Review

Various stress proteins protect gastric mucosal cells against non-steroidal anti-inflammatory drugs

T. Mizushima

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan, Tel: ++81-96-371-4323, e-mail: mizu@gpo.kumamoto-u.ac.jp

Received 12 July 2006; accepted 1 September 2006

Abstract. Gastric mucosal cell death induced by non-steroidal anti-inflammatory drugs (NSAIDs) is suggested to be involved in NSAID-induced gastric lesions. Therefore, cellular factors that suppress this cell death are important for protection of the gastric mucosa from NSAIDs. When cells are exposed to various stressors, including NSAIDs, they induce a number of proteins, so-called stress proteins, in order to protect themselves against such stressors. Stress proteins contain cytosolic molecular chaperons (such as heat shock proteins), endoplasmic reticulum molecular chaperons (such as glucose-regulated proteins) and heme oxygenase-1. We recently showed that (i) these stress proteins are up-regulated by NSAIDs both in vitro and in vivo; (ii) these up-regulation make gastric mucosal cells resistant to NSAIDs in vitro; (iii) these up-regulation protects the gastric mucosa from NSAID-induced gastric lesions in vivo. In this review, I summarize these results and propose that non-toxic inducers of these stress proteins are therapeutically beneficial as anti-ulcer drugs.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a useful family of therapeutics, accounting for nearly 5% of all prescribed medications (Smalley et al., 1995). The anti-inflammatory actions of NSAIDs are mediated through their inhibitory effects on cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the other hand, NSAID use is associated with gastrointestinal complications (Hawkey, 2000), with about 15–30% of chronic users of NSAIDs suffering from gastrointestinal ulcers and bleeding (Barrier and Hirschowitz, 1989; Fries et al., 1989).

Although PGs have a strong protective effect on gastrointestinal mucosa, the inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side-effects of NSAIDs (Lichtenberger, 2001). We have recently demonstrated that NSAIDs induce apoptosis in primary cultures of gastric mucosal cells in a manner independent of COX inhibition (Tanaka et al., 2005; Tomisato et al., 2001 & 2004a; Tsutsumi et al., 2004). As for 2001 the molecular mechanism governing this apoptosis, we recently proposed that permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca^{2+} influx which in turn induces production of the C/EBP homologous transcription factor (CHOP), and activates calpain, a Ca^{2+}-dependent cysteine protease, both of which have apoptosis-inducing ability (Tanaka et al., 2005). Furthermore, we suggested that both COX-inhibition and NSAID-induced cell death (such as apoptosis) in gastric mucosa are required for production of NSAID-induced gastric lesions in vivo (Tomisato et al., 2004b). Cellular factors that suppress NSAID-induced apoptosis are therefore important for protection of gastric mucosa from NSAID-induced gastric lesions.

When cells are exposed to various stressors, including NSAIDs, they induce a number of proteins, so-called stress proteins, in order to protect themselves against such stressors. Molecular chaperons are representative stress proteins. Their up-regulation in cells confers resistance to various stressors as the chaperons re-fold or degrade denatured proteins produced by stressors (Mathew and Morimoto, 1998). Molecular chaperons can be divided into cytosolic molecular chaperons (such as heat shock proteins (HSPs)) and endoplasmic reticulum (ER) molecular chaperons (such as glucose-regulated proteins (GRPs)). Heme oxygenase-1 (HO-1) is another type of stress protein. Not only its substrate, heme, but also various stressors such as oxidative stressors, ultraviolet irradiation, inflammatory cytokines and heavy metals, have been reported to induce HO-1 production (Maines, 1997; Ponka, 1999; Tenhunen et al., 1969). HO-1 degrades heme to carbon monoxide (CO), free iron and biliverdin. Biliverdin is subsequently converted into bilirubin by biliverdin reductase (Maines, 1997; Ponka, 1999; Tenhunen et al., 1969). Bilirubin and biliverdin are potent antioxidants and CO has anti-apoptotic activity. Therefore, up-regulation of HO-1 in cells makes cells resistant to apoptosis induced...
by various stressors (Brouard et al., 2000; Maines, 1997; Tenhunen et al., 1969).

Based on these results, we consider a possibility that various stress proteins are up-regulated by NSAIDs and this up-regulation contributes to suppress NSAID-induced apoptosis and NSAID-induced gastric lesions.

**Experimental procedures**

**Gastric Damage Assay** – Gastric damage assays were performed as described previously (Tomisato et al., 2004b). Rats, which had had been fasted for 24 h, were intraperitoneally injected with SnMP (dissolved in 0.1 N NaOH, adjusted to pH7.6 with HCl). One hour later, indomethacin in 1 % methylcellulose was orally administered. Three hours after administration, the rats were sacrificed by decapitation under light anesthesia with ethyl ether and the stomachs were removed and scored for hemorrhagic damage by an observer unaware of the treatment the rats had received. Calculation of the scores involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index.

**Cell Culture, Transfection and Cell Viability Assay** – Gastric mucosal cells were isolated from guinea pig fundic glands, as described previously (Hirakawa et al., 1996; Tomisato et al., 2002). Isolated gastric mucosal cells were cultured for 12 h in RPMI 1640 containing 0.3 % v/v FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in type-I collagen-coated plastic culture plates in 5 % CO2/95 % air at 37 °C. After removing non-adherent cells by washing with RPMI 1640, cells that were attached to the plate at approximately 50 % confluence were used. Guinea pig gastric mucosal cells prepared under these conditions have been previously characterized, with the majority (about 90 %) of such cells being identified as pit cells (Hirakawa et al., 1996; Tomisato et al., 2002).

Human gastric carcinoma cells (AGS) were cultured in RPMI1640 medium supplemented with 10 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in 5 % CO2/95 % air at 37 °C. Unless otherwise noted, cells (0.8 × 104 cells per well in 24-well plates, 4 × 104 cells per well in 6-well plates, 6 × 104 cells in 100-mm plates) were cultured for 24 h and then used in the experiments. Transfection of cells with plasmid was carried out using Lipofectamine (TM2000) according to the manufacturer’s instructions. Transfected cells were used for experiments after a 24 h recovery period. Transfection efficiency was determined in parallel plates by transfection of cells with the pEeGFP-C1 control vector. Transfection efficiency was more than 80 % in all experiments.

NSAIDs were dissolved in DMSO or Na2CO3 (for indomethacin only) and control experiments (without NSAIDs) were performed in the presence of the same concentrations of DMSO or Na2CO3. Cells were exposed to NSAIDs by changing the medium. Cell viability was determined by the MTT method.

**Immuno-blotting Analysis** – Whole cell extracts and nuclear extracts were prepared as described previously (Schreiber et al., 1989; Tsutsumi et al., 2002). The protein concentration of samples was determined by the Bradford method. Samples were applied to 8 % (HSP72 and GRP78), 10 % (lamin B, Nfr2, p38 MAPK and actin) or 12 % (HO-1) polyacrylamide SDS gels, subjected to electrophoresis, and proteins then immuno-blotted with appropriate antibodies.

**Histological and Immunohistochemical Analysis** – Gastric tissue samples were fixed in 4 % buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were stained first with Mayer’s hematoxylin and then with 1 % eosin alcohol solution for histological examination (hematoxylin and eosin (HE) staining). Samples were mounted with Malinol and inspected using microscopy (Olympus IX70).

For immunohistochemical analysis, sections were blocked with 2.5 % goat serum for 10 min and then incubated for 12 h with antibody against HO-1 (1:500 dilution) in the presence of 2.5 % BSA, and finally incubated for 1 h with Alexa Fluor 488 goat anti-mouse immunoglobulin G. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX70).

**TdT-mediated dUTP-biotin End Labeling (TUNEL) Assay** – Gastric tissue samples were fixed in 4 % buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were first incubated with proteinase K (10 µg/ml) for 15 min at 37 °C, then with TdTase and biotin-14-ATP for 1 h at 37 °C and finally with Alexa Fluor 488 conjugated with streptavidin for 1 h. Sections were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX70).

**Statistical Analysis** – All values are expressed as the mean ± standard deviation (S.D). One-way analysis of variance (ANOVA) followed by Scheffe’s multiple comparison test was used for evaluation of differences between groups. The Student’s t-test for unpaired results was used for the evaluation of differences between two groups. Differences were considered to be significant for values of P < 0.05.

**Results and discussion**

**NSAIDs Up-regulate Various Stress Proteins** – Up-regulation of various stress proteins was examined in primary cultures of guinea pig gastric mucosal cells. This type of cell has been used as an in vitro model for physiological and pathological studies of gastric mucosa because various characteristic features of gastric mucosal cells in vivo (such as vigorous secretion of mucus) are reproduced in this system (Hirakawa et al., 1996). As shown in Figure 1A, treatment of cells with indomethacin up-regulated HO-1 very rapidly (within 3 h of the addition of indomethacin) and transiently (HO-1 levels returned to pre-treatment levels 24 h after the addition) (Aburaya et al., 2006). Indomethacin also up-regulates other stress proteins (HSP72 and GRP78) (Fig. 1A). The results in Figure 1A show that up-regulation of HO-1 by indomethacin occurs prior to that of HSP72 and GRP78. Figure 1B shows the effects of different concentrations of indomethacin on HO-1 up-regulation. Up-regulation of HO-1 was just apparent at 25–50 µM indomethacin and was distinct at 200–400 µM indomethacin. These concentrations of indomethacin did not affect cell viability.