1-Diarylchidonoyl-sn-glycero-3-phosphoethanolamine (DAPE) induces both necrosis/necroptosis and apoptosis of NCI-H28 malignant pleural mesothelioma (MPM) cells. The present study was conducted to understand the mechanism for DAPE-induced apoptosis of NCI-H28 cells. DAPE induced caspase-independent apoptosis of NCI-H28 malignant pleural mesothelioma (MPM) cells, and the effect of DAPE was prevented by antioxidants or an inhibitor of NADPH oxidase (NOX). DAPE activated reactive oxygen species (ROS) and inhibited activity of thioredoxin (Trx) reductase (TrxR). DAPE decreased an association of apoptosis signal-regulating kinase 1 (ASK1) with thioredoxin (Trx), thereby releasing ASK1. DAPE activated p38 mitogen-activated protein kinase (MAPK), which was inhibited by an antioxidant or knocking-down ASK1. In addition, DAPE-induced NCI-H28 cell death was also prevented by knocking-down ASK1. Taken together, the results of the present study indicate that DAPE stimulates NOX-mediated ROS production and suppresses TrxR activity, resulting in the decrease of reduced Trx and the dissociation of ASK1 from a complex with Trx, allowing sequential activation of ASK1 and p38 MAPK, to induce apoptosis of NCI-H28 MPM cells.

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1. Introduction

MPM is an aggressive malignant tumor with very poor prognosis, and exposure to asbestos fibers is the major pathogenic factor for MPM as well as fibrotic lung disease and lung cancer (1). Asbestos causes DNA double strand breaks, chromosomal aberrations, and abnormal chromosome segregation (2,3). Asbestos induces genotoxicities directly or indirectly by producing ROS (4). Asbestos affects expression of genes involved in integrin-mediated signaling pathways, MAPK pathways, and IκB/NFκB pathways (5).

Asbestos stimulates secretion of tumor necrosis factor α (TNFα) and activates NFκB cascades, to prevent MPM cells from apoptosis, i.e., to promote proliferation/differentiation of MPM cells (6). MPM cells are resistant to ROS-induced oxidative stress or radiation. This may be due to the potential antioxidant mechanisms in MPM cells. Expression of mitochondrial manganese superoxide dismutase and enzymes associated with glutathione metabolism such as glutathione-S-transferase and γ-glutamylcysteine synthetase is upregulated in MPM cells (7—11). In addition, Trx may be implicated in the resistance to oxidants and cytotoxic drugs. In response to a variety of factors such as oxidative stress, tumor-promoting agents, and cytotoxic drugs expression of Trx is induced (12—14). Expression of Trx and TrxR is upregulated in MPM cells (15), and higher concentrations of serum Trx are found in MPM patients (16).

The Trx system, which is composed of NADPH, cytosolic and mitochondrial Trx, and TrxR, plays a crucial role in the regulation of cancer cell proliferation and invasion (17—20). TrxR reduces and activates Trx, and thioredoxin-interacting protein (TXNIP) negatively regulates Trx. Trx is a 12 kD multifunctional protein and has an active site with two redox-active cysteine residues (–Trp-Cys-Gly-Pro-Cys–) catalyzing protein disulfide reductions. Trx keeps the cellular redox state and regulates cell proliferation by acting as an electron donor of ribonucleotide reductase, essential for DNA synthesis (21,22). Trx, alternatively, regulates p53-mediated p21 activity and modulates gene expression through thiol redox control over transcription factors such as NFκB and AP-1 (23—26).
In our previous study, the phosphatidylethanolamine 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) induced apoptosis of NCI-H28 MPM cells by enhancing activities of protein phosphatase 2A (PP2A) and protein tyrosine phosphatase 1B (PTP1B) (27). We have lately found that another phosphatidylethanolamine DAPE-induced necrosis/necroptosis and apoptosis of NCI-H28 MPM cells (28). DAPE-induced necrosis/necroptosis was mediated by RIP1 kinase and caused by cyclophilin D-dependent mitochondrial damage in association with ROS generation, to reduce intracellular ATP levels (28). The mechanism underlying DAPE-induced apoptosis of NCI-H28 cells, however, remains to be elucidated as yet. The present study was designed to answer this issue. We show here apoptosis of NCI-H28 MPM cells by enhancing activities of protein phosphatase 2A (PP2A) and protein tyrosine phosphatase 1B (PTP1B) along an ASK1/MKK(3/6)/p38 MAPK axis under the regulation of Trx.

2. Materials and methods

2.1. Cell culture

NCI-H28 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 0.003% (w/v) L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO2 and 95% air at 37 °C.

2.2. Assay of cell viability

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed by the method as previously described (27).

2.3. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

TUNEL staining was performed by the method as previously described (27).

2.4. Enzymatic assay of caspase activity

Caspase activity was measured by the method as previously described (29). The substrate peptides used were as follows: Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide, Ac-Leu-Glu-Val-Asp-AFC for a caspase-4 substrate peptide, Ac-Ile-Glu-Thr-Asp-MCA for a caspase-8 substrate peptide, and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide.

2.5. Monitoring of ROS

Cells were incubated in the culture medium containing 10 μM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) (Life Technologies, Tokyo, Japan) for 30 min at 37 °C. ROS generation in living cells was monitored at 488-nm argon laser with a confocal scanning laser microscope (LSM 510, Carl Zeiss Co., Ltd.) before and after bath-application with DAPE and/or GSH or VAS. The fluorescent signal intensity in each cell was measured and summed as an index of ROS production.

2.6. Assay of TrxR activity

TrxR activity was monitored using a TrxR activity colorimetric assay kit (Biovision, Milpitas, CA, USA). In this assay, TrxR catalyzes reduction of 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB2-), and in this reaction process TNB2- generates a strong yellow color. Cells were harvested before and after treatment with DAPE for periods of time, and then centrifuged at 800 g for 5 min at 4 °C. The pellet was homogenized on ice in an assay buffer, and then centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was reacted with DTNB and NADPH at 25 °C for 20 min, and the reaction intensity as a TrxR activity was measured at an absorbance of 412 nm.

2.7. Immunoprecipitation

Cells were lysed in a lysis buffer [150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1% (v/v) Triton X-100 and 20 mM Tris, pH 7.5] containing 1% (v/v) protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and then centrifuged at 10,000 g for 15 min at 4 °C. Supernatants were incubated with an anti-ASK1 antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4 °C. Then, protein G sepharose 4 beads (GE Healthcare, Piscataway, NJ, USA) was added to the extracts and incubated for 2 h at 4 °C. Pellets were washed 5 times with the lysis buffer and dissolved in an SDS sample buffer for Western blotting.

2.8. Western blotting

Western blotting was performed by the method as previously described (29) using antibodies against p38, ERK, and phospho-ERK (p-ERK) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA); Trx1 (GeneTex, Inc., Irvine, CA, USA); ASK1, phospho-ASK1(p-ASK1), MKK3, phospho-MKK3 (p-MKK3), MKK4, phospho-MKK4 (p-MKK4), phospho-p38 (p-p38), JNK, and phospho-JNK (p-JNK) (Cell Signaling Technology); and β-actin (SIGMA, Missouri, SL, USA).

2.9. Construction and transfection of siRNA

The siRNA silencing the ASK1-targeted gene (ASK1 siRNA) and the negative control siRNA (NC siRNA) were obtained from Invitrogen (Carlsbad, CA, USA). The sequences of ASK1 siRNA used here were 5’-UUAAUAGCAUUGACUGUUGGUUGC-3’ and 5’-GAACAC-GAAGCAAGUUGCUAAUA-3’. The NC siRNA contained the scrambled sequence with the same GC content and nucleic acid composition. ASK1 siRNA and NC siRNA were reverse-transfected into cells using a Lipofectamine reagent (Invitrogen). Cells were used for experiments 48 h after transfection.

2.10. Statistical analysis

The data presented were mean ± standard error of the mean (SEM). Statistical differences were analyzed by unpaired t-test and analysis of variance (ANOVA) followed by a Bonferroni correction. Values of p < 0.05 were considered statistically significant.

3. Results

In the MTT assay, DAPE reduced NCI-H28 cell viability in a treatment time (24–48 h)- and concentration (1–100 μM)-dependent manner (Fig. 1A). In the TUNEL staining, DAPE obviously increased the number of TUNEL-positive NCI-H28 cells as compared with control (Fig. 1B). In the caspase assay, no significant activation of caspase-3, -4, -8, and -9 was obtained with DAPE (Fig. 1C). Taken together, these results indicate that DAPE induces caspase-independent apoptosis of NCI-H28 cells.

In the real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis, DAPE had no significant effect on expression of mRNAs for FasL, Fas, Fas-associated death domain protein (FADD), TNFα, TNFα receptor 1 (TNFR1), TNFR2, TNFR1-associated death domain protein (TRADD), TNF-related apoptosis-inducing...
ligand (TRAIL), TNF-related weak inducer of apoptosis (TWEAK), death receptor 3 (DR3), DR4, DR5, and DR6 in NCI-H28 cells (Fig. S1). This suggests that DAPE-induced apoptosis of NCI-H28 cells is not mediated through death receptors bearing activation of caspase-8 followed by the effect caspase-3.

Apoptosis-inducing factor (AIF) and AIF-homologous mitochon-drion-associated inducer of death (AMID) induce caspase-independent apoptosis by accumulating in the nucleus. Endonuclease G (EndoG), alternatively, induces caspase-independent apoptosis by cleaving DNA. DAPE had no effect on total expression of AIF, AMID, and EndoG in NCI-H28 cells (Fig. S2A–C) or nuclear localization of AIF, AMID, and EndoG in NCI-H28 cells (Fig. S2D–F). This indicates that AIF, AMID, and EndoG are not implicated in DAPE-induced caspase-independent apoptosis of NCI-H28 cells.

X-linked inhibitor of apoptosis protein (XIAP) inhibits caspase-3, -7, and -9. In response to apoptotic stimuli Smac/DIABLO is released from the mitochondria and neutralizes inhibition of caspase-3, -7, and -9 due to XIAP. Expression of XIAP and Smac/DIABLO in NCI-H28 cells was not affected by DAPE (Fig. S3), indicating no implication of XIAP and Smac/DIABLO in DAPE-induced caspase-independent apoptosis of NCI-H28 cells.

We next examined whether oxidative stress is implicated in DAPE-induced apoptosis of NCI-H28 cells. DAPE-induced NCI-H28 cell death was significantly inhibited by glutathione (GSH), an antioxidant (Fig. 2A). In addition, the effect of DAPE was clearly prevented by N-acetyl-cysteine (NAC), an antioxidant that is a precursor of reduced GSH (Fig. 2B). These results suggest that DAPE induces apoptosis of NCI-H28 cells by producing oxidants. DAPE-induced NCI-H28 cell death was also attenuated by VAS-2870 (VAS), an inhibitor of NOX, or NSC23766 (NSC), an inhibitor of Rac (Fig. 2C,D). Collectively, DAPE appears to stimulate NOX-mediated ROS generation, responsible for apoptosis of NCI-H28 cells.

To obtain further evidence for this, we monitored ROS production using carboxy-H$_2$DCFDA. DAPE produced ROS in NCI-H28 cells in a treatment time (1–20 min)-dependent manner, and the effect was inhibited by GSH (Fig. 3A). DAPE-induced ROS production, alternatively, was abrogated by VAS (Fig. 3B). Taken together, these results imply that DAPE stimulates NOX-mediated ROS production in NCI-H28 cells and that produced ROS is reduced by GSH.

ROS, produced by DAPE, might inhibit TrxR followed by Trx reduction. To obtain evidence for this, we assayed TrxR activity in NCI-H28 cells. Expectedly, DAPE suppressed TrxR activity in NCI-H28 cells in a treatment time (10–120 min)-dependent manner, reaching approximately 60% of basal levels at 120 min (Fig. 4).

Trx-SH$_2$, which is reduced by TrxR, associates with ASK1, but otherwise Trx-S$_2$, a non-reduced from of Trx, dissociates and activates ASK1. To address this issue, we examined an association of Trx with ASK1 in immunoprecipitants using an anti-ASK1 antibody from NCI-H28 cells. DAPE significantly decreased immunoreactive signals for Trx in a treatment time (10–120 min)-dependent manner, reaching almost half of basal levels at 120 min (Fig. 5). This indicates that DAPE decreases an association of ASK1 with Trx, i.e., DAPE increases free ASK1. Overall, these results show that DAPE promotes NOX-mediated ROS production, causing a decrease in the reductive form of Trx due to TrxR inhibition, allowing dissociation of ASK1 from a complex with Trx.

Our next attempt was to see whether ASK1, dissociated from a complex with Trx, is activated. ASK1 is activated by being phosphorylated at Thr845. DAPE apparently increased phosphorylation of Trx.
ASK1 at Thr845 in NCI-H28 cells in a bell-shaped treatment time (10–120 min)-dependent manner (Fig. 6A,B). This indicates that DAPE activates ASK1 by dissociating ASK1 from a complex with Trx. ASK1 phosphorylates and activates MKK3/6, which in turn, phosphorylates and activates p38 MAP kinase. ASK1, alternatively, phosphorylates and activates MKK4/7, which phosphorylates and activates stress-activated protein kinases (SAPK)/c-Jun N-terminal kinase (JNK). DAPE did not significantly increase MKK3 phosphorylation at Ser189 (Fig. 6A,C), but a striking increase in p38 MAPK phosphorylation at Thr180/Tyr182 was obtained (Fig. 6A, E). DAPE significantly increased JNK phosphorylation at Thr183/Tyr185 only at 30-min treatment (Fig. 6A,F), while no remarkable increase in the phosphorylation of MKK4 at Ser257/Thr261 was found (Fig. 6A,D). DAPE did not enhance phosphorylation of the other MAPK extracellular signal-regulated kinase 1/2 (ERK1/2) at Thr202/Tyr204 (Fig. 6A,G). 1,2-Dilinoleoyl-sn-glycero-3-phosphoethanolamine (DLPE) as well as DAPE increased ASK1 phosphorylation in NCI-H28 cells, while DPPE and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) had no effect (Fig. S4A,B). DLPE also increased p38 MAPK phosphorylation, with much lesser potential than that for DAPE, but otherwise no increase was obtained with DOPE and DPPE (Fig. S4A,C). These results indicate that the effect of DAPE on phosphorylation of ASK1 and p38 MAPK is not due to non-specific action of phosphatidylethanolamine.

Fig. 3. DAPE promotes NOX-mediated ROS production in NCI-H28 cells. After 30-min loading of carboxy-H2DCFDA (10 μM), cells were treated DAPE (100 μM) in the presence and absence of GSH (1 mM) (A) or VAS (1 μM) (B) for periods of time as indicated, and the fluorescent signal intensities were measured. Typical fluorescent images are shown in the upper panel and time-course profiles of ROS production are shown in the lower panel. Scale bars, 50 μm. In the graphs, each point represents the mean ± SEM ROS production (n = 4 independent experiments).
GSH abolished DAPE-induced phosphorylation of p38 MAPK in NCI-H28 cells (Fig. 7A,D). GSH also decreased DAPE-induced phosphorylation of ASK1, ERK, and JNK, but not significantly (Fig. 7B,C,E). These results suggest that DAPE activates p38 MAPK in a ROS-dependent manner, possibly as mediated via ASK1.

To obtain further evidence for ASK1-dependent p38 MAPK activation, we knocked-down ASK1 using the siRNA. Expression of ASK1 was clearly suppressed in NCI-H28 cells transfected with the siRNA silencing the ASK1-targeted gene, but the expression levels were not affected by DAPE (Fig. 8A,B). DAPE-induced increase in p38 MAPK phosphorylation was clearly inhibited by knocking-down ASK1 (Fig. 8A,C), although DAPE-induced JNK phosphorylation was not affected (Fig. 8A,D). DAPE-induced NCI-H28 cell death was significantly prevented by knocking-down ASK1 (Fig. 8E). Overall, these results indicate that DAPE activates p38 MAPK and induces NCI-H28 cell death in an ASK1-dependent manner.

4. Discussion

In the present study, DAPE induced caspase-independent apoptosis of NCI-H28 MPM cells. DAPE-induced NCI-H28 cell death was inhibited by the antioxidants GSH and NAC or the NOX inhibitor VAS. This suggests that DAPE stimulates NOX-mediated ROS production, which triggers to induce apoptosis of NCI-H28 cells. In the NOX assay, DAPE actually increased ROS production in NCI-H28 cells and the effect was canceled by VAS. This provides evidence that DAPE stimulates NOX activity followed by ROS production. ROS, alternatively, is recognized to be produced through damaged mitochondria. DAPE perturbed mitochondrial membrane potentials in NCI-H28 cells, but ROS production preceded mitochondrial damage (28). This implies that DAPE generates ROS by activating NOX, but not due to mitochondrial damage. The NOX family includes NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2, which are composed of several subunits (30). Of them NOX1 and NOX2 contain the small G-protein Rac1 in the subunits, to regulate NOX activation (30). DAPE-induced NCI-H28 cell death was significantly attenuated by the Rac1 inhibitor NSC, suggesting that DAPE may activate NOX by targeting Rac1.

Trx and TrxR are recognized to play a crucial role in the regulation of cancer cell proliferation and invasion (20). TrxR reduces Trx-S2 to Trx-Sh2, which associates and inhibits ASK1; conversely, Trx-S2, a non-reduced form of Trx, dissociates and activates ASK1, to induce apoptosis (31–33). We postulated that ROS produced by DAPE might suppress TrxR activity. Indeed, DAPE attenuated TrxR activity in NCI-H28 cells and significantly decreased association of ASK1 with Trx1. Moreover, DAPE enhanced phosphorylation of ASK1 at Thr845 in NCI-H28. Taken together, these results indicate that DAPE activates NOX to generate ROS, which inhibits TrxR, allowing decrease in reduced Trx and dissociation of ASK1 from a complex with Trx, and then leading to activation of ASK1.

ASK1 activates MKK3/6 followed by p38 MAPK or MKK4/7 followed by SAPK/JNK through sequential phosphorylation, and both of p38 MAPK and SAPK/JNK are implicated in apoptosis (34,35). DAPE markedly enhanced phosphorylation of p38 MAPK at Thr180/Tyr182 in NCI-H28 cells, although no significant increase in the phosphorylation of MKK3 at Ser189 was obtained. DAPE, on the other hand, enhanced phosphorylation of JNK at Thr183/Tyr185 only at 30-min treatment, but phosphorylation of MKK4 at Ser257/Thr261 was not affected. DAPE-induced increase in the phosphorylation of p38 MAPK, but not JNK, was abolished by GSH and clearly prevented by knocking-down ASK1. In addition, DAPE-induced NCI-H28 cell death was significantly inhibited by knocking-down ASK1. Overall, these results suggest that DAPE induces apoptosis of NCI-H28 cells dominantly through an ASK1/MKK(3/6)/p38 MAPK pathway. The questions raised are as to: why in spite of significant...
Fig. 6. DAPE enhances phosphorylation of ASK1 and p38 MAPK in NCI-H28 cells. Cells were treated with DAPE (100 μM) for periods of time as indicated, followed by Western blotting. Typical blotting images are shown in (A). In the graphs, each column represents the mean (±SEM) signal intensity for p-ASK1 (B), p-MKK3 (C), p-MKK4 (D), p-p38 (E), p-JNK (F), or p-ERK (G) relative to that for each protein (n = 4 independent experiments). P value, ANOVA followed by a Bonferroni correction. NS, not significant.
activation of p38 MAPK DAPE did not activate MKK3 significantly and why in spite of significant inhibition of DAPE-induced p38 MAPK activation GSH did not inhibit DAPE-induced ASK1 activation. This may represent an additional DAPE-induced direct p38 MAPK activation pathway, regardless of MKK3/6.

In summary, the results of the present study show that DAPE stimulates NOX-mediated ROS production, which suppresses TrxR activity and decreases reduced Trx, allowing ASK1 release from an association with Trx, resulting in the sequential activation of ASK1/MKK(3/6)/p38 MAPK, to induce caspase-independent apoptosis of...
Fig. 8. DAPE-induced phosphorylation of p38 MAPK and cell death are dependent upon ASK1 in NCI-H28 cells. Cells, transfected with the NC siRNA or the ASK1 siRNA, were untreated and treated with DAPE (100 μM) for 30 min, followed by Western blotting. (A) Typical blotting images. (B) In the graphs, each column represents the mean (±SEM) signal intensity for ASK1 normalized by that for β-actin (n = 4 independent experiments). (C) (D) The signal intensities for p-p38 (C) and p-JNK (D) were normalized by those for each unphosphorylated protein. In the graphs, each column represents the mean (±SEM) signal intensity for p-p38 (C) and p-JNK (D) relative to that for each (n = 4 independent experiments). (E) Cells, transfected with the NC siRNA and the ASK1 siRNA, were treated with DAPE (100 μM) for 48 h, followed by MTT assay. In the graph, each column represents the mean (±SEM) percentage of basal levels (MTT intensities for cells before treatment with DAPE) (n = 4 independent experiments). P values, unpaired t-test. NS, not significant.
NCI-H28 MPM cells. This may establish a new apoptotic pathway relevant to DAPE signaling.

Conflicts of interest

The authors declare that they have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2015.10.003.

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