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Aspirin Induces Apoptosis through the Inhibition of Proteasome Function*

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Aspirin and other nonsteroidal anti-inflammatory drugs inhibit cell proliferation and induce apoptosis in various cancer cell lines, which is considered to be an important mechanism for their anti-tumor activity and prevention of carcinogenesis. However, the molecular mechanisms through which these compounds induce apoptosis are not well understood. Here we have found that aspirin treatment of the mouse Neuro 2a cells impaired the proteasome function and caused severe mitochondrial abnormalities. Treatment with aspirin lead to a dose- and time-dependent decrease in proteasome activity and an increase in the accumulation of ubiquitylated proteins in the cells, which correlated with its effect on cell death. Aspirin exposure also resulted in an increase in the half-life of pd1EGFP, a model substrate of proteasome, as well as various intracellular substrates like Bax, IκB-α, p53, and p27^{kip1}. Aspirin-induced proteasomal malfunction might be responsible, at least in part, for the down-regulation of NF-κB activity and neurite outgrowth. Finally, we have shown that aspirin treatment caused changes in the mitochondrial membrane potential, release of cytochrome c from mitochondria, and activation of caspase-9 and -3, which could be because of the proteasomal dysfunction.

Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs)[3] are well known to inhibit cyclooxygenase activity, the key enzyme in prostaglandin biosynthesis (1). However, several clinical observations and epidemiological and experimental studies found aspirin and other NSAIDs to be promising anti-cancer agents. Prolonged use of NSAIDs has been reported to reduce the risk of cancer of the colon and other gastrointestinal organs as well as cancer of the breast, prostrate, lung, and skin (2–6). The chemopreventive properties of NSAIDs are the result of their ability to induce apoptosis and are possibly independent of their ability to inhibit cyclooxygenase activity

(7–14). Several mechanisms have been proposed for aspirin-induced apoptosis, including activation of caspases (11–14), down-regulation of NF-κB activity (15–17), activation of ceramide pathway (18), up-regulation of several proapoptotic proteins (8, 11, 14, 19, 20), and generation of endoplasmic reticulum (ER) and oxidative stress (21, 22). Aspirin has been found to induce both the extrinsic (the death receptor) and intrinsic (the mitochondrial) pathway of caspase activation (10, 12, 14), but how it does so is not well understood. Aspirin can induce up-regulation of Bax and down-regulation of Bcl-X$_L$ (11, 14, 19, 23). The alteration of the ratio of proapoptotic to antiapoptotic molecules could activate the mitochondrial pathway of apoptosis.

The ubiquitin proteasome system (UPS) is the major extra lysosomal pathway of the cells responsible for the intracellular protein degradation in eukaryotes (24). A protein to be degraded by this pathway is first covalently attached with multiple molecules of ubiquitin, and then the polyubiquitylated proteins are degraded by the 26 S proteasome. The 26 S proteasome is a multicatalytic proteinase complex, which contains 20 S proteasome as its proteolytic core. The 20 S proteasome has three different types of proteolytic activities. Those are chymotrypsin-like (Tyr or Phe at P1), trypsin-like (Arg or Lys at P1), and post-glutamyl peptidyl hydrolytic-like (Glu at P1) activities. The normal function of UPS is necessary for the cell growth by regulating the cell cycle through timely degradation of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors (24, 25). The proteasome is also associated with many other important functions including degradation of short-lived and misfolded proteins and several transcription factors (24).

Because the UPS is involved in the degradation of many short-lived proteins that are required for cell survival, it is expected that the dysfunction of this pathway will promote cell death. In fact, the pharmacological inhibition of proteasome function has been found to induce the dual apoptotic signaling pathways, depending on the cell types and conditions (26–32). Proteasomal inhibitors also inhibit NF-κB activity, induce oxidative and ER stress response, and activate various stress kinases (31, 33, 34). In the present investigation, we demonstrate that aspirin inhibits the proteasome function, induces mitochondrial abnormalities, and activates intrinsic pathway of apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Aspirin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactacystin, *N*-acetylcysteine (NAC), MG132, cycloheximide, proteasome and caspase substrates, rabbit polyclonal anti-ubiquitin, mouse monoclonal

[3] The abbreviations used are: NSAIDs, nonsteroidal anti-inflammatory drugs; ER, endoplasmic reticulum; UPS, ubiquitin proteasome system; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; JC-1, 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide; HA, hemagglutinin; IKKβ, IκB kinase-β; GFP, green fluorescence protein; EGFP, enhanced green fluorescence protein; PBS, phosphate-buffered saline; Hsp, heat shock protein; MCA, methylcoumarinamide.

anti-β-tubulin, and all cell culture reagents were obtained from Sigma. Lipofectamine 2000 and JC-1 (5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide) were purchased from Invitrogen and Molecular Probes, respectively. Dual luciferase reporter gene assay kit was purchased from Promega. Mouse monoclonal anti-GFP and anti-HA were from Roche Applied Science; mouse monoclonal anti-cytochrome c and anti-p27^{kip1} were from Pharmingen; and mouse monoclonal anti-IκB kinase-β (IKKβ), rabbit polyclonal anti-IκB-α, anti-phospho-IκB-α, anti-Bax, and anti-p53 were from Santa Cruz Biotechnology. Goat anti-rabbit IgG-fluorescein isothiocyanate was purchased from Vector laboratories, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Amersham Biosciences. The source of plasmids ubiquitin (with HA tag), pd1EGFP-N1, Hsp70, and Hsp40 are described elsewhere (29, 35). IκB-α-EGFP plasmid was purchased from BD Biosciences.

Cell Culture, Treatments, and Viability Assay—Neuro 2a, COS-1, and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin antibiotics. For routine experiments, the cells were plated onto a 6-well tissue culture plate at subconfluent density. After 24 h of plating, cells were treated with different doses of aspirin and MG132 for different time periods and then processed for immunoblotting experiments. For cell viability assay, cells (5×10^3 cells/well) were seeded into 96-well plates, and 24 h after seeding, medium was replaced and cells were treated with different doses of aspirin alone or along with NAC. In some experiments, the cells were plated onto 48-well tissue culture plates. On the following day, cells were transfected with Hsp40 and Hsp70 plasmids. Twenty-four hours later, cells were treated with different doses of aspirin and then processed for cell viability assay. Cell viability was measured by MTT assay and trypan blue dye exclusion method. Statistical analysis was performed using Student's t test, and $p < 0.05$ was considered to indicate statistical significance.

Co-immunoprecipitation and Immunoblotting Experiment—Twenty-four hours after transfection of pd1EGFP plasmids, cells were treated with different doses of aspirin and MG132 for 8 h. Cells were then washed with cold PBS, scraped, pelleted by centrifugation, and lysed on ice for 30 min with Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, complete protease inhibitor mixture). Cell lysates were briefly sonicated, centrifuged for 10 min at 15000 × g at 4 °C, and the supernatants (total soluble extract) were used for immunoprecipitation. Protein concentration was measured according to the method of Bradford using bovine serum albumin as a standard (36). For each immunoprecipitation experiment, 200-μg protein in 0.2 ml of Nonidet P-40 lysis buffer was incubated with 5 μl (2.5 μg) of GFP antibody. After overnight incubation at 4 °C with rotation, 20 μl of protein G-agarose beads were added, and incubation was continued at 4 °C for 5 h. The beads were washed six times with Nonidet P-40 lysis buffer. Bound proteins were eluted from the beads with SDS (1×) sample buffer, vortexed, boiled for 5 min, and analyzed by immunoblotting. The total cell lysate or the immunoprecipitated proteins were separated through SDS-polyacrylamide gel elec-

trophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were successively incubated in blocking buffer (5% skim milk in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween)) with primary antibody in TBST and then with secondary antibody conjugated with horseradish peroxidase in TBST. Detection was carried out with enhanced chemiluminescence reagent. All primary antibodies were used in 1:1000 dilutions for immunoblotting.

In Vitro Kinase Assay—Neuro 2a cells were transiently transfected with the IκB-α-EGFP plasmid, and 24 h later the cells were collected and lysed in 400 μl of buffer A (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% deoxycholate, 5 mM EGTA, 150 mM NaCl, 10 mM NaF, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and complete protease inhibitor mixture). Cell lysates were immunoprecipitated with IKKβ and GFP antibodies. Immune complex was washed twice with buffer A and then equal amounts of immunoprecipitated proteins were incubated with or without different doses of aspirin for 30 min at 4 °C, and *in vitro* kinase assay was performed at 30 °C for 30 min as described elsewhere (37). The samples were then subjected to immunoblotting using phospho-IκB-α and IKKβ antibodies.

Measurement of Mitochondrial Membrane Potential and Cytochrome c Release—Neuro 2a cells were plated onto a 60-mm tissue culture plate at subconfluent density. Twenty-four hours later, cells were exposed to different doses of aspirin and MG132 for 12 h and then incubated with 5 μM JC-1 fluorescence dye for 30 min in the CO_2 incubator and washed several times with PBS prewarmed at 37 °C. Mitochondrial membrane potential was evaluated qualitatively under a fluorescence microscope using 568-nm filter.

To study the release of cytochrome c from mitochondria, Neuro 2a cells grown on chamber slides were treated with different doses of aspirin and MG132. Twelve h after treatment, cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed extensively and then blocked with 5% nonfat dried milk in TBST for 1 h. Primary antibody (anti-cytochrome c) incubation was carried out overnight at 4 °C. After several washings with TBST, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody for 4 h, washed several times, and visualized using a fluorescence microscope. The digital images were assembled using Adobe Photoshop. To evaluate the release of cytochrome c from mitochondria by immunoblotting, Neuro 2a cells were grown on tissue culture dishes, and the cells were then treated with different doses of aspirin for 12 h. The cells were collected by scraping, washed in the PBS followed by sucrose buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride containing 250 mM sucrose), and resuspended in the same buffer. After 1 h of incubation on ice, cells were lysed by Dounce homogenizer of B-type pestle with 30 strokes. Homogenates were centrifuged at 750 × g for 10 min at 4 °C, and the supernatants were recentrifuged at 10,000 × g for 15 min at 4 °C. The final supernatants were used for immunoblotting experiments of cytochrome c.
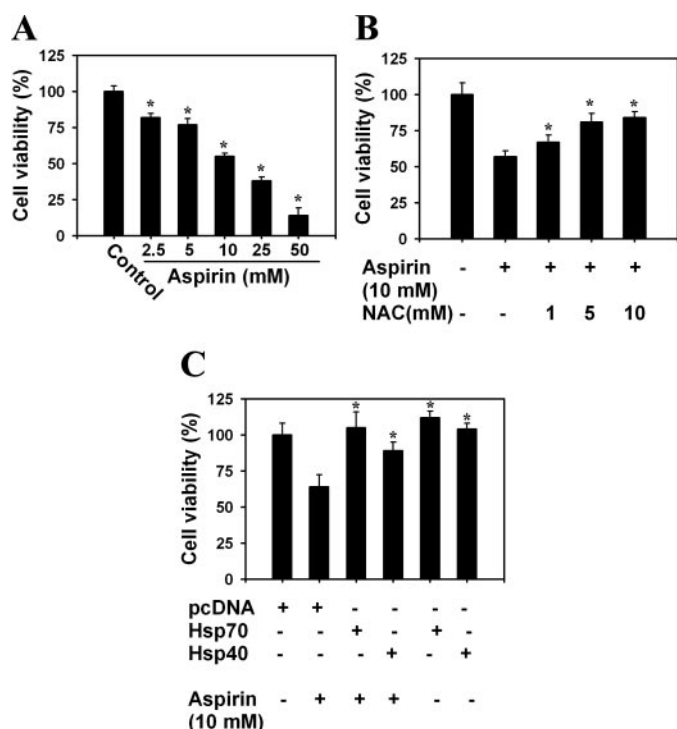
FIGURE 1. **Aspirin induces cell death.** *A*, mouse Neuro 2a cells were plated onto 96-well tissue culture plates. Cells were left untreated or treated with different doses of aspirin for 24 h. Cell viability was measured by MTT assay. *, $p < 0.01$ as compared with control. *B*, cells were plated as described in *A* and then exposed to either aspirin alone or along with different doses of NAC for 24 h. Cell viability was assayed by trypan blue dye exclusion method. *, $p < 0.01$ as compared with aspirin-treated experiments. *C*, cells were plated onto 48-well tissue culture plates and transiently transfected with empty pcDNA, Hsp40, and Hsp70 expression plasmids. Twenty-fours hours later, cells were left untreated or were treated with different doses of aspirin for another 24 h. Cell viability was determined by MTT assay. *, $p < 0.01$ as compared with empty pcDNA-transfected and aspirin-treated experiments.

Assay of Proteasome, and Caspase-9- and Caspase-3-like Protease Activity—The cells were plated onto a 6-well tissue culture plate, and on the following day, cells were treated with varying doses of aspirin and MG132 for different time periods. Cells were then isolated and processed for proteasome activity assay as described earlier (29). The substrates Succinyl-Leu-Leu-Val-Tyr-MCA and benzyloxycarbonyl-Leu-Leu-Glu-MCA were used to determine chymotrypsin and post-glutamyl peptidyl hydrolytic-like activity, respectively. To evaluate the direct effect of aspirin on the protease activity of proteasome, pure 20 S proteasome (250 ng/reaction) was used instead of cell supernatant in the protease activity assay buffer. Protease activities at a particular time point (30 min) within the linear range were used to calculate the data. The fluorescence intensity was measured at 380 nm excitation and 460 nm emissions using a fluorescence plate reader. The caspase-9- and -3-like protease activities were determined as described earlier (32). The substrates Ac-Asp-Glu-Val-Asp-MCA and Ac-Leu-Glu-His-Asp-MCA were used to determine the caspase-3- and -9-like protease activity, respectively.

Cycloheximide-Chase Experiment—Neuro 2a cells were plated onto a 6-well tissue culture plate and on the following day, cells were chased with 15 μg/ml of cycloheximide for different time periods in the presence or absence of aspirin. Cells collected at each time point were then processed for immuno-
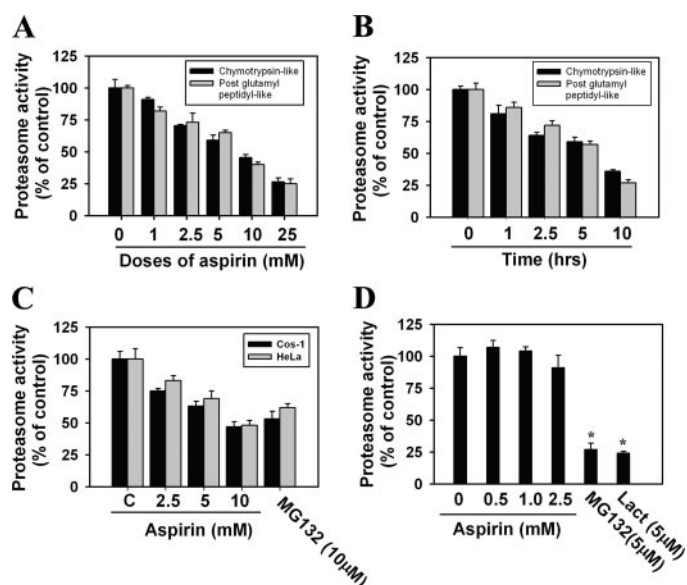

FIGURE 2. **Aspirin inhibits proteasome activity.** Neuro 2a cells were exposed to different doses of aspirin for 8 h (*A*) or treated with 10 mM aspirin for different time periods (*B*). Cells were then collected and processed for proteasome activity assays (chymotrypsin and post-glutamyl peptidyl hydrolytic-like protease activity) as described under "Experimental Procedures." *C*, COS-1 and HeLa cells were treated with either different doses of aspirin or MG132 for 8 h. Cells were collected and subjected to proteasome activity (chymotrypsin-like) assay. *D*, partially purified 20 S proteasome was incubated with different doses of aspirin and proteasome inhibitors MG132 and lactacystin (*Lact*) in the presence of chymotrypsin-like substrate of proteasome. In *A*, *B*, and *C*, all the treatment groups show significant inhibition ($p < 0.01$) of proteasome activity as compared with control. *, $p < 0.01$ as compared with control.

blotting using antibodies against Bax, IκB-α, p53, p27^{kip1}, and β-tubulin. In some experiments, Neuro 2a cells were transiently transfected with pd1EGFP plasmids, and 24 h later, cells were chased with cycloheximide as described above. The collected cells at each time points were then processed for immunoblotting using anti-GFP.

Reporter Gene Assay—Neuro 2a cells were plated onto 6-well tissue culture plates at a subconfluent density. Twenty-four hours later, cells were transiently transfected with NF-κB-luciferase and pRL-SV40 plasmids together using Lipofectamine 2000 reagent according to the manufacturer's instructions. Transfection efficiency was about 80–90%. Twenty-four hours posttransfection, cells were treated with different doses of aspirin and then processed for luciferase assay. Luciferase activity was measured using a dual luciferase reporter assay system according to the manufacturer's instructions. pRL-SV40 plasmid was used for co-transfection to normalize the data, and it was transfected at a very low concentration (150-fold lower than NF-κB luciferase plasmid). The data were represented as relative luciferase activity (the ratio of firefly to *Renilla* values).

RESULTS

Aspirin Inhibits Growth and Induces Apoptosis in Neuro 2a Cells—Aspirin and other NSAIDs have been found to induce apoptosis in many cell types. Here we first tested the effect of aspirin on the viability of mouse Neuro 2a neuroblastoma cells. The cells were plated onto 96-well tissue culture plates, and on the following day, the cells were exposed to the different doses of aspirin for 24 h. The cell viability was determined by MTT

assay. As shown in Fig. 1, treatment with aspirin dose dependently reduced the cell viability. Aspirin at a dose of 2.5 mM (the concentration that can be found in the serum of patients treated with aspirin for chronic inflammatory diseases (38)) significantly increased the cell death to about 15 and 35% after 24 and 48 h of exposure, respectively, but how aspirin induced the cell death program was not very clear. NSAIDs have been found to induce the expression of heat shock proteins (Hsps) and gener-

FIGURE 3. **Aspirin treatment leads to increased accumulation of ubiquitylated proteins.** *A*, Neuro 2a cells were treated with different doses of aspirin for 8 h, and cell lysates were made and processed for immunoblotting using ubiquitin and β-tubulin antibodies. *B*, Neuro 2a cells were transiently transfected with HA-ubiquitin expression plasmid. Twenty-four hours later, cells were treated with different doses of aspirin as described in *A*. The cell lysates were processed for immunoblotting using anti-HA and anti-β-tubulin.

FIGURE 4. **Aspirin exposure decreases the turnover of the destabilized EGFP.** *A*, Neuro 2a cells were transiently transfected with pd1EGFP plasmid as described under "Experimental Procedures." Twenty-four hours posttransfection, cells were treated with cycloheximide (15 μg/ml) and chased in the presence or absence of 10 mM aspirin for different time periods. Blot was detected with GFP and β-tubulin antibodies. *B*, quantitation of the d1EGFP protein levels in the chase experiment as described in *A* is from two independent experiments, each performed in duplicate. Quantitation was performed using NIH Image analysis software. Data were normalized against β-tubulin. Aspirin treatment significantly increased ($p < 0.01$) the levels of d1EGFP protein in comparison with control at all the time points tested. *C*, Neuro 2a cells were transiently transfected and treated with different doses of aspirin as described in *A*. Cell lysates were made and subjected to immunoprecipitation using anti-GFP. Blot was sequentially detected with ubiquitin and GFP antibodies.

ate the oxidative and ER stress (21, 22). Therefore, we presumed that aspirin-induced apoptosis might be because of the induction of stress responses. To test this hypothesis, we checked the role of antioxidant NAC and the overexpression of various heat shock proteins on aspirin-induced cell death. The treatment of NAC dose dependently rescued aspirin-induced cell death (Fig. 1*B*). Aspirin-induced cell death was also prevented upon overexpression of Hsp70 and Hsp40 chaperones (Fig. 1*C*).

Aspirin Inhibits the Proteasome Function—We next tried to explore the mechanism of aspirin-induced stress responses in the cells. Because the inhibition of proteasomal function induces the stress responses inside the cells, we checked the possible role of aspirin on proteasomal dysfunction. As shown in Fig. 2, *A* and *B*, dose and time dependently, aspirin treatment of Neuro 2a cells inhibits various protease activities of proteasome. Aspirin exposure at doses of 2.5, 5, and 10 mM for 8 h caused about 25, 40, and 60% inhibition of proteasome activity, respectively. Aspirin also inhibited the proteasome activity in HeLa and COS-1 cells (Fig. 2*C*). In many experiments, we used 10 mM aspirin to reduce the time of exposure.

Because aspirin inhibits the proteasome activity in cell culture, we were further interested to know the possible mechanism of proteasome inhibition. Therefore, we tested the direct effect of aspirin on proteasome activity. The partially purified 20 S proteasome was incubated with varying doses of aspirin in the presence of chymotrypsin-like protease substrate. Fig. 2*D* shows that aspirin had no effect on the protease activity of the purified 20 S proteasome. Proteasome inhibitors MG132 and lactacystin were used as positive controls, and they dramatically decreased the proteasome activity. Thus our results suggest that aspirin-induced proteasomal inhibition is mediated indirectly.

To reconfirm the aspirin-induced proteasomal malfunction in cell culture, we checked the ubiquitylation profile because inhibition of proteasome function would lead to the increased accumulation of ubiquitylated derivatives of various proteins. Fig. 3 shows that the exposure to aspirin caused a dose-dependent increase in the accumulation of ubiquitylated derivatives of various cellular proteins. To further confirm the accumulation of ubiquitylated proteins upon aspirin treatment, we transiently transfected Neuro 2a cells with HA-ubiquitin plasmids and then treated them with different doses of aspirin. The blot was detected with HA antibody. In this case, we also detected an increased accumulation of HA-tagged ubiquitylated proteins and
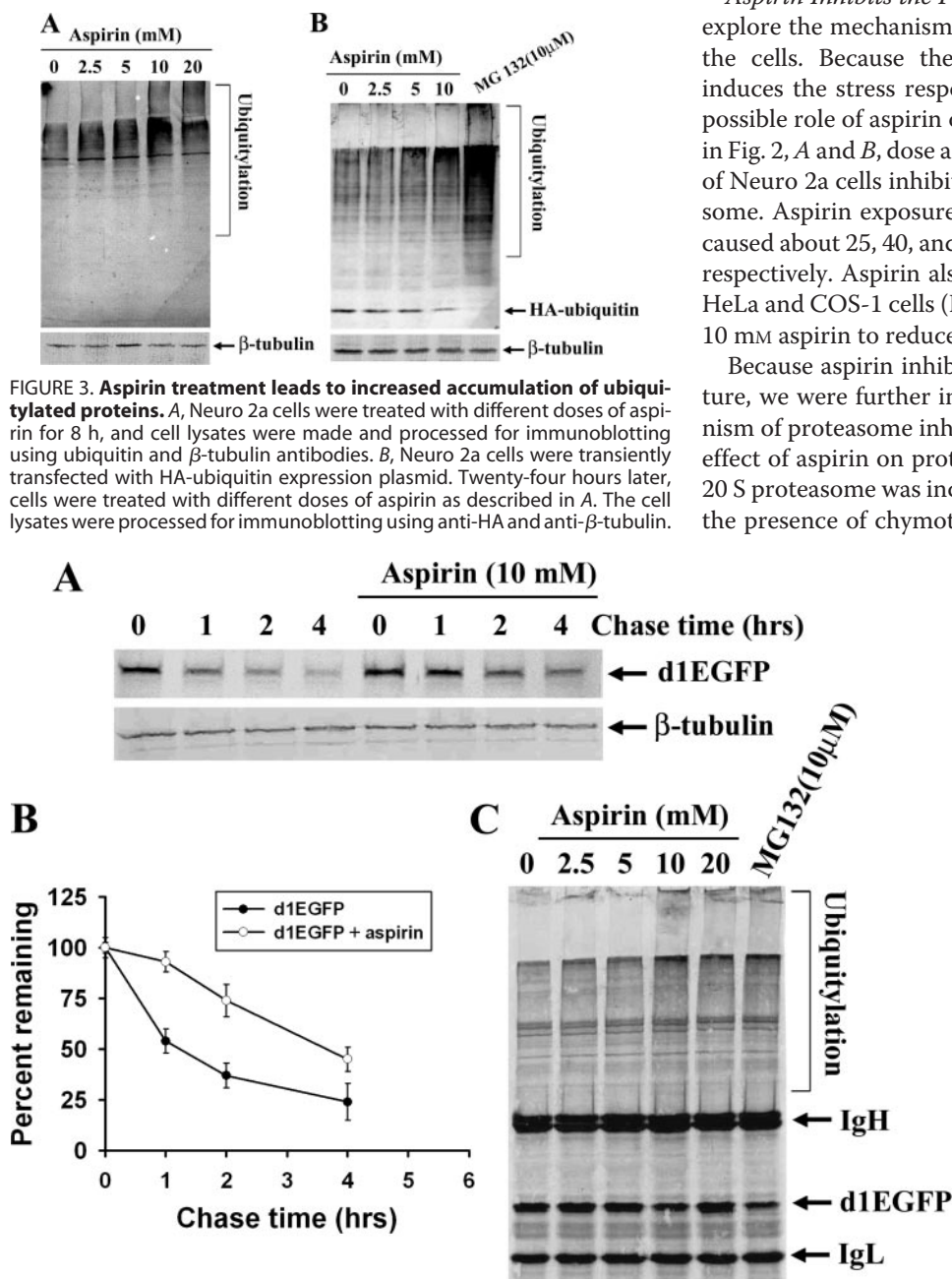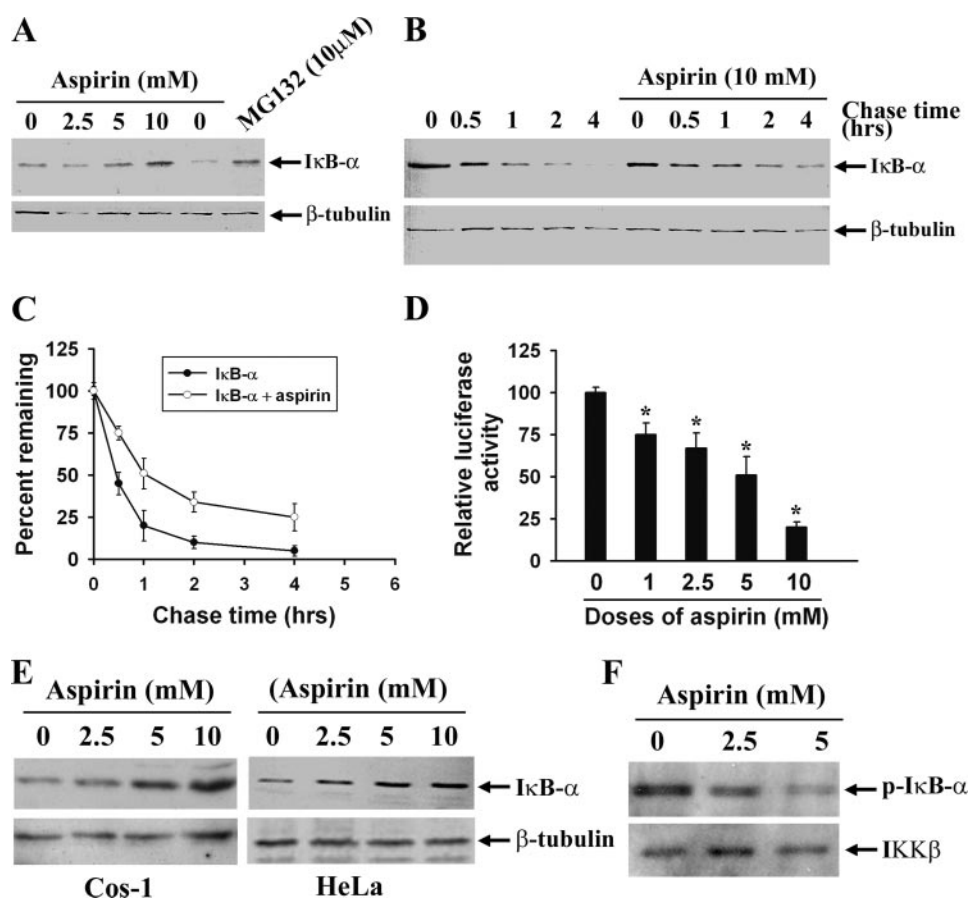
FIGURE 5. **Aspirin treatment increases the stability of IκB-α and down-regulates the NF-κB activity.** *A*, Neuro 2a cells were treated with different doses of aspirin and MG132 for 8 h. The cells were collected and processed for immunoblotting using IκB-α and β-tubulin antibodies. *B*, the cells were treated with 15 μg/ml cycloheximide and chased in the presence and absence of aspirin for different time periods. The blot was detected with IκB-α and β-tubulin antibodies. *C*, quantitation of IκB-α protein levels in the chase experiment as described in *B* is from three independent experiments. Data were normalized against β-tubulin. Aspirin treatment significantly increased ($p < 0.01$) the accumulations of IκB-α in comparison with control at all the time points tested. *D*, Neuro 2a cells were transiently transfected with NF-κB luciferase and pRL-SV40 plasmids. Twenty-four hours after posttransfection, cells were treated with different doses of aspirin for 8 h. Cells were then collected and processed for dual luciferase reporter gene assay. *, $p < 0.01$ as compared with control. *E*, COS-1 and HeLa cells were treated with different doses of aspirin as described in *A*. Cells were collected and processed for immunoblotting using IκB-α and β-tubulin antibodies. *F*, effect of aspirin on IKKβ activity. Neuro 2a cells were transiently transfected with IκB-α-EGFP construct. Twenty-four hours later, the cells were lysed, and IKKβ and IκB-α-EGFP proteins were immunoprecipitated using their respective antibodies. Equal amounts of immunoprecipitated proteins were incubated with or without 2.5 and 5 mM aspirin for 30 min at 4 °C, and then an *in vitro* kinase assay was performed at 30 °C for 30 min as described under "Experimental Procedures." The samples were then processed for immunoblotting using phospho-IκB-α and IKKβ antibodies.

a subsequent decrease in the free HA-tagged ubiquitin levels (Fig. 3*B*).

Aspirin Treatment Decreases the Turnover of a Model Substrate of Proteasome—Aspirin-induced proteasomal dysfunction was further confirmed by using destabilized enhanced green fluorescent protein (d1EGFP), a model substrate for proteasome with 1 h of half-life. The pd1EGFP vector contains multiple proline, glutamic acid, serine, and threonine signal sequence in its C terminus, which can be targeted for degradation by proteasome. The proteasomal malfunction will increase the half-life of this protein. Neuro 2a cells were transiently transfected with the pd1EGFP expression plasmids, and then the cells were chased with cycloheximide in the presence or absence of 10 mM aspirin. Results show that the treatment with 10 mM aspirin increased the half-life of d1EGFP proteins by about 3.5-fold (Fig. 4, *A* and *B*). In another experiment, Neuro

2a cells were transiently transfected with pd1EGFP plasmids and then treated with varying doses of aspirin and MG132. The cell lysates were then processed for co-immunoprecipitation experiment using anti-GFP, and the blot was detected sequentially with ubiquitin and GFP antibody. As expected, aspirin treatment, like MG132, resulted in increased accumulations of ubiquitylated d1EGFP proteins (Fig. 4*C*).

Exposure to Aspirin Increases the Half-life of Various Cellular Substrates of Proteasome—Because aspirin increases the half-life of a model substrate of proteasome, we next tested its effect on the half-life of other cellular target substrates. First, we checked the effect of aspirin on the accumulations and degradations of IκB-α and the down-regulation of NF-κB activity. As shown in Fig. 5, *A–C*, the treatment with aspirin caused a dose-dependent increase in accumulation and increased half-life of IκB-α. This increased half-life of IκB-α might be involved, at least in part, in the down-regulation of the NF-κB-dependent transcriptional activity (Fig. 5*D*). Aspirin-induced increased accumulation of IκB-α is not only observed in Neuro 2a cells but also can be detected in COS-1 and HeLa cells (Fig. 5*E*). Aspirin at a dose of 5 mM caused about 3-fold increase in the accumulation of IκB-α in COS-1 and HeLa cells. However, an earlier report demonstrated the enhanced degradation of IκB-α in aspirin-treated colon cancer cells (39).

Therefore, the effect of aspirin on IκB-α might be cell-specific. Interestingly, aspirin-induced NF-κB inhibition was much more dramatic than the proteasomal inhibition. This suggests that proteasomal dysfunction is not the only reason for aspirin-induced NF-κB inhibition. In fact, earlier report demonstrated the specific inhibition of IKKβ induced by aspirin both *in vitro* and also in COS-1, HeLa, and LNCaP cells (15). We have also found similar results in Neuro 2a cells (Fig. 5*F*). Therefore, it is possible that aspirin-induced inhibition of IKKβ and proteasome activity both might be involved in the down-regulation of NF-κB activity.

Next, we checked the half-life of three other known substrates of proteasome, Bax, p53, and p27^{kip1}. As expected the half-life of Bax, p53, and p27^{kip1} were dramatically increased upon treatment with aspirin (Fig. 6). Aspirin at a dose of 10 mM increased the half-life of Bax, p53, and p27^{kip1} by about 4-fold.
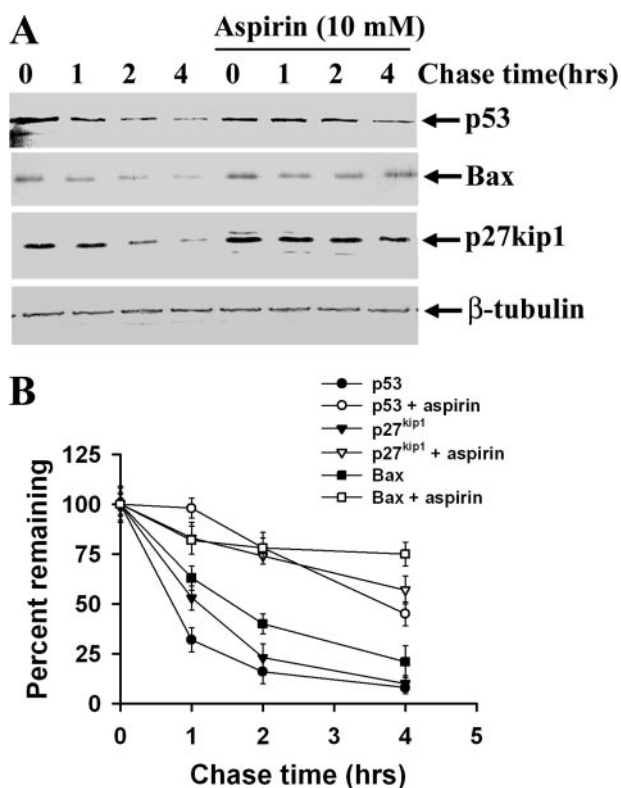
FIGURE 6. **Aspirin treatment increases the half-life of Bax, p27^{kip1}, and p53.** *A*, Neuro 2a cells were treated with cycloheximide and chased in the presence and absence of 10 mM aspirin for different time periods as described in the legend to Fig. 5. Cells were collected and subjected to immunoblotting using antibodies against Bax, p27^{kip1}, p53, and β-tubulin. *B*, quantitation of Bax, p27^{kip1}, and p53 protein levels in the chase experiment as described in *A* from three independent experiments. Data were normalized against β-tubulin. Aspirin treatment significantly increased ($p < 0.01$) the accumulations of p53, p27^{kip1}, and Bax in comparison with control at all the time points tested.

The increased accumulation of Bax, p53, and p27^{kip1} might result in the cell cycle arrest and apoptosis.

Aspirin Induces Mitochondrial Membrane Depolarization, Cytochrome c Release, and Activation of Caspases—Because proteasomal inhibition induces the mitochondrial pathway of apoptosis, we next checked the effect of aspirin on the mitochondrial membrane depolarization and cytochrome *c* release. Neuro 2a cells were exposed to different doses of aspirin for different time periods and then subjected to JC-1 staining to study the changes in the mitochondrial membrane potential or processed for immunofluorescence staining of cytochrome *c*. JC-1 is a voltage-sensitive fluorescence dye that specifically detects the polarized mitochondria as red, and the depolarized membrane becomes green. The treatment with aspirin dose dependently decreased the mitochondrial membrane potential as evident from the loss of red-dotted mitochondrial staining (Fig. 7, *top panel*). Aspirin treatment also dose dependently induced the cytochrome *c* release from the mitochondria (Fig. 7, *bottom panel*, and Fig. 8*A*).

Because the caspase-9 activation is initiated with the release of cytochrome *c* from mitochondria followed by binding to apoptosis protease-activating factor-1, we further examined the activation of caspase-9 and subsequent events upon aspirin treatment. As shown in Fig. 8*B*, aspirin exposure dose dependently increased the
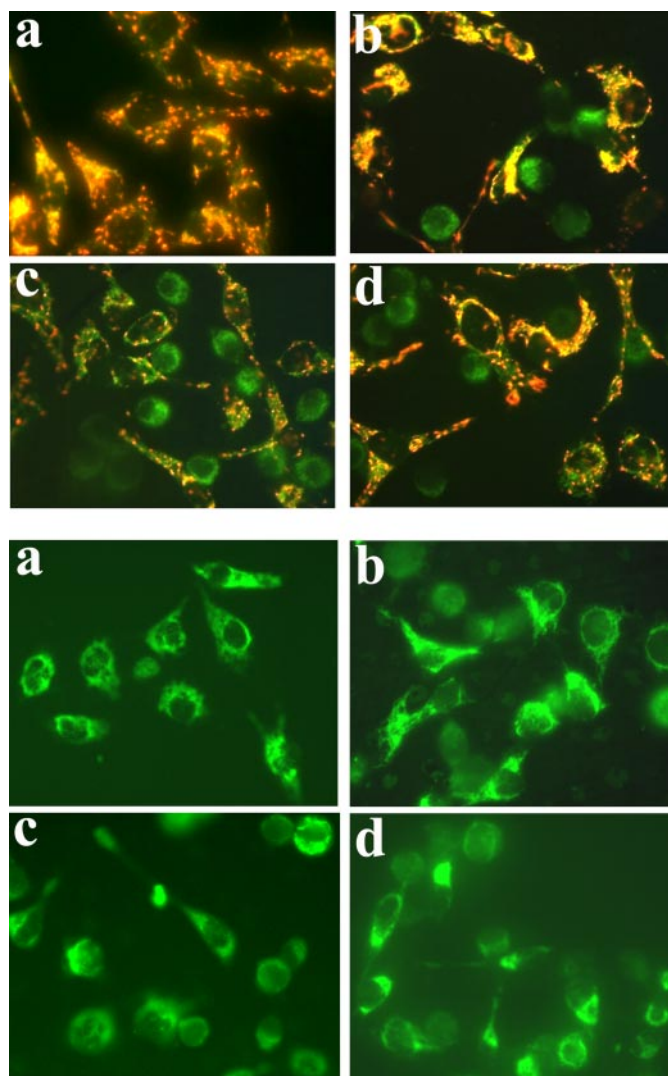

FIGURE 7. **Aspirin exposure results in dose-dependent changes in the mitochondrial membrane potential and release of cytochrome c.** Neuro 2a cells were treated with either different doses of aspirin or MG132 for 12 h and then subjected to JC-1 staining to study the changes in mitochondrial membrane potential (*top panel*) and to immunofluorescence staining using cytochrome *c* antibody (*bottom panel*). *a*, control; *b*, aspirin (2.5 mM); *c*, aspirin (10 mM); *d*, MG132 (5 μM).

activity of both caspase-9 and -3. The MG132 was used as a positive control.

Treatment with Aspirin Induces Neurite Outgrowth—The impairment of proteasome function is also known to induce the neurite outgrowth. Because aspirin inhibits the proteasome function, we further tested its possible effect on neurite outgrowth. As shown in Fig. 9, aspirin exposure induced the neurite outgrowth like proteasome inhibitor, MG132, depending upon the dose. Approximately 50% of the cells looked bipolar (having two neurites), and the rest were round at a 10 mM dose. Aspirin-induced neurite outgrowth could be because of the increased accumulation of p27^{kip1} or ubiquitylated proteins.

DISCUSSION

Apart from the classical anti-inflammatory function, aspirin and other NSAIDs exhibit an antiproliferative effect because of
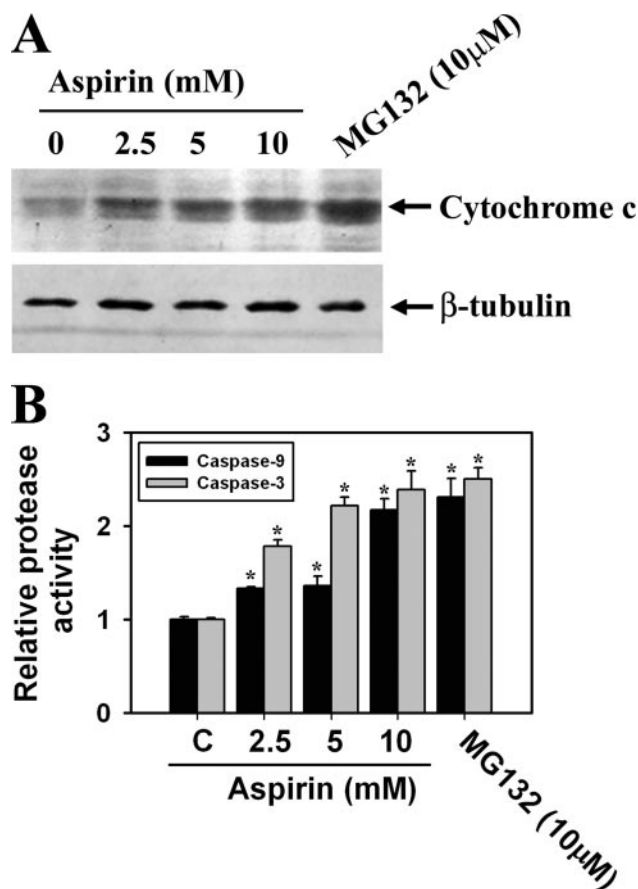
FIGURE 8. **Aspirin treatment induces cytochrome *c* release from mitochondria and activates caspase-9 and -3.** *A*, Neuro 2a cells were treated with either different doses of aspirin or MG132 for 12 h as described in the legend to Fig. 7. The cells were then collected and subjected to subcellular fractionation. The cytosolic fractions were processed for immunoblotting using cytochrome *c* and β-tubulin antibodies. *B*, Neuro 2a cells were plated onto 6-well tissue culture plates, and on the following day, the cells were treated with either different doses of aspirin for 24 h or 10 mM MG132 for 12 h. The cells were then collected and processed for caspase-9 and -3 activity assay. *, $p < 0.01$ as compared with control.

FIGURE 9. **Treatment with aspirin induces neurite outgrowth.** Neuro 2a cells were plated onto the chamber slides at subconfluent density. The cells were then treated with different doses of aspirin and MG132 for 8 h. The morphology of the cells was then observed under the microscope.

their ability to induce apoptosis on a variety of cancer cells (7–14), but how they induce apoptosis is not well understood. Here we report that aspirin inhibits the proteasome function and causes severe mitochondrial abnormalities. This inhibition of proteasome function by aspirin play an important role in the induction of apoptotic signals and anti-inflammatory responses.

Firstly, we have shown that the exposure to aspirin dose and time dependently inhibits the cellular proteasome function and increases the accumulations of ubiquitylated derivatives of various cellular proteins. The inhibition of proteasome function induced by aspirin correlates well with its effect on cell death. Secondly, we have found that treatment with aspirin inhibits the degradation of destabilized enhanced green fluorescence proteins and also various other cellular substrates of proteasome like Bax, IκB-α, p53, and p27^{kip1}. Finally, we have demonstrated the dramatic changes of mitochondrial membrane potential, release of cytochrome *c* from mitochondria into cytosol, and activation of caspase-9 and -3 upon aspirin exposure, which might be because of the proteasomal malfunction. The dose of aspirin (1–5 mM) we used can be measured in the serum of patients treated with aspirin for various chronic inflammatory diseases (38).
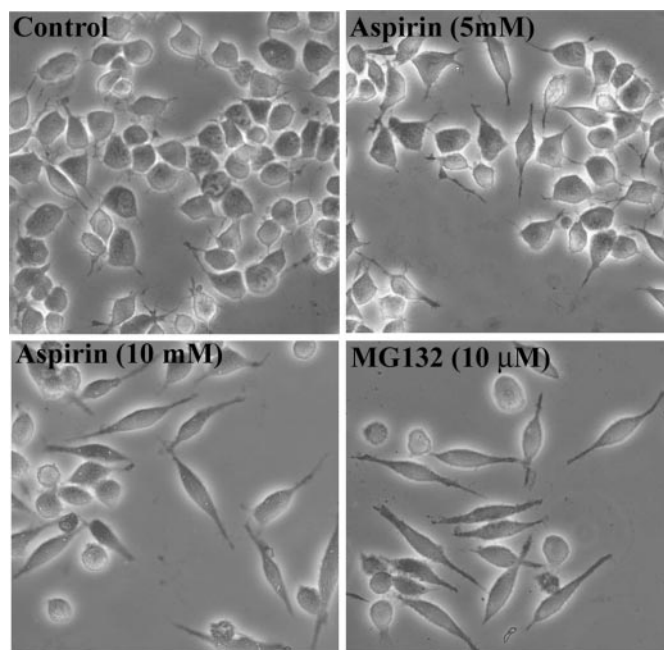
The released cytochrome *c* into the cytosol binds with the apoptosis protease-activating factor-1 and forms the apoptosome complex, which in turn leads to the sequential activation of caspase-9 and -3. In fact, aspirin, like other proteasome inhibitors, has been reported to activate caspase-9 and -3 by inducing the release of mitochondrial cytochrome *c* (10–12, 40, 41). Aspirin, like other proteasome inhibitors, also induces the extrinsic pathway of caspase activation (14, 40, 41).

How can aspirin induce mitochondrial cytochrome *c* release? Aspirin is reported to up-regulate the expression of Bax (11, 14), which can then translocate from cytosol to the outer mitochondrial membrane and make pores in the mitochondrial membrane to leak cytochrome *c*. Bax is a substrate of proteasome, and its expression is induced by p53, which is also a substrate of proteasome. Therefore, aspirin-induced proteasomal dysfunction could conceivably cause mitochondrial cytochrome *c* release by up-regulating Bax and p53 levels because aspirin treatment causes up-regulation of these proteins (8, 11, 14).

Besides Bax and p53, there are several other proteins involved in regulating transcription; growth and apoptosis are substrates of proteasome. Proteasome inhibition also up-regulates the expression of various proteins implicated in the pro-apoptotic pathway and down-regulates various transcripts involved in cell growth and survival pathways (40, 41). The altered degradation or altered expression of those proteins will definitely affect cell survival and promote apoptosis. Here we have shown that treatment with aspirin leads to an increase in the half-life of Bax, p53, p27^{kip1}, and IκB-α because of the impaired proteasome function.

Aspirin-induced proteasomal dysfunction could explain several earlier reported effects of aspirin including inhibition of NF-κB activity, cell cycle arrest, and induction of stress response (7–9, 15–17, 21, 22). All these effects can be observed

during proteasomal inhibition. Aspirin is known to inhibit the NF-κB pathway, which is involved in the pathogenesis of inflammatory responses. Earlier reports have demonstrated that aspirin inhibits the NF-κB pathway by inhibiting the IKKβ (15). Here we provide additional evidence that aspirin-induced proteasomal dysfunction prevents the degradation of IκB-α and thereby blocks the nuclear translocation and transactivation of NF-κB. We have also found that aspirin, like other proteasome inhibitors, increases the accumulation of p27^{kip1}. p27^{kip1} acts as an inhibitor of cdk2 and cdk4 and is highly regulated by UPS. Increased accumulation of p27^{kip1} results in cell cycle arrest at the G$_1$/S phase and apoptosis (42, 43).

How does aspirin disturb the cellular proteasome function? It does not inhibit the proteasome function by directly inhibiting the enzyme activity. However, aspirin could impair the proteasome function indirectly by inhibiting the expression of various subunits of proteasome. Oxidative and ER stress can also promote aspirin-induced proteasomal malfunction because aspirin treatment generates oxidative and ER stress, and both of them have been reported to induce the proteasomal malfunction (33, 44, 45). Aspirin-induced mitochondrial damage would be able to generate the oxidative stress inside the cell (12–14). Oxidative stress can again damage mitochondria and thus generate a vicious cycle. Oxidative stress would be able to disturb the proteasome function either by producing excessive levels of damaged protein or by reducing the levels of ATP production.

Altogether, our results suggest that aspirin inhibits the proteasome function, and the impairment of proteasome function might be associated at least in part with its anti-inflammatory function as well as the induction of apoptosis. Proteasome inhibitors are considered to be promising anticancer agents, and recently the Food and Drug Administration approved the first proteasome inhibitor, bortezomib (Velcade), for the treatment of multiple myeloma (46, 47). Therefore, aspirin has enormous potential in the prevention and therapy of cancer apart from its popular use as an anti-inflammatory drug.

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