, the denitration (nitrite anion release) was observed only in the case of nitroglycerin or isosorbide dinitrate which show slight depression of lipid peroxidation in the microsomes. Furthermore, our preliminary study showed that more nitric oxide was formed from nitroglycerin or isosorbide dinitrate than SG-75 or nipradilol. Thus, SG-75 seemed to have an antioxidative action through a mechanism other than nitric oxide formation. Pieper and Gross (2) reported that the nicotinamide
moiety of SG-75 was involved in its anti-free radical property. It is therefore likely that the antioxidative effect of SG-75 depends upon the molecular structure containing the nitro group, but not nitric oxide formation. Although SG-75 acts as a K⁺-channel opener (17, 18), the K⁺-channel opener analog pinacidil did not inhibit the lipid peroxidation (data not shown), suggesting that the antioxidative action of SG-75 is not mediated via a K⁺-channel. Thus it is expected that SG-75 produces the antioxidative action through forcing structural interactions with lipid membranes and the nitro group of SG-75 may play a role in the interaction. Concerning the antioxidant action of nipradilol, it may be attributable to a molecular structure with β-adrenergic receptor blocking action as seen in other β-blockers (19).

Thus an antioxidative effect of SG-75 was established in vitro and in vivo. This action of SG-75 may involve a structure-membrane association but is not due to the interaction with a K⁺-channel or release of nitric oxide. In addition to its vasodilative action, the antioxidative effect of SG-75 may be favorable for the treatment of ischemic diseases, like angina pectoris.

Acknowledgments

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