Evidence of Oxidant-induced Injury to Epithelial Cells during Inflammatory Bowel Disease

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Abstract

Evidence of in vivo oxidant-induced injury in inflammatory bowel disease (IBD) is largely indirect. Colon epithelial crypt cells (CEC) from paired specimens of histologically normal and inflamed bowel from IBD patients with active disease were examined for altered protein thiol redox status as an indicator of oxidative damage. When CEC preparations from 22 IBD patients were labeled with the reduced-thiol-specific probe [14C]-iodoacetamide (IAM), there was decreased labeling of a number of proteins indicating oxidation of thiol groups in CEC from inflamed mucosa compared to paired normal mucosa, especially the loss of thiold labeling of a 37-kD protein which was almost completely lost. The loss of reduced protein thiol status for the 37-kD band was paralleled by loss of epithelial cell glycerdehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) enzyme activity, an enzyme known to contain an essential reduced cysteine (Cys349) at the active site. The identity of the 37-kD protein as GAPDH monomer was confirmed by NH2-terminal amino acid sequence analysis.

To examine whether this type of in vivo injury could be attributed to biologically relevant oxidants produced by inflammatory cells, CEC prepared from normal mucosa were exposed to H2O2, OCl-, nitric oxide (NO), and a model chlorine molecule chloramine T (ChT) in vitro. Dose-dependent loss of IAM labeling and GAPDH enzyme activity was observed. The efficacy (IC50) against IAM labeling was OCl- >> ChT >> H2O2 >> NO (52±3, 250±17, 420±12, 779±120 μM oxidant) and OCl- >> ChT >> NO >> H2O2 (89±17, 256±11, 407±105, 457±75 μM oxidant), respectively, for GAPDH enzyme activity.

This study provides direct evidence of in vivo oxidant injury in CEC from inflamed mucosa of IBD patients. Oxidation and inhibition of essential protein function by inflammatory cells is a potential mechanism of tissue injury that may contribute to the pathogenesis of the disease and supports the exploration of compounds with antioxidant activity as new therapies for IBD. (J. Clin. Invest. 1996; 98:136–141.) Key words: glycerdehydephosphate dehydrogenase (E.C.1.2.1.12) • protein oxidation • protein thiol • reactive oxygen species • inflammatory bowel disease

Introduction

The mucosal lesion in inflammatory bowel disease (IBD) is characterized by a dense inflammatory cell infiltrate mainly comprising neutrophils, macrophages, and lymphocytes. Although many inflammatory mediators secreted by these cells, including the cytokines IL-1, IL-6, IL-8, IL-10, TNF-α, and leukotrienes together with luminal bacterial products such as bacterial lipopolysaccharide (LPS) and the chemotactic peptide fMLP, have been implicated in the mucosal injury observed in IBD (1), the molecules that mediate tissue damage remain poorly understood (2). Recent indirect evidence has, however, implicated reactive oxygen and nitrogen species (RONS) such as nitric oxide (NO), superoxide (O2−), peroxynitrite (ONOO−), hydrogen peroxide (H2O2), and hypochlorite (OCl−) in the pathogenesis of the mucosal lesion (3–9).

Increased production of RONS has been observed after in vitro stimulation of whole colonic mucosa, mucosal macrophages, and peripheral blood monocytes of IBD patients (3–5). Furthermore, the coproduct of NO generation, citrulline, has been observed at greater than normal levels in the colonic lumen of patients suffering from active IBD (7) and of primates with chronic colitis (9). RONS scavengers and inhibitors of RONS production including superoxide dismutase (10–13), dimethylsulfoximide, and allopurinol (10), and N6-nitro-l-arginine (14) have shown some antiinflammatory effects in limited clinical trials and in animal models of IBD. These findings support a role for RONS in the pathogenesis of the mucosal lesion in IBD, yet direct evidence that RONS actually injure the inflamed mucosa in IBD is lacking. Among the many potential targets of RONS-induced injury, reduced thiols (sulfhydryl) groups are particularly sensitive (15). The thiol-containing enzyme glycerdehyde-3-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.12) has been shown to be particularly susceptible to oxidation of reduced thiol groups both in vitro (16, 17) and during carageenin-induced arthritis (18).

The purpose of this study was to examine colon epithelial crypt cells (CEC) harvested directly from inflamed lesions of both Crohn’s disease and ulcerative colitis patients for markers

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1. Abbreviations used in this paper: CD, Crohn’s disease; CEC, colon epithelial crypts; ChT, chloramine T; GSH, glutathione (reduced); GSSG, glutathione disulfide (oxidized); IBD, inflammatory bowel disease; IAM, [14C] iodoacetamide; OCl−, hypochlorite; RONS, reactive oxygen and nitrogen species; UC, ulcerative colitis.
of RONS-induced injury by determining whether GAPDH thiols were oxidized in vivo. We report here evidence of the oxidation of GAPDH in CEC from inflamed, but not noninflamed, IBD mucosa, establishing direct evidence of RONS-induced mucosal injury in vivo in IBD.

Methods

Specimens. CEC from inflamed and noninflamed mucosa were prepared from paired specimens of inflamed and noninflamed mucosa obtained at colonoscopy or from surgically resected material from IBD patients (Crohn’s disease [CD], n = 13; ulcerative colitis [UC], n = 9). For in vitro studies, histologically normal mucosa was obtained from material resected from cancer-bearing colons (≥ 10 cm away from any histologically abnormal mucosa). In every case the diagnosis of IBD was verified by standard clinical, radiological, endoscopic, and histological criteria. The inflammatory or noninflamed status of the paired mucosal samples from IBD patients was confirmed histologically using tissue taken from an area immediately adjacent to that used for this study. The patient cohort therapy regime was grouped as follows: (a) steroids with no 5-aminosalicylic acid (5-ASA) base compounds (six patients); (b) 5-ASA–based compounds, no steroids (two patients); (c) both steroids and 5-ASA–based compounds (seven patients); (d) no treatment (one patient); or other therapies including azathioprine, pain suppressants, and bacteriostatic compounds (two patients) and (e) unknown treatment (seven patients). Many patients in groups (a) and (b) were also prescribed compounds categorized as in (d). Biopsy material from patients diagnosed with ischemic colitis and quiescent or healed colitis was available during the course of this study and served as disease controls. Tissue samples used for experimental purposes were taken and used in accordance with approval of the ACT Health Institutions Ethics Committee and John Curtin School of Medical Research Ethics Committee.

Isolation of colon epithelial crypts (CEC). Primary cultures of CEC were prepared as described previously by this laboratory with minor modifications (19). Briefly, fresh mucosal biopsies and resected colo-rectal material were transferred to HBSS without phenol red or glucose. Biopsies or mucosa scraped from resected material using sterile microscope slides were minced finely using a crossed scalpel techni-que, incubated (60 min, 37°C, ≥ 2 g tissue/10 ml) in a solution containing Dispase I (Boehringer Mannheim Biochemicals, Indianapolis, IN) 1.2 U/ml and collagenase IV (Worthington Biochemical Corp., Freehold, NJ) 50 U/ml in RPMI medium (Cytosystems, Sydney, Aus-tralia) followed by trituration through a 21 gauge needle. The digest was washed in HBSS (75 g for 3 min) then passed through a coarse stainless steel mesh (300–400 μm gauge) to remove mucus and undigested fragments of lamina propria. The resulting suspension was enriched for whole and partial CEC using at least six slow speed sedi-mentation steps (60 g for 3 min) to remove single cells. The purified CEC were then resuspended in Dulbecco’s minimum essential me-dium (MEM; Gibco Laboratories, Grand Island, NY) supplemented by 20% NuSerum (Collaborative Research Inc., Bedford, MA), 4 mM l-glutamine, 4% Luria broth, 20 mM Hepes, 50 μM gentamycin (Delta West, Australia), and 50 μM penicillin (CSL, Australia). Primary cultures of CEC in supplemented MEM were incubated (37°C, 5% CO2) for 16–20 h before use. Cell viability was assessed by trypan blue exclusion as assessed by a method of trypsinization, 10 mM. Enzyme activity was then determined spectrophotometrically by monitoring the oxidation of NADH by the reverse reaction of GAPDH coupled with phosphoglycerate kinase.

In vitro oxidant exposure of CEC preparations. Primary cultures of normal CEC obtained from cancer-bearing colons were washed twice and resuspended to 106 cells/ml in HBSS. Various concentra-tions (125–2,000 μM) of H2O2 (BDH Chemicals, Melbourne, Aus-tralia), NaOCl (Ajax Chemicals, Australia), chloramphenicol (Merck, Darmstadt, Germany), and NO (in the form of diethylamine NONOate; Cayman Chemical Co., Ann Arbor, MI) in HBSS were added to CEC at 106 cells/ml and then incubated at 37°C for 30 min with constant gentle agitation. Cells were washed to remove any unreacted oxidant and resuspended in HBSS for protein thiol analysis with C14-IAM or resuspended in Tris/EDTA for the determination of GAPDH enzyme activity. Decomposition of diethylamine NONOate occurs with a half-life of 2.1 min at pH 7.4 resulting in > 95% decomposition within 10 min (21).

Statistical analysis. The Student’s t test was used to assess the sta-tistical significance of patient and in vitro data. Statistical significance was accepted when P < 0.05.

Results

Protein thiol oxidation in CEC. To analyze the redox status of protein thiols in CEC from IBD patients [14C]-idoacetamide ([14C]-IAM) was used to label reduced thiol groups selectively. Proteins concentrated from the CEC lysates were then sepa-rated by SDS-PAGE and labeled proteins were detected by autoradiography. When the proteins labeled by [14C]-IAM in ly-sates of CEC from inflamed IBD mucosa were compared to those from paired normal colon mucosa, the most striking obser-vation was the almost complete loss of [14C]-IAM binding to a 37-kD protein from inflamed CEC. Decreased labeling of proteins of minor bands at 27, 69, 72, and 86 kD and very faint bands at 30, 31, 34, 46, 54, and 61 kD were also observed in ly-sates of CEC from inflamed mucosa (Fig. 1a). Protein staining by Coomassie brilliant blue of the SDS polyacrylamide gel

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Oxidation of GAPDH thiols in CEC from IBD patients. Identification of oxidized protein thiols in inflamed mucosa. (a) Purified CEC from the noninflamed and inflamed mucosa of an IBD patient were incubated with \(^{14}\)C-IAM, and then labeled proteins were separated by 10% SDS-PAGE and detected by autoradiography. (b) Coomassie blue staining of a SDS-PAGE gel of samples from the same patient. Arrows indicate the 37-kD protein.

TABLE I. Comparison of NH\(~{2}\)-terminal Amino Acid Sequences of the 37-kD Protein and Human GAPDH

<table>
<thead>
<tr>
<th>Sample source</th>
<th>NH(~{2})-terminal amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human erythrocyte</td>
<td>GKVKV GVNGF GRIGR LVTRA</td>
</tr>
<tr>
<td>Noninflamed mucosa</td>
<td>GKVKV GVNGF GRIGG LLDCD</td>
</tr>
<tr>
<td>Inflamed mucosa</td>
<td>GKVKV GVNGF GRIGG LLWRA</td>
</tr>
</tbody>
</table>

The NH\(~{2}\)-terminal amino acid sequences of the 37-kD protein purified from CEC from both noninflamed and inflamed colonic mucosa showed almost complete homology with the published sequence of human GAPDH. The deviations from the known sequence (underlined) indicated limitations in amino acid detection due to sample size.

In CEC from paired samples of inflamed and noninflamed IBD mucosa. As shown in Fig. 3, there was a significant loss of GAPDH activity in CEC from inflamed mucosa from both CD and UC patients compared to CEC from paired normal mucosa \((P < 0.05)\) that corresponded to the decreases seen in \(^{14}\)C-IAM binding to the 37-kD protein (Fig. 2). There was no significant difference in CEC GAPDH activity between CD and UC mucosa nor between CEC preparations from previously inflamed or healed IBD (quiescent colitis, \(n = 3\); healed ischemic colitis, \(n = 1\)) mucosa compared to paired noninflamed mucosa (data not shown).

Although the numbers of patients in each category of drug treatment were small (see Methods), there was no apparent effect on \(^{14}\)C-IAM-binding or GAPDH enzyme activity.

Oxidation and inhibition of GAPDH function by exposure to CEC to oxidants in vitro. To determine the mechanism of oxidation and inactivation of GAPDH observed in CEC from inflamed IBD mucosa, CEC prepared from normal colon resection material were exposed to the biologically relevant oxidants: OCl\(^-\), H\(_2\)O\(_2\), NO, and chloramine-T (ChT), to simulate the oxidative stress that might occur during active mucosal inflammation in IBD. As demonstrated in Fig. 4, in vitro exposure to these oxidants caused a dose-dependent loss of IAM-binding that was statistically significant when compared to control binding of IAM by CEC not exposed to oxidants. The

![Figure 1](image1.png)  
![Figure 2](image2.png)
order of efficacy of the oxidants in decreasing IAM binding calculated from the IC\textsubscript{50} was OCl\textsuperscript{−} >> ChT > H\textsubscript{2}O\textsubscript{2} > NO (Table II). Consistent with these findings, exposure to each of the oxidants markedly decreased GAPDH enzyme activity of CEC in a dose-dependent manner (Fig. 5). The order of efficacy of the oxidants in inhibiting GAPDH activity calculated from the IC\textsubscript{50} was OCl\textsuperscript{−} >> ChT > NO > H\textsubscript{2}O\textsubscript{2} (Table II).

**Discussion**

Oxidation of GAPDH active-site thiols and subsequent inhibition of enzyme activity were consistent observations in preparations of CEC from inflamed mucosa of Crohn’s disease and ulcerative colitis patients whereas there were no significant differences between GAPDH thiol redox status and enzyme activity of CEC from healed (i.e., previously inflamed) or from paired samples of unaffected mucosa.

RONS produced by activated neutrophils and macrophages (23) are the most likely cause of the oxidative inhibition of GAPDH detected in the inflamed mucosa. The capacity of RONS to initiate this observed thiol oxidation was confirmed in vitro by exposure of primary CEC cultures to H\textsubscript{2}O\textsubscript{2}, OCl\textsuperscript{−}, NO, and ChT. This exposure resulted in oxidation and inhibition of GAPDH as observed in CEC from the inflamed mucosa of IBD patients. The 37-kD monomer of GAPDH contains three thiol groups, two of which are involved in disulfide linkages between monomers to form the GAPDH tetramer, while the remaining reduced thiol group at C\textsubscript{ys\textsubscript{149}} is an essential component of the enzyme active site (24). The C\textsubscript{ys\textsubscript{149}} thiol must be reduced to act as a nucleophile during the NAD\textsuperscript{+} catalyzed dehydrogenation of the substrate glycer-aldehyde-3-phosphate. Inhibition of GAPDH activity can be mediated by direct oxidation of the C\textsubscript{ys\textsubscript{149}} by reaction with oxidants and regenerated in the presence of reduced glutathione (GSH) (16, 24). Oxidant stress is known to increase the levels of glutathione disulfide (GSSG) (25), which, if not reduced or exteriorized, may participate in protein S-thiolation reactions forming mixed disulfides with reduced protein thiols (26) and possibly with the C\textsubscript{ys\textsubscript{149}} in GAPDH. The normal homeostatic mechanism of exteriorization of GSSG is an energy-dependent process (27), and the decreased NADPH and ATP levels (28) and mitochondrial function (29) in inflamed mucosa from IBD patients are likely to compromise both the re-reduction and/or transport of excessive GSSG and to increase the opportunity for participation in intracellular S-thiolation reactions with proteins. Indeed, exposure of rat small intestine to luminal H\textsubscript{2}O\textsubscript{2} causes an increase in epithelial cell GSSG content and an associated increase in protein mixed disulfide formation (30). However, S-thiolation of GAPDH in endothelial cells after exposure to H\textsubscript{2}O\textsubscript{2} in vitro and subsequent enzyme inhibition was unlikely to occur via a simple reaction between formed GSSG and protein thiol groups since S-thiolation of GAPDH did not occur in the presence of appreciable concentrations of GSSG (17). The authors suggested that GSH may react directly with GAPDH via the formation of a thyl radical intermediate, as

**Table II. Order of Oxidant Inhibition of IAM Binding and GAPDH Activity**

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>Inhibition of [\textsuperscript{14}C]-IAM binding</th>
<th>Inhibition of GAPDH activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC\textsubscript{50}</td>
<td>OCl\textsuperscript{−} &gt;&gt; ChT &gt; H\textsubscript{2}O\textsubscript{2} &gt; NO</td>
<td>OCT\textsuperscript{−} &gt;&gt; ChT &gt; NO &gt; H\textsubscript{2}O\textsubscript{2}</td>
</tr>
<tr>
<td>±SD (n = 3)</td>
<td>52 250 420 779</td>
<td>89 256 407 457</td>
</tr>
</tbody>
</table>

Units, μM oxidant.
has been reported for the S-thiolation of hemoglobin within erythrocytes (31). Physiologically, these events may provide a rationale for the observed decrease in GSH in active IBD lesions compared to paired normal mucosa (32, 33).

The inhibition of GAPDH in vivo is likely to be more complex than the simple interaction between RONS and the active-site thiol group. Direct inhibition of GAPDH activity by \( \mathrm{H}_2\mathrm{O}_2 \) is followed by autoreduction of the enzyme (24). However, in CEC from inflamed IBD mucosa, the loss of IAM-labeling capacity and inhibition of GAPDH activity did not recover despite incubation for 16 h in complete media under nonoxidative conditions (see Methods), suggesting that the occurrence of other reactions contribute to the oxidation of the reduced thiol. Direct oxidation by NO leads to the formation of an irreversible reaction with the essential cofactor NAD\(^+\) via RONS-induced activation of the DNA repair enzyme poly-(ADP ribose) polymerase (PARP) (38). While this would not have influenced the determination of GAPDH enzyme activity in the presence of excess cofactor as in this study, the increased PARP activity detected in IBD patients (39) may exacerbate the marked thiol-mediated inhibition of GAPDH in CEC seen in inflamed IBD mucosa. The importance of the observed inhibition of glycolysis via inhibition of GAPDH in IBD, however, remains unclear as CEC from normal colonic mucosa derive the majority of their energy requirements from \( \beta \)-oxidation of short-chain fatty acids (40).

The development of new strategies for therapies for IBD has been hampered by the lack of a clear understanding of the molecular basis of its pathogenesis. Intervention by targeting therapies to specific immunoregulatory mediators is often thwarted by the multiple overlapping functions of proinflammatory cytokines that are known to be involved in IBD. A more direct therapeutic strategy aimed at blocking the chemical species that cause actual tissue injury offers the prospect of more targeted therapies. In this context, the evidence presented here of RONS-induced injury to the colonic mucosa of IBD patients indicates considerable potential for new therapies based on the prevention of oxidative injury.

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