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Protective effects of glutathione on oxidative injury induced by hydrogen peroxide in intestinal epithelial cells

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ARTICLE INFO

Article history:
Received 1 June 2017
Received in revised form
21 August 2017
Accepted 28 September 2017
Available online xxx

Keywords:
GSH
IEC-6
Apoptosis
NF-κB
P38 MAPK
IL-1β
ROS
Hydrogen peroxide (H_2O_2)

ABSTRACT

Background: Reactive oxygen species are increased in multiple gastrointestinal diseases and contribute to their pathogenesis. glutathione (GSH) is an antioxidant that helps to prevent reactive oxygen species–mediated mucosal damage. This study examines the mechanisms by which GSH attenuates hydrogen peroxide (H_2O_2)–induced injury in intestinal epithelial cells.

Methods: IEC-6 cells were cultured and treated with H_2O_2 ± GSH. Inflammation was measured by nuclear factor kappa-B (NF-κB) P65 expression, NF-κB nuclear translocation, iκBα phosphorylation, and interleukin 1 beta secretion. Terminal deoxynucleotidyl transferase–mediated UTP end-labeling staining and cleaved caspase-3 were used to assess apoptosis. The role of P38 mitogen-activated protein kinase (P38 MAPK) signaling was examined using the P38 MAPK agonist U46619 and inhibitor SB203580 in H_2O_2 and GSH-treated cells. Phosphorylated and total P38 MAPKs and cleaved caspase-3 were measured by Western blot. Data are means ± standard deviation, statistical significance $P < 0.05$ by student's t-test, or one-way analysis of variance.

Results: Pretreatment with GSH attenuates the activation of NF-κB and P38 MAPK signaling pathways by H_2O_2. GSH also decreased H_2O_2-mediated increases in interleukin 1 beta secretion, cleaved caspase-3 activation, and apoptosis in IEC-6 cells. SB203580 attenuated the increase in apoptosis and cleaved caspase-3 in H_2O_2-treated cells. The increase in apoptotic index and cleaved caspase-3 observed in U46619-treated cells was also diminished by GSH.

Conclusions: GSH appears to ameliorate oxidative injury in intestinal epithelial cells by attenuating H_2O_2-mediated activation of NF-κB and P38 MAPK signaling pathways that regulate intestinal inflammation and apoptosis.

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0022-4804/$ – see front matter
https://doi.org/10.1016/j.jss.2017.09.041

Introduction

Reactive oxygen species (ROS) including peroxides, superoxide, hydroxyl free radical, and singlet oxygen are short-lived, highly reactive molecules that are byproducts of normal cellular metabolism. In normal conditions, ROS are thought to be essential for regulating physiological functions involved in development and modulation of immune system through activation of various cellular signaling pathways. However, during oxidative stress higher levels of ROS are produced which cause damage to proteins, nucleic acids, lipids, membranes, and subcellular organelles contributing to cell damage and may activate apoptosis and other pathways resulting in tissue injury.[1,2]

The gastrointestinal tract is an important source of ROS that are involved in the pathogenesis of many intestinal diseases including the following: *Helicobacter pylori*–associated gastritis, nonsteroidal antiinflammatory drug-induced enteritis, celiac disease, necrotizing enterocolitis, ischemia-reperfusion injury, and inflammatory bowel disease.[3] Damage of the intestinal epithelia during oxidative stress is thought to be caused by redox stress due to elevated levels of ROS and decreased antioxidant defenses.[3-7] The maintenance of physiological redox balance is important for attenuating tissue injury in diseases characterized by pathologic oxidative stress. Antioxidants such as dexpanthenol and gallic acid have been utilized to lower the severity of intestinal damage in an experimental model of gut injury including necrotizing enterocolitis and dextran sulfate sodium-induced experimental colitis in mice.[8,9]

The tripeptide glutathione (GSH) is composed of three amino acids: L-cysteine (Cys) , L-glutamic acid, and glycine which serves as a major cellular antioxidant.[10] ROS may react chemically with GSH, whereas hydrogen peroxide (H_2O_2) is enzymatically degraded by catalase or glutathione peroxidase (GPX) resulting in the oxidized GSH species glutathione disulfide (GSSG).[11] Normally, mucosal integrity is maintained by the luminal redox status of the GSH/GSSG and Cys/cystine couples, which modulate intestinal cell transition through proliferation, differentiation, or apoptosis and govern the regenerative potential of the mucosa.[12] Several intestinal conditions of the intestine-like inflammatory bowel disease and necrotizing enterocolitis are characterized by decreased GSH and GSH-dependent enzymes.[13,14] Suffice it to say GSH appears to play a major role in redox signaling, regulation of antioxidant defense, cell proliferation, and apoptosis.[15] Collectively these studies provide evidence that decreased GSH levels and alterations in GSH-dependent enzymes like GST, GPX, glutathione reductase (GR), and gamma-glutamate transpeptidase contribute to redox-mediated tissue injury in many diseases.[16] H_2O_2-induced oxidative stress has been shown to activate multiple signaling pathways in intestinal epithelial cells including the following: extracellular-related kinase 1/2, nuclear factor kappa-B (NF-κB), and P38 mitogen-activated protein kinase (P38 MAPK) resulting in increased apoptotic cell death in several studies.[17-20] However, little is known about the direct effects of GSH on oxidative stress-mediated injury to the intestinal epithelia.

In the current study, we use H_2O_2 to induce oxidative stress in the IEC-6 intestinal epithelial cells' cell line. IEC-6 cells have been widely employed as an *in vitro* model to study oxidative stress.[17-24] We hypothesize that GSH attenuates H_2O_2-induced oxidative stress in intestinal epithelia by improving cell viability, attenuating inflammation, and apoptotic cell death. Our results provide evidence that GSH can attenuate the effects of H_2O_2 on NF-κB activation, interleukin 1 beta (IL-1β) secretion, P38 MAPK phosphorylation, and apoptosis in intestinal epithelia cells.

Methods

Culture and treatments of IEC-6

IEC-6 cells (rat small intestinal epithelial) were grown in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin with streptomycin, and 0.1% of human insulin. The cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Pilot studies were performed by exposing IEC-6 cells to different concentrations of H_2O_2 (0-800 μM) in FBS-free medium for different times (2 h, 4 h, and 6 h), assessing cell viability and apoptosis. Oxidative stress was induced by exposing IEC-6 cells to H_2O_2 (0-800 μM) in FBS-free medium for 2 h, 4 h, and 6 h. The experimental conditions used in subsequent studies (200 μM of H_2O_2 treatment for 4 h) were chosen as optimal oxidative stress conditions based on these experiments and previously published literature .[20,25] The effects of GSH were examined by adding 10 mM of GSH to the media 1 h before H_2O_2 challenge. This dose of GSH was determined based on previous studies and pilot studies examining the effects of GSH on cell viability. Then, cultures were incubated for the time (4 h) noted. Signaling pathway inhibitors, including P38 MAPK (SB203580: SB, Cat. No: sc-3533, Santa Cruz) and NF-κB inhibitor (Parthenolide, Cat. No: sc-3523, Santa Cruz) were added to the culture medium 1 h before the addition of H_2O_2 (200 μM). IEC-6 cells were treated with U46619, (Cat. No: sc-201242, Santa Cruz), the P38 MAPK activator for 4 h to stimulate P38 MAPK and GSH pretreatment was 1 h before as in all other experiments.

Immunocytochemistry measurement of total and nuclear NF-κB protein

For immunocytochemistry (ICC), primary antibody (goat anti-NF-κB p65 subunit, 1:100 dilution, Santa Cruz) was used with a donkey anti-goat 1:200 secondary antibody and 5% normal donkey serum in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 used as blocking buffer. IEC cells seeded on 96-well plate were treated and fixed in 4% formaldehyde (10 min, at room temperature) and washed with PBS containing 0.1% Triton X-100 (30 min), and incubated with primary antibody (4°C overnight). The following day, the cells were washed with PBS + Tween 20 after removing primary antibody and incubating with secondary antibody (1 h in a dark environment). Mounting with Fluoroshield medium with 4,6-diamino-2-phenylindole (Cat. no. 104139; Abcam) was