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Nonsteroidal Anti-Inflammatory Drugs Induced Endothelial Apoptosis by Perturbing Peroxisome Proliferator-Activated Receptor-δ Transcriptional Pathway

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ABSTRACT

Recent studies have shown that use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with an increased risk of myocardial infarction. To explore whether NSAIDs may induce endothelial apoptosis and thereby enhance atherothrombosis, we treated human umbilical vein endothelial cells (HUVECs) with sulindac sulfide (SUL), indomethacin (IND), aspirin (ASA), or sodium salicylate (NaS), and we analyzed apoptosis. SUL and/or IND significantly increased annexin V-positive cells, cleaved poly(ADP-ribose) polymerase (PARP) and caspase-3. ASA and NaS at 1 mM did not induce PARP cleavage or caspase-3 and at 5 mM, ASA but not NaS increased apoptosis. Because peroxisome proliferator-activated receptor δ-mediated 14-3-3ε up-regulation was reported to play a crucial role in protecting against apoptosis, we determined whether NSAIDs suppress this transcriptional pathway. SUL, IND, and ASA (5 mM) suppressed PPARδ and 14-3-3 proteins in a manner parallel to PARP cleavage. Neither ASA nor NaS at 1 mM interfered with PPARδ or 14-3-3ε expression. SUL inhibited PPARδ promoter activity, which correlated with 14-3-3ε promoter suppression. Suppression of 14-3-3ε was associated with increased Bad translocation to mitochondria. Neither carbaprostacylin nor 4-(3-(2-propyl-3-hydroxy-4-acetyl)-phenoxy)propyloxyphenoxy acetic acid (L-165041) prevented HUVECs from SUL-induced apoptosis. Because of suppression of ectopic PPARδ by sulindac, adenoviral PPARδ transduction failed to restore 14-3-3ε or prevent PPAR cleavage. Our findings suggest that NSAIDs, but not aspirin (<1 mM) induce endothelial apoptosis via suppression of PPARδ-mediated 14-3-3ε expression.

Nonsteroidal anti-inflammatory drugs (NSAIDs) make up a group of compounds of diverse chemical structures distinct from corticosteroids, but they possess common steroid-like anti-inflammatory actions (Simon and Mills, 1980). Recent reports reveal that NSAIDs such as sulindac and indomethacin prevent carcinogen-induced tumors in rats (Narisawa et al., 1981; Pollard and Luckert, 1983; Piazza et al., 1995) and reduce adenomas in Min mice, which spontaneously develop adenomatous polyposis (Boolbol et al., 1996; Jacoby et al., 1996). Epidemiological and controlled human clinical trials have confirmed that NSAID use reduces the risk of cancers and reduces adenomas in familial adenomatous polyposis (Kune et al., 1988; Giardiello et al., 1993; Steinbach et al., 2000). Although the mechanisms by which NSAIDs control cancer growth are not completely understood, several reports suggest that they induce cancer cell apoptosis (Shiff et al., 1995). NSAIDs induce apoptosis by tilting the balance between antiapoptotic and proapoptotic Bcl-2 family proteins toward the proapoptotic members, notably, Bax and Bad (Sheng et al., 1998; Zhang et al., 2000). Results from our laboratory suggest that the antitumor actions of sulindac and indomethacin are attributable to suppression of poly(ADP-ribose) polymerase (PPAR)-δ) and PPARδ-mediated 14-3-3ε expression, resulting in reduction of cytosolic 14-3-3ε proteins and consequently and increase in Bad translocation to mitochondria in which it induces apoptosis (Liou et al., 2007b).

Aspirin has been reported to possess similar antitumor

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; PPAR, peroxisome proliferator-activated receptor; COX, cyclooxygenase; HUVEC, human umbilical vein endothelial cell; $cPGI_2$, carbaprostacyclin; L0165041, 4-(3-(2-propyl-3-hydroxy-4-acetyl)phenoxy)propyloxyphenoxy acetic acid; HSP60, 60-kDa heat shock protein; PPRE, peroxisome proliferator response element; Ad, adenovirus; GFP, green fluorescent protein; pNA, *p*-nitroaniline; CHO, aldehyde; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; PG, prostaglandin; SUL, sulindac sulfide; IND, indomethacin; ASA, aspirin; NaS, sodium salicylate.

properties as NSAIDs. Aspirin was efficacious in preventing carcinogen-induced colon tumors in rats (Craven and DeRubertis, 1992; Reddy et al., 1993). Epidemiological studies show that aspirin use is associated with a significant reduction in cancers, notably colon cancers in humans (Kune et al., 1988; Thun et al., 1991). A randomized double-blind clinical trial has shown that aspirin reduces adenomatous polyposis in familial adenomatous polyposis patients (Baron et al., 2003). Aspirin and sodium salicylate at very high concentrations (>10 mM) were reported to induce apoptosis of human leukemia and lymphoma cells (Bellosillo et al., 1998; Klampfer et al., 1999; Pique et al., 2000). However, it remains unknown whether aspirin at therapeutic concentrations (≤1 mM) causes cell damage. Furthermore, mechanism by which high, suprapharmacological concentrations of aspirin and salicylate (>5 mM) induce apoptosis has not been elucidated.

It is well recognized that selective cyclooxygenase (COX)-2 inhibitors are associated with increased rick of myocardial infarction (Mukherjee et al., 2001; Fitzgerald, 2004). Recent reports indicate that all classes of NSAIDs are also associated with risk of myocardial infarction (Chan et al., 2006). The reasons for the adverse cardiovascular complications of NSAIDs are not entirely clear. Because NSAIDs induce cancer cell apoptosis, we postulated that their cardiovascular toxicity may be due to endothelial cell apoptosis. In this report, we evaluated the effect of sulindac and indomethacin as well as aspirin and sodium salicylate on PARP cleavage and/or other apoptotic markers in human umbilical vein endothelial cells (HUVECs), and we determined changes in PPARδ and 14-3-3ε expressions. The results show that sulindac and indomethacin induced apoptosis and suppressed PPARδ and 14-3-3ε expressions in a correlative manner. Aspirin and sodium salicylate at 1 mM had no effect, but aspirin at 5 mM exerted a similar apoptotic action as NSAIDs.

Materials and Methods

Pharmacological Reagents. Sulindac sulfide and indomethacin were purchased from Calbiochem (San Diego, CA), and aspirin and sodium salicylate were from Sigma-Aldrich (St. Louis, MO). All reagents except sodium salicylate were dissolved in ethanol. Sodium salicylate was dissolved in filtered distilled water. Carbaprostacylin ($cPGI_2$; 50 μM) and L-165041 (50 μM) were obtained from Cayman Chemical (Ann Arbor, MI). They were dissolved in ethanol. The final concentrations of ethanol vehicle were <0.1%.

Cell Culture. HUVECs were collected from fresh umbilical veins and cultured as described previously (Xu et al., 1999). In all experiments, only cultures up to five passages were used before experiments. HUVECs were washed and cultured in serum-free medium containing the testing drugs for 24 h. Because it was difficult to transfect HUVECs with conventional liposome-based carriers, we used ECV304 cells for selected transfection experiments. ECV304 cells are immortalized cells derived from HUVECs. They retain cobble stone-like morphology and stain positively for von Willebrand factor. Like HUVECs, they possess enzymes for prostacyclin synthesis. ECV304 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Preparation of Mitochondrial Fraction. HUVECs treated with sulindac sulfide for 4 h were washed three times with phosphate-buffered saline and harvested by centrifugation. Mitochondrial fractions were prepared by a mitochondria isolation kit (Sigma-Aldrich) as described previously (Liou et al., 2006). The mitochondrial and cytosolic fractions were purified by two-step gradient centrifugation and stored at −20°C. Mitochondrial and cytosolic Bad protein levels were analyzed by Western blotting. The 60-kDa heat shock protein (HSP60) was used as a mitochondrial marker.

Western Blot Analysis. HUVECs were lysed with radioimmunoprecipitation assay buffer containing protease inhibitors. Western blots were performed as described previously (Liou et al., 2006). For cell apoptosis evaluation, 20 μg of lysate protein was applied to each lane. A rabbit PARP antibody (1:1000 dilution) (Cell Signaling Technology Inc., Danvers, MA) was used to detect full-length PARP (116 kDa) and cleaved PARP (carboxyl-terminal catalytic fragment, 89 kDa). For 14-3-3ε analysis, 20 μg of lysate protein was applied to each lane. 14-3-3ε was detected with a rabbit polyclonal antibody (1:2000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For PPARδ protein analysis, 30 μg of cell lysate proteins was loaded to each lane. PPARδ was detected with a rabbit polyclonal antibody against PPARδ (1:500 dilution) (Cayman Chemical). Donkey anti-rabbit IgG conjugated with horseradish peroxidase was purchased from Santa Cruz Biotechnology, Inc. Protein bands were visualized by enhanced chemiluminescence (Pierce Chemical, Rockford, IL).

Plasmid Constructs and Luciferase Reporter Assay. The human 14-3-3ε promoter (−1625 to +24) was subcloned into pGL3 luciferase reporter as described previously (Liou et al., 2006). PPRE reporter conjugated to luciferase was kindly provided by Drs. K. W. Kinzler and B. Vogelstein (Johns Hopkins University, Baltimore, MD; He et al., 1999). To achieve high transfection efficiency, the endothelial-like ECV304 cells were treated with a mixture containing reporter constructs and FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) for 24 h, washed, and replaced with fresh medium containing the testing drugs for an additional 24 h. Cells were lysed, and the luciferase activity was measured using a kit from Promega (Madison, WI). The emitted light was determined in a luminometer. Protein concentrations of cell lysates were determined by a protein assay kit (Bio-Rad, Hercules, CA). Luciferase activity was expressed as relative light units per microgram of protein.

Recombinant Adenoviral Vectors. Adenoviral vectors containing green fluorescent protein (GFP) coding sequence (Ad-GFP) were amplified in 293 cells as described previously (Shyue et al., 2001). Ad-PPARδ was kindly provided by Drs. K. W. Kinzler and B. Vogelstein (Johns Hopkins University). The amplified recombinant adenoviruses were purified by CsCl density-gradient centrifugation, and the viral titers were determined by a plaque assay as described previously (Shyue et al., 2001). Based on our previous experimental results (Shyue et al., 2001), we infected HUVECs with recombinant adenoviruses at 50 multiplicity of infection (or plaque-forming units/cell) for 48 h followed by treatment with sulindac for an additional 24 h.

Cytotoxicity Assay. Cell viability was assessed by using trypan blue dye and a hemocytometer. HUVECs were trypsinized, resuspended (1×10^5 cells/ml), and mixed with trypan blue (1:1 ratio with 0.4% stock; Invitrogen, Carlsbad, CA) for 5 min. The cell suspension was loaded into a hemocytometer, and nonviable (stained in blue color) and viable (opaque) cells were counted.

Caspase-3 Activity Assay. Caspase-3 activity was analyzed by caspase-3 colorimetric activity assay according to the manufacturer's protocol (Millipore Corporation, Billerica, MA). In brief, the activities of caspases were assessed by recognizing the sequence of DEVD. The chromophore *p*-nitroaniline (*p*NA) was quantified by using an enzyme-linked immunosorbent assay reader at 405 nm after cleavage from the labeled substrate DEVD-*p*NA. One unit of caspase-3 activity is defined as cleavage of 1 nmol of *p*NA per hour at 37°C.

Flow Cytometry. Apoptosis was analyzed by flow cytometry, which measures cells positively stained with annexin V and propidium iodide as described previously (Liou et al., 2007a). HUVECs incubated with sulindac sulfide for different time points were harvested by trypsin, centrifuged at 500*g* for 10 min, washed with phosphate-buffered saline, and incubated with fluorescein isothiocyanate-labeled annexin V antibody and propidium iodide (BD

Pharmingen, San Diego, CA) in the dark at room temperature for 30 min. The labeled cells were measured by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA) and analyzed by CellQuest software (BD Biosciences; http://www.bdbiosciences.com/). Percentages of cells with positive stain for annexin V were calculated.

Statistical Analysis. Analysis of variance software was used to determine statistical differences. A p value <0.05 is considered to be statistically significant.

Results

Induction of Endothelial Apoptosis by NSAIDs. Sulindac sulfide at a concentration that induces colon cancer cell apoptosis (160 μM) increased annexin V-positive HUVECs in a time-dependent manner (Fig. 1A). Annexin V-positive cells were significantly increased at 16 h and further increased at 24 h (Fig. 1A). The effect of sulindac and indomethacin on HUVEC apoptosis was supported by PARP cleavage. Cleaved PARP fragment was not detected in native HUVECs or HUVECs treated with vehicle alone (Fig. 1B). Sulindac at 160 μM and indomethacin at 800 μM induced a significant level of cleaved PARP (Fig. 1B). Aspirin at 1 mM did not induce PARP cleavage, but at 5 mM it induced a significant level of cleaved PARP (Fig. 1B). Like aspirin, sodium salicylate at 1 mM did not induce PARP cleavage. However, sodium salicylate at 5 mM also had no effect on PARP cleavage (Fig. 1B). Caspase-3 activity was highly elevated by sulindac (Fig. 1C). Indomethacin and aspirin at 5 mM significantly increased caspase-3 activity, whereas neither aspirin at 1 mM nor sodium salicylate at 1 or 5 mM had an effect (Fig. 1C).

Suppression of PPARδ and 14-3-3ε Expression by NSAIDs. PPARδ and 14-3-3ε play important roles in protecting cells from apoptosis. We determined whether the effect of NSAIDs and aspirin on HUVEC apoptosis is attributable to suppression of PPARδ and 14-3-3ε proteins. HUVECs were treated with sulindac sulfide, indomethacin, or salicylates for 24 h, and PPARδ or 14-3-3ε protein levels were analyzed by Western blotting. Sulindac and indomethacin suppressed PPARδ proteins (Fig. 2A). Aspirin and sodium salicylate at 1 mM had no effect. However, aspirin at 5 mM suppressed PPARδ, whereas sodium salicylate at 5 mM had no effect (Fig. 2A). Suppression of 14-3-3ε proteins by sulindac and indomethacin correlated with that of PPARδ inhibition (Fig. 2B). Likewise, the concentration-dependent effect of aspirin on PPARδ correlated with that on 14-3-3ε proteins (Fig. 2B). We next compared sulindac-induced PPAR cleavage with PPARδ and 14-3-3 suppression. Sulindac-induced PARP cleavage was correlated with decline of PPARδ and 14-3-3ε proteins in a time-dependent manner (Fig. 2C). Treatment of HUVECs with sulindac for 16 to 24 h resulted in marked PARP cleavage accompanied by almost complete elimination of PPARδ and 14-3-3ε proteins.

Sulindac Disrupted PPARδ-Mediated 14-3-3ε Transcription. The promoter region of 14-3-3ε harbors three contiguous PPREs that are responsive to PPARδ activation (Liou et al., 2006). Deletion of PPREs abrogated PPARδ-mediated 14-3-3ε up-regulation (Liou et al., 2006). To determine whether NSAIDs block this transcription pathway thereby suppressing 14-3-3ε, we evaluated the effects of sulindac on PPARδ transcriptional activity and 14-3-3ε promoter activity. To analyze PPARδ transactivation, we transfected endothelial cells with a PPRE-containing promoter construct conjugated to luciferase. Sulindac treatment resulted in a concentration-dependent re-

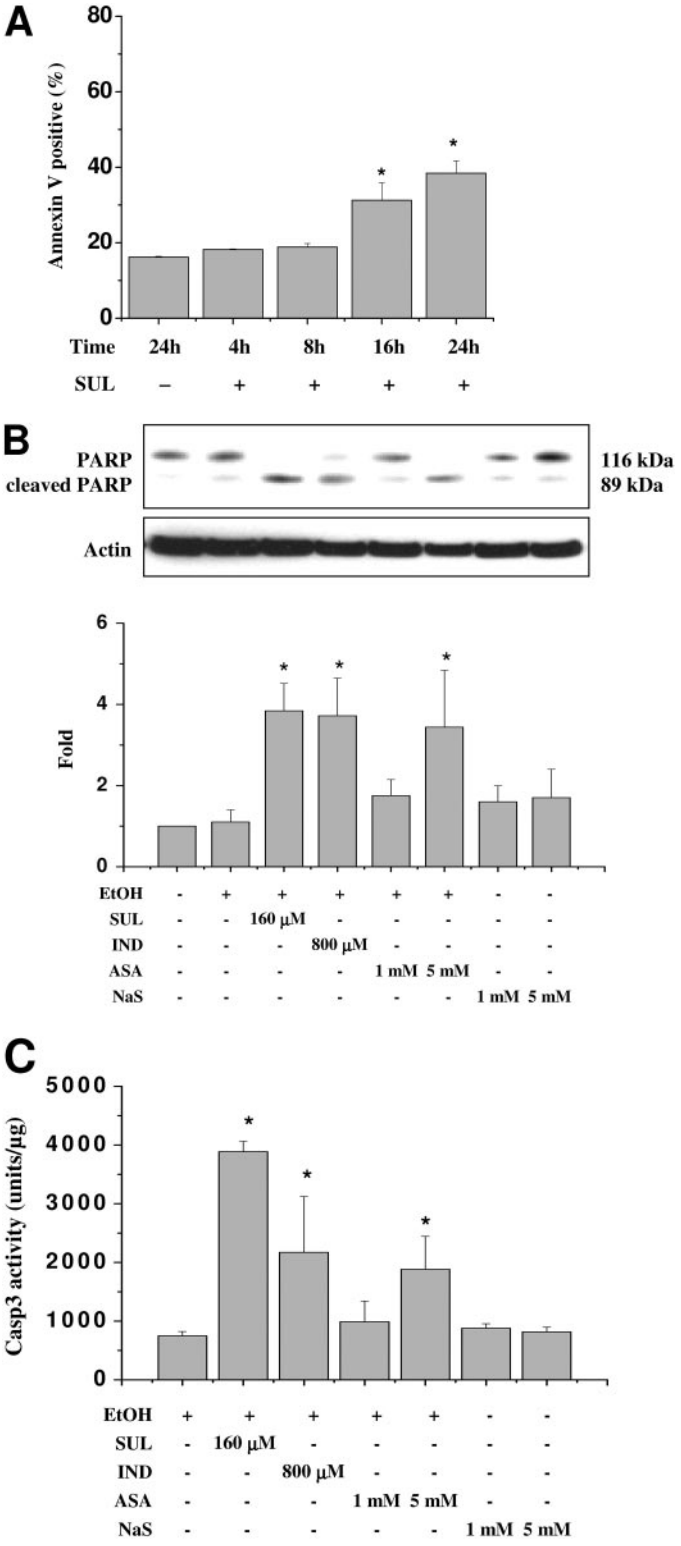

Fig. 1. NSAIDs induced HUVEC apoptosis. A, HUVECs were treated with sulindac (SUL; 160 μM) for 4 to 24 h and annexin V-positive cells were measured by flow cytometry. Each bar denotes mean ± S.D. (n = 3). *, $p < 0.05$ compared with control (SUL−; 24 h). B and C, HUVECs were treated with SUL, indomethacin (IND), aspirin (ASA), or sodium salicylate (NaS) for 24 h. PARP cleavage (B) and caspase-3 activity (C) were analyzed by Western blotting and activity assay, respectively. B, Top, representative blot. Bottom, densitometric analysis of the 89-kDa cleaved PARP (n = 3). Each bar denotes mean ± S.D. (n = 3). *, $p < 0.05$, compared with control.

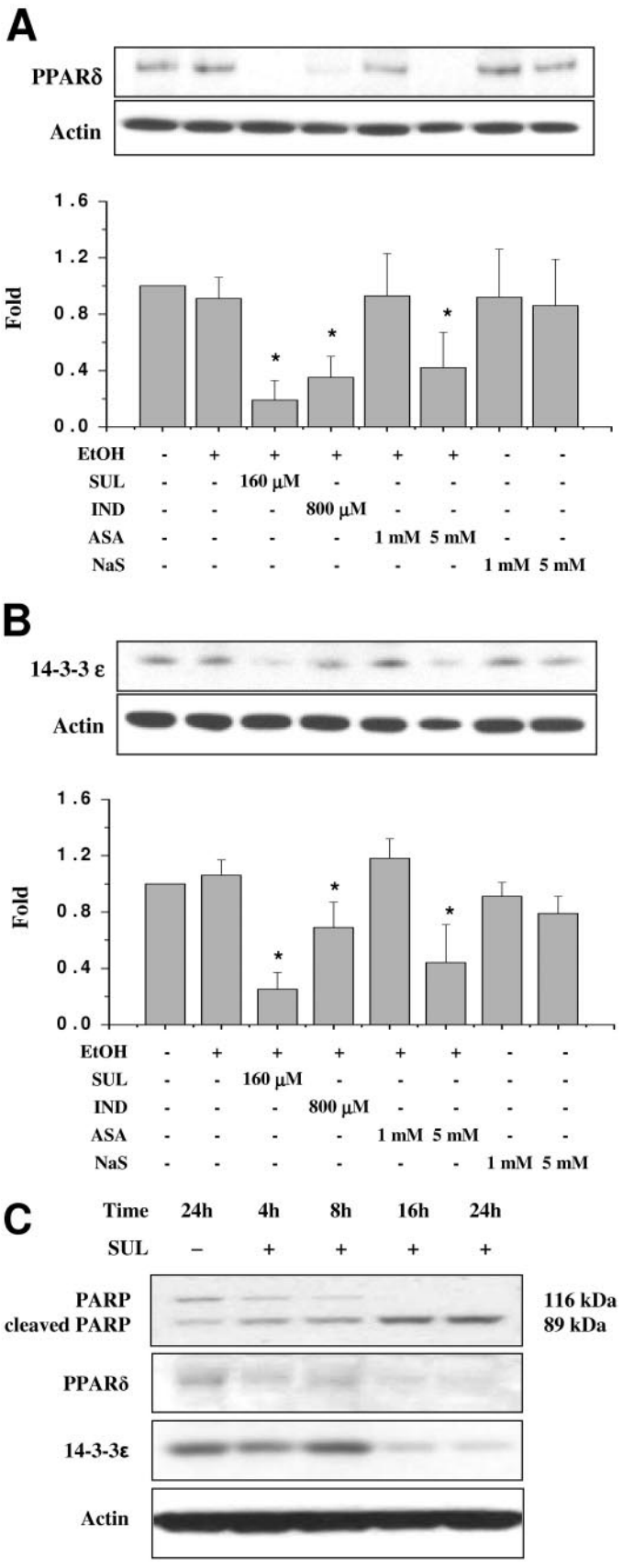

Fig. 2. NSAIDs suppressed PPARδ and 14-3-3ε. PPARδ (A) and 14-3-3ε (B) proteins in HUVECs treated with the indicated compounds for 24 h were analyzed by Western blots. Top, representative Western blots. Bottom, densitometric analysis ($n = 3$). Each bar denotes mean ± S.D. ($n = 3$). *, $p < 0.05$. C, HUVECs were treated with or without SUL (160 μM), and the indicated proteins were analyzed by Western blots at the indicated time points.

duction in luciferase expression (Fig. 3A), which is correlated with a concentration-dependent suppression of PPARδ proteins by sulindac (Fig. 3B). PPARδ mRNA measured with real-time quantitative polymerase chain reaction was reduced by sulindac in a time-dependent manner (Fig. 3C). By contrast, sodium salicylate did not have a significant effect on PPARδ mRNA. We next analyzed 14-3-3ε promoter activity by transfecting a human 14-3-3ε promoter construct (−1625 to +24) conjugated to luciferase. Sulindac caused a concentration-dependent suppression of luciferase activity (Fig. 4A). Sulindac-induced reduction of 14-3-3ε proteins correlated with that of 14-3-3ε promoter activity (Fig. 4B).

Sulindac Increased Bad in Mitochondria and Influenced Bcl-2 Levels. 14-3-3ε binds and sequesters Bad and thereby prevents Bad translocation to mitochondria. Because 14-3-3ε proteins were severely reduced in sulindac-treated cells, we determined whether Bad translocation to mitochondria is increased. We isolated mitochondrial fractions from HUVECs treated with sulindac or vehicle for 4 h and analyzed Bad and HSP60, which serves as a mitochondrial marker. Trace Bad was detected in the mitochondrial fraction of cells treated with vehicle, whereas a heavy band of Bad was detected in the mitochondrial fraction of cells treated with sulindac (80 μM) (Fig. 4C). Conversely, Bad in cytosol was reduced (Fig. 4C). We have shown previously by immunoprecipitation that 14-3-3ε binds Bad in cytosolic fraction and that Bad translocation to mitochondria is influenced by 14-3-3ε binding of Bad (Liou et al., 2006, 2007b). Together, these results support the notion that sulindac-induced 14-3-3ε suppression is associated with reduced Bad sequestration and increased Bad translocation to mitochondria.

We next evaluated the effect of NSAIDs on selective Bcl-2 family proteins (Bcl-2, Bad, and Bax) in the cell lysates of HUVECs treated with various NSAIDs for 24 h. Sulindac and indomethacin reduced Bcl-2 and Bad but not Bax, whereas aspirin at 5 mM but not at 1 mM reduced Bcl-2 and Bax, but not Bad. Sodium salicylate at 1 or 5 mM did not significantly alter the Bcl-2 protein levels (Fig. 5).

PPARδ Ligands Did Not Prevent Sulindac-Induced Cytotoxicity and PPAR Cleavage. It was reported previously that $cPGI_2$ and L-165041, a selective PPARδ ligand, activate PPARδ-mediated promoter activity and 14-3-3ε upregulation and protect endothelial cells from H_2O_2-induced apoptosis (Liou et al., 2006). In this study, we determined whether these two ligands could protect endothelial cells from sulindac-induced cell death. Neither $cPGI_2$ nor L-165041 was able to attenuate PPAR cleavage induced by sulindac (Fig. 6A) or to reduce sulindac-induced cytotoxicity (Fig. 6B). These results are consistent with the interpretation that PPARδ ligands, which protect cells from cytotoxicity and apoptosis by ligating PPARδ, lose the protective action when PPARδ is suppressed by sulindac.

Adenoviral PPARδ Transduction Failed to Rescue Sulindac-Induced 14-3-3ε Suppression and PARP Cleavage. We next determined whether Ad-PPARδ transduction was capable of rescuing 14-3-3ε proteins and apoptosis from sulindac. PPARδ proteins that were highly elevated by Ad-PPARδ transduction for 48 h were suppressed by pretreatment of cells

with sulindac in a concentration-dependent manner (Fig. 7A). 14-3-3ε proteins were also elevated by Ad-PPARδ and were concentration-dependently suppressed by sulindac (Fig. 7A). Sulindac at 160 μM eliminated PPARδ and 14-3-3ε proteins (Fig. 7A).

PPARδ proteins in Ad-PPARδ-transduced cells were increased by MG-132, a proteasome inhibitor (Fig. 7B). Sulindac at 80 μM attenuated the increase by MG-132 and at 160 μM abrogated the increase (Fig. 7B). Likewise, DEV-CHO, a caspase inhibitor, increased PPARδ proteins in Ad-PPARδ-transduced cells, which were attenuated by sulindac at 80 μM and abolished by sulindac at 160 μM (Fig. 7C). These results suggest that PPARδ overexpression via adenoviral gene transfer is controlled by protein degradation via proteasome and caspase. Sulindac suppresses adenoviral-transduced PPARδ in a manner independent of proteasome and caspases.

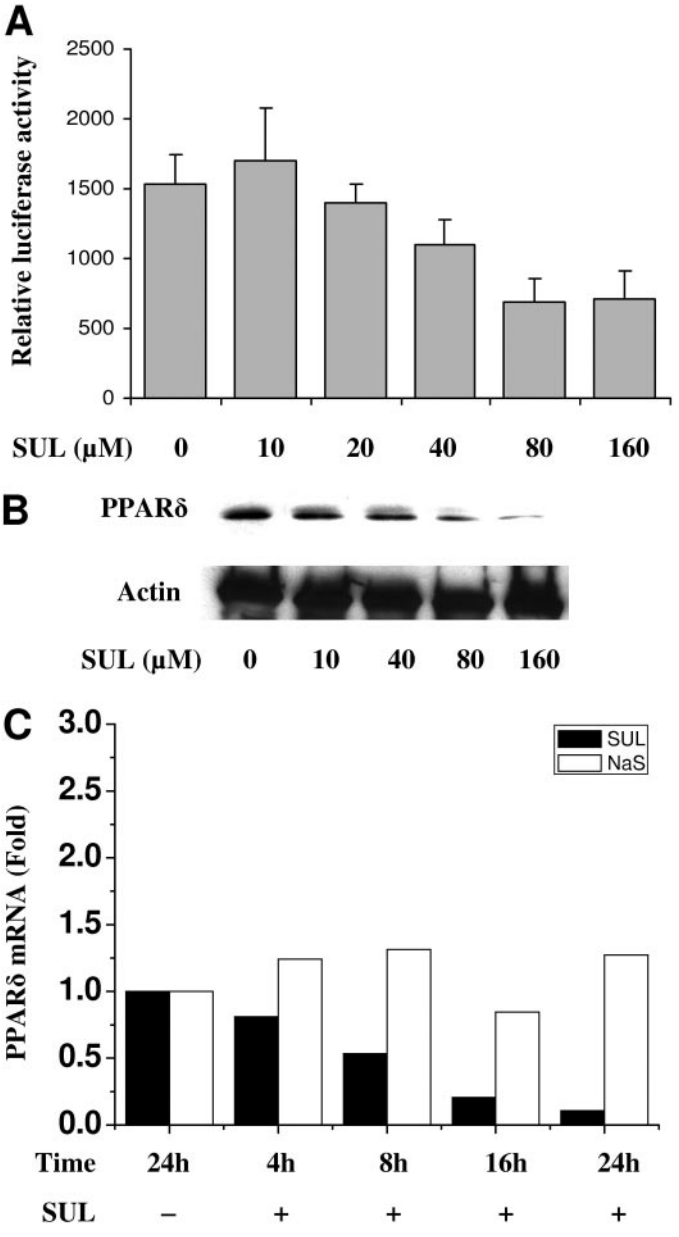

Fig. 3. Sulindac inhibited PPRE promoter in a concentration-dependent manner. A, ECV304 cells were transfected with PPRE reporter for 24 h and treated with different concentrations of SUL for an additional 24 h. Luciferase activities were expressed as relative light units (RLU)/μg protein. Each bar represents mean ± S.D. of three independent experiments. B, HUVECs were treated with sulindac at increasing concentrations for 24 h, and PPARδ was analyzed by Western blotting. A representative blot from three experiments is shown. C, HUVECs were treated with SUL (160 μM) or NaS (5 mM), and PPARδ mRNA was measured by real-time quantitative polymerase chain reaction. Each bar represents mean of two experiments.

Discussion

A major finding of this study is that sulindac and indomethacin induce endothelial cell apoptosis with a correlative suppression of PPARδ and 14-3-3ε expressions. Results from time course experiments reveal a significant suppression of PPARδ and 14-3-3ε proteins accompanied by a significant increase in annexin V-positive cells after HUVECs had been treated with sulindac sulfide for 16 and 24 h. Furthermore, suppression of PPARδ is correlated with that of 14-3-3ε in a concentration-dependent manner. Both PPARδ and 14-3-3ε proteins were partially suppressed by 80 μM and almost eliminated by 160 μM sulindac. Sulindac at 80 μM partially cleaved PARP and at 160 μM almost completely cleaved PARP. PARP cleavage by sulindac and indomethacin is correlated with

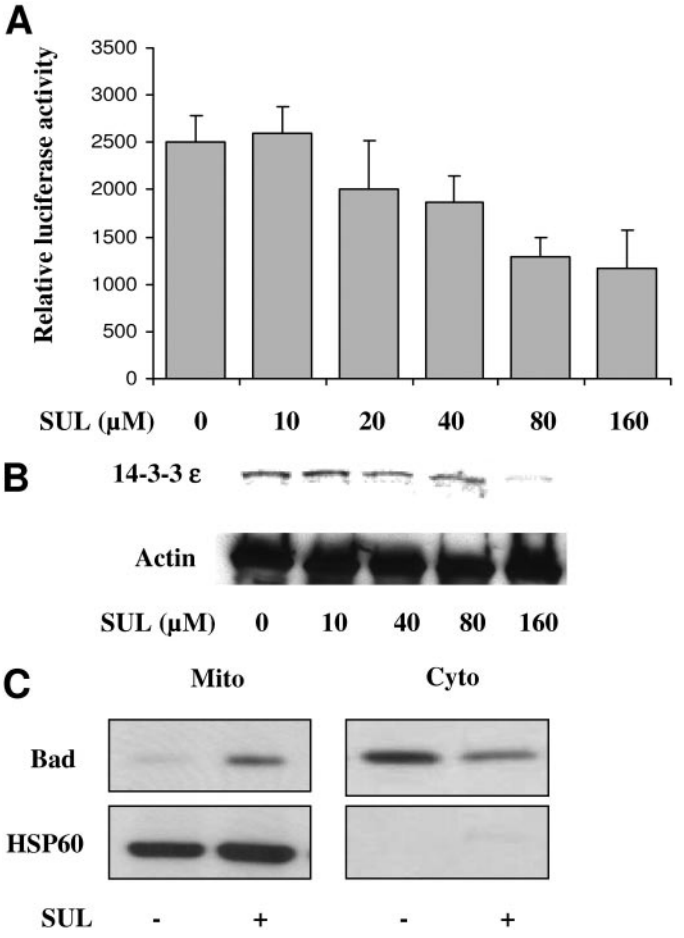

Fig. 4. Sulindac inhibited 14-3-3ε promoter activity and protein expression and increased Bad translocation to mitochondria. A, 14-3-3ε promoter activity expressed in ECV304 cells treated with sulindac. Each bar denotes mean ± S.D. of three experiments. B, a representative Western blot of 14-3-3ε proteins in HUVECs treated with sulindac under identical experimental conditions as described in A. C, HUVECs were treated with sulindac (80 μM) for 4 h. Mitochondrial and cytosolic fractions were prepared. Bad proteins and HSP60 were analyzed.

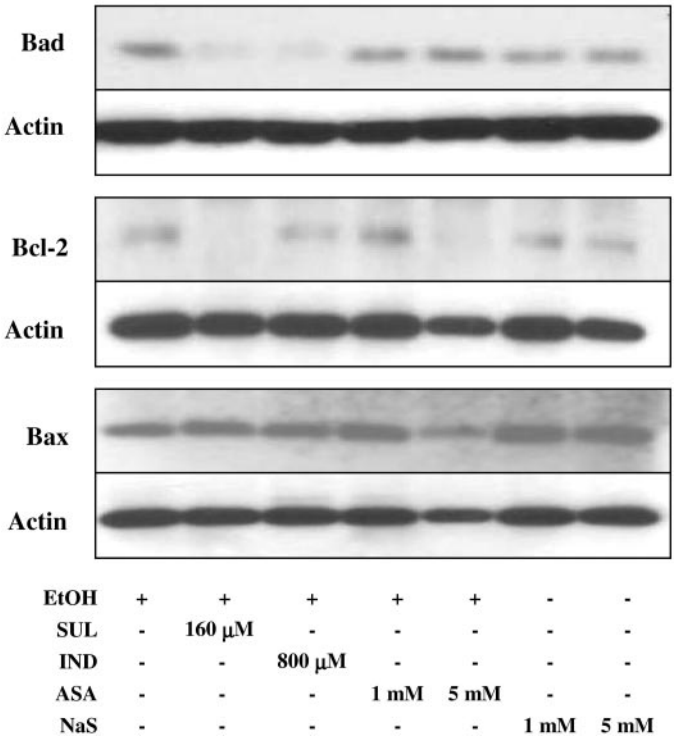

Fig. 5. Effect of NSAIDs on Bcl-2 proteins. HUVECs were treated with various NSAIDs for 24 h, and the Bcl-2 proteins (Bad, Bcl-2, and Bax) in cell lysates were analyzed by Western blots. Actin was concurrently analyzed as reference.

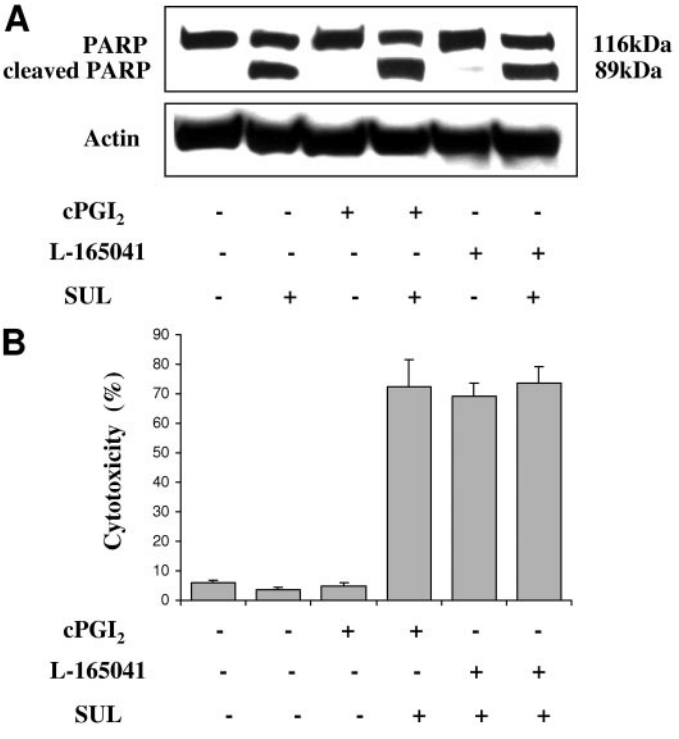

Fig. 6. PPARδ ligands failed to reverse NSAIDs-induced HUVEC death. HUVECs were treated with $cPGI_2$ (50 μM) or L-165041 (50 μM) for 4 h before addition of sulindac sulfide. A, PARP cleavage was analyzed by Western blotting. B, cytotoxicity was determined by trypan blue staining. Each bar denotes mean ± S.D. of three experiments.

caspase-3 activation. These results indicate a close relationship between PPARδ suppression, 14-3-3ε down-regulation, and apoptotic changes, including caspase-3 activation, PARP cleavage, and annexin V expression. It has been reported previously that ligand-activated PPARδ mediates 14-3-3ε expression at the transcriptional level (Liou et al., 2006). Upon activation by PGI_2 analogs or selective PPARδ agonists such as L-165041, PPARδ binds to PPREs located at −1348 to −1625 of human 14-3-3ε gene and promotes the transcriptional activation, thereby increasing 14-3-3ε proteins in endothelial cells (Liou et al., 2006). 14-3-3ε binds phosphorylated Bad, sequesters Bad in the cytoplasm, and thereby reduces Bad translocation to mitochondria to induce apoptosis (Fu et al., 2000; Tzivion Avruch, 2002). In this study, the results show that suppression of 14-3-3ε by sulindac was associated with increased Bad transloca-

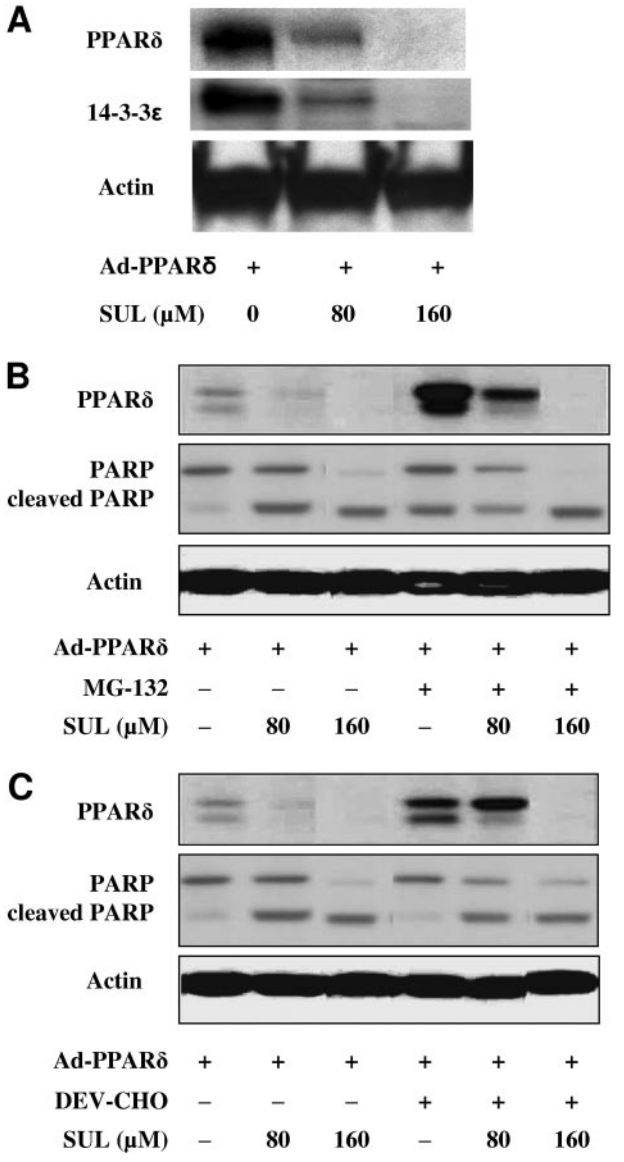

Fig. 7. Effects of Ad-PPARδ transduction on PPARδ, 14-3-3ε, and PARP cleavage. A, HUVECs transduced with Ad-PPARδ (50 multiplicity of infection) for 48 h were treated with SUL for 24 h. PPARδ and 14-3-3ε protein levels were analyzed by Western blots. B and C, Ad-PPARδ-transduced cells were treated with SUL in the presence or absence of MG-132 (10 μM) (B) or DEV-CHO (50 μM) (C) for 24 h, and PPARδ and PARP were analyzed by Western blotting.

tion to mitochondria consistent with the notion that cellular 14-3-3ε quantities play an important role in controlling apoptosis via the mitochondrial pathway. Together, findings of this study suggest that NSAIDs such as sulindac sulfide and indomethacin induce endothelial apoptosis by suppressing PPARδ and thereby attenuating PPARδ-mediated expression of 14-3-3ε and increasing Bad-induced apoptosis.

It is unclear how NSAIDs suppress PPARδ expression. It is suggested that sulindac may disrupt the β-catenin signaling pathway and inhibit binding of β-catenon/T-cell factor to the promoter/enhancer region of PPARδ in colon cancer cells (Gardner et al., 2004). Results from our laboratory confirm that sulindac, indomethacin, and selective COX-2 inhibitors suppress PPARδ proteins and PPARδ transactivation activities in colon cancer cells (Liou et al., 2007b). We have extended the study to show that by suppressing PPARδ, NSAIDs, and COX-2 inhibitors inhibit PPARδ-mediated 14-3-3ε expression in colon cancer cells, which leads to increased Bad translocation to mitochondria and colon cancer cell apoptosis (Liou et al., 2007b). It is possible that sulindac and indomethacin may suppress PPARδ expression in endothelial cells by a similar mechanism. NSAIDs may interfere with endothelial Wnt signaling to liberate β-catenin from the glycogen synthase kinase-3β, adenomatous polyposis coli, and axin complex or directly interfere with the transactivation activity of β-catenin/T-cell factor as have been reported in cancer cells (Hawcroft et al., 2002; Dihlmann et al., 2003; Lu et al., 2005).

PPARδ overexpression by Ad-PPARδ was accompanied by elevation of 14-3-3ε protein expression and reduction of PARP cleavage. However, PPARδ overexpression was unable to rescue 14-3-3ε protein suppression and PARP cleavage from sulindac insults, probably due to suppression of Ad-PPARδ-mediated PPARδ protein expression by sulindac. The reason for suppressing Ad-cytomegalovirus promoter-driven PPARδ by sulindac is unclear. It is unlikely to be that induction of PPARδ protein degradation via proteasome or caspases, because neither proteasome nor caspase inhibitors block the suppressing effect of sulindac. Because sulindac at 160 μM abolished PPARδ proteins regardless of whether proteasome or caspase inhibitors were present, it may be assumed that sulindac inhibits cytomegalovirus-driven PPARδ transcription. PPARδ proteins are expressed at very low levels in native untransduced HUVECs, which were not enhanced by MG-132 or DEV-CHO (data not shown), suggesting that low PPARδ abundance is largely attributed to a low level of basal transcription in HUVECs. However, when PPARδ is overexpressed, it is degraded via proteasome, suggesting that PPARδ is controlled by ubiquitin-proteasome degradation pathway in a concentration-related manner. Our results also reveal degradation of PPARδ by caspases in Ad-PPARδ-transduced cells, whereas caspase inhibitor DEV-CHO had minimal effect on native PPARδ proteins. It is possible that adenoviral transduction induces caspase activation, which targets overexpressed PPARδ for degradation.

Bcl-2 family proteins include antiapoptotic members such as Bcl-2 and proapoptotic members such as Bad and Bax (Green and Reed, 1998; Gross et al., 1999). The balance between antiapoptotic and proapoptotic Bcl-2 members is crucial in controlling apoptosis via the mitochondrial pathway (Decaudin et al., 1997). Because Bad is sequestered by 14-3-3ε and its translocation to mitochondria is regulated by 14-3-3ε levels, we measured Bad in mitochondrial and cytosolic fractions as well as in cell lysates. Sulindac increases Bad in the mitochondrial fraction and reciprocally reduces its level in cytosol, supporting the notion that decline in 14-3-3ε proteins results in reduced Bad sequestration and increased Bad translocation to mitochondria. Sulindac also reduces Bcl-2 but has no effect on Bax levels. Our results suggest that sulindac induces apoptosis via the mitochondrial pathway by reducing the antiapoptotic Bcl-2 and inducing translocation of Bad to mitochondria, tilting the balance toward proapoptosis.

It was reported that aspirin (> 5 mM) induces leukemia cell apoptosis (Bellosillo et al., 1998). Aspirin at 10 mM was reported to induce cytochrome *c* release and trigger caspase activation (Pique et al., 2000). Our results shed light on the potential underlying mechanism. The results indicate that aspirin at 5 mM induces apoptosis by suppressing PPARδ-mediated 14-3-3ε expression, which leads to increased Bad at the mitochondria. The results further show that aspirin at 5 mM inhibits Bcl-2 expression, leading to unopposed injury to mitochondria by Bad. The underlying mechanism of aspirin-induced apoptosis closely resembles that of sulindac. It is noteworthy that aspirin at 1 mM does not cause endothelial cell apoptosis. Because the present-day aspirin therapeutic concentrations are <1 mM, aspirin use in cardiovascular and stroke prevention would not be expected to induce endothelial apoptosis. Sodium salicylate at concentrations >5 mM induces leukemia cell apoptosis in a manner similar to aspirin (Bellosillo et al., 1998). It was reported that sodium salicylate at 1 mM had no effect on apoptosis but potentiates apoptosis and cytotoxicity mediated by mitochondrial permeability transition (Oh et al. 203). In several leukemia cell lines, sodium salicylate at 5 mM and higher was reported to induce caspase-3 activation and PARP cleavage (Klampfer et al., 1999). Our results did not reveal caspase-3 activation, or PARP cleavage in endothelial cells treated with 1 or 5 mM sodium salicylate. These findings suggest that susceptibility to salicylate-induced apoptosis is cell type-specific. Endothelial cells may be more resistant to salicylate than leukemia cells.

Besides a significant difference in inducing endothelial cell apoptosis between sodium salicylate and aspirin at 5 mM, sodium salicylate differs from aspirin in lacking an effect on Bcl-2 and Bax levels. The reason for the differences is unclear. It may be speculated that these differences could be attributed to the acetylation property of aspirin, which may modify proteins in the signaling and transcriptional pathways.

Based on extensive characterization of eicosanoid binding to PPARδ, it has been suggested that PGI_2 may be an active endogenous ligand of PPARδ (Forman et al., 1997). PGI_2 is a major product of endothelial cells. It is synthesized from arachidonic acid via COX enzymes, which catalyze the formation of prostaglandin endoperoxides, PGG_2 and PGH_2, and PGH_2 is in turn converted to PGI_2 by a specific isomerase, PGI synthase. There is increasing evidence that COX-2-derived PGI_2 plays an important role in protecting vascular integrity and function (Fitzgerald, 2004). Both sulindac and indomethacin are nonselective COX inhibitors. They are capable of inhibiting COX-2-derived PGI_2. Furthermore, despite its selective inhibition of COX-1, aspirin at high concentrations is also capable of inhibiting COX-2-derived PGI_2 formation. Another COX-derived prostaglandin, PGE_2, was reported to stimulate β-catenin (Castellone et al., 2005) and

therefore may be involved in PPARδ transcriptional activation. Together, the results imply that the potential mechanism by which NSAIDs suppress 14-3-3ε proteins and induce apoptosis is mediated through inhibition of COX-derived PGI_2 and PGE_2, thereby suppressing PPARδ activities.

Our findings have important clinical implications. The finding that NSAIDs induce endothelial cell apoptosis may explain the association of chronic use of NSAIDs with an increased risk of myocardial infarction. Vascular endothelial cells play a critical role in protecting arterial damage by producing active molecules that protect against arterial damage, inhibit platelet aggregation, and control arterial constriction. Loss of the endothelial barrier and its ability to produce the protective molecules as a result of apoptosis caused by NSAID use may lead to vascular damage, atherosclerosis, and thrombosis. Another important implication is that aspirin at low doses such as the commonly used doses of 81 to 325 mg, which yield a blood concentration of aspirin or salicylate below 1 mM, is devoid of the proapoptotic action. At those doses, aspirin is efficacious in preventing recurrence of myocardial infarction and ischemic stroke primarily by inhibiting COX-1 derived thromboxane A_2. Because a large population is now routinely taking a low dose of aspirin daily, it is reassuring to learn that at low aspirin and salicylate concentrations, they do not induce endothelial apoptosis. Aspirin at 5 mM induces apoptosis and reductions in PPARδ and 14-3-3ε proteins, whereas sodium salicylate at 5 mM does not. The reason for this disparity is unclear. Because aspirin is capable of acetylating macromolecules, it is possible that aspirin at high concentrations may exert its actions by acetylating a target gene or a signaling molecule that is involved in PPARδ and 14-3-3ε transcriptional pathway.

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