Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells

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Redlak, Maria J., Jacinda J. Power, and Thomas A. Miller. Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells. Am J Physiol Gastrointest Liver Physiol 289: G731–G738, 2005. First published June 23, 2005; doi:10.1152/ajpgi.00150.2005.—This study was undertaken to determine whether the Bcl-2 family proteins and Smac are regulators of aspirin-mediated apoptosis in a gastric mucosal cell line known as AGS cells. Cells were incubated with varying concentrations of acetylsalicylic acid (ASA; 2–40 mM), with or without preincubation of caspase inhibitors. Apoptosis was characterized by Hoechst staining and DNA-histone-associated complex formation. Antiapoptotic Bcl-2, proapoptotic Bax and Bid, Smac, and cytochrome-c oxidase (COX IV) were analyzed by Western blot analyses from cytosol and mitochondrial fractions. ASA downregulated Bcl-2 protein expression and induced Bax translocation into the mitochondria and cleavage of Bid. In contrast, expression of Smac was significantly decreased in mitochondrial fractions of ASA-treated cells. Bax and Bid involvement in apoptosis regulation was dependent on caspase activation, because caspase-8 inhibition suppressed Bax translocation and Bid processing. Caspase-9 inhibition prevented Smac release from mitochondria. Additionally, increased expression of the oxidative phosphorylation enzyme COX IV was observed in mitochondrial fractions exposed to ASA at concentrations >5 mM. Although caspase-8 inhibition had no effect on aspirin-induced apoptosis and DNA-histone complex formation, caspase-9 inhibition significantly decreased both of these events. We conclude that Bcl-2 protein family members and Smac regulate the apoptotic pathway in a caspase-dependent manner. Our results indicate also that mitochondrial integration and oxidative phosphorylation play a critical role in the pathogenesis of apoptosis in human gastric epithelial cells.

Caspases; Bcl-2 family proteins; Smac

Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to induce apoptosis in human colorectal tumor cells (4, 9, 32, 38), B cell chronic lymphocytic leukemia cells (2, 21), fibroblasts (23, 25), and human gastric mucous cells (50–53). We have previously reported that the proapoptotic Bcl-2 family members play a crucial role in apoptotic mitochondrial changes that include loss of the mitochondrial membrane potential (Δψm), directly targeting the mitochondrial outer membrane voltage-dependent anion channel (VDAC), and allowing cytochrome c to pass through this channel (17, 39). Antiapoptotic Bcl-2 and Bcl-xL prevent cytochrome c release by closing the VDAC channel. It has been shown that cytoplasmic Bid is cleaved by caspases and that the truncated Bid protein translocates to the mitochondria when apoptosis is induced (12, 40). In addition, Bax has also been shown to translocate to the mitochondria during apoptosis (28, 49), and this process is dependent on caspases (11). Other proteins, such as Smac/Diablo (hereafter referred to as Smac), are released from the intermembrane space to the cytosol in response to apoptotic stimuli, leading to the promotion of caspase activation by binding and neutralizing members of the inhibitor of apoptosis protein family (IAPs) (8, 36, 45). Precisely how the efflux of proapoptotic molecules from the mitochondrial intermembrane space occurs is not clear. However, available evidence suggests that the death-promoting caspase-cleaved Bid (tBid) induces conformational change and the homooligomerization of death promoting molecules Bax and/or Bak and their insertion into the outer mitochondrial membrane (7, 10). Bax or Bak oligomers may then form channels within the mitochondrial outer membrane, allowing the release of proapoptotic proteins such as cytochrome c and Smac (8, 46).

The main aim of the present study was to determine the role of the mitochondrial pathway in aspirin-induced apoptosis using gastric epithelial cells from a human cell line. In particular, we investigated the expression of Bcl-2, Bax, tBid, and Smac in cytosol and mitochondrial fractions and found that Bax translocation from the mitochondria into the cytosol, Smac expression in the mitochondria, and Bid processing are dependent on caspase activation. Inhibition of caspase-8 prevented

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Bid cleavage and mitochondrial translocation of Bax. Inhibition of caspase-9 changed Smac processing mediated by acetysalicylic acid (ASA). In addition, caspase-mediated change in distribution of Bcl-2 family proteins and Smac decreased the apoptotic death induced by aspirin in AGS cells.

MATERIALS AND METHODS

Chemicals and reagents. ASA and protease inhibitor cocktail were obtained from Sigma (St. Louis, MO). Stock solution of aspirin in 1 M Tris·HCl, pH 8.0, was prepared fresh for each experiment. Anti-Bcl-2, -Bax, -tBid, -Smac/Diablo, and -XIAP polyclonal antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Cytochrome c oxidase (Complex IV) and porin antibodies and Hoechst 33342 were obtained from Molecular Probes. Pan-caspase inhibitor I (z-VAD-fmk), caspase-8-inhibitor I, and caspase-9-inhibitor-I were purchased from Calbiochem.

Cell culture and drug treatment. A human gastric epithelial cell line (AGS) was purchased from the American Type Culture Collection (Manassas, VA) and cultured in Ham’s F-12 culture medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were kept in 75-cm² culture flasks and 3.5-cm² dishes as a confluent monolayer at 37°C in a humidified atmosphere containing 95% air and 5% carbon dioxide.

Cells were incubated with 40 mM ASA or other concentrations as appropriate in the absence or presence of caspase inhibitor for indicated times. After treatment, adherent cells were removed by scraping and combined with the floating cells in the medium. Cells were pelleted by centrifugation for further analysis.

Subcellular fractionation and Western blot analysis. Cytoplasmic and mitochondrial extracts were prepared according to recommendations as described in the mitochondrial isolation kit (Pierce). Cytosolic fractions were concentrated using spin columns (Microcon, Amicon). Purity of both fractions was determined by immunoblotting with antibodies against mitochondrial proteins cytochrome-c oxidase (subunit IV, mouse IgG2a, monoclonal, Molecular Probes), porin (mouse, monoclonal IgG, Molecular Probes), and pyruvate dehydrogenase E2 (mouse, monoclonal IgG, Molecular Probes). No protein expression was detected in the cytosolic fractions, indicating no mitochondrial contamination in the preparations. Equal amounts of protein were loaded in each lane for 4–20% SDS-polyacrylamide gel electrophoresis, and blots were probed with appropriate primary antibodies. Proteins were visualized by using CDP-Star chemiluminescence reagent (Perkin-Elmer).

Determination of apoptotic cells. To determine the level of apoptosis after aspirin treatment, with or without the presence of caspase inhibitors, cells were fixed with 1% vol/vol of formaldehyde/PBS, pH 7.2, for 1 h. Cells were then washed with PBS, and cellular DNA was stained with 5 μg/ml Hoechst 33342 for 1 h at 37°C, washed with cold PBS, and examined under fluorescence microscopy. Apoptotic cells were counted according to changes in nuclear morphology, including shrinkage, condensation, margination, and fragmentation of chromatin. For detection of histone-associated DNA fragments, a cell death detection ELISA kit (Roche Molecular Biochemicals) was used according to the manufacturer’s instructions.

Protein concentration assay. Protein concentration was determined by the Bradford method with BioRad reagent and BSA as a standard (3).

Statistical analysis. Data represent at least three independent experiments and are expressed as the means ± SE. Analysis was performed by a paired Student’s t-test, with significant differences determined at P < 0.05.

RESULTS

Effect of aspirin on Bcl-2 and IAP family protein expression in cytosol and mitochondrial fractions of AGS cells. We have previously shown that the caspase cascade and mitochondrial involvement are a part of apoptotic activation in aspirin-induced apoptosis (31). To further elucidate the mitochondrial role in this process, we evaluated the expression of Bcl-2 and IAP family proteins in the cytosol and mitochondrial fractions isolated from AGS cells treated with 40 mM aspirin from 1 to 4 h. As shown in Fig. 1A, expression of Bcl-2, the antiapoptotic protein, decreased in a time-dependent manner in cytosol fractions of aspirin-treated cells. The expression of the proapoptotic protein Bax decreased in cytosol fractions over time during incubation with aspirin and profoundly increased in mitochondrial fractions, indicating the translocation of this protein from the cytosol into the mitochondrial compartment. Another proapoptotic protein, Bid, was processed in the mitochondria of aspirin-treated cells, and its truncated 15-kDa fragment was detected as early as 1 h after treatment with aspirin (Fig. 1A).

We also examined the effect of aspirin on members of the IAP family, in particular, the expression of XIAP, which is known to bind and inhibit several caspases (i.e., caspase-3, -7, and -9) involved in the mediation of apoptosis (8, 45). We did not observe any change in the expression of XIAP protein in cytosol or mitochondrial fractions isolated from aspirin-treated cells (data not shown).

Effect of aspirin on Smac expression in cytosol and mitochondrial fractions of AGS cells. Recent studies associated with cytochrome c have shown that another mitochondrial protein, Smac, is released into the cytosol on mitochondrial stress and apoptosis. The interaction of Smac with IAPs relieves the inhibitory effects of the IAPs on caspases (8, 45). To investigate the role of Smac in AGS cells undergoing ASA-induced apoptosis, we determined cytosolic and mitochondrial Smac by immunoblotting fractionated cell lysates. We noted that Smac was significantly decreased in mitochondrial fractions exposed as early as 1 h to ASA treatment compared with the untreated control sample, and longer exposure to ASA, up to 4 h, resulted in an almost complete disappearance of this protein in mitochondria (Fig. 1A). Because decreased expression of Smac in cytosol fractions treated with ASA was also observed, we speculate that the disappearance of Smac from the mitochondrial compartment of ASA-treated cells may indirectly indicate its release into the cytosol where further processing and/or degradation likely occurred.

Cytochrome c oxidase expression in the mitochondrial fraction of ASA-treated cells. Aspirin treatment caused a significant increase in cytochrome c oxidase (COX IV) expression over the basal value in the mitochondrial fractions treated with 40 and 10 mM aspirin. As shown in Fig. 1B, this rapid increase was noted as early as 1 h and increased over the time of aspirin treatment. At lower concentrations, COX IV expression did not significantly change over a time of 3 h but had begun to increase by 4 h in the samples treated with 5 mM aspirin. NO COX IV expression was measured in cytosol fractions isolated from AGS cells treated with aspirin (data not shown).
Mitochondrial porin, an outer-membrane protein that forms voltage-dependent anionic channels between the cytosol and the mitochondrial intermembrane space, was also increased in mitochondrial fractions isolated from aspirin-treated AGS cells (data not shown).

**Effect of caspase inhibitors on Smac, Bax, and Bid expression in cytosol and mitochondrial fractions isolated from ASA-treated AGS cells.** In addition to the processing of Smac noted above, we have shown previously that caspase-9 is activated in aspirin-induced apoptosis in AGS cells (31). It has been reported that Smac-induced apoptosis can be mediated via a pathway that involves caspase-9 activation (8). To examine whether Smac processing in the mitochondria is dependent on caspase activation, cells were pretreated for 1 h with a caspase-9 inhibitor before adding 40 mM ASA, and cytosolic and mitochondrial Smac expression were determined. As shown in Fig. 2A, >80% of protein expression disappeared from mitochondria as early as 1 h after aspirin treatment compared with the untreated control. In the presence of caspase-9 inhibitor (i.e., 150 μM), this process was significantly altered. Indeed, only 20% of protein expression disappeared after 1 h and 60% after 4 h. No significant change was observed in Smac expression in cytosol fractions isolated from cells treated with aspirin in the absence of caspase-9 inhibitor (Fig. 2A).

It has been reported that Bax and Bid require caspase-8 as an upstream regulator (30) and that Bax translocation to the mitochondria is dependent on this caspase (11). Therefore, we examined Bax and Bid expression in cytosol and mitochondrial fractions isolated from cells treated with ASA in the presence of caspase-8 inhibitor. Cells were pretreated for 1 h with caspase-8 inhibitor (i.e., 50 μM) before the addition of 40 mM ASA, and they were harvested at the indicated times. Cytosolic and mitochondrial fractions were then examined. Caspase-8 inhibitor completely blocked ASA-induced Bax translocation from the cytosol to the mitochondria (Fig. 2B). In addition, caspase-8 inhibitor pretreatment prevented Bid cleavage induced by ASA in AGS cells (data not shown).

**Effect of caspase inhibitors on ASA-induced apoptosis.** The proportion of apoptotic cells was calculated according to changes in nuclear morphology in Hoechst-stained cells (Fig. 3). Pretreatment of AGS cells with caspase-8 inhibitor did not change the extent of apoptosis induced by 40 mM ASA over a period of 1–8 h. Caspase-9 inhibitor, however, caused a significant decrease in the percentage of apoptotic cells incubated with ASA for 4 (17%) and 8 (15%) h. The most effective inhibitor was z-VAD-fmk, a broad-range caspase inhibitor that reduced the percentage of apoptotic cells exposed to ASA for 3, 4, and 8 h by ~37.8, 40.3, and 28.6%, respectively (Fig. 4A).

Another method we used to assay apoptotic death was through DNA-histone complex formation. ASA (40 mM) induced a time-dependent increase of DNA damage over a period of 1–8 h. The proportion of DNA-histone complex formation was not affected by caspase-8 inhibitor. Conversely, caspase-9 inhibitor and z-VAD-fmk significantly reduced the DNA double-strand cleavage between nucleosomes as assessed by ELISA assay (Fig. 4B).
DISCUSSION

The Bcl-2-related protein family comprises one of the major biological apoptosis regulatory gene products, including pro- and antiapoptotic regulators, which in many pathways are responsible for signal transduction between the cytoplasm and mitochondria. Apoptosis-inducing signals cause the translocation of proapoptotic proteins of the Bcl-2 family, such as Bax, from the cytoplasm to the outer mitochondrial membrane and facilitate the release of some components including cytochrome c and Smac from mitochondria into the cytosol (1, 19). On the other hand, the antiapoptotic Bcl-2 family proteins interfere with cytochrome c release and caspase activation (37).

In this study, we examined Bcl-2 protein family expression during aspirin-induced apoptosis in gastric mucosal cells. Of those tested, the antiapoptotic Bcl-2 protein decreased in the cytosol of aspirin-treated cells. Several studies suggest a link between pathologically altered ratios of pro- to antiapoptotic Bcl-2 family proteins and the development and progression of malignant tumors. In many malignant conditions, including gastric cancers, Bcl-2 overexpression has been reported, which is believed to influence the resistance of such cells to apoptotic death (22). Overexpression of Bcl-2 protein in gastric cancer cell lines has been reported to block apoptosis by inhibiting cytochrome c release from mitochondria, thus inhibiting caspase-3 activation (29). More than half of colon and gastric cancers with DNA mismatch-repair defects carry mutations in the Bax gene (16), and Bax overexpression induces cytochrome c release in vivo and in vitro (34). This sensitizes cells to chemotherapeutic agent-induced apoptosis by enhancing the release of cytochrome c from mitochondria (20, 35). Some studies have shown that Bax translocates from a predominantly cytoplasmic location to the mitochondria on induction of apoptosis (11, 28, 49). In this report, we present evidence that the proapoptotic proteins Bax and Bid play an important role in signal transduction during aspirin-induced apoptosis. Bax translocates from the cytosol to the mitochondrial compartment, and Bid is processed to its active truncated form, tBid. Our data show that mitochondria are the site where Smac processing takes place. In our experimental model, apoptotic death of gastric epithelial cells and mitochondrial translocation of regulatory proteins, such as Bax and Smac, are dependent on

Fig. 2. Smac/Diablo and Bax expressions in cytosol and mitochondrial fractions isolated from AGS cells treated with aspirin in the absence or presence of caspase inhibitor. Cells were incubated with 40 mM ASA without or with 150 μM caspase-9 inhibitor (A) or 50 μM caspase-8 inhibitor (B) for indicated times, harvested, and fractionated. Smac/Diablo and Bax expressions were detected on Western blots in the lysates of cytosolic (20 μg) and mitochondrial fractions (10 μg) with anti-Smac or anti-Bax antibodies. Blots were examined, and relative density for each sample was calculated and expressed as a percentage of control. Results are means ± SE from 3 experiments. Statistical significance was determined using the t-test for nonpaired samples. *P < 0.02, **P < 0.005, ***P < 0.001.

Fig. 3. Nuclear morphology of Hoechst-stained AGS cells treated with aspirin.
caspase activation. Thus caspase-dependent processing of Bax, Bid, and Smac appears to regulate the magnitude of apoptosis mediated by ASA and is critical to the induction of this process.

Other important steps involved in apoptosis associated with mitochondria are the disruption of electron transport in oxidative phosphorylation and the opening of a large-conductance channel, the permeability transition pore. NSAIDs, including aspirin, have been shown to uncouple oxidative phosphorylation in the respiratory chain in both the gastrointestinal tract (18, 33, 47) and liver mitochondria (43). This biochemical mechanism contributes to the “topical” toxicity of NSAIDs (41). Uncoupling of oxidative phosphorylation has been documented in vitro with some NSAIDs (42) and in vivo in the case of aspirin (26). This process is accompanied by an important change in the permeability of the mitochondrial membrane, resulting in lipid peroxidation and damage. In experiments with rats receiving indomethacin administration directly into the small intestine, a significant increase in the activity of cytochrome c oxidase, succinate dehydrogenase, and citrate synthase was observed in the rat jejunum. No intestinal damage or change in activity of these three mitochondrial marker enzymes was observed when aspirin was administrated orally (42).

In the present study, we have provided evidence that aspirin at concentrations >5 mM increases the mitochondrial expression of cytochrome c oxidase in a time-dependent manner. We speculate that this “topical” effect is responsible for the gastric toxicity to AGS cells and the resultant mitochondrial disintegration, such as depolarization and permeability of the outer membrane, gives rise to the release of proapoptotic proteins such as Smac from the mitochondria into the cytosol, and induces translocation of Bax and Bid from cytosol into the mitochondria. Our results further suggest that Bax has an effect on components of the inner membrane, which interferes with respiration and/or ATPase activation and induces matrix alcalinization and cytosolic acidification, as previously observed (15, 24). Bax has also been shown to interact with the permeability transition pore to induce permeability transition in isolated mitochondria (27). Therefore, it is our hypothesis that increased expression of cytochrome c oxidase in the mitochondrial fractions of aspirin-treated gastric mucosal cells (at concentrations >5 mM) can be an indicator of Bax-induced permeabilization of the outer mitochondrial membrane and Bax-induced disturbance in the enzymatic chain reaction of mitochondrial oxidative phosphorylation. Further studies will obviously be needed to confirm this speculation.

Two methods were used to measure apoptosis in our studies. The early marker was histone-associated DNA fragmentation; the late-term marker was identification of apoptotic bodies using fluorescent microscopy. Although all markers are associated with some degree of subjectivity, measurement of DNA fragmentation employs a very sensitive filter elution methodology that enables a reproducible ability to quantitate DNA breaks with minimal interobserver variability. Its major drawback is that DNA fragmentation per se does not always lead to apoptosis. In contrast, whereas fluorescent imaging with Hoechst staining is quite capable of distinguishing an apoptotic cell from a nonapoptotic one, quantifying the proportion of cells that are apoptotic on the microscopic examination of a prepared sample can be very labor intense and carries with it a certain degree of subjectivity, because all cells are not oriented the same and what may appear normal may actually be apoptotic.

Notwithstanding these drawbacks, both methods paralleled each other in terms of results (Fig. 4). In contrast to aspirin alone, which induced a time-related increase in DNA fragmentation and apoptosis, both caspase-9 inhibition and pan-caspase inhibition significantly decreased these events. In fact, when using DNA fragmentation as an index of early apoptosis, both of these inhibitors profoundly decreased aspirin-induced fragmentation that was not different from control levels. Surprisingly, caspase-8 inhibition had no significant effect on either caspase-dependent processing of Bax and Bid (Fig. 2) but had no obvious effects on apoptosis is not immediately apparent from our data. Three possible explanations are invoked. First, activation of caspase-8 by aspirin may preferentially direct its action toward stimulation of Bax and Bid without a corresponding initiator...
activation of caspase-3 and subsequent apoptosis. We are unaware, however, of any experimental support for this contention. Second, activation of caspase-9 through the mitochondrion may have had such a profound effect on the initiation of apoptosis that it overrode any parallel effect by caspase-8 activation. Thus any inhibition of caspase-8 would not have significantly altered the magnitude of apoptosis. Supporting this hypothesis is our findings with caspase-9 inhibition, in which aspirin-induced apoptosis was decreased to a level not significantly different from control. Third, stimulation of the Bax and Bid pathways by caspase-8 may be redundant, meaning that the mitochondrial activation of caspase-9 would have occurred anyway, whether the Bax and Bid pathways were intact or not. This would mean that induction of Bax and Bid by caspase-8 was sufficient to activate the mitochondrion but not necessary. Further study is needed to determine whether any of these explanations is experimentally valid.

For most of the studies described in this report, a 40 mM concentration of ASA was used. The rationale underlying this is based on the pioneering work of Davenport (5, 6), who showed that a 20 mM concentration is physiologically relevant because it is equivalent to two conventional tablets of aspirin, each having a 325-mg dosage. In view of the fact that patients with various inflammatory disorders, such as rheumatoid or osteoarthritis, require two to four times this concentration to maintain pain relief (14, 48), a 40 mM ASA concentration would be more in line with what this patient population routinely receives. Accordingly, an understanding of the gastric effects of this higher ASA concentration has special relevance for this clinical scenario.

Additionally, it would appear that different concentrations of aspirin may induce different mechanisms of regulation of the apoptotic pathway. We base this contention on results published by Gu et al. (13), who treated AGS cells with a much lower concentration of aspirin (namely, 2 mM) for a much longer period of time (24 and 48 h) before apoptosis was observed. They found that suppression of caspase-8 with the specific inhibitor z-IETD-fmk, prevented Bid cleavage and subsequent apoptosis. Bax translocation and its conformational change, however, were not dependent on caspase-8 activation and did not appear to influence apoptosis. On the basis of our findings in the present study, apoptosis induced by much higher physiological concentrations of aspirin appears to be regulated and modified mostly by a caspase-9-dependent mechanism.

In conclusion, our results demonstrate that physiologically relevant concentrations of aspirin induce apoptosis in the gastric cell line used, with mitochondrial activation playing a prominent role. Important findings induced by aspirin included downregulation of cytosolic Bcl-2, translocation of Bax from the cytosol into the mitochondria, and Bid processing. The changes in Bax and Bid appeared to be dependent on caspase-8 activation because inhibition of this caspase blocked Bax and Bid processing. We further observed that ASA induced a decrease in Smac expression in mitochondrial fractions that was dependent on caspase-9 activation. Under our experimental conditions, the disappearance of Smac from mitochondria in response to an aspirin stimulus may indicate a change of Smac distribution and/or Smac processing/degradation in the cytosol. Although inhibition of caspase-8 had no effect on the magnitude of apoptosis or histone-associated DNA fragmentation in aspirin-treated cells, inhibition of caspase-9 significantly inhibited apoptosis and profoundly decreased aspirin-induced DNA fragmentation, suggesting an important role for this caspase in aspirin injury. Stress-induced pathways have been recently identified, in which caspase-8 stimulates the release of proapoptotic proteins such as cytochrome c from mitochondria through the degradation of Bid, which results in the activation of caspase-9 (54). Thus it is possible that apoptosis induced by NSAIDs, including aspirin, is mediated by this pathway, because caspase-8 is activated by aspirin (31) and by indomethacin (44) in gastric mucosal cells. On the basis of the findings presented in this report, a schematic representation of the possible pathways through which aspirin may trigger apoptosis in AGS cells is shown in Fig. 5. In summary, our results support the notion that the mitochondrion plays a critical role.

![Diagram illustrating how mitochondrial pathway proteins Bax, Bid, and Smac integrate into the caspase cascade in aspirin-induced apoptosis in AGS cells. Caspase-8-dependent translocation of Bax and Bid processing and caspase-9-dependent Smac activation are directly involved in the generation of a mitochondrial response.](Fig. 5)
in the pathogenesis of aspirin-induced gastric injury and apoptosis in human gastric epithelial cells.

GRANTS
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