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Role of heat shock proteins in oxygen radical–induced gastric apoptosis

Anna M. Leung, MD,[1] *Maria J. Redlak, PhD, and Thomas A. Miller, MD*[*]

Department of Surgery, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia

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ABSTRACT

Background: The generation of reactive oxygen species (ROS) and their resultant oxidative damage is a common pathway for gastric mucosal injury. Developing strategies to protect the gastric epithelium against oxygen free radical damage is of profound pathophysiological interest. We have previously shown caspase-mediated apoptosis as a major cause of ROS-induced cell death in gastric mucosa. Because heat shock proteins (Hsps) confer protection against many cytotoxic agents, this study was undertaken to determine whether modulation of Hsps was protective against oxidative damage.

Materials and methods: AGS cells (human gastric mucosal cell line) received either no pretreatment, heat shock pretreatment (1 h at 42 ± 1°C), or pretreatment with an Hsp modulating drug (geldanamycin or quercetin). Cells were then exposed to hydrogen peroxide (H_2O_2), a representative ROS (1 mM, a physiologically relevant concentration), for 24 h. Caspase-3 activation and Poly ADP Ribose Polymerase (PARP) inactivation, as well as DNA-histone complex formation were used as measures of apoptosis. Inducible Hsps (Hsp70 and Hsp90) were detected using Western blot analysis.

Results: Results showed heat shock pretreatment induced increased expression of Hsp70 without change in Hsp90. In response to H_2O_2 exposure alone, there was significant increase in DNA-histone complex formation as well as caspase-3 activation and PARP cleavage in gastric epithelium. Heat shock pretreatment resulted in statistically significant prevention in these measures of apoptosis. Geldanamycin increased Hsp70, but elicited cleavage of Hsp90 and subsequently resulted in an increase in H_2O_2-induced apoptosis. Quercetin decreased Hsp70 and resulted again in increased H_2O_2-induced apoptosis.

Conclusions: These findings indicate that heat shock pretreatment protects gastric mucosal cells against H_2O_2-induced apoptosis and that Hsp70 and Hsp90 may play key roles in this process. These results further suggest that perturbations in Hsp metabolism may induce mucosal injury in response to oxygen free radicals.

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1. Introduction

Current knowledge implicates oxygen free radicals as important mediators of gastric mucosal injury in response to episodes of ischemia–reperfusion, and following exposure to ethanol, bile acids, *Helicobacter pylori*, and nonsteroidal anti-inflammatory drugs [1–3]. The result of such injury involving the gastric epithelium can range from simple gastric irritation to frank

[*] *Corresponding author*. Department of Surgery, Medical College of Virginia Campus, Virginia Commonwealth University, Surgical Service (112), 1201 Broad Rock Blvd., Richmond, VA 23249. Tel.: +1 804 675 5112; fax: +1 804 675 5390.
E-mail address: thomas.miller3@med.va.gov (T.A. Miller).
[1] Dr. Leung is currently associated with the Department of Surgery, Penn State Hershey Medical Center, Hershey, Pennsylvania
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ulcer formation and the potential sequellae of hemorrhage and perforation. We have previously shown that the free radical hydrogen peroxide mediates its damaging effects on gastric mucosal cells through an apoptotic pathway [4]. Thus, targeting this method of death provides a potential strategy to protect the gastric mucosa against free radical-induced injury.

One approach that has been used to protect gastric mucosa against oxygen radical–induced injury has focused on increasing intracellular antioxidants. In this regard, induction of heat shock proteins (Hsps) has been proposed as a novel means of preventing injury induced by free radicals [5]. Among the various Hsps, 70-kDA Hsp (Hsp70) appears to exhibit broad cytoprotective functions. Increased expression of Hsp70, for example, has been shown to protect against increased cellular damage induced by hyperthermia, endotoxin, ultraviolet radiation, nitric oxide, and ischemia–reperfusion [6–8].

The present study was designed to test the hypothesis that increased expression of Hsp70 protects cultured gastric cells against oxygen free radical damage. We used hydrogen peroxide (H_2O_2) as the reactive oxygen species (ROS) to induce injury in a concentration of 1 mM, which was shown previously to be an apoptosis-inducing concentration and having physiological relevance [4]. A human gastric surface cell line, known as AGS cells, was used to accomplish these goals. We have used this cell line for many years, as have many other investigators, to study various aspects of gastric epithelial biology [9–11].

2. Materials and methods

2.1. Gastric epithelial cell culture

A human gastric epithelial cell line (AGS), derived from gastric adenocarcinoma cells and purchased from American Type Culture Collection (Manassas, VA), was used in these experiments. The cells were seeded in 75-cm^2 culture flasks (Corning, New York) and maintained in Ham's F-12 (Cellgro) culture medium supplemented with 10% fetal bovine serum, 100-μg/mL penicillin, 100-μg/mL streptomycin, and 0.25-μg/mL amphotericin B. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Experiments were performed in 3.5-cm^2 dishes (2.5 million cells per dish) or in six-well tissue culture plates, (300,000 cells per well; Costar; Sigma-Aldrich, St. Louis, MO). Before treatment, media were aspirated and cells were washed with phosphate-buffered saline.

2.2. Chemicals and reagents

A stock solution of 9.8 M H_2O_2 (Fisher Scientific, Pittsburgh, PA) was maintained at 4°C and diluted freshly before each use. Geldanamycin (17-AAG), the Hsp90 inhibitor (Sigma, St. Louis, MO), and Quercetin ($C_{15}H_{10}O_7$), the Hsp70 inhibitor (Sigma), were both dissolved in 100% dimethyl sulfoxide (DMSO) and maintained at −20°C. The stock solution of DMSO for these agents was 100 mM with the final concentration of DMSO in medium not exceeding 0.05% when used for experiments.

2.3. Heat shock treatment

Cultured cells were counted and plated equally in six-well tissue culture plates overnight. They were then subjected to hyperthermia of 42 ± 1°C for 1 h with a water bath. As a control, cells were cultured under normal conditions without hyperthermia. Cells were then routinely allowed to recover for 6 h at 37°C in a humidified atmosphere containing 5% CO_2. After recovery, cells were used for appropriate studies.

2.4. Quercetin treatment

Cultured cells were counted and plated equally in six-well tissue culture plates overnight. They were then incubated with quercetin 100 μM for 4 h. As a control, cells were cultured under normal conditions without quercetin. Cells were then used for appropriate studies. The dose of quercetin used for these experiments is commonly used in cell culture studies [12].

2.5. Geldanamycin treatment

Cultured cells were counted and plated equally in six-well tissue culture plates overnight. They were then incubated with geldanamycin 0.2–1 μM for 24 h. As a control, cells were cultured under normal conditions without geldanamycin. Cells were then used for appropriate studies. The dose range for geldanamycin used for these experiments is commonly used in cell culture studies [13].

2.6. Western blot analysis of caspase-3, Hsp70, Hsp90, β-actin, and Poly ADP Ribose Polymerase

After H_2O_2 treatment, whole cell lysates were extracted with lysis buffer containing 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM ethylene glycol tetra-acetic acid, 1 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40. An equal amount of cell lysate (50 μg) was solubilized in sample buffer, boiled for 5 min, and electrophoresed on a 4%–20% Tris-glycine gel (Invitrogen; Fisher Scientific, Pittsburgh, PA). Proteins were then transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Nonspecific binding was blocked with 20 mM Tris-HCl buffered saline (pH 7.6) plus 0.05% Tween-20 (TBS-T) containing 5% nonfat dry milk and 1% bovine serum albumin. After incubation with the appropriate primary antibody of Hsp70 (Cat no. 4876), Hsp90 (Cat no. 4877), β-actin (Cat no. 8457), caspase-3 (Cat no. 9665), or Poly ADP Ribose Polymerase (PARP) (Cat no. 9532) (Cell Signaling Technology, Danvers, MA), membranes were washed with TBS-T and then incubated with horseradish peroxidase anti-rabbit immunoglobulin G in the second reaction. Enhanced chemiluminescence reagent was used in accordance with the manufacturer's recommendations, and the resulting membranes were exposed to Kodak AR film and developed using a Kodak (Eastman Kodak Company, Rochester, NY) X-OMAT processor. Caspases that are involved in the execution of apoptosis exist in living cells as inactive zymogens that become activated through cleavage of intracellular caspase cascades [14,15]. The effect of hyperthermia on H_2O_2-induced apoptosis was assessed by Western blot analysis on caspase-3 and PARP cleavage. This caspase was chosen for measurement because it is the most important member of the effector caspases responsible for orchestrating apoptosis [14,15].