The Role of Cationized Catalase and Cationized Glucose Oxidase in Mucosal Oxidative Damage Induced in the Rat Jejunum*

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The successful prevention of hydrogen peroxide-induced damage to the rat jejunal mucosa by cationized catalase is described in this study. Biological damage was induced in a closed circulating intestinal loop of the rat by hydrogen peroxide and by hydroxyl radicals induced in situ via the metal-mediated Haber-Wies reaction. The mucosal activity of lactate dehydrogenase and the amount of potassium ions were used to quantitatively characterize the tissue damage. Catalase was cationized by reacting it with N,N'-dimethyl-1,3-propanediamine to give a soluble product or with polyhistidine to give an insoluble product. The activity of the modified enzymes was assessed, and their ability to protect the rat jejunal mucosa against oxidative stress was studied. It was found that in all cases the cationized enzymes were superior to the native catalase in their shield capability. A significant protection against Fe(II)/H2O2 and ascorbic acid/copper ion-mediated damage was obtained when the cationized enzymes were used.

In the presence of glucose, native glucose oxidase failed to cause damage in the rat jejunal mucosa; however, the cationized enzyme caused profound tissue injury. These findings indicate the potential therapeutic merit of cationized enzymes for the treatment of pathological processes in the intestine, whenever oxidative stress is involved.

Reactive oxygen species such as hydrogen peroxide, superoxide radicals, hydroxyl radicals, and lipid peroxidation products are initiators of gastrointestinal epithelium injuries associated with inflammatory and ischemic bowel diseases, gastric ulceration, radiation enteritis, or colon cancer (1-4). Ischemic and post-ischemic processes in the intestinal vasculature, ingested food, catalase-negative bacteria, oxidases and substrate from sloughed cells, saliva, and cigarette smoke are possible sources for the hazardous oxygen metabolites (2). It was reported that the mucous lining of the gastrointestinal tract and of the tracheobronchial tissue possesses antioxidant properties (2, 5). However, a continued exposure to efflux of oxygen-reactive species may cause biological damage to the epithelial cells of the stomach and intestine (2). Hydrogen peroxide can cause a direct damage to proteins and other cellular macromolecules (6). It may also mediate the generation of the highly reactive hydroxyl radicals via the metal-mediated Haber-Wies reaction (7). The hydroxyl radical has been shown to cause a reduction in the viscosity of mucin, thus reducing its ability to protect the gastrointestinal epithelium (2).

We suggest that it is possible to increase the mucosal protection against damage mediated by hydrogen peroxide by shielding the tissue with a suitable defending agent. The enzyme catalase is an obvious candidate for that purpose (8). Catalase inhibits cell killing by leukocytes in vitro (9, 10). Together with superoxide dismutase and peroxidase it prevents oxidative damage in vivo (11-14). Since hydrogen peroxide production may occur in the immediate surrounding of the epithelium cells, we hypothesized that tissue protection by catalase may be more efficient if the enzyme could be anchored to a desired location in the intestine, where the oxidative damage is expected.

Attachment of catalase to epithelial cells could be accomplished by a chemical modification of the enzyme, namely coating it with a polycationic substance, which will interact with the anionic sites of the epithelium cells to form a positively charged complex (8, 15) without damaging its catalytic activity. Alternatively, the catalase carboxyl groups can be coupled to an amino reagent. Various attempts to attach cationized proteins to mammal tissues have been described (16). Attachment of modified enzymes to the intestinal mucosa to prevent oxidative stress in vivo has, to the best of our knowledge, never been reported.

Quantitative evaluation of mucosal injury can be detected by following enterocyte level of potassium ions and lactate dehydrogenase activity. It has been shown that damage to the mucosa will lead to leakage of these biochemical markers from the cell (17).

The purposes of this study were as follows: (a) to characterize the biological damage induced by hydrogen peroxide and hydroxyl radical in vivo in the rat jejunal mucosa; (b) to protect the rat jejunal mucosa from oxidative stress by tissue binding of cationized catalase; and (c) to induce oxidative damage in the jejunal mucosa by the enzymatic production of hydrogen peroxide via tissue binding of cationized glucose oxidase.

**MATERIALS AND METHODS**

All materials and reagents were purchased from Sigma, unless otherwise mentioned in the text. All solvents were analytical grade.

**Perfusion System**

Male Sabra rats, 200-230 g each, were anesthetized by an intraperitoneal injection of Equitensine solution (equivalent to 6 mg/100
Cationized Enzymes and Intestinal Oxidative Damage

**Hydrogen Peroxide**—Hydrogen peroxide was introduced to the perfused jejunal mucosa by the following ways: (a) direct administration of 176 mM H₂O₂ to the perfusion system (30 min); (b) circulation of glucose oxidase (GO) \(^1\) (EC 1.1.3.4) (132 units) in 1 mM phosphate buffer, pH 7.2, for 30 min followed by a 30-min perfusion of glucose (60 mM) (c) circulation of a mixture of glucose (60 mM) and GO (132 units) in phosphate buffer, pH 7, for 60 min (Table I). In the two last methods H₂O₂ was induced enzymatically. The in situ production of H₂O₂ was ascertained by the Thurman reaction (19). In a separate experiment, 10 mM diethylenetriamine pentaacetic acid (DTPA) was added to the perfused hydrogen peroxide solution. This was done to clarify whether metal ions are involved in the expected mucosal damage caused by the hydrogen peroxide.

**Iron Levels in the Rat Jejunal Mucosa**

The amount of iron ions was determined in the scraped mucosa by atomic absorption. A 1-ml sample from the homogenized tissue was acidified with 250 μl of 9 N HCl, vortexed, and diluted to 3 ml with distilled water. The iron content was measured (Zeeam SpectAA 300, Varian) against a standard curve of Fe(II) and normalized to tissue dry weight.

**Characterization of the Induced Tissue Damage**

At the end of each perfusion study, the perfused jejunal segment was separated from the anesthetized animal and cut open. The mucosal epithelial layer was delicately scraped with the aid of a microscope slide and rapidly transferred into a tissue-homogenizing test tube containing 5 ml of saline, kept in an ice bath. The contents of the test tube was homogenized manually for 2 min and centrifuged at 3,000 rpm for 15 min, and the supernatant liquid was separated. Two portions of 1 ml were taken for tissue damage assessment, and one portion of 1 ml was used for tissue dry weight measurements (complete dehydration after heating at 80 °C was validated by no weight loss on further drying). The induced tissue damage was biochemically characterized by: (a) measuring the activity of the enterocytes enzyme lactate dehydrogenase (LDH) at 340 nm (Uvikon 930, Kontron Instruments, Switzerland), using pyruvic acid as substrate and NADH as an electron donor, and (b) measuring the intracellular glutathione levels. In this method a 1-ml sample from the homogenized tissue was dissolved in HCl and analyzed for GSH content by atomic absorption as described elsewhere (17). In both cases results were normalized to the tissue dry weight.

Each experiment was repeated at least four times, and the results are reported as the mean value ± standard deviation.

**Cationization of Catalase**

Two methods were used to cationize the enzyme catalase (EC 1.11.1.6). The "soluble method" was performed as described by Schalkwijk et al. (16) and Danon et al. (22). Two ml of 2 M 1,6-diaminohexane (hexamethyldiamine) (HMD) solution in double-distilled water (the reaction activator) was mixed with 8 ml of double-distilled water containing 10 mg of catalase, followed by the addition of 200 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) as nucleophile. The reaction mixture was stirred overnight, and the pH was adjusted to 6.5 with 2 N HCl during the first 2 h. In this way the free carboxyl groups of the catalase were coupled to the amino groups of HMD to form soluble modified catalase (sm-catalase) (22). The cationized catalase thus formed was mounted in a dialysis bag and rinsed with 0.15 M NaCl over 24 h.

The "insoluble method" was performed as described by Gibbs et al. (8). At first, stock solutions of catalase in saline (10 mg/ml), poly-L-histidine in saline (10 mg/ml), and HBSS buffered with 1 M HEPES buffer, pH 7.5, were prepared. A 0.1-ml aliquot of a stock catalase solution was then vortexed with 0.4 ml of saline in a 5-ml test tube. While vortexing, 0.1 ml of poly-L-histidine stock solution was added.

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\(^1\) The abbreviations used are: GO, native glucose oxidase; DTPA, diethylenetriamine pentaacetic acid; AA, ascorbic acid; LDH, lactate dehydrogenase; sm-catalase, soluble modified catalase; im-catalase, insoluble modified catalase; smGO, soluble modified glucose oxidase; imGO, insoluble modified glucose oxidase; ATZ, 3-amino-1,2,4-triazole; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; HMD, hexamethyldiamine; HBSS, Hanks' buffered saline solution.

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**Table I**

| Code   | Protocol                        | Perfusion time before damage measured | Time before damage measured |
|--------|---------------------------------|--------------------------------------|-----------------------------|
|        |                                 | min                                  | min                         |
| Control| Saline                          | 120                                  | 120                         |
| D-I    | H₂O₂ (176 mM)                   | 30                                   | 30                          |
| D-II   | H₂O₂ (176 mM) + DTPA (10 mM)   | 30                                   | 30                          |
| D-III  | 1. Fe(II) (10 mM), EDTA (1 mM) | 15                                   | 15                          |
|        | 2. Saline                       | 15                                   | 15                          |
|        | 3. H₂O₂ (88 mM)                 | 30                                   | 60                          |
| D-IV   | 1. Copper sulfate (10 mM)       | 15                                   | 15                          |
|        | 2. Saline                       | 15                                   | 15                          |
|        | 3. AA (20 mM)                   | 30                                   | 60                          |
| D-V    | 1. GO (132 units)               | 30                                   | 30                          |
|        | 2. Glucose (60 mM)              | 30                                   | 60                          |
| D-VI   | GO (132 units) + glucose (60 mM)| 30                                   | 60                          |
| D-VII  | 1. GO, smGO, or imGO (132 units)| 30                                   | 30                          |
|        | 2. Saline                       | 15                                   | 15                          |
|        | 3. Glucose (60 mM)              | 30                                   | 75                          |
| P-P    | 1. Fe(II) (10 mM), EDTA (1 mM)  | 15                                   | 15                          |
|        | 2. Saline                       | 15                                   | 15                          |
|        | 3. Catalase or sm-catalase       | 30                                   | 30                          |
|        | or im-catalase (0.01 mg/ml)     |                                       |                             |
|        | 4. Saline                       | 15                                   | 15                          |
|        | 5. H₂O₂ (88 mM)                 | 30                                   | 115                         |
| P-II   | 1. Copper sulfate (10 mM)       | 15                                   | 15                          |
|        | 2. Saline                       | 15                                   | 15                          |
|        | 3. Catalase or sm-catalase       | 30                                   | 30                          |
|        | or im-catalase (0.01 mg/ml)     |                                       |                             |
|        | 4. Saline                       | 15                                   | 15                          |
|        | 5. AA (20 mM)                   | 30                                   | 115                         |

* D, damage.

* P, protection.
Cationized Enzymes and Intestinal Oxidative Damage

21351

Hepatic mucosa (HENKS buffer) was added to bring the mixture to a final volume of 1 ml. The addition of the buffer resulted in a pH of ~7.0, at which point a precipitate was formed. The mixture was allowed to stand for 5 min, then the poly-l-histidine catalase complex (im-catalase) was pelleted by centrifugation and washed twice by 10 ml of HENKS buffer. At the end, the im-catalase pellet was resuspended in 1 ml of HBSS.

Cationization Yield Assessment—in different experiments, various concentrations of catalase, sm-catalase, and im-catalase (ranging from 0 to 2.5 mg in 1.5 ml of phosphate buffer, pH 8) were reacted with fluorescamine in acetone for 90 min (23). The fluorescence of the solutions was measured in a Jasco FP-770 spectrofluorometer at excitation wavelength of 390 nm and emission of 475 nm, and the slopes of the linear plots of the fluorescence values versus enzyme concentrations were compared. The cationization yield values were found to be 75% for the sm-catalase and 85% for the im-catalase.

Measurement of Catalase Activity—The activity of catalase, sm-catalase, and im-catalase was measured by monitoring their ability to degrade hydrogen peroxide as described elsewhere (18). The activity of sm-catalase was determined as 75% as compared to catalase, while that of im-catalase was determined as 70% compared to catalase.

Jejunal Mucosa Protection by Catalase, sm-Catalase, and im-Catalase

The ability of the three types of catalase products to protect the jejunal mucosa against damage generated by hydroxyl radicals was assessed by conducting two different experimental protocols. In the first one the hydroxyl radicals were induced by chelated Fe(II) (10 mM) and hydrogen peroxide (88 mM), whereas in the second protocol the induction was achieved by perfusing copper ions (10 mM) and AA (20 mM). These protocols included perfusion studies as follows: (a) perfusion of Fe(III):EDTA (10:1 mM) for 15 min followed by perfusion of saline for 15 min, followed by perfusion of 0.01 mg/ml catalase, or sm-catalase, or im-catalase for 30 min, followed by saline rinsing for 15 min, followed by 88 mM H2O2 for 30 min; (b) perfusion of 10 mM copper ions for 15 min, followed by perfusion of saline for 15 min, followed by perfusion of 0.01 mg/ml catalase, sm-catalase, or im-catalase for 30 min, followed by saline rinsing for 15 min, followed by 20 mM AA (30 min). At the end of each study the mucosal integrity was evaluated by measuring the enterocyte activity of LDH and level of potassium ions, as described above (Table I).

Verification of the Cationized Catalase Attachment to the Jejunal Epithelium

The specific binding of the two types of cationized catalase products to the perfused jejunal epithelium was verified by measuring the enzyme activity in the perfusate solution in the beginning and at the end of the perfusion session in which catalase, sm-catalase, or im-catalase was perfused for 30 min. The enzyme activities were assessed as mentioned above and compared to each other. Each experiment was repeated at least four times, and the results are reported as the mean value ± standard deviation.

Induction of Oxidative Damage by Cationized Glucose Oxidase

In order to demonstrate the concept of in vivo adherence of enzymes to the jejunal mucosa, GO was modified similarly to catalase. The activity of the cationized GO with HMD was determined by the Thurman reaction (19) and found to be 55% of the native glucose oxidase. In the same manner, the activity of the GO-poly-l-histidine complex was determined to be 45%.

It was expected that upon perfusion of smGO and imGO to the rat jejunum, an enhanced mucosal damage, as a result of tissue attachment of the cationized glucose oxidase products, would be observed compared with the damage caused by GO alone.

The ability of the three types of GO enzymes to induce oxidative damage to the jejunal mucosa was assessed by conducting the following perfusion studies; 132 units of GO, smGO, or imGO were perfused for 30 min, followed by saline rinsing for 15 min, followed by perfusion of 69 mM glucose for 30 min. At the end of each study the mucosal integrity was evaluated by measuring the enterocyte activity of LDH and the levels of potassium ions, as described in Table I.

Each experiment was repeated at least four times, and the results are reported as the mean value ± standard deviation.

Statistical Analysis

A Kruskal-Wallis test was performed to check whether the various groups of rats were from different populations. A difference was considered to be statistically significant when the p value was less than 0.05. When the difference between the groups was validated, a Mann-Whitney U test was used to analyze the significance of the differences between the obtained data (p < 0.05).

RESULTS

Oxidative Stress-induced Damage to the Rat Jejunal Mucosa—Perfusion of the rat jejunum with hydrogen peroxide caused biological damage to the epithelial mucosa (Fig. 1). The damage was characterized by a significant decrease in the cellular LDH activity and potassium level of the tissue, which was in intimate contact with the perfused solution (from 5.1 ± 0.5 to 1.8 ± 0.3 (units/ml)/mg × 10−4, and from 7.0 ± 0.14 to 5.0 ± 0.16 μM/mg, respectively). To find out whether the damage induced by hydrogen peroxide is mediated by mucosal iron ions, the tissue was analyzed for iron content by atomic absorption and was found to be 1.22 μM/mg. Therefore, the same study was repeated with the addition of chelating agent (DTPA) in the perfused solution containing hydrogen peroxide. In this case the measured LDH activity was 5.1 ± 0.5 (units/ml)/mg × 10−4, and the potassium level was 6.7 ± 0.34 μM/mg (Fig. 1), indicating that damage was prevented by DTPA. Fig. 1 also shows that hydrogen peroxide, which was produced enzymatically by the reaction between GO and glucose (subsequent perfusions), did not cause mucosal damage as indicated by the LDH activity (5.0 ± 1.1 (units/ml)/mg × 10−4) and the cellular potassium level (6.6 ± 0.34 μM/mg). Similar results were obtained when the rat jejunum was perfused with a mixture of GO and glucose (LDH activity of 5.0 ± 1.5 (units/ml)/mg × 10−4, and potassium levels of 6.8 ± 0.56 μM/mg) (Fig. 1).

Hydroxyl radicals were produced in situ by perfusing ferrous sulfate chelated with EDTA in the jejunal loop. This was followed by a perfusion of saline and a perfusion of the same intestinal loop with hydrogen peroxide (D-III in Table I). In this experiment a significant decreases in the cellular LDH activity and potassium level was observed (from 5.2 ± 0.5 to 0.3 ± 0.04 (units/ml)/mg × 10−4 and from 7.0 ± 0.14 to 2.8 ± 0.4 μM/mg, respectively) indicating a sever damage to the mucosal tissue (Fig. 2). Alternatively, hydroxyl radicals were produced by the combination of copper ions and AA. A perfusion of copper ions was followed by a perfusion of saline and a perfusion of AA (D-IV in Table I). The results in Fig. 3 show a sever damage to the jejunal mucosa as indicated by a significant decrease in the cellular LDH activity (from 4.6 ± 0.4 to 0.83 ± 0.2 (units/ml)/mg × 10−4) and potassium levels (from 5.5 ± 1.1 to 3.8 ± 0.16 μM/mg).

![Fig. 1. The activity of LDH (empty columns) and the potassium level (filled columns) in the rat jejunal mucosa at the end of varying perfusions. Perfusions were as follows: 1, saline (120 min); 2, 176 mM hydrogen peroxide (30 min); 3, 176 mM H2O2 and 10 mM DTPA (30 min); 4, 132 units of GO (30 min) followed by 30 mM of glucose (30 min); 5, mixture of 60 mM glucose and 132 units of GO (60 min).](image-url)
The activity of LDH (empty columns) and the potassium level (filled columns) in the rat jejunal mucosa at the end of various perfusions. The perfusions were as follows. 1, saline (120 min); 2, 10 mM Fe(II) (15 min); saline (15 min); 88 mM H$_2$O$_2$ (30 min). 3, 101 mM Fe(II)-EDTA (15 min); saline (15 min); 0.01 mg/ml catalase (CAT, 30 min); saline (15 min); 88 mM H$_2$O$_2$ (30 min). 4, 101 mM Fe(II)-EDTA (15 min); saline (15 min); 0.01 mg/ml sm-catalase (smCAT, 30 min); saline (15 min); 88 mM H$_2$O$_2$ (30 min). 5, 101 mM Fe(II)-EDTA (15 min); saline (15 min); 0.01 mg/ml im-catalase (imCAT, 30 min); saline (15 min); 88 mM H$_2$O$_2$ (30 min). 6, 101 mM Fe(II)-EDTA (15 min); saline (15 min); 0.01 mg/ml sm-catalase (smCAT, 30 min); saline (15 min); 88 mM H$_2$O$_2$ and 10 mM 3-amino-1,2,4-triazole (30 min).

Prevention of the Oxidative Stress Damage by Cationized Catalase—The rat jejunum was perfused with catalase to examine whether native catalase can reduce the mucosal damage caused by hydrogen peroxide. This was followed by a perfusion of the same jejunal segment with saline, after which hydrogen peroxide was perfused (D-III in Table I). No protection was observed as reflected by the LDH activity (0.34 ± 0.09 compared with control (saline perfusion) levels of 5.2 ± 0.5 (units/ml)/mg × 10$^{-4}$) and potassium level (3.3 ± 1.2 compared with control level of 7.0 ± 0.14 μM/mg) (Fig. 2). The results did not differ significantly from those obtained when EDTA-chelated ferrous sulfate, followed by perfusion with hydrogen peroxide was performed (Fig. 2). However, when sm-catalase or im-catalase was perfused prior to the perfusion with hydrogen peroxide, the recorded biological damage in the jejunal mucosa was significantly lower, i.e., the LDH activity and potassium level were significantly higher than the values obtained when the untreated enzyme was perfused. Fig. 2 shows that in the case of sm-catalase the recovery (compared with the saline control experiments) was 95% as measured by the activity of the LDH (4.7 ± 1.04 compared with 5.2 ± 0.5 (units/ml)/mg × 10$^{-4}$), and 100% as measured by the amounts of the cellular potassium (7.1 ± 1.2 compared with 7.0 ± 0.14 μM/mg). In the case of im-catalase, the recovery was 40% as measured by the activity of the LDH (2.92 ± 0.064 compared with 5.2 ± 0.5 (units/ml)/mg × 10$^{-4}$) and 120% as measured by the amounts of cellular potassium (8.2 ± 2.3 compared with 7.0 ± 0.14 μM/mg) (Fig. 2).

Perfusion of copper ions, which was followed by perfusion of AA, resulted in a 82% reduction in the cellular LDH activity (from 4.6 ± 0.4 (saline perfusion) to 0.83 ± 0.2 (units/ml)/mg × 10$^{-4}$) and a 32% decrease in the cellular potassium levels (from 5.5 ± 1.08 (saline perfusion) to 3.8 ± 0.16 μM/mg) (Fig. 3). Treatment with catalase following the copper ions perfusion and prior to the perfusion with AA was unable to protect the mucosa as analyzed by the LDH activity (0.76 ± 0.18 compared with 0.83 ± 0.2 (units/ml)/mg × 10$^{-4}$) and the potassium level (3.9 ± 0.54 compared with 3.8 ± 0.16 μM/mg for the damaged tissue, or 5.5 ± 1.1 μM/mg for the control experiment) (Fig. 3). Repeating the above experiment with sm-catalase and im-catalase showed partial protection as analyzed by the LDH activity (1.87 ± 0.8 (units/ml)/mg × 10$^{-4}$ for the sm-catalase, and 1.7 ± 0.34 (units/ml)/mg × 10$^{-4}$ for the im-catalase compared with 4.6 ± 0.4 (units/ml)/mg × 10$^{-4}$ for the saline perfusion) (Fig. 3) and full protection as indicated by measuring the cellular potassium levels (5.8 ± 1.4 μM/mg for the sm-catalase and 5.7 ± 0.8 μM/mg for the im-catalase compared with 5.5 ± 1.1 μM/mg for the saline perfusion) (Fig. 3).

To verify that the observed protection was not resulted by either the poly-L-histidine residues or by a nonspecific shield of the cationized protein, we pretreated the rat with poly-L-histidine together with heat-inactivated catalase. This was followed by perfusion with hydrogen peroxide and chelated ferrous ions in the protocol mentioned above. No protection against mucosal damage was observed in any of these experiments (data not shown). To strength this point we performed an additional experiment, in which 10 mM of 3-amino-1,2,4-triazole (ATZ) was introduced to the perfusion system together with H$_2$O$_2$ (88 mM) following a perfusion with chelated ferrous ions (10 mM Fe(II), 1 mM EDTA), saline rinse, sm-catalase, and finally saline rinse, as shown in Fig. 2 (column 6). Previous to this experiment we tested the inhibition effect of ATZ on the activity of cationized catalase in vitro. ATZ (1 mM) decreased the activity of catalase sm-catalase by 84 and 82%, respectively. The in vivo results indicated that in the presence of the inhibitor, the cationized catalase failed to protect the rat jejunal mucosa from the damage induced by the combination of Fe(II) and H$_2$O$_2$ (Fig. 2, column 6). These results may indicate that the protection obtained by the cationized enzymes against oxidative damage is the result of the their catalatic activity and is not the result of other nonspecific effects.

Quantification of the Cationized Catalase Attachment to the Jejunal Epithelium—As shown in Table II, when catalase was perfused, no attachment between catalase and the perfused mucosa was observed. However, when a perfusion of sm-catalase or im-catalase was performed, 89 and 50% attachment of the enzyme to the rat jejunal mucosa was observed, respectively. These findings can be deduced from calculating the differences between the enzyme activities in the beginning and the end of each perfusion. Moreover, when saline was perfused following the perfusion with the various enzymes, no enzyme activity could be detected in the perfusate, suggesting a strong attachment of these enzymes to the rat jejunal mucosa.

Cationization of Glucose Oxidase and Its Mucosal Attachment Evaluation—An opposite approach to the catalase cationized was taken by the cationization of GO. In the presence
The enzyme activity was analyzed in the perfusate, in the beginning and at the end of each perfusion experiment. Native catalase or cationized enzyme (sm-catalase or im-catalase) were perfused in a closed loop in the rat jejunum over 30 min. This was followed by 30 min of saline perfusion. The activities were assessed by measuring the ability of the various enzymes to decompose H$_2$O$_2$. This was measured via the Thurman reaction (19). The higher the A value at 419 nm, the less active was the enzyme in the mixture.

| Type of enzyme | $A_{419}$ in the beginning of the perfusion | $A_{419}$ at the end of the perfusion | Percent bound |
|---------------|------------------------------------------|--------------------------------------|--------------|
| Catalase      | 0.21 ± 0.009                             | 0.22 ± 0.006                         | 0            |
| sm-catalase   | 0.21 ± 0.009                             | 1.90 ± 0.016                         | 89           |
| im-catalase   | 0.09 ± 0.006                             | 0.18 ± 0.009                         | 50           |

**Fig. 4.** The activity of LDH (empty columns) and the potassium level (filled columns) in the rat jejunal mucosa at the end of varying perfusions. 1, saline (120 min); 2, a mixture of 50 mM glucose and 132 units of GO (60 min); 3, 132 units of GO (30 min); saline rinse (15 min); 4, 132 units of smGO (30 min); saline rinse (15 min); 5, 132 units of imGO (30 min); saline rinse (15 min); 6, mM glucose (30 min). of glucose, this enzyme produces hydrogen peroxide in situ. Fig. 1 demonstrates that the attempt to induce oxidative damage in the jejunal epithelium by GO and glucose failed, as analyzed by the LDH activity (5.3 ± 1.1 for the mixture and 5.2 ± 1.8 for the consequent perfusions compared with 5.2 ± 0.5 (units/ml)/mg × 10$^{-4}$ of the saline perfusion) and the cellular potassium level (6.8 ± 0.34 for the mixture and 6.8 ± 0.56 for the consequent perfusions compared with 7.0 ± 0.13 μM/mg of the saline perfusion). No reduction in the LDH activity was noted even in the presence of Fe(II) in the perfusion mixture (Figs. 1 and 2). On the other hand, no damage could be recorded when hydrogen peroxide was perfused together with the chelating agent DTPA. Moderate damage was recorded when hydrogen peroxide was introduced alone (Fig. 1). These results suggest the involvement of ferrous ions via the metal-mediated Haber-Wiess reaction (7) in which hydrogen radicals are produced in the lumen. The damage induced by hydrogen peroxide alone may be explained by the presence of loosely bound transition metals on the surface of the epithelial cells (26). A significant amount of iron in the rat jejunal mucosa was found (122 μM/mg). This iron can be reduced to the ferrous state by the metabolic activity of the anaerobic bacteria in the intestinal flora, which generate substances such as H$_2$S. This process may be hazardous, especially in the colon where fecal materials are continually brought into intimate contact with the mucosa as a result of the differences in solubility in the perfused solutions.

**DISCUSSION**

Bacteria, ischemic and post-ischemic processes, and food ingredients can induce oxidative stress injury to the intestinal epithelium (2, 24, 25). This damage is a result of reactive oxygen metabolites such as hydroxyl radicals, superoxide radicals, lipid peroxidation products, and hydrogen peroxide, which are produced in large quantities in the lumen (2). It has been suggested that these reactive oxygen species may play an important role in the pathogenesis of several intestinal disorders such as inflammatory bowel diseases, gastric ulceration, and colon cancer (24). Hydrogen peroxide, although not very reactive by itself, may serve as precursor for the highly reactive hydroxyl radicals (26) in a virtue of the Fenton reaction. In addition, hydrogen peroxide can diffuse relatively easily, as compared with other radicals, through the epithelial cell membrane (26).

Catalase has been suggested as a therapeutic means to treat pathological processes in which oxygen reactive metabolites are involved (13). Indeed, several attempts were made to protect cells by catalase from oxidative damage (27). However, its therapeutic action is limited to those organs where the resident time is limited in the vicinity of the desired location (e.g. the GI lumen). Despite some studies in which catalase was attached to the surface of cells in culture media (8), attempts to bind the enzyme to a whole tissue in vivo like the intestinal mucosa are scarce.

We showed that, in contrast to the native enzyme, cationized catalase is able to prevent oxidative damage induced in the rat jejunal mucosa by hydrogen peroxide and hydroxyl radicals. Anionic sites on the cell surface have been shown to exhibit strong affinity for cationic agents (16, 28). In this study, catalase was positively charged, to enable it to adhere to the negatively charged epithelial cells' membrane of the rat jejunum. The attachment mechanism is probably an interaction that occurs between the cations and negatively charged proteoglycans, present abundantly in basal membranes (16). The high catalase-tissue attachment shown in Table II (89% for the sm-catalase and 50% for the im-catalase) can explain the observed protection against the induced oxidative damage. The differences in the enzyme-tissue binding between the two cationized products may be a result of the differences in their solubility in the perfused solutions.

The mucosal damage induced by hydrogen peroxide was significantly increased when chelated ferrous ions were added to the perfusion mixture (Figs. 1 and 2). On the other hand, no damage could be recorded when hydrogen peroxide was perfused together with the chelating agent DTPA. Moderate damage was recorded when hydrogen peroxide was introduced alone (Fig. 1). These results suggest the involvement of ferrous ions via the metal-mediated Haber-Wiess reaction (7) in which hydrogen radicals are produced in the lumen. The damage induced by hydrogen peroxide alone may be explained by the presence of loosely bound transition metals on the surface of the epithelial cells (26). A significant amount of iron in the rat jejunal mucosa was found (122 μM/mg). This iron can be reduced to the ferrous state by the metabolic activity of the anaerobic bacteria in the intestinal flora, which generate substances such as H$_2$S. This process may be hazardous, especially in the colon where fecal materials are continually brought into intimate contact with the mucosa as a result of the long residence time and contractile activity (24).

Hydrogen peroxide was also produced in our system by GO and glucose interaction. Fig. 1 shows that no damage was observed, probably because of dilution effect. However, when the GO was cationized and then perfused, an injury was observed most reasonably because of the attachment of the modified enzyme to the epithelial surface (Fig. 4). In the presence of glucose, hydrogen peroxide was produced locally by the bound enzyme, which, together with mucosal transition metals, resulted in damage to the jejunal epithelium. This attachment, between the cationized GO and jejunal mucosa points on an attractive possibility in which enzymes or other macromolecules capable of producing oxidative damage could be targeted to diseased tissues. For example, cationized GO may be injected into solid tumors. Its binding to the diseased tissue will result in a local production of hydrogen peroxide.
at the site of the tumor only. This hypothesis requires further experimental verification.

Introducing catalase to the perfusion system prior to the oxidative stress induction did not protect the jejunal mucosa (Figs. 2 and 3). Table II shows that tissue attachment of catalase did not occur. Catalase is a negatively charged protein and therefore is repelled from the epithelial membrane (8). When the enzyme was cationized (retaining 70–75% of its activity), attraction between the enzyme and the epithelium was observed (Table II). The specific attachment of small catalase and im-catalase to the jejunal mucosa prevented the mucosal damage resulted by the hydroxyl radicals produced by the reaction between Fe(II) and H₂O₂ (Fig. 2). When catalase inhibitor was added to the perfusion system, a significant nucosal damage was recorded, indicating that the protection effect of catalase is due to its catalatic activity and not to other effects of the cationized enzyme (Fig. 2). A less profound, but yet significant, protection of small catalase and im-catalase was achieved in the system when damage was induced by the reaction between copper ions and AA (Fig. 3). Oxidants originating in the diet are major contributors to the induced oxidative damage in the intestine (2). It is well documented that a diet rich in fat is correlated with colon and breast cancer, or deleterious processes (29). In our study, ascorbic acid, a common dietary component, was shown to generate oxidative damage to the rat jejunal epithelium. Although being a natural antioxidant AA may act as prooxidant in the presence of transition metals such as iron (21, 26, 30). When AA was perfused alone, no tissue damage could be detected (data not shown). Perfusion of copper ions prior to the AA perfusion resulted in severe mucosal damage (Fig. 3). When AA was perfused alone, no tissue damage could be detected. The use of cationized enzymes may be useful in the treatment of those clinical disorders where oxidative tissue damage is produced locally. In these cases specific binding of protective enzymes such catalase, superoxide dismutase, and peroxidase to defined tissues is beneficial. Such cationized proteins have been found to be efficient in preventing experimental arthritis when injected in rats (16) and to be capable of protecting endothelial cells in culture (8). Furthermore, cationized albumin and cationized immunoglobulin have been demonstrated to be capable of penetrating the blood brain barrier (31, 32). We have demonstrated that cationized catalase can prevent oxidative stress tissue damage, and cationized glucose oxidase can induce local oxidative stress damage. Further experiments are required to elucidate the binding kinetics and the pharmacological potential of cationized proteins in medicine.

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