Inhibition of Gastric Glucosamine Synthetase Activity by Oxygen Radicals: A Possible Cause of Decreased Mucosal Protective Capacity

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ABSTRACT—To clarify the role of oxygen radicals in the mucus metabolism of the gastrointestinal tract, the effect of oxygen radicals on the activity of glucosamine synthetase, the rate-limiting enzyme of mucus synthesis, was investigated using homogenate derived from rat gastric mucosa. The simultaneous addition of both xanthine and xanthine oxidase caused a significant inhibition of the enzyme activity, and this decrease was counteracted by catalase, but not by superoxide dismutase. Hydrogen peroxide also caused a significant decrease in the enzyme activity; and this effect of hydrogen peroxide was counteracted by catalase and dithiothreitol, but not by mannitol, dimethyl sulfoxide and reduced glutathione. The inhibition of glucosamine synthetase activity by oxygen radicals is considered to be caused by the oxidation of sulfhydryl groups of the enzyme molecule. The present results also suggest that oxygen radicals in the gastrointestinal tract may induce the suppression of a protective mechanism of the gastric mucosa by inhibiting glucosamine synthesis activity.

Recent studies have demonstrated that oxygen-derived free radicals play important roles in the pathogenesis of ischemia-induced mucosal lesions (1) and these lesions are attributed to the degeneration of deoxyribonucleic acids, enzymes and biomembranes by oxygen radicals. On the other hand, gastric mucus is known as one of the protective factors against gastric mucosal damage, and its quantitative and qualitative changes have been reported to be associated with the formation of gastric ulcers induced by several experimental procedures (2). Furthermore, it has been reported that the alteration in the enzyme activities involved in mucus metabolism also plays an important role in the occurrences of experimental ulcer (3) as well as various diseases in humans (4). One of the mechanisms underlying such protective properties of gastric mucus on various gastrointestinal damages is the ability of gastric mucus to scavenge oxygen radicals (5). Actually, no data is available, however, that indicates a direct effect of oxygen radicals on mucus metabolism in the alimentary tract. Therefore, in the present study, we examined the effect of oxygen radicals on the activity of glucosamine synthetase, the rate-limiting enzyme of mucus synthesis, using a crude homogenate of gastric mucosa.
MATERIALS AND METHODS

Enzyme extract
Male Wistar rats weighing 200 g were used. Rats were housed in our animal facilities under standardized conditions for one week and were not fasted before the experimental procedure. Rats were killed by an overdose of ether, and the glandular part of the stomach was removed. The whole wall of gastric tissue was homogenized by a Polytron homogenizer (dial 6, 30 sec) in ice-cold solution containing 12 mM glucose-6-phosphate, 1 mM ethylenediamine tetraacetic acid, and 154 mM KCl (pH 7.5). The homogenate was then centrifuged (100,000 × g, 4°C, 30 min), and the resultant supernatant was used as the enzyme extract.

Assay of glucosamine synthetase activity
Glucosamine synthetase activity was measured according to the method of Sander et al. (3), using fructose-6-phosphate and glutamine as substrates. The reaction mixture consisting of 0.5 ml of the enzyme extract and 0.5 ml of 80 mM sodium phosphate buffer (6 mM D-fructose 6-phosphate, 12 mM L-glutamine, 1 mM EDTA, pH 7.5) was incubated at 37°C for 2 hours. The reaction was stopped by placing the reaction mixture in boiling water for 1 min. Glucosamine-6-phosphate, the product formed in the reaction catalyzed by glucosamine synthetase, was measured using the method of Neuhaus and Letzring (6), and enzyme activity was expressed as micrograms of product formed per milligram of protein per hour. Protein was measured according to the method of Lowry et al. (7), using bovine serum albumin as a standard.

Experiment 1. Effects of xanthine and xanthine oxidase on the glucosamine synthetase activity
Xanthine (0.875 mM) and xanthine oxidase (5, 50 mU/ml) were simultaneously added into the enzyme extract and incubated at 37°C for 40 min. The reaction was terminated by the addition of catalase (40 μg/ml) and superoxide dismutase (200 μg/ml), and then glucosamine synthetase activity was measured. The same procedure was also carried out in the presence of xanthine alone, xanthine oxidase alone, or distilled water. The activity obtained in the presence of distilled water instead of xanthine and xanthine oxidase was defined as the control value.

Experiment 2. Effects of catalase and superoxide dismutase on the inhibition of glucosamine synthetase activity induced by xanthine and xanthine oxidase
Immediately after the addition of catalase (0.4, 4 μg/ml) or superoxide dismutase (2, 20, 200 μg/ml), xanthine (0.875 mM) and xanthine oxidase (5, 50 mU/ml) were simultaneously added into the enzyme extract and incubated at 37°C for 40 min. The reaction was terminated by the addition of catalase (40 μg/ml) and superoxide dismutase (200 μg/ml), and then glucosamine synthetase activity was measured. The same procedure was also carried out with distilled water instead of catalase, superoxide dismutase, xanthine and xanthine oxidase. The activity obtained in the presence of distilled water was defined as the control value.

Experiment 3. Effect of hydrogen peroxide on the glucosamine synthetase activity
Hydrogen peroxide was added into the enzyme extract at the concentrations of 10⁻⁴ to 10⁻² M and incubated at 37°C for 3, 5, or 10 min. The reaction was terminated by adding catalase (40 μg/ml), and glucosamine synthetase activity was measured. The same procedure was done with distilled water instead of hydrogen peroxide, and the activity obtained with distilled water was defined as the control value.

Experiment 4. Effect of various drugs on the inhibition of glucosamine synthetase activity induced by hydrogen peroxide
Immediately after the addition of catalase (1, 10, 40 μg/ml), mannitol, dimethyl sulfoxide, dithiothreitol, or reduced glutathione (10⁻⁴, 10⁻³, 10⁻² M), hydrogen per-
oxide was added at the concentration of $10^{-2}$ M into the enzyme extract and incubated at 37°C for 10 min. Incubation was stopped by the addition of catalase (40 μg/ml), and glucosamine synthetase activity was measured. The same procedure was done with distilled water instead of various drugs and hydrogen peroxide, and the activity obtained in the presence of distilled water was defined as the control value.

**Statistics**
Data are expressed as a percentage of the control value and presented as the means ± S.E. obtained from three separate experiments. Dunnett's test was used to determine statistical significance.

**Reagents**
Catalase (from bovine liver) and superoxide dismutase (SOD) (from bovine erythrocytes) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Xanthine oxidase (from cow milk) was obtained from Boehringer Mannheim GmbH (Mannheim, F.R.G.). Other chemicals used were available locally and of analytical grade.

**RESULTS**

**Effect of xanthine and xanthine oxidase on glucosamine synthetase activity**
The simultaneous addition of xanthine and xanthine oxidase caused a significant decrease of glucosamine synthetase activity. Xanthine or xanthine oxidase alone had no effect on the enzyme activity (Fig. 1).

**Effect of catalase and superoxide dismutase on xanthine and xanthine oxidase-induced inhibition of glucosamine synthetase activity**
The decrease of glucosamine synthetase activity induced by xanthine and xanthine oxidase was abolished by catalase, in a concentration-dependent manner (Fig. 2), whereas SOD had no effect on the decrease of glucosamine synthetase activity (Fig. 3).

**Effect of hydrogen peroxide on glucosamine synthetase activity**
The addition of hydrogen peroxide significantly inhibited glucosamine synthetase activity in a concentration-dependent manner (Table 1). The extent of the decrease in enzyme activity in the 3-min incubation was similar to those in the 5- and 10-min incubations (Table 2). Based on these experimental results, $10^{-2}$ M of hydrogen peroxide and 10 min of incubation were chosen as the experimental conditions for determining the effects of various drugs on glucosamine synthetase activity.

**Effect of various drugs on glucosamine synthetase activity in the presence of hydrogen peroxide**
The inhibition of glucosamine synthetase
Fig. 2. Effect of catalase on the decreased glucosamine synthetase activity induced by xanthine (0.875 mM) and xanthine oxidase (50 mU/ml). *P < 0.01, compared with the value without catalase (Dunnett's test). Each number in this figure is the mean ± S.E. obtained from three separate experiments. The control value was 6.2 ± 0.3 μg/mg protein/hr.

Fig. 3. Effect of superoxide dismutase on the decreased glucosamine synthetase activity induced by xanthine (0.875 mM) and xanthine oxidase (50 mU/ml). Each number in this figure is the mean ± S.E. obtained from three separate experiments. The control value was 6.2 ± 0.3 μg/mg protein/hr.

Table 1. Effect of various concentrations of hydrogen peroxide on glucosamine synthetase activity

<table>
<thead>
<tr>
<th>Hydrogen peroxide (M)</th>
<th>Glucosamine synthetase activity (% of control)</th>
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<tbody>
<tr>
<td>10^{-4}</td>
<td>98.2 ± 7.9</td>
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<tr>
<td>5 × 10^{-3}</td>
<td>82.9 ± 6.6</td>
</tr>
<tr>
<td>10^{-3}</td>
<td>68.4 ± 4.1*</td>
</tr>
<tr>
<td>10^{-2}</td>
<td>34.0 ± 0.8*</td>
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</tbody>
</table>

Incubation was carried out with various concentrations of hydrogen peroxide for 10 min. *P < 0.01, compared with the control value (Dunnett's test). Each number in this table is the mean ± S.E. obtained from three separate experiments. The control value was 9.0 ± 2.9 μg/mg protein/hr.

Table 2. Effect of hydrogen peroxide on glucosamine synthetase activity

<table>
<thead>
<tr>
<th>Hydrogen peroxide (M)</th>
<th>Glucosamine synthetase activity (% of control)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3 min</td>
</tr>
<tr>
<td>10^{-3}</td>
<td>63.4 ± 4.5</td>
</tr>
<tr>
<td>10^{-2}</td>
<td>27.2 ± 1.3</td>
</tr>
</tbody>
</table>

Incubation was carried out with 10^{-3} or 10^{-2} M of hydrogen peroxide for 3, 5, 10 min as indicated. Each number in this table is the mean ± S.E. obtained from three separate experiments. The control value was 12.7 ± 7.3 μg/mg protein/hr.
activity induced by hydrogen peroxide was counteracted by both catalase and dithiothreitol, while such antagonistic effects were not found in the case of mannitol and dimethyl sulfoxide. On the other hand, reduced glutathione \((10^{-3} - 10^{-2} \text{ M})\) induced a further decrease of enzyme activity (Fig. 4).

**DISCUSSION**

Glucosamine synthetase (\(L\)-glutamine-\(D\)-fructose-6-phosphate aminotransferase, EC. 2.6.1.16) is a cytoplasmic enzyme that catalyzes the synthesis of glucosamine-6-phosphate from fructose-6-phosphate and glutamine. It is well-known that glycoproteins consist of a protein skeleton on which complex carbohydrate side chains are assembled. In mucus, approximately half of such carbohydrate components is \(N\)-acetylglucosamine, \(N\)-acetylgalactosamine or sialic acid, and these particular sugars are known to be supplied to the glycoprotein molecule from UDP-\(N\)-acetylglucosamine. Since there is feedback inhibition of glucosamine synthetase by UDP-\(N\)-acetylglucosamine, this enzyme is considered to be the rate-limiting step in the biosynthesis of both UDP-\(N\)-acetylglucosamine and glycoprotein (8). This enzyme is known to be distributed in the submaxillary gland, cartilage, liver and intestine, all of which synthesize and export large amounts of glycoproteins or mucopolysaccharides. The modulation of this enzyme activity by thyroid hormone (9), testosterone (10), epidermal growth factor (EGF) (11), anti-inflammatory agents (12), bacilysin (13) and an atherogenic diet (14) was also reported. Because the decrease of this enzyme activity was

![Fig. 4. Effect of various drugs on the decreased glucosamine synthetase activity induced by hydrogen peroxide \((10^{-2} \text{ M})\). *P < 0.01, compared with each value obtained in the presence of hydrogen peroxide \((10^{-2} \text{ M})\) alone (Dunnett's test). Each number in this figure is the mean ± S.E. obtained from three separate experiments. The control value was \(9.1 ± 1.7 \mu \text{g/mg protein/hr.}\)](image)
found in the stomach under cold restraint of rats (3) and in the colon of patients with ulcerative colitis and Crohn's disease (4), it has been considered that the changes in this enzyme activity may play an important role in the formation of mucosal injury of the gastrointestinal tract.

Concerning the interaction between oxygen radicals and gastric mucin, Grisham et al. (5) have reported that mucin scavenges oxidants in vitro and that it does so at the expense of its viscoelastic properties. Although the presence of a close relationship between them is assumed, no report has been available until the present one on the effect of oxygen radicals on the enzymes related to glycoprotein metabolism. Therefore, the effect of oxygen radicals on glucosamine synthetase activity in vitro was investigated in this study.

At first, xanthine and the xanthine oxidase system was used for generating oxygen radicals. Although xanthine and xanthine oxidase themselves had no effects on glucosamine synthetase activity, simultaneous addition of both xanthine and xanthine oxidase induced a significant decrease of the enzyme activity. These results suggest that oxygen radicals derived from this experimental system may affect the enzyme activity. To determine what kinds of oxygen radicals affect the enzyme activity, SOD, a scavenger for superoxide radicals, and catalase, a scavenger for hydrogen peroxide, were added into this experimental system. Since it has been found that only catalase antagonizes the decrease of enzyme activity induced by xanthine and xanthine oxidase, the oxygen radical involved in the decrease of glucosamine synthetase activity has been considered to be hydrogen peroxide or its derivatives but not superoxide radical. In fact, xanthine and xanthine oxidase system has been reported to generate superoxide radical and/or hydrogen peroxide under the experimental conditions used in this study (15). Furthermore, Hiraishi et al. (16) have reported that the cytotoxicity induced by xanthine and the xanthine oxidase system in cultured rat gastric mucosal cells is antagonized by catalase, but not by SOD.

The effect of hydrogen peroxide on glucosamine synthetase activity was further confirmed by the addition of hydrogen peroxide itself to the enzyme extract. The enzyme activity was markedly decreased by the addition of hydrogen peroxide, and this inhibitory effect was found to reach its maximum level within 3 min. Since the decrease of the enzyme activity induced by hydrogen peroxide was abolished by dithiothreitol, a protective agent for sulfhydryl groups, this decrease was considered to be due to the oxidation of sulfhydryl groups in the enzyme molecule. The cloning study has demonstrated that the derived sequence of glucosamine synthetase contains 609 amino acids and cysteine is localized in residue 2 of the sequence (17). These findings are likely to support the present results that the oxidation of the sulfhydryl group may be involved in the hydrogen peroxide-induced decrease of glucosamine synthetase activity. Wills (18) have also reported that succinyl peroxide is a powerful inhibitor for several sulfhydryl-containing enzymes and the site of reaction of peroxide with enzymes is the sulfhydryl groups of the cysteine residues or the sulphur of the methionine residues, either or both of which may be oxidized. The results presented in this study are in essential agreement with these descriptions of Wills (18).

Reduced glutathione was not effective for preventing the decrease of the enzyme activity induced by hydrogen peroxide, although reduced glutathione is known as a reducing agent widely distributed in animals. Liebler et al. (19) have also reported that reduced glutathione acts as an oxidative agent in the presence of Fe$^{2+}$. Therefore, the lack of effect of reduced glutathione on the hydrogen peroxide-induced decrease of glucosamine synthetase activity may also be due to the presence of Fe$^{2+}$ in the assay system used in this study, although the exact reason for this lack of the effect of reduced glutathione is unclear at present.

The decrease of the enzyme activity was clearly antagonized by catalase, a scavenger of
hydrogen peroxide, but not by mannitol and dimethyl sulfoxide, scavengers of hydroxyl radicals. From these results, it seems likely that the observed decrease of the enzyme activity may be due to hydrogen peroxide itself and not the hydroxyl radicals evolved. Considering the high reactivity of hydroxyl radicals and weak specificity of scavengers used, further examination, however, may be necessary to clarify the exact cause of the differential effects of these scavengers on glucosamine synthetase activity.

Both production and scavenging systems for oxygen radicals are known to be distributed separately in various cells and organs. For example, catalase is mainly localized in peroxisomes. Since the centrifuged supernatant of the stomach tissue homogenate was used in this experiment, it seems possible that the results obtained in this study may not be directly applicable to the gastric glucosamine synthetase activity in vivo. However, taking into consideration that the cell membrane has a high permeability to hydrogen peroxide, it seems likely that this type of modulatory effect of hydrogen peroxide on the enzyme activity may also occur in vivo. In fact, it has been reported that the activity of xanthine oxidase, one of the oxygen radical derived systems, is high (20) in gastrointestinal tissues, while that of SOD, one of the oxygen radical scavenging systems is low (21). Therefore, these tissues are considered to be damaged easily by oxygen radicals and subsequently decrease mucus synthesis, which in turn, induces the decrease in mucosal protective capacity.

REFERENCES


