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Nonsteroidal Anti-inflammatory Drugs Induced Endothelial Apoptosis by Perturbing PPAR-δ Transcriptional Pathway

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Running title: NSAIDs induce endothelial apoptosis

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ABSTRACT

Recent studies have shown that use of NSAIDs is associated with an increased risk of myocardial infarction. To explore if 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 analyzed apoptosis. SUL and/or IND significantly increased annexin V positive cells, cleaved 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. As peroxisome proliferator-activated receptor δ (PPARδ)-mediated 14-3-3ε upregulation was reported to play a crucial role in protecting against apoptosis, we determined whether NSAIDs suppress this transcriptional pathway. SUL, IND and ASA (5mM) 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 PGI_2 nor L-165041 prevented HUVEC from SUL-induced apoptosis. Adenoviral PPARδ transduction failed to restore 14-3-3ε or prevent PPAR cleavage due to suppression of ectopic PPARδ by sulindac. Our findings suggest that NSAIDs but not aspirin (<1 mM) induce endothelial apoptosis via suppression of PPARδ-mediated 14-3-3ε expression.

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) comprise a group of compounds of diverse chemical structures distinct from corticosteroids but 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 (Jacoby et al., 1996; Boolbol et al., 1996). Epidemiological and controlled human clinical trials have confirmed that NSAID use reduce the risk of cancers and reduce adenomas in familial adenomatous polyposis (FAP) (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 anti-apoptotic and pro-apoptotic Bcl-2 family proteins towards the pro-apoptotic members notably Bax and Bad (Zhang et al., 2000; Sheng et al., 1998). Results from our laboratory suggest that the anti-tumor actions of sulindac and indomethacin are attributable to suppression of peroxisome proliferator-activated receptor-δ (PPARδ) and PPARδ-mediated 14-3-3ε expression, resulting in reduction of cytosolic 14-3-3ε proteins and consequently increase in Bad translocation to mitochondria where it induces apoptosis (Liou et al., 2007b).

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Aspirin has been reported to possess similar anti-tumor 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 FAP 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 (Pique et al., 2000; Bellosillo et al., 1998; Klampfer et al., 1999). However, it remains unknown if aspirin at therapeutic concentrations (≦ 1mM) causes cell damage. Furthermore, mechanism by which high, supra-pharmacological concentrations of aspirin and salicylate (> 5 mM) induce apoptosis has not been elucidated.

It is well recognized that selective cyclooxygenase-2 (COX-2) inhibitors are associated with increased rick of myocardial infarction (MI) (Fitzgerald, 2004; Mukherjee et al., 2001). Recent reports indicate that all classes of NSAIDs are also associated with risk of MI (Chan et al., 2006). The reasons for the adverse cardiovascular complications of NSAIDs are not entirely clear. As 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 poly(ADP-ribose)polymerase (PARP) cleavage and/or other apoptotic markers in human

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umbilical vein endothelial cells (HUVECs) and 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 fashion. Aspirin and sodium salicylate at 1 mM had no effect but aspirin at 5 mM exerted a similar apoptotic action as NSAIDs.

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Materials and Methods

Pharmacological reagents. Sulindac sulfide and indomethacin were purchased from Calbiochem (San Diego, CA), and aspirin and sodium salicylate were from Sigma (St. Louis, MO). All 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 previously described previously (Xu et al., 1999). In all experiments, only cultures up to 5 passages were used prior to experiments. HUVECs were washed and cultured in serum-free medium containing the testing drugs for 24 h. As it was difficult to transfect HUVECs with conventional liposome-based carriers, we employed ECV304 cells for selected transfection experiments. ECV 304 cells are immortalized cells derived from HUVEC. They retain cobble stone-like morphology and stain positively for von willebrand factor. Like HUVEC, they possess enzymes for prostacyclin synthesis. ECV304 cells were maintained in DMEM containing 10% FBS.

Preparation of mitochondrial fraction. HUVECs treated with sulindac sulfide for 4h were washed thrice with PBS and harvested by centrifugation. Mitochondrial fractions were prepared by a mitochondria isolation kit (Sigma, St. Louis, MO) as described previously

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(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. Heat shock protein 60 (HSP 60) was used as a mitochondrial marker.

Western Blot Analysis. HUVECs were lysed with RIPA buffer containing protease inhibitors. Western blots were performed as previously described (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 Signal Technology, Danvers, MA) was used to detect full length PARP (116 kDa) and cleaved PARP (carboxy-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, 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. Protein bands were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

Plasmid Constructs and Luciferase Reporter Assay. The human 14-3-3ε promoter (–1625 to +24) was subcloned into pGL3 luciferase reporter as previously described (Liou et al., 2006). PPRE reporter conjugated to luciferase was kindly provided by Drs. Kinzler and

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Vogelstein at Johns Hopkins University (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, 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/μg protein.

Recombinant Adenoviral Vectors. Adenoviral vectors containing GFP coding sequence (Ad-GFP) were amplified in 293 cells as described previously (Shyue et al., 2001). Ad-PPARδ was kindly provided by Drs Kinzler and Vogelstein at 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 MOI (multiplicity of infection or pfu/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 ($1x10^5$ cells/ml), and mixed with trypan blue (1:1 ratio with 0.4 % stock, Invitrogen) for 5 min. The cells were then filled to a

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hemocytometer and non-viable (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 protocols (Millipore, 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 ELISA reader at 405nm after cleavage from the labeled substrate DEVD-*p*NA. One unit of caspase-3 activity is defined as cleavage of one 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 previous described (Liou et al 2007a). HUVECs incubated with sulindac sulfide for different time points were harvested by trypsin, centrifuged at 500 g for 10 minutes, washed with phosphate-buffered saline (PBS), and incubated with fluorescein isothiocyanate-labeled annexin V antibody and propidium iodide (PI) (BD Pharmingen^{TM}) in the dark at room temperature for 30 minutes. The labeled cells were measured by flow cytometry (BD FACSCalibur^{TM}) and analyzed by CellQuest^{TM} software (BD bioscience, http://www.bdbiosciences.com/). Percentages of cells with positive stain for annexin V were calculated.

Statistical Analysis. ANOVA software was used to determine statistical differences. A P value <0.05 is considered to be statistically significant.

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Results

Induction of endothelial apoptosis by NSAIDs. Sulinsac 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 HUVEC or HUVEC 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 1mM 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 if 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

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indomethacin suppressed PPARδ proteins (Fig. 2A). Aspirin and sodium salicylate at 1mM had no effect. However, aspirin at 5 mM suppressed PPARδ while 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). Similarly, 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 – 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 which are responsive to PPARδ activation (Liou et al 2006). Deletion of PPREs abrogated PPARδ-mediated 14-3-3ε upregulation (Liou et al 2006). To determine if 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 reduction in luciferase expression (Fig. 3A), which is correlated with a concentration-dependent suppression of PPARδ proteins by sulindac (Fig. 3B).

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PPARδ mRNA measured with real time qPCR 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. As 14-3-3ε proteins were severely reduced in sulindac-treated cells, we determined if 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 previously shown by immunoprecipitation that 14-3-3ε binds Bad in cytosolic fraction and Bad translocation to mitochondria is influenced by 14-3-3ε binding of Bad (Liou et al 2006; Liou et al 2007b). Taken 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.

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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 & Bad but not Bax while 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 previously reported 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 if 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 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 adenoviral PPARδ (Ad-PPARδ) transduction was capable of resucing 14-3-3ε proteins and apoptosis from sulindac. PPARδ proteins which were highly elevated by Ad- PPARδ transduction for 48 h were suppressed by pretreatment of cells with sulindac in a concentration-dependent manner (Fig.

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7A). 14-3-3ε proteins were also elevated by Ad-PPARδ and were concentration-dependently suppressed by sulindac (Fig. 7A). Sulindac at 160 μM completely 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 entirely (Fig. 7B). Similarly, DEV-CHO, a caspase inhibitor, increased PPARδ proteins in Ad-PPARδ trensduced 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 capase. Sulindac suppresses adenoviral transduced PPARδ in a manner independent of proteasome and caspases.

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Discussion

A major finding of this study is that sulindac and indomethacin induce endothelial cell apoptosis with a correlative suppression of PPAR-δ and 14-3-3ε expressions. Results from time-course experiments reveal a significant suppression of PPAR-δ 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 h and 24 h. Furthermore, suppression of PPAR-δ is correlated with that of 14-3-3ε in a concentration-dependent manner. Both PPAR-δ and 14-3-3ε proteins were partially suppressed by 80 μM and almost completely eliminated by 160 μM of 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 caspase 3 activation. These results indicate a close relationship between PPAR-δ suppression, 14-3-3ε downregulation and apoptotic changes including caspase 3 activation, PARP cleavage and annexin V expression. It has been reported previously that ligand-activated PPAR-δ mediates 14-3-3ε expression at the transcriptional level (Liou et al 2006). Upon activation by PGI_2 analogs or selective PPAR-δ agonists such as L-165041, PPAR-δ 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 et al 2002). In this

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study, the results show that suppression of 14-3-3ε by sulindac was associated with increased Bad translocation to mitochondria consistent with the notion that cellular 14-3-3ε quantities play an important role in controlling apoptosis via the mitochondrial pathway. Taken together, findings of this study suggest that NSAIDs such as sulindac sulfide and indomethacin induce endothelial apoptosis by suppressing PPAR-δ and thereby attenuating PPAR-δ-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 (Tcf) 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 (COXIBs) 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 COXIBs 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 (GSK-3β), APC and axin complex or directly interfere with the transactivation activity of β-catenin/Tcf as have been reported in cancer cells (Hawcroft et al., 2002; Lu et al., 2005;

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Dihlmann et al., 2003).

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-CMV promoter driven PPARδ by sulindac is unclear. It is unlikely to be because of induction of PPARδ protein degradation via proteasome or capases, as neither proteasome nor caspase inhibitors block the suppressing effect of sulindac. Since sulindac at 160 μM completely abolished PPARδ proteins regardless whether proteasome or caspase inhibitors were present, it may be assumed that sulindac inhibits CMV-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 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 while 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.

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Bcl-2 family proteins comprise antiapoptotic members such as Bcl-2 and proapoptotic members such as Bad and Bax (Gross et al 1999; Green and Reed 1998). The balance between antiapoptotic and proapoptotic Bcl-2 members is crucial in controlling apoptosis via the mitochondrial pathway (Decaudin et al 1997). Since 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 result 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 pro-apoptosis.

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 lights 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

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resembles that of sulindac. Importantly, aspirin at 1 mM does not cause endothelial cell apoptosis. As 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 1mM 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 mM or 5 mM of 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).

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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 non-selective 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. Taken 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 due to NSAID use may lead to vascular damage, atherosclerosis and thrombosis. Another important implication

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is that aspirin at low doses such as the commonly used doses of 81-325 mg which yield a blood concentration of aspirin or salicylate below 1 mM is devoid of the pro-apoptotic 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. As 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 induce apoptosis and reductions in PPARδ and 14-3-3ε proteins while sodium salicylate at 5 mM do not. The reason for this disparity is unclear. As 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.

Acknowledgment

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Footnotes

Jun-Yang Liou and Chia-Ching Wu contributed equally to this work.

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Figure Legends

Fig. 1. NSAIDs induced HUVEC apoptosis. **A)** HUVECs were treated with sulindac (SUL, 160 μM) for 4-24 h and annexin V positive cells were measured by flow cytometry. Each bar denotes mean ± SD. (n=3). * $p < 0.05$ compared to control (SUL-, 24 h). **B) & C)** HUVECs were treated with sulindac (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)** The upper panel shows a representative blot and the lower panel, the densitometric analysis of the 89 kDa cleaved PARP (n=3). Each bar denotes mean ± SD (n=3). * denotes $p<0.05$, compared to control.

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

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 sulindac sulfide (SUL) for an additional 24 h. Luciferase activities were expressed as relative light unit (RLU)/μg protein. Each bar represents mean ± S.D. of 3 independent

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experiments. **B)** HUVECs were treated with sulindac at increasing concentrations for 24 h and PPARδ was analyzed by Western blotting. A representative blot from 3 experiments is shown. **C)** HUVECs were treated with SUL (160 μM) or NaS (5 mM) and PPARδ mRNA was measured by real time qPCR. Each bar represents mean of two experiments.

Fig. 4. Sulindac inhibited 14-3-3ε promoter activity and proteins expression, and increased Bad translocation to mitochondria. **A)** 14-3-3ε promoter activity expressed in ECV treated with sulindac. Each bar denotes mean ± S.D. of 3 experiments. **B)** A representative Western blot of 14-3-3ε proteins in HUVECs treated with sulindac under identical experimental condition as 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 & 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 ± SD of three experiments.

Fig. 7. Effects of Ad-PPARδ transduction on PPARδ, 14-3-3ε and PARP cleavage. **A)** HUVECs transduced with Ad-PPARδ (50 moi) for 48 h were treated with sulindac sulfide

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(SUL) for 24 h. PPARδ and 14-3-3ε protein levels were analyzed by Western blots. **B) & C)** Ad-PPARδ transduced cells were treated with SUL in the presence or absence of **B)** MG-132 (10 μM) or **C)** DEV-CHO (50 μM) for 24 h and PPARδ and PARP were analyzed by Western blotting.

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Figure 1

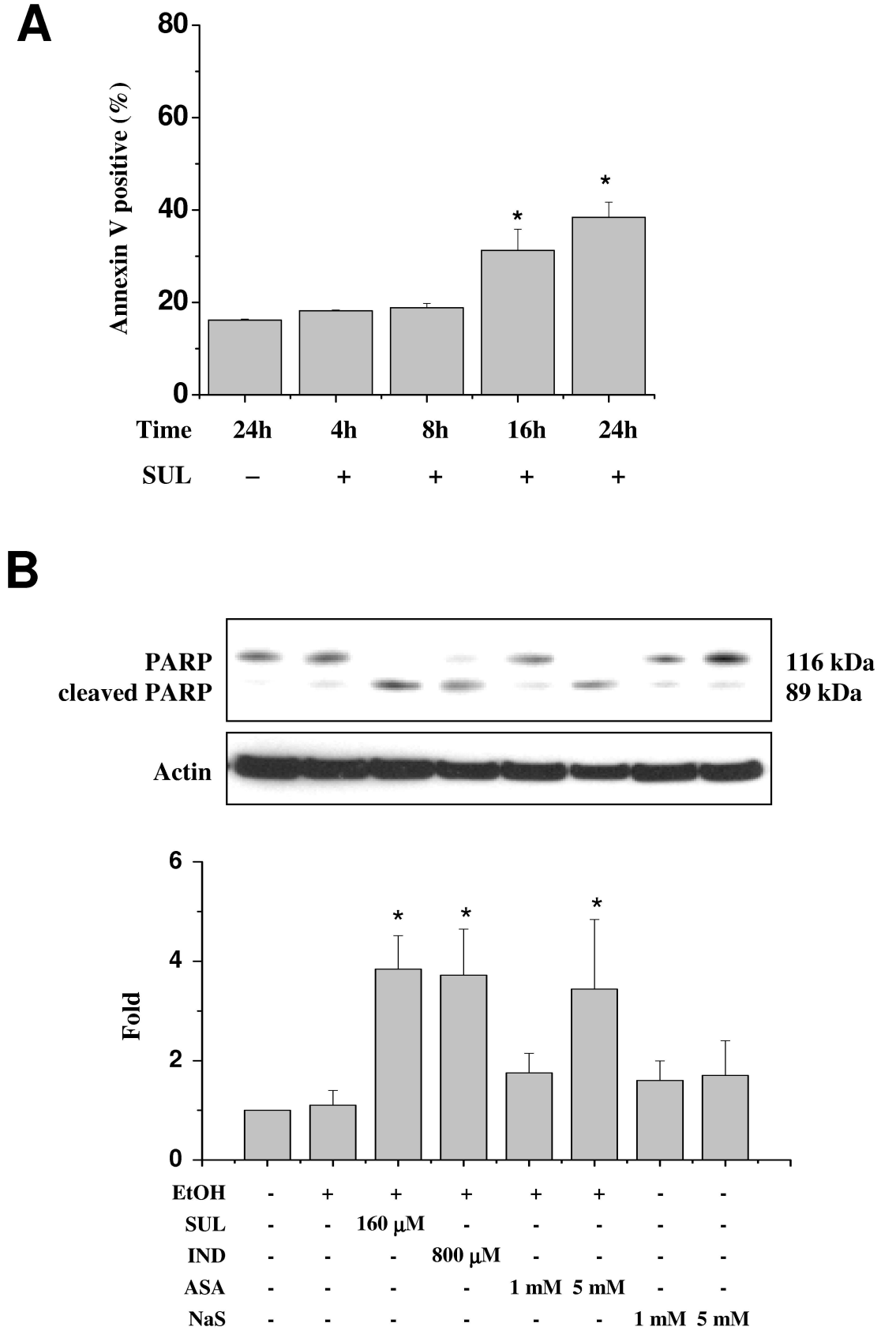

A
Annexin V positive (%)
80
60
40
20
0
*
*
Time 24h 4h 8h 16h 24h
SUL – + + + +
B
PARP
cleaved PARP
116 kDa
89 kDa
Actin
Fold
6
4
2
0
*
*
*
EtOH - + + + + + - -
SUL - - 160 μM - - - - -
IND - - - 800 μM - - - -
ASA - - - - 1 mM 5 mM - -
NaS - - - - - - 1 mM 5 mM

Figure 1

C

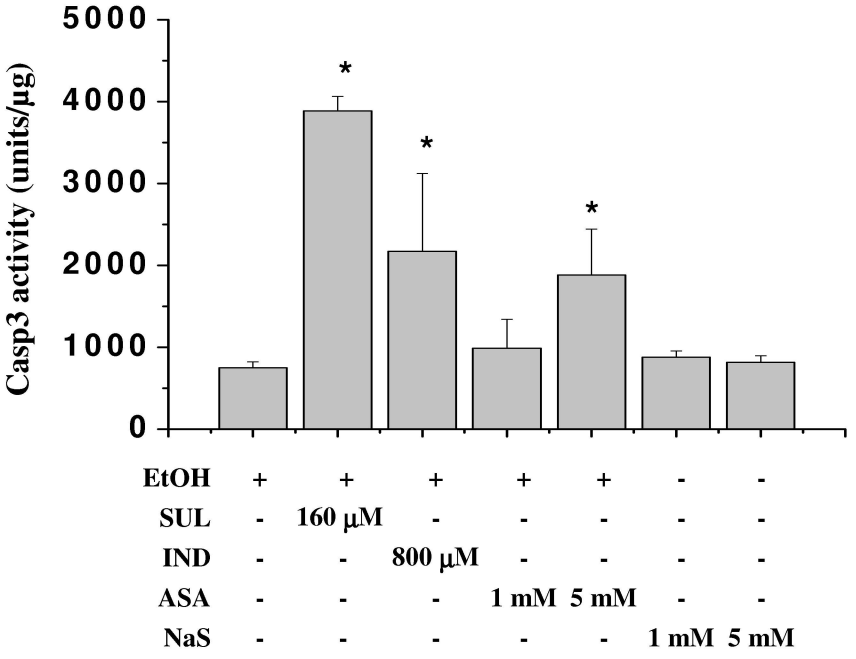

Casp3 activity (units/µg)
5000
4000
3000
2000
1000
0
*
*
*
EtOH + + + + + - -
SUL - 160 µM - - - - -
IND - - 800 µM - - - -
ASA - - - 1 mM 5 mM - -
NaS - - - - - 1 mM 5 mM

Figure 2

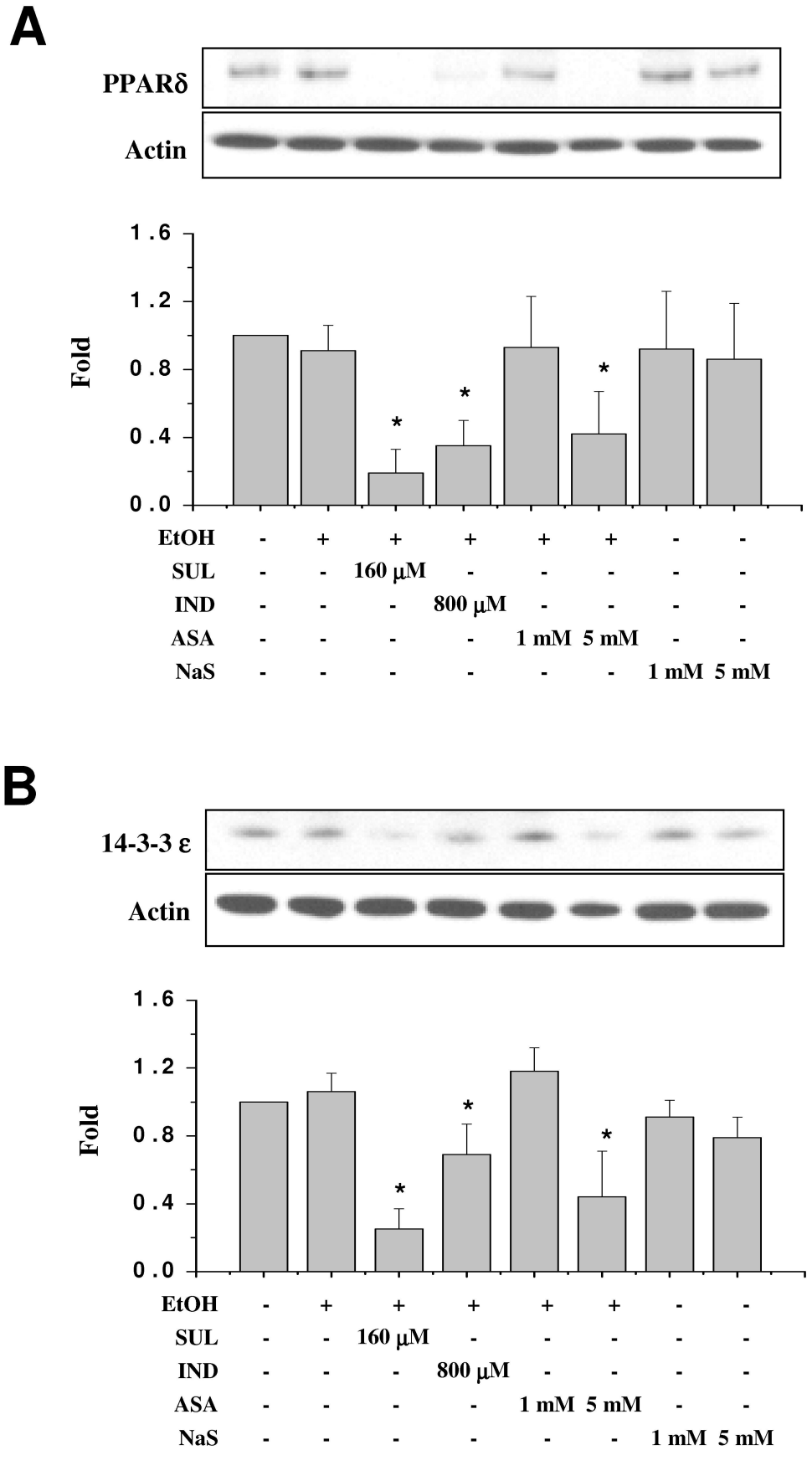

A
PPARδ
Actin
Fold
1.6
1.2
0.8
0.4
0.0
EtOH - + + + + + - -
SUL - - 160 μM - - - - -
IND - - - 800 μM - - - -
ASA - - - - 1 mM 5 mM - -
NaS - - - - - - 1 mM 5 mM
B
14-3-3 ε
Actin
Fold
1.6
1.2
0.8
0.4
0.0
EtOH - + + + + + - -
SUL - - 160 μM - - - - -
IND - - - 800 μM - - - -
ASA - - - - 1 mM 5 mM - -
NaS - - - - - - 1 mM 5 mM

Figure 2

C

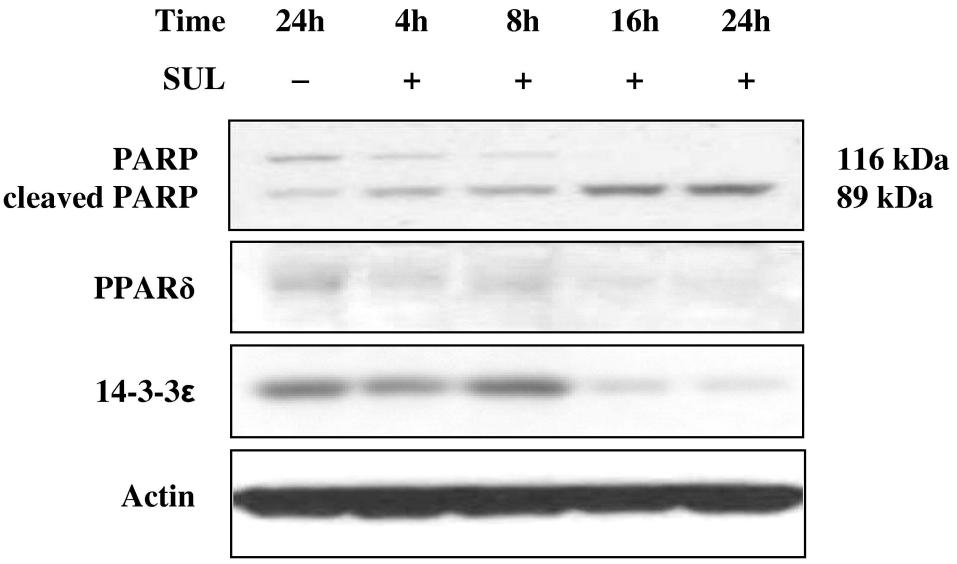

Time
24h
4h
8h
16h
24h
SUL
–
+
+
+
+
PARP
cleaved PARP
116 kDa
89 kDa
PPARδ
14-3-3ε
Actin

Figure 3

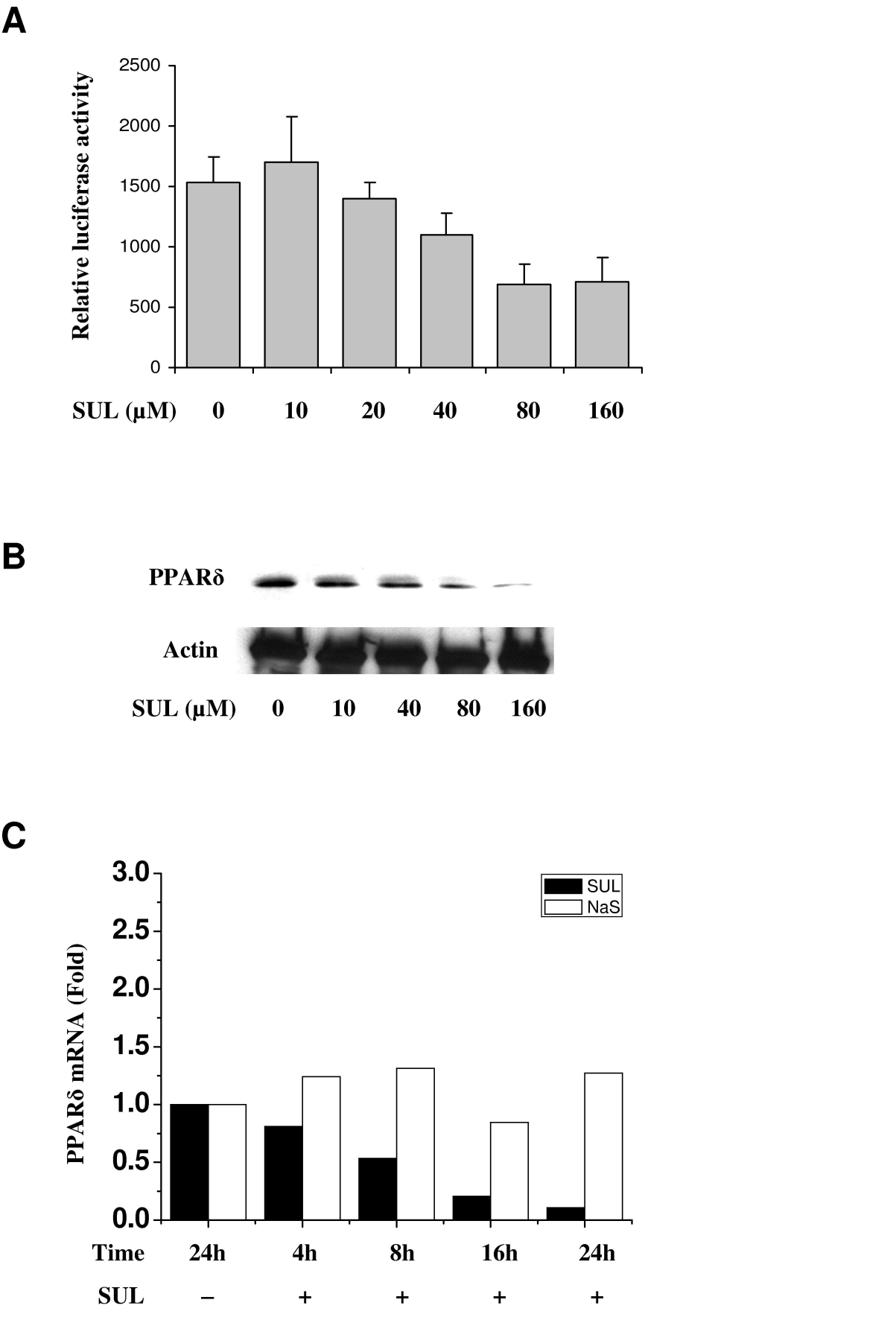

A
Relative luciferase activity
2500
2000
1500
1000
500
0
SUL (μM) 0 10 20 40 80 160
B
PPARδ
Actin
SUL (μM) 0 10 40 80 160
C
PPARδ mRNA (Fold)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
SUL
NaS
Time 24h 4h 8h 16h 24h
SUL – + + + +

Figure 4

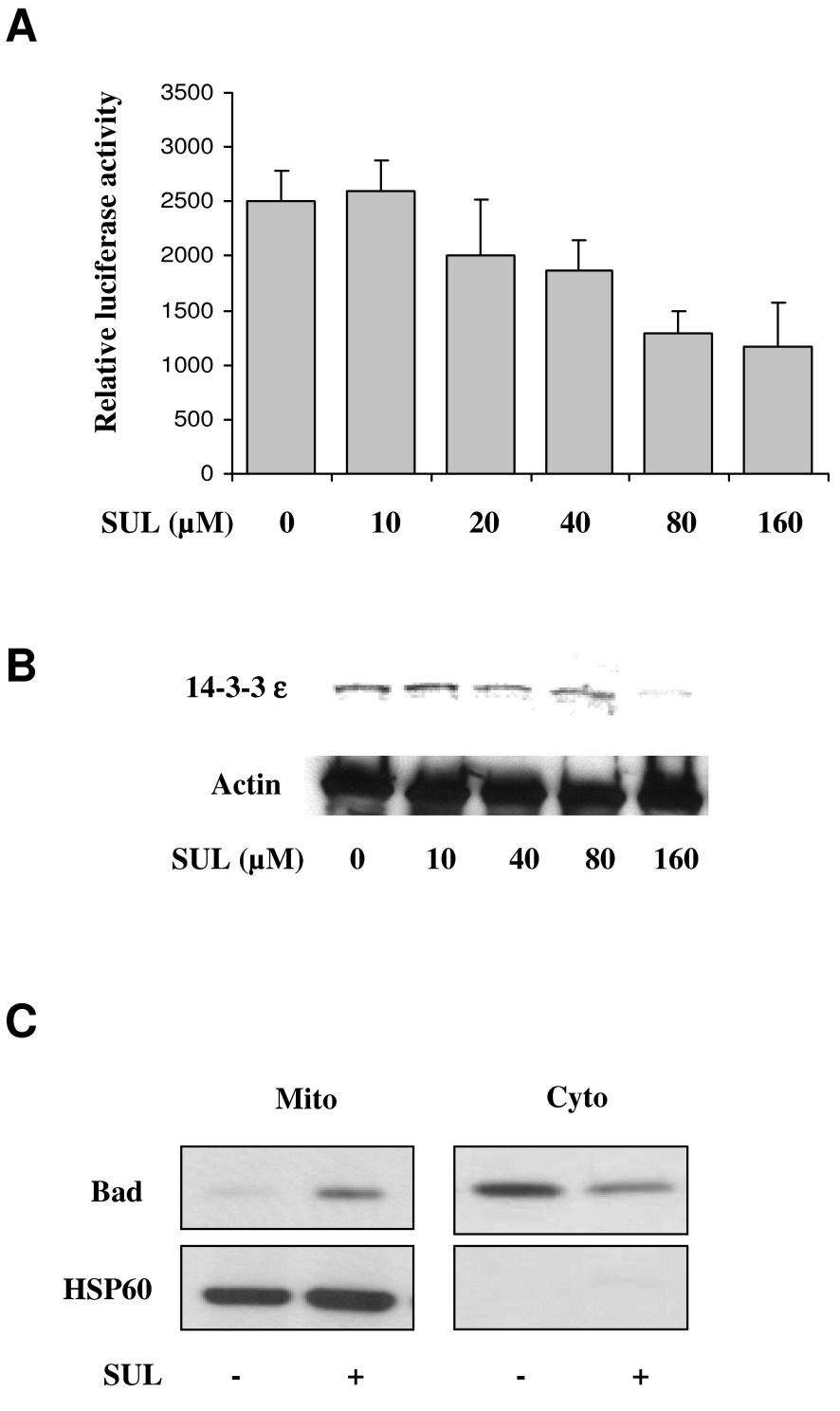

A
Relative luciferase activity
3500
3000
2500
2000
1500
1000
500
0
SUL (μM) 0 10 20 40 80 160
B
14-3-3 ε
Actin
SUL (μM) 0 10 40 80 160
C
Mito
Cyto
Bad
HSP60
SUL - + - +

Figure 5

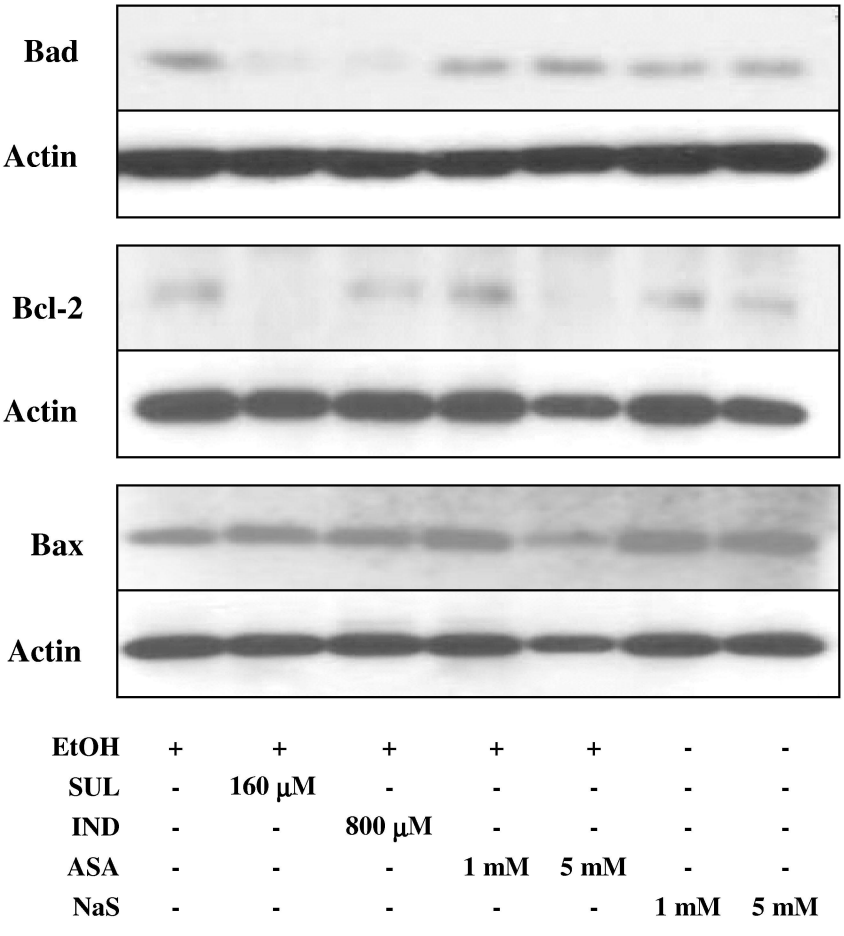

Bad
Actin
Bcl-2
Actin
Bax
Actin
EtOH + + + + + - -
SUL - 160 μM - - - - -
IND - - 800 μM - - - -
ASA - - - 1 mM 5 mM - -
NaS - - - - - 1 mM 5 mM

Figure 6

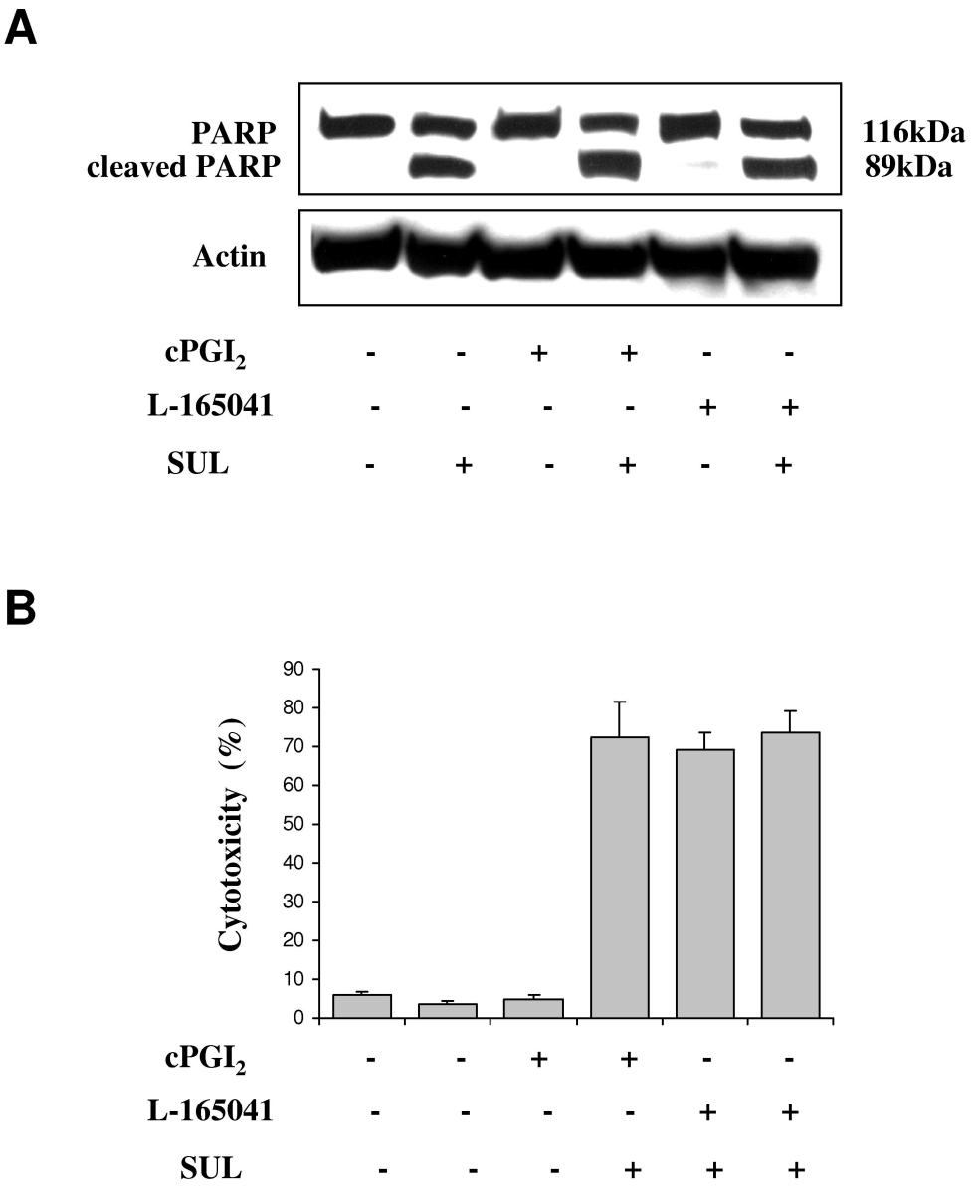
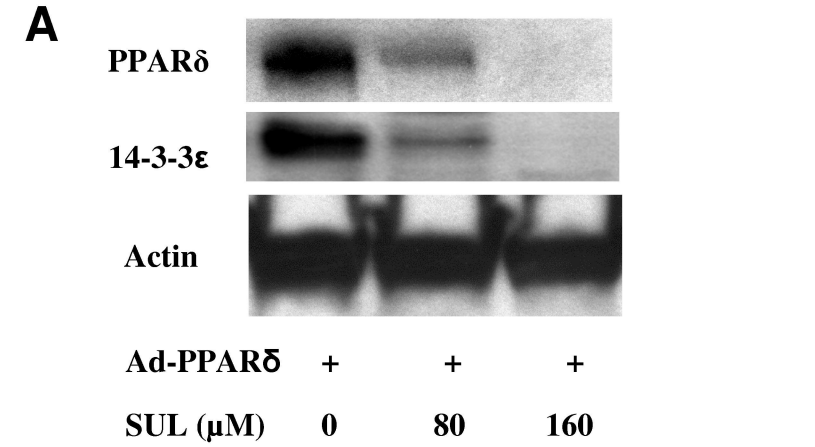
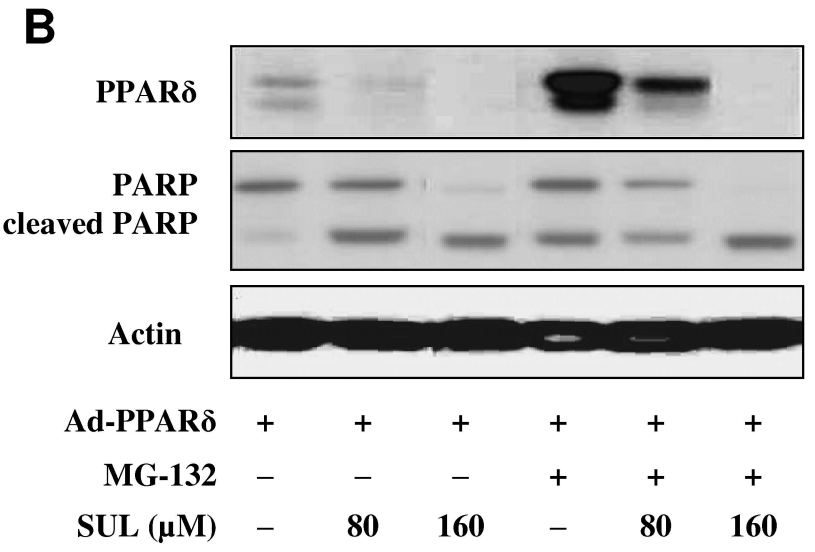
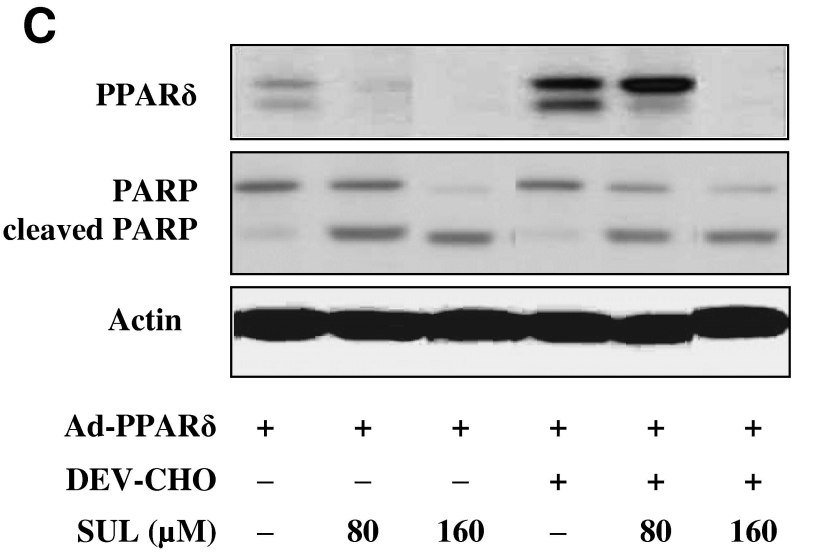

A
PARP
cleaved PARP
116kDa
89kDa
Actin
cPGI2 - - + + - -
L-165041 - - - - + +
SUL - + - + - +
B
Cytotoxicity (%)
90
80
70
60
50
40
30
20
10
0
cPGI2 - - + + - -
L-165041 - - - - + +
SUL - - - + + +

Figure 7


A
PPARδ
14-3-3ε
Actin
Ad-PPARδ + + +
SUL (μM) 0 80 160


B
PPARδ
PARP
cleaved PARP
Actin
Ad-PPARδ + + + + + +
MG-132 – – – + + +
SUL (μM) – 80 160 – 80 160


C
PPARδ
PARP
cleaved PARP
Actin
Ad-PPARδ + + + + + +
DEV-CHO – – – + + +
SUL (μM) – 80 160 – 80 160