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Suppression of growth arrest and DNA damage-inducible 45 alpha
(GADD45α) expression confers resistance to sulindac and indomethacin-
induced gastric mucosal injury

Shiun-Kwei Chiou, Amy Hodges, Neil Hoa

Department of Veterans Affairs Medical Center, 5901 E 7th st., Long Beach,
California, 90822, USA
Running Title Page

a) **Running Title:** Suppression of GADD45α inhibits gastric damage by NSAIDs.

b) **Correspondence to:** Shiun-Kwei Chiou, Ph.D., Department of Veterans Affairs Medical Center, 5901 E 7th St, Long Beach, CA 90822-5201, phone: (562) 862-8000 Ex 4910, fax: (562) 826-5675, e-mail: skchiou@yahoo.com

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as sulindac and indomethacin are a major cause of gastric erosions and ulcers. Induction of apoptosis by NSAIDs is an important mechanism involved. Understanding how NSAIDs affect genes that regulate apoptosis is useful for designing therapeutic or preventive strategies, and evaluating the efficacy of safer drugs being developed. We investigated whether GADD45α, a stress signal response gene involved in regulation of DNA repair and induction of apoptosis, plays a part in NSAID-induced gastric mucosal injury and apoptosis in vivo in mice, and in vitro in cultured human AGS and rat RGM-1 gastric epithelial cells. Intraperitoneal administration of sulindac and indomethacin both resulted in up-regulation of GADD45α expression and induction of significant injury and apoptosis in gastric mucosa of wildtype mice. GADD45α−/− mice were markedly more resistant to both sulindac and indomethacin-induced gastric mucosal injury and apoptosis than wildtype mice. Sulindac sulfide and indomethacin treatments also concentration-dependently increased GADD45α expression and apoptosis in AGS and RGM-1 cells. Anti-sense suppression of GADD45α expression significantly reduced sulindac and indomethacin-induced activation of caspase 9 and apoptosis in AGS cells. Pretreatments with exogenous prostaglandins and siRNA suppression of COX-1 and -2 did not affect up-regulation of GADD45α by sulindac sulfide and indomethacin in AGS cells. These findings indicate that GADD45α up-regulation is a COX-independent mechanism that is required for induction of severe gastric mucosal apoptosis and injury by NSAIDs, likely via a caspase 9-dependent pathway of programmed cell death.
Introduction

NSAIDs such as indomethacin, sulindac and others are commonly utilized drugs worldwide. However, they frequently cause gastric erosions and ulcers. NSAIDs can cause gastric injury through a variety of mechanisms, many of which culminate in induction of apoptosis. A well known mechanism is inhibition of prostaglandin synthesis by inhibiting cyclooxygenase (COX) -1 and COX-2 enzymes. Inhibition of both COX-1 and COX-2 appears crucial for effective induction of gastric injury, since non-selective NSAIDs (i.e. indomethacin, sulindac) cause severe gastric erosions while COX-2 selective NSAIDs (e.g. celecoxib) produce significantly less injury (Tanaka et al, 2001; Wallace et al, 2000). Although selective NSAIDs are less damaging to gastric mucosa, their adverse cardiovascular effects have prompted their recent withdrawal from the market.

Several strategies are currently being developed to overcome gastrointestinal toxicities associated with NSAIDs while minimizing the adverse cardiovascular effects. One strategy involves attachment of small chemical components to nonselective NSAIDs such that release of these moieties upon administration of the drug would produce gastroprotective effects (e.g. nitric oxide-releasing NSAIDs (Singh et al, 2009), and hydrogen sulfide-donating NSAIDs (Fiorucci et al, 2006), etc.). Another strategy being considered is selective inhibition of microsomal prostaglandin E(2) synthase (mPGES)-1 derived prostaglandin (PGE2) formation as an alternative to general inhibition of physiologically relevant prostanoid synthesis by NSAIDs (Koberle and Werz, 2009). Excessive PGE2 formation from COX-2 and mPGEs-1 activities has been linked to inflammation, pain, fever, atherosclerosis, and tumorigenesis. Inhibition of
mPGE-1 is expected to reduce inflammation, fever and pain while allowing continued biosynthesis of gastroprotective prostanoids.

Mounting evidence indicates that NSAIDs also exert COX/prostaglandin-independent effects, such as inhibition of EGF signaling, regulation of cell cycle proteins such as p21 and cyclins, inhibition of the MAP-kinase pathway (Ishikawa et al, 1998; Tegeder et al, 2001), and increased ubiquitination of proteins such as HIF-1α and β-catenin in T cells for degradation by the ubiquitin-proteasome (Jones et al, 2002; Dihlman et al, 2003). NSAIDs also directly activate pro-apoptotic factors such as caspases and Bax (Zhou et al, 2001), and down-regulate levels of survivin, an anti-apoptosis protein, both in vivo in human and rat gastric mucosa and in vitro in rat gastric epithelial RGM-1 cells (Chiou et al, 2005). Our recent studies showed that exogenous PGE2 treatments do not alter survivin levels in the rat gastric mucosa or gastric epithelial cells, suggesting that regulation of survivin by NSAIDs occurs through COX/prostaglandin-independent pathways (Chiou et al, 2005). Further understanding of how non-selective NSAIDs affect genes involved in the regulation of apoptosis remains crucial for designing new therapeutic or preventive strategies against induction of gastric mucosal injury, and is also useful for evaluating the efficacy of new drugs being developed.

GADD45α is a stress signal response gene involved in regulation of DNA repair and apoptosis (Hollander et al, 1999; Hildesheim et al, 2002). GADD45α null mice exhibit severe genomic instability characterized by aneuploidy, centrosome amplification, aberrant mitosis, and
cytokinesis (Hollander et al, 1999). More strikingly, mice lacking GADD45α are susceptible to carcinogenesis induced by ionizing radiation, UV irradiation, and dimethylbenzanthracene (Hollander et al, 2001). In addition, GADD45α is important in cellular response to a variety of stress stimuli and genotoxic agents that are known to induce apoptosis, such as hypoxia, growth factor withdrawal, ionizing radiation, hydroxyurea, and methanesulfonate (Fornace et al, 1992). UV radiation-induced apoptosis is deficient in GADD45α null mice (Hollander et al, 1999). Rebamipide, a gastro-protective agent that inhibits indomethacin-induced apoptosis, also down-regulates GADD45α expression (Naito et al, 2005), suggesting that NSAIDs may activate GADD45α expression as a mechanism of gastric cell apoptosis and gastric damage. The role of GADD45α in NSAID-induced gastric mucosal injury and apoptosis has not been explored. In this study, we investigated: 1) Whether GADD45α expression is up-regulated by sulindac and indomethacin treatments both in vitro in human gastric AGS and normal rat gastric RGM-1 cells and in vivo in mouse gastric mucosa. 2) Whether anti-sense suppression of GADD45α expression in AGS cells confers resistance to NSAIDs-induced apoptosis, and whether GADD45α null mice are resistant to NSAID-induced gastric injury. 3) Whether exogenous PGE2 treatment and siRNA suppression of COX-1 and -2 could inhibit up-regulation of GADD45α by sulindac and indomethacin in AGS cells. 4) Whether NSAIDs induce growth inhibition and caspase 9 activation in gastric epithelial cells, and whether they are associated with GADD45α up-regulation.
Methods

Animals, treatments and tissues

These studies were approved by the Subcommittee for Animal Studies of the Long Beach Department of Veterans Affairs Medical Center. GADD45α -/- B6129F1 mice (Hollander C, 1999, MMHCC Repository, Frederick MD), their wildtype (WT) age-matched siblings and WT age-matched C57BL6 mice (Jackson Laboratories, Bar Harbor ME) weighing 20-24g were fasted for 16 hours. Groups of 12 mice each were then administered the following via intraperitoneal injections: 1) vehicle (.2 mol/L Na2CO3 +NaH2PO4 in 4:1 ratio), 2) 15mg/kg indomethacin (Sigma Chemicals, St Louis MO), or 3) 30mg/kg sulindac (Sigma Chemicals, St Louis MO). The indomethacin concentration used has previously been utilized to effectively induce experimental gastric mucosal injury in rodents within a few hours. The sulindac concentration was chosen because the daily recommended dose in humans for sulindac is double that of indomethacin. Four, eight and twenty four hours after administration of drugs, the mice were euthanized with 200mg/kg Nembutal, and laparotomy was immediately performed to excise the stomachs. Gastric samples for biochemical studies were prepared by scraping the mucosa and freezing it immediately in liquid nitrogen. Gastric samples for immunohistochemical studies were frozen and sectioned.

Quantitation of macroscopic injury in gastric mucosa

The stomachs were opened along the greater curvature and photographed using a Canon Powershot A70 digital camera (Canon USA inc., One Canon Plaza Lake Success, NY). The area of visible mucosal erosions was measured using Photoshop CS3 (Adobe Systems Inc, San Jose,
Mucosal damage was expressed as percent damage of the entire gastric mucosa and calculated as follows:

\[
\% \text{ Gastric Mucosal Damage} = \left( \frac{\text{Total Lesion Area}}{\text{Total Area of the Gastric Mucosa}} \right) \times 100
\]

**Analysis of apoptosis in the gastric mucosa (caspase-3 activity assay)**

Frozen gastric mucosal sections from 4 hours post NSAIDs administration were incubated with anti-Caspase-3 or anti-active-caspase-3 rabbit polyclonal antibodies (BioVision Inc, Mountain View, CA), followed by Texas Red-conjugated goat anti-rabbit secondary antibodies (Vector Laboratories, Burlingame, CA). Staining was visualized using Nikon Optiphot epifluorescence microscope (Nikon, Inc, Melville, NY) with Omega filter fluorescein isothiocyanate/Texas red. Percent of apoptotic cells within and around the periphery of each gastric mucosal erosion was determined as follows:

\[
\% \text{ apoptotic cells/injury} = \frac{\text{number of active caspase-3 positive (red) cells}}{\text{500 gastric mucosal cells in and around erosion cross sections (blue Hoechst counter stained)}} \times 100
\]

Sections from 5 different gastric mucosal erosions were analyzed per treatment condition. Five randomly selected intact gastric mucosal areas were similarly analyzed per treatment condition. Quantitation of fold gastric mucosal caspase-3 activity was performed using gastric mucosal scrapings and the colorimetric CasPASE apoptosis assay kit (Geno Technologies, inc, Maryland Heights, MO) following manufacturer’s instructions and as previously described (Chiou et al, 2005). Gastric mucosal scrapings from 6 mice were utilized per condition and treatment.

**Cell lines and treatments**
Human gastric mucosal AGS cells were cultured in RPMI-1640 medium (Sigma, St Louis MO) supplemented with 10% FBS and 1% antibiotics. Normal rat gastric epithelial RGM-1 cells were cultured in DMEM:F12 medium (Sigma, St Louis MO) supplemented with 20% FBS. For quantification of GADD45α mRNA expression, cells were grown in 6 well culture plates until they were about 70% confluent, incubated in serum free medium overnight, then treated with vehicle only (dimethylsulfoxide), 0.2mM, 0.1mM, or 0.05mM Indomethacin, or 0.1mM, 0.05mM, or 0.01mM sulindac sulfide for 3 hours. Then total RNA was purified for Realtime RT-PCR analysis. We utilized the sulindac metabolite since sulindac is a prodrug that is insoluble in culture medium.

Sulindac sulfide concentrations utilized were lower than those of indomethacin because this active sulindac metabolite was very potent in inducing apoptosis. Once cells were dead, they were no longer useful for subsequent biochemical assays.

For determination of GADD45α protein up-regulation, cells were grown and serum starved as above, then treated with vehicle only, 0.2mM indomethacin or 0.1mM sulindac sulfide for 6 hours. Then total protein was extracted for Western Blot analysis.

To examine effects of exogenous prostaglandins on GADD45α up-regulation by NSAIDs, cells were treated with 1 μM, or 10 μM of PGE2 (Cayman Chemicals, Ann Arbor, MI) for 6 hours or pretreated with the same concentrations of PGE2 for 30 minutes and then treated with 0.2mM indomethacin or 0.1mM sulindac sulfide. Vehicle only, 0.2mM indomethacin and 0.1mM sulindac sulfide treatments were utilized as controls. At 6 hours after NSAID treatment, before onset of significant apparent cellular damage, cells were collected for Western blot analysis.

To examine the effects of siRNA suppression of COX-1 and COX-2 on GADD45α up-regulation by NSAIDs, 10nM of siRNA targeting COX-1 (Santa Cruz Biotechnologies, Santa
Cruz CA), COX-2 (Santa Cruz Biotechnologies, Santa Cruz CA), or both, were transfected into AGS cells at 50% confluence using RNAiMax transfection reagent (Invitrogen, Carlsbad CA), following the manufacturer’s instructions. Mock transfection utilizing only the transfection reagent but no siRNA, and transfection using a control RNA (Santa Cruz Biotechnologies, Santa Cruz CA) that does not silence mammalian mRNAs were included as controls. The control RNA is conjugated to a green fluorescent dye for monitoring transfection efficiency with fluorescence microscopy (percentage of fluorescent cells per 500 total cells counted). 48 hours post transfection, cells were treated with vehicle only, sulindac sulfide (0.1mM) and indomethacin (0.2mM) for 4 hours. Then total RNA was isolated for RT-PCR analysis of COX-1, COX-2, GADD45a and β-actin expressions, utilizing gene specific primers following the manufacturer’s instructions (Santa Cruz Biotechnologies, Santa Cruz CA).

**Real-time RT-qPCR**

Total RNA was isolated utilizing the RNeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. 0.3 μg of total RNA from each sample was used in a random primed reverse transcription (RT) reaction utilizing the MMLV reverse transcriptase (Applied Biosystems, Foster City, CA) to synthesize first strand cDNA. Quantitation of mRNA expression (first strand cDNA) was performed by Realtime qPCR using custom made gene specific primers (Invitrogen, Carlsbad, CA). The Human Gadd45α primer sequences were previously described (Chiou and Hoa, 2009). Survivin primers were also previously described (Chiou et al, 2003). Realtime qPCR data was analyzed using a method described by M.W. Pfaffl (Pfaffl, 2001). This method accounts for variations in amplification efficiency in the following formula:
Ratio = \left( \frac{E_{\text{target}}}{E_{\text{ref}}} \right)^{\Delta \text{Ct target (control-treated)}} / \left( \frac{E_{\text{ref}}}{E_{\text{target}}} \right)^{\Delta \text{Ct ref (control-treated)}}

Target refers to Gadd45\(\alpha\) or survivin, and beta-actin is used as reference to which all Gadd45\(\alpha\) and survivin PCR products were normalized. \(E\) refers the amplification efficiency based on the slope of the standard dilution curve. Realtime qPCR analysis was performed on the iCycler (Biorad, Hercules, CA) using IQ SYBR Green Supermix (Biorad, Hercules, CA). Each PCR reaction was performed in triplicate, and results were presented as average fold expression \(\pm\) standard deviation.

**Western blot analysis**

Cells were lysed with protein lysis buffer (20 mM Tris-HCl (pH 7.9), 1.5 mM MgCl\(_2\), 550 mM NaCl, 0.2 mM EDTA, 2 mM DTT and 20 % (v/v) glycerol). 100 \(\mu\)g protein per sample was separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were blocked in skim milk and incubated with rabbit-polyclonal anti-Gadd45\(\alpha\), rabbit polyclonal anti-survivin, rabbit polyclonal anti-pro Caspase 9, and rabbit polyclonal active caspase 9 p10 antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) at 4°C overnight. The membrane was then washed and incubated for 1 h with peroxidase conjugated goat-anti-rabbit secondary antibody (Sigma, St. Louis, MO) for 1 h. The protein signals were visualized using ECL chemo luminescence reagent (Amersham Life Science, Piscataway, NJ) and by exposure to Kodak X-omat film (Eastman Kodak, Pittsburgh, PA). The membranes were stripped and re-incubated with monoclonal anti-\(\beta\)-actin antibodies (Sigma, St. Louis, MO), then with peroxidase conjugated anti-mouse antibodies (BD Transduction Laboratories, Lexington, KY). Protein signal densities were quantified using a Metamorph Imaging System, version 3.0.
(Universal Imaging Corp., Downingtown, PA). Protein signal densities were subtracted from background densities and normalized to the corresponding β-actin signal densities.

**Anti-sense suppression of Gadd45α**

Morpholino anti-sense oligonucleotides were designed to block expression of human Gadd45α mRNA (Gene Tools, Philomath, OR). The oligonucleotide sequence used was previously described (Chiou and Hoa, 2009). AGS cells were seeded in 6 well tissue culture plates to obtain 70% confluence the next day. Endoporter transfection reagent (Gene Tools, Philomath, OR) was used alone (mock transfection), or to deliver either negative control (inverse sequence to the antisense) or the antisense oligonucleotide at 5μM concentration into cells. 48 hours later Western blot analysis was utilized to determine the extent of suppression of Gadd45α protein. At this time, cells were serum starved overnight for treatment with vehicle only, 0.2mM or 0.1mM indomethacin or 0.1mM or 0.05mM sulindac sulfide for 24 hours followed by apoptosis assay and Western blot analysis for caspase 9 activation.

**Determination of Growth Inhibition and Apoptosis in response to indomethacin and sulindac sulfide treatments in cell culture studies**

For growth inhibition assays, 10⁴ AGS and RGM-1 cells per well were seeded and grown in reduced-serum medium (0.5% FBS) overnight, then triplicate wells were treated with vehicle only, 0.002mM, 0.01mM, 0.02mM and 0.2mM indomethacin, or 0.001mM, 0.005mM 0.01mM and 0.1mM sulindac sulfide. Proliferation assays were performed using the CyQUANT® Cell Proliferation Assay Kit (Molecular Probes Inc., Eugene, OR), according to the manufacturer’s instructions, at 0, 24, 48 and 72 hours, with renewal of culture medium containing NSAIDs.
every 24 hours. Fluorescence intensity data was obtained using NOVASTar (BMG LABTECH, Durham, NC), and converted to number of cells according to the manufacturer’s instructions. For apoptosis assays, AGS and RGM-1 cells were grown and serum starved as described above, then treated with vehicle only, 0.2mM, 0.1mM or 0.05mM Indomethacin, or 0.1mM, 0.05mM or 0.01mM sulindac sulfide. 24 hours later, the number of cells undergoing apoptosis was measured using BD ApoAlert apoptosis detection kit (BD Biosciences, Mountain View, CA), following the manufacturer’s instructions, and flow cytometry analysis as described previously (Ge et al., 2009). Briefly, induced cells were trypsinized, gently washed, and labeled with annexin V per manufacturer’s protocol. Ten thousand cells were analyzed by flow Cytometry using the BD FACSCalibur (BD Biosciences, San Jose, CA) with single laser emitting excitation wavelength at 488 nm.

**Statistical analysis**

Student's two-tailed t test was used to compare data between two groups. One-way analysis of variance and Bonferroni’s correction were used to compare data between three or more groups. P-value ≤ 0.05 was considered statistically significant.
Results

Gastric mucosal injury and apoptosis in NSAID treated WT versus GADD45α-/- mice

Vehicle only treatment resulted in low basal amounts of visible gastric mucosal injury in WT mice at all the time points examined (Figure 1A). Sulindac and indomethacin treatments caused severe visible gastric lesions in WT mice at all the time points examined, compared to vehicle only treatment (Figure 1A). The extent of gastric mucosal injury was similar at 4 and 8 hours, and slightly less at 24 hours after NSAIDs were administered (Figure 1A). Vehicle only treatment also resulted in low basal amounts of visible gastric mucosal injury in GADD45α-/- mice at all the time points examined (Figure 1B). Compared to vehicle only treatment, treatment with sulindac did not cause significantly greater amounts of visible gastric mucosal injury at all the time points examined in GADD45α-/- mice (Figure 1B). Indomethacin treatment caused slightly greater amounts of visible gastric mucosal injury in GADD45α-/- mice but the increase was not statistically significant (Figure 1B).

Immunofluorescence staining of gastric mucosal sections showed that caspase-3 is expressed almost ubiquitously in both the WT and GADD45α-/- mouse gastric mucosa treated with vehicle only, sulindac or indomethacin (Figure 2, upper panels). The white dotted lines demarcate the borders of gastric mucosal lesions found in sulindac and indomethacin treated mice (Figure 2, upper panels), and showed that caspase-3 is also detectable in areas surrounding the lesions. This result is consistent with others’ findings that caspase-3 expression is widely detected in normal human gastric mucosa (Kania et al, 2003), and enabled us to measure caspase-3 activity in the mouse gastric mucosa as a method to determine the extent of apoptosis induction by NSAIDs. Basal caspase-3 activity was detected in the gastric mucosa of vehicle
only treated WT mice (Figure 2, left upper graph). Sulindac and indomethacin treatments increased caspase-3 activity 3.1 +/- 0.2 and 4.2 +/- 0.2 fold, respectively, in gastric mucosa of WT mice (Figure 2, left upper graph). Basal caspase-3 activity in gastric mucosa of GADD45α-/- mice treated with vehicle only was slightly lower than that in gastric mucosa of vehicle only treated WT mice (0.79 +/- 0.1 fold; Figure 2 right upper graph). Sulindac and indomethacin treatments increased caspase-3 activity 1.2 +/- 0.2 and 1.4 +/- 0.3 fold, respectively, in gastric mucosa of GADD45α-/- mice (Figure 2, right upper graph). The increases in caspase-3 activity due to indomethacin and sulindac treatments in the GADD45α-/- gastric mucosa were significant, but drastically less than in WT gastric mucosa.

To determine the percentage of apoptotic cells in intact gastric mucosa and erosions induced by sulindac and indomethacin, cross sections of intact mucosa and erosions were stained with active caspase-3 antibodies, and the percentage of positive cells were determined. Basal amounts of apoptotic cells were detected in WT intact gastric mucosa with vehicle only treatment (8.1 +/- 0.7%; Figure 2 left lower graph). Compared to vehicle treatment, sulindac and indomethacin treatments slightly but significantly increased the percentage of apoptotic cells in the intact areas of the gastric mucosa in WT mice (12.3 +/- 0.8 and 11.5 +/- 0.4, respectively; Figure 2 left lower graph). Both sulindac and indomethacin-induced erosions contained significantly greater proportions of apoptotic cells (32.1 +/- 4.3 and 38.5 +/- 3.3 %, respectively; Figure 2 left lower graph). Basal amounts of apoptotic cells were also detected in GADD45α-/- intact gastric mucosa with vehicle only treatment (5.7 +/- 1.2%; Figure 2 right lower graph). Sulindac and indomethacin treatments also slightly increased the percentage of apoptotic cells in the intact areas of the gastric mucosa in GADD45α-/- mice (8.1 +/- 0.7 and 7.6 +/- 0.5, respectively; Figure
Both sulindac and indomethacin-induced erosions contained significantly higher proportions of apoptotic cells (15.1 +/- 1.3 and 18.0 +/- 0.9 %, respectively; Figure 2 right lower graph). Although the percentage of apoptotic cells were significantly increased with NSAIDs treatments in gastric mucosa and erosions in GADD45α-/- mice, they were markedly lower than that in WT mice.

**Up-regulation of GADD45α expression in gastric mucosa of WT mice**

Gastric mucosal scrapings from WT mice treated with sulindac and indomethacin were analyzed for GADD45α protein expression compared to those treated with vehicle only. Both sulindac and indomethacin treatments significantly increased GADD45α protein levels in the gastric mucosa compared to vehicle only treatments (2.5 +/- 0.2 fold and 3.8 +/- 0.9 fold increase, respectively; Figure 3 graphs). Western blots showing representative GADD45α protein levels found in gastric mucosal scrapings from vehicle only, sulindac and indomethacin treated mice were shown in top panels of Figure 3.

**Up-regulation of GADD45α mRNA and protein expression by sulindac and indomethacin treatments in human gastric mucosal AGS and normal rat gastric epithelial RGM-1 cells in culture**

AGS and RGM-1 cells were treated with vehicle only, and increasing concentrations of sulindac sulfide and indomethacin. GADD45α mRNA expression levels were then measured by quantitative Realtime PCR. Both sulindac sulfide and indomethacin treatments significantly increased GADD45α mRNA levels in both cell lines, and the greater the NSAID concentration utilized, the greater the fold increase (Figure 4A,B). Our treatments with Sulindac sulfide down-
regulated survivin mRNA expression in both cell lines and the fold down-regulation increased with increasing NSAID concentrations (Figure 4A). Survivin mRNA down-regulation was measured as independent confirmation of the effectiveness of our sulindac sulfide treatments, since others have shown that sulindac treatment down-regulates survivin via transcriptional control in cultured cells (Zhang et al, 2004). Survivin mRNA was not measured in indomethacin treated cells since we have previously shown that indomethacin treatment does not down-regulate survivin at the mRNA level, but enhances survivin protein degradation (Chiou and Mandayam, 2007).

Treatments with sulindac sulfide and indomethacin both up-regulated GADD45α protein levels compared to vehicle only treatment in both cell lines (Figure 4C). Treatments with sulindac sulfide and indomethacin both down-regulated survivin protein levels compared to vehicle only treatment in both cell lines (Figure 4C), verifying that our sulindac sulfide and indomethacin treatments were effective in these cells.

**Induction of apoptosis in AGS and RGM-1 cells by sulindac sulfide and indomethacin treatments**

Treatments of both AGS and RGM-1 cells with indomethacin and sulindac sulfide induced significant amounts of apoptosis compared with vehicle only treatment, as shown by Annexin V binding assay and flow cytometry (Figure 5). The amount of apoptosis induction increased with increasing indomethacin and sulindac sulfide concentrations utilized (Figure 5 C and D, respectively). Thus, there is a positive association between the extent of apoptosis induction and the extent of GADD45α up-regulation by indomethacin and sulindac sulfide treatments in both cell lines.
Inhibition of sulindac sulfide and indomethacin-induced apoptosis, and caspase 9 activation, in AGS cells by anti-sense suppression of GADD45α expression

To suppress GADD45α expression in AGS cells, we transfected anti-sense oligonucleotides (oligo) into the cells, and monitored GADD45α protein levels by Western blot analysis. Compared to mock transfection and transfection with the negative control oligo, transfection with anti-sense GADD45α oligos resulted in approximately 60% reduction of GADD45α protein levels (Figure 6A).

Flow cytometric quantitation of annexin V bound apoptotic cells revealed that both low and high concentrations of sulindac sulfide and indomethacin induced significant amounts of apoptosis in mock transfected cells, and the fold apoptosis induction increased with increasing NSAID concentrations utilized (Figure 6B and C, respectively). Significant apoptosis was also induced by all concentrations of sulindac sulfide and indomethacin in control oligo transfected cells, at levels comparable to or slightly lower than that of mock transfected cells (Figure 6B and C, respectively). In contrast, cells transfected with anti-sense GADD45α oligos were resistant to sulindac sulfide and indomethacin-induced apoptosis. Only the high concentrations of these NSAIDs utilized were able to induce significant amounts of apoptosis, and the fold inductions were dramatically lower than that in mock and control oligo transfected cells (Figure 6B and C).

We investigated whether antisense GADD45α suppression affects the caspase 9 pathway of apoptosis. Western blot analysis showed that low levels of procaspase 9 were expressed in the cells, and treatments with sulindac sulfide and indomethacin did not alter procaspase 9 levels compared to vehicle treatment (Figure 7A). Basal levels of active caspase 9 p10 subunit were
detected in mock and control oligo transfected cells treated with vehicle only, and anti-GADD45α transfection reduced the basal levels of active caspase 9 (Figure 7A). Sulindac sulfide and indomethacin treatments dramatically increased active caspase 9 p10 subunit levels in mock and control oligo transfected cells, but not in anti-GADD45α oligo transfected cells, compared to vehicle only treatment (Figure 7A).

**Analysis of anti-proliferative effects of sulindac sulfide and indomethacin treatments in AGS and RGM-1 cells**

Increased GADD45α expression is associated with cell growth inhibition, and deletion of GADD45α frequently results in uncontrolled proliferation (Siafakas and Richardson, 2009). Therefore, we examined the effects of sulindac sulfide and indomethacin on AGS and RGM-1 cell proliferation, and its relationship to GADD45α up-regulation. Vehicle only treated AGS and RGM-1 cells multiplied at a rate of 2x10³ cells/day and 8x10³ cells/day, respectively (Figure 7B). Indomethacin treatment at lower than 0.02mM did not have any detectable effect on proliferation of either cell line (negative data, not shown). At 0.02 mM, indomethacin treatment reduced proliferation of both AGS and RGM-1 cells to 0.5X10³ cells/day and 4x10³ cells/day, respectively (Figure 7B). This concentration of indomethacin was insufficient to induce apoptosis, and did not up-regulate GADD45α mRNA expression in either cell line (negative data, not shown). Indomethacin treatment at 0.2mM was included in this assay as positive control for up-regulation of GADD45α and loss of cell numbers due to induction of cell death (Figures 4 and 7B). Sulindac sulfide treatment at concentrations lower than 0.01mM did not up-regulate GADD45α expression, induce apoptosis, or have any detectable effect on AGS and
RGM-1 proliferation (negative data, not shown). Sulindac sulfide treatment at 0.01mM reduced proliferation of AGS and RGM-1 cells and induced apoptosis (Figure 4 and 7B). Sulindac sulfide at 0.1mM was included as positive control for up-regulation of GADD45α expression and loss of cell numbers due to induction of cell death (Figure 4 and 7B). Thus indomethacin but not sulindac sulfide treatment at concentrations below what is necessary to induce apoptosis had growth inhibitory effects on gastric epithelial cells, but did not up-regulate GADD45α expression.

**Exogenous PGE2 treatment and siRNA suppression of COX-1 and -2 did not inhibit up-regulation of GADD45α expression by sulindac sulfide and indomethacin**

To determine whether up-regulation of GADD45α by sulindac sulfide and indomethacin occurred through prostaglandin/COX-dependent pathways, we examined if pretreatment with exogenous PGE2, and siRNA suppression of COX-1 and -2 expression, would inhibit up-regulation of GADD45α expression by sulindac sulfide and indomethacin treatments in AGS cells. As expected, sulindac sulfide treatment dramatically up-regulated GADD45α protein levels compared to vehicle only treatment (Figure 8A). Treatments with PGE2 alone did not affect GADD45α protein levels compared to vehicle only treatment, and pretreatment with PGE2 also did not affect up-regulation of GADD45α protein levels by sulindac sulfide (Figure 8A). Similarly, indomethacin treatment significantly up-regulated GADD45α protein levels compared to vehicle only treatment (Figure 8B). Pretreatment with PGE2 also did not affect up-regulation of GADD45α protein levels by indomethacin (Figure 8B). Thus up-regulation of
GADD45α expression by sulindac sulfide and indomethacin treatments did not occur through prostaglandin/COX-dependent pathways.

Compared to mock transfections with no siRNAs, transfections with control RNA did not affect the expression of COX-1, COX-2 or GADD45α, in AGS cells (Figure 8C, left panels). Transfections with siRNAs targeting COX-1 and COX-2 effectively reduced COX-1 and COX-2 expressions, respectively, to almost undetectable levels (Figure 8C, left top and middle panels). Co-transfection of COX-1 and COX-2 siRNA effectively reduced both COX-1 and -2 to undetectable levels, as visualized by using both COX-1 and COX-2 gene specific primers in the same PCR reaction (Figure 8C, left bottom panel). Effective suppression of COX-1, COX-2 or both by their respective siRNAs did not affect GADD45α levels (Figure 8C, left panels). As expected, sulindac sulfide and indomethacin treatments markedly increased GADD45α mRNA levels compared to vehicle only treatment in control RNA transfected AGS cells (Figure 8C, right top panel). Transfections with siRNA targeting COX-1, COX-2 or both did not inhibit up-regulation of GADD45α mRNA expression by sulindac sulfide and indomethacin (Figure 8C, right middle and bottom panels).
Discussion

In this study, we showed that: 1) GADD45α expression was up-regulated by sulindac and indomethacin treatments both in vivo in mouse gastric mucosa and in vitro in AGS and RGM-1 cells. 2) Apoptotic cells constitute a significant percentage of cells in and around NSAID-induced gastric mucosal erosions in vivo. 3) Up-regulation of GADD45α expression by NSAID treatments in AGS and RGM-1 cells was accompanied by apoptosis. 4) GADD45α null mice were resistant to NSAID-induced gastric injury and apoptosis, and anti-sense suppression of GADD45α expression in AGS cells inhibited NSAIDs-induced caspase 9 activation and apoptosis. 5) Pretreatments with PGE2 and siRNA suppression of COX-1 and -2 did not inhibit up-regulation of GADD45α by sulindac sulfide and indomethacin in AGS cells. These data suggest that GADD45α up-regulation is a COX-independent mechanism that is necessary for induction of severe gastric injury and caspase 9-dependent apoptosis pathway by sulindac and indomethacin.

In WT mice, indomethacin treatment up-regulated GADD45α expression more effectively (3.8 +/- 0.9 fold) than sulindac treatment (2.5 +/- 0.2 fold), and induced greater gastric mucosal injury (17.7 +/- 14.6 percent at 4 hours) and apoptosis (4.2 +/- 0.2 fold versus vehicle only) than sulindac treatment (14.1 +/- 8.4 percent injury at 4 hours and 3.1 +/- 0.2 fold versus vehicle only). This positive association of the magnitude of GADD45α up-regulation with extent of gastric mucosal injury and apoptosis suggests a causative effect between GADD45α up-regulation and induction of injury and apoptosis in gastric mucosa. However, GADD45α up-
regulation is not the sole mechanism responsible, since, in gastric mucosa of GADD45α -/- mice, treatments with sulindac and indomethacin could still induce caspase-3 activation to significantly higher levels than treatment with vehicle only, and a moderate amount of apoptotic cells in and around gastric mucosal erosions. These indicated that both sulindac and indomethacin treatments induced apoptosis pathways that did not require the presence of GADD45α gene expression.

In agreement with our previous experience that acute mucosal damage by NSAIDs is rapidly produced via systemic effects, in this study, sulindac and indomethacin treatments produced severe gastric mucosal damage in WT mice as early as 4 hours after intraperitoneal injection. The extent of injury was sustained for at least 8 hours after NSAID administration. The decrease in gastric mucosal damage observed at 24 hours after NSAIDs were administered was likely due to healing of the gastric mucosa in the absence of repeat NSAID treatment after the initial injection. Sulindac and indomethacin treatments did not induce significant gastric mucosal damage at all time points up to 24 hours in GADD45α null mice, suggesting that inhibition of NSAID-induced gastric mucosal injury in GADD45α null mice was not due to a delay in injury induction. Further, the effective resistance of GADD45α null mice to NSAID-induced gastric mucosal damage suggested that deletion of GADD45α may also inhibit other non-apoptotic cell deaths that also contribute to gastric mucosal injuries, since our data showed that only about a third of gastric mucosal cells in and around NSAID-induced erosions were apoptotic cells.
We utilized the human gastric carcinoma AGS cells as our in vitro human model because at present there is no normal human gastric mucosal cell model available. Normal human gastric epithelial cells have poor viability in culture, making it difficult to establish a cell line for culture studies. We utilized an immortalized non-neoplastic rat gastric epithelial cell line, RGM-1, as a normal cell model.

As in the \textit{in vivo} mouse model, in both AGS and RGM-1 cells the extent of apoptosis increased with greater GADD45\textsubscript{\alpha} up-regulation by higher concentrations of both sulindac sulfide and indomethacin treatments. In RGM-1 cells, indomethacin treatment up-regulated GADD45\textsubscript{\alpha} and induced apoptosis to greater extent than sulindac sulfide treatment. In AGS cells, however, sulindac sulfide treatments up-regulated GADD45\textsubscript{\alpha} expression to comparable levels as indomethacin treatments, but induced markedly greater apoptosis than did indomethacin treatment. An explanation for this is that, in addition to the GADD45\textsubscript{\alpha} pathway, sulindac sulfide induced more numbers of and/or more potent apoptosis pathways than did indomethacin in AGS cells. An alternative explanation is that GADD45\textsubscript{\alpha} may play a greater role in sulindac sulfide-induced than indomethacin-induced apoptosis pathway(s) in AGS cells. Our data support the latter supposition, since consistent levels of GADD45\textsubscript{\alpha} suppression by anti-sense oligos in both sulindac sulfide and indomethacin treated cells resulted in greater inhibition of sulindac sulfide-induced apoptosis (approximately 2.7 fold decrease relative to mock transfected) than indomethacin-induced apoptosis (approximately 1.6 fold decrease relative to mock transfected).
Results from our AGS studies were inconsistent with our *in vivo* data showing that indomethacin is a more potent drug than sulindac in up-regulation of GADD45α expression and induction of gastric mucosal injury and gastric cell apoptosis. These differences may be due in part to the fact that we utilized sulindac, a prodrug that requires further breakdown *in vivo* to produce active metabolites, in our animal studies, while we utilized sulidac sulfide, the active metabolite of sulindac, directly in our cell culture studies. After the breakdown of sulindac *in vivo*, the resulting concentration of the active sulfide metabolite may be greatly diminished relative to the concentration of sulindac we administered. Another explanation is that the gastric carcinoma AGS cells responded to each drug differently than non-neoplastic gastric epithelial cells. Our data showing that indomethacin treatment is more potent than sulindac sulfide treatment in up-regulating GADD45α expression and inducing apoptosis in RGM-1 cells support this supposition. A third possibility remains that human gastric epithelial cells responded differently to each drug than rodent gastric epithelial cells.

These studies were the first to demonstrate that GADD45α expression is required for induction of severe gastric mucosal injury and apoptosis by NSAIDs. They are consistent with our previous findings and those of others showing that GADD45α expression is up-regulated in various cancer cells in response to NSAIDs, and that GADD45α is involved in injury of other tissues: We have previously shown that indomethacin and sulindac treatments up-regulate GADD45α expression in colon cancer cells in culture (Chiou et al., 2009). GADD45α up-regulation has also been observed in various prostate, breast and stomach cancer cells in culture in association with induction of interleukin-24 by sulindac, aspirin, ibuprofen, acetaminophen...
and naproxen, resulting in cancer cell apoptosis and/or growth arrest (Zerbini et al., 2006).

GADD45\(\alpha\) up-regulation is also observed in adult primary sensory and motor neurons after peripheral axonal injury (Befort et al., 2003), and GADD45\(\alpha\) has been shown to participate in lipopolysaccharide and ventilator-induced inflammatory lung injury (Meyer et al., 2009).

NSAIDs are known to have anti-proliferative effects on a variety of cell types. GADD45\(\alpha\) overexpression has been shown to lead to growth arrest (Hollander and Fornace, 2002). Therefore, the possibility arises that up-regulation of GADD45\(\alpha\) expression may mediate NSAID-induced growth inhibition of gastric epithelial cells. The relationship between GADD45\(\alpha\) up-regulation and growth inhibition of gastric epithelial cells by NSAIDs is unknown. This study showed that low concentrations of indomethacin, but not sulindac sulfide, that was insufficient to induce cell death had growth inhibitory effects on AGS and RGM-1 cells, but did not up-regulate GADD45\(\alpha\) expression. This indicated that in the gastric epithelial cells we utilized, GADD45\(\alpha\) is not involved in inhibition of cell proliferation in response to NSAID treatments.

Our current study provided evidence that GADD45\(\alpha\) up-regulation by NSAIDs triggers a caspase 9-dependent pathway of apoptosis. Caspase 9 is the initiator caspase involved in the intrinsic or mitochondrial pathway of apoptosis (Li et al, 1997). Once activated, it cleaves and activates downstream effector caspases such as caspase 3 and/or 7 to induce apoptosis (Taylor et al, 2008). Our finding does not preclude the possibility that other caspase pathways are triggered
by GADD45α up-regulation. The mechanism of how GADD45α up-regulation leads to apoptosis in gastric mucosa, and what caspase pathways are involved requires further investigation.

Our current data support the idea that, in addition to inhibition of COX enzymes, COX-independent mechanisms of NSAID action are also important contributors to induction of gastric mucosal damage and apoptosis. Disruption of COX-independent pathways such as genetic ablation of GADD45α expression in vivo and anti-sense suppression of GADD45α expression in cultured cells could largely inhibit NSAID-induced gastric mucosal injury and gastric cell apoptosis. Our current data also suggest that effective strategies may be developed for minimizing NSAID-induced gastric injury by utilizing methods that result in inhibition of GADD45α expression, such as siRNA or anti-sense oligo treatment.

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References


Footnotes

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Figure Legends

Figure 1. Sulindac and indomethacin-induced gastric mucosal injury in WT versus GADD45α -/- mice. A. Upper panels show representative WT mouse stomachs at 4 hours after NSAIDs were administered, mucosal side and opened along the greater curvature. Minimal visible injury was detected with vehicle only treatment. Extensive visible injury was present with either sulindac or indomethacin treatment. Lower graph shows average percent injury +/- stdev at the indicated time points with the indicated treatments (* significantly higher percent injury relative to vehicle only treatment, p<0.05). B. Upper panels show representative null mouse stomachs at 4 hours after NSAIDs were administered. Compared to vehicle only treatment, neither sulindac nor indomethacin induced extensive visible mucosal injury at all time points examined.

Figure 2. Caspase-3 activity (apoptosis) in WT versus GADD45α -/- mice. Upper panels: Caspase-3 expression was detected almost ubiquitously in gastric mucosa of both WT and GADD45α -/- mice by immunofluorescence staining, including the area around the NSAID-induced erosions. White dotted lines demarcate the borders of erosions. Left upper graph: Compared to vehicle only treatment, both sulindac and indomethacin (INDO) treatments increased caspase-3 activity in gastric mucosa of WT mice. Right upper graph: Sulindac and INDO also increased caspase-3 activity in gastric mucosa of GADD45α -/- mice, but at much lower levels than in WT mice. Left lower graph: Compared to vehicle only treatment, both sulindac and INDO treatments increased % apoptotic cells in intact gastric mucosa (I) and in and around erosions (E) in WT mice. Right lower graph: Compared to vehicle only treatment, both
sulindac and INDO treatments also increased % apoptotic cells in intact gastric mucosa (I) and in and around erosions (E) in GADD45α-/- mice, though at much lower levels than in WT mice. (* significant fold increase in caspase-3 activity and % apoptotic cells relative to vehicle only treatment, p<0.05.)

**Figure 3. GADD45α protein levels in sulindac and indomethacin treated versus vehicle only treated gastric mucosa of WT mice.** Western blotting followed by densitometry analysis of protein bands showed that GADD45α protein levels were up-regulated in gastric mucosa of sulindac versus vehicle only treated mice (A.) and indomethacin (INDO) versus vehicle only treated mice (B.). Two representative vehicle treated samples and three sulindac or indomethacin treated samples are shown in the upper panels. (*significant up-regulation of GADD45α by sulindac and indomethacin treatments, compared to vehicle only treatment, p<0.05.)

**Figure 4. GADD45α mRNA and protein levels in sulindac sulfide and indomethacin treated versus vehicle only treated AGS and RGM-1 cells.** Realtime RT-PCR analysis of: A. GADD45α and survivin mRNA expression in response to increasing concentrations of sulindac sulfide (S.S.); and B. GADD45α mRNA expression in response to increasing concentrations of indomethacin (INDO). C. Western blot analysis of GADD45α and survivin protein levels in vehicle only, S.S. (0.1mM) and INDO (0.2mM) treated cells. (* significant up-regulation of GADD45α mRNA by sulindac sulfide and indomethacin treatments compared with vehicle only treatment, p<0.05.)
treatment, and down-regulation of survivin mRNA by sulindac sulfide treatment compared with vehicle only treatment, p<=0.01).

Figure 5. Analysis of apoptosis induction by increasing concentrations of indomethacin and sulindac sulfide treatments in AGS and RGM-1 cells. Amount of apoptotic cells per 10,000 cells were counted by flow cytometry analysis of annexin V-fluorescein binding. High concentrations of indomethacin (INDO) treatment (A.) and sulindac sulfide (S.S.) treatment (B.) significantly increased the amounts of fluorescence signal. Fold apoptotic cells relative to vehicle only treatment increased with increasing concentrations of INDO (C.) and S.S. (D.) treatments. (*significant increases in the amount of apoptotic cells in indomethacin and sulindac sulfide treated samples relative to that in vehicle treated samples, p<=0.001.)

Figure 6. Effects of anti-sense suppression of GADD45α expression on apoptosis induction by sulindac sulfide and indomethacin in AGS cells. A. Cells were mock transfected (1), transfected with negative control oligos (2) or with anti-sense GADD45α oligos (3). Western blot analysis (left panels) and densitometry analysis of protein bands (right graph) were performed to determine the level of GADD45α suppression. (* significant reduction of GADD45α protein levels relative to that in mock transfected cells, p<0.01.) Fold apoptosis induction in cell populations treated with high concentrations of sulindac sulfide (S.S., B.) and indomethacin (INDO, C.) relative to those treated with vehicle only were determined by flow
cytometry analysis of annexinV-fluorescein binding. (* significant fold apoptosis induction relative to vehicle only treated cell populations, p<0.01.)

**Figure 7. Analysis of caspase 9 activation and cell growth inhibition by sulindac sulfide and indomethacin treatments.** A. Western blots showing that compared to vehicle treatment, sulindac sulfide (S.S.) and indomethacin (INDO) treatments did not alter procaspase 9 levels, but markedly increased active caspase 9 p10 levels in mock (1) and control oligo (2) transfected AGS cells but not in anti-sense GADD45α oligo (3) transfected AGS cells. Transfection efficiency >85% for all transfections. B. Cell proliferation curves showing that INDO and S.S. treatments reduced cell growth relative to vehicle only treatment. Top graphs: AGS cells. Bottom graphs RGM-1 cells.

**Figure 8. Effects of exogenous PGE2 treatments and siRNA suppression of COX-1 and COX-2 expression on up-regulation of GADD45α expression by sulindac sulfide and indomethacin in AGS cells.** Western blots showing that: A. Sulindac sulfide (S.S.) treatment up-regulated GADD45α protein levels relative to vehicle only treatment, treatments with various PGE2 concentrations did not alter GADD45α protein levels, and pretreatment with same PGE2 concentrations did not affect up-regulation of GADD45α protein levels by sulindac sulfide. B. Indomethacin (indo) treatment up-regulated GADD45α protein levels relative to vehicle only treatment, and pretreatment with PGE2 did not affect up-regulation of GADD45α protein levels by indomethacin. C. Left panels: Compared to mock transfections (M), transfections with
control RNA (Ctl) did not affect the expression of COX-1, COX-2 or GADD45α. Transfections with siRNA targeting COX-1 (siCOX-1), COX-2 (siCOX-2), or both (siCOX-1+2) were effective in suppressing the expression of COX-1, COX-2 or both, respectively, but did not affect GADD45α expression. Right panels: GADD45α mRNA levels were markedly increased by S.S. and indo treatments in Ctl transfected cells, and siCOX-1, si-COX-2 or siCOX-1+2 transfections did not inhibit this increase. Transfection efficiency > 85% for all transfections.
Figure 1A

% Injury

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Figure 1B

B.

GADD45α -/- Vehicle

GADD45α -/- sulindac

GADD45α -/- indomethacin

% Injury

4h 8h 24h 4h 8h 24h 4h 8h 24h
Vehicle sulindac indomethacin
**Figure 2**

The images depict immunofluorescence staining of WT and +/- gastric mucosa with different treatments:

- **WT Vehicle**
- **WT sulindac**
- **WT indomethacin**
- **/- Vehicle**
- **/- sulindac**
- **/- indomethacin**

**Graphs**

- **WT gastric mucosa**
  - **Fold caspase 3 activity**
    - Vehicle
    - sulindac
    - INDO
  - **% Apoptotic cells**
    - Vehicle
    - sulindac
    - INDO

- **/- gastric mucosa**
  - **Fold caspase 3 activity**
    - Vehicle
    - sulindac
    - INDO
  - **% Apoptotic cells**
    - Vehicle
    - sulindac
    - INDO
Figure 3

A.  

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GADD45α
β-actin

B.  

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GADD45α
β-actin

Fold GADD45α protein

Veh  sulindac

Veh  INDO

*
Figure 4
Figure 5

A. AGS

Vehicle
INDO (0.2 mM)

B. AGS

Vehicle
SS (0.1 mM)

C. AGS

RGM-1

Fold apoptosis

Veh 0.05 0.10 0.20

D. AGS

RGM-1

Fold apoptosis

Veh 0.01 0.05 0.10

* p <= 0.001
Figure 6

A. Western blot analysis showing GADD45α and β-actin levels.

B. Graph showing fold apoptosis with Vehicle, S.S. (0.05 mM), and S.S. (0.10 mM) treatments.

C. Graph showing fold apoptosis with Vehicle, INDO (0.10 mM), and INDO (0.20 mM) treatments.
A. 

<table>
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→ procaspase 9
→ active caspase 9 p10
→ β-actin

Figure 7A
Figure 7B
Figure 8