1521-0111/12/8201-27–36$25.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 82:27–36, 2012

Vol. 82, No. 1
77149/3775160

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Fyn Inhibition by Cycloalkane-Fused 1,2-Dithiole-3-thiones Enhances Antioxidant Capacity and Protects Mitochondria from Oxidative Injury

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Received December 8, 2011; accepted April 2, 2012

ABSTRACT

Fyn kinase has emerged as a regulator of diverse pathological processes. However, therapeutic Fyn inhibitors are not available. This study investigated the potential of a series of cycloalkane-fused dithiolethiones (CDTs) or other congeners to increase antioxidant capacity in association with Fyn inhibition, as well as the molecular basis for this effect. Treatment of HepG2 cells with each agent protected the mitochondria from oxidative injury elicited by arachidonic acid and iron, which increased cell viability; 4,5,6,7-tetrahydrobenzo-1,2-dithiole-3-thione (SNU1A) and 5,6-dihydro-4*H*-cyclopenta-1,2-dithiole-3-thione (SNU2A) were the most effective, whereas 5-methyl-1,2-dithiole-3-thione (SNU3A) was less active. 5-(Quinolin-2-yl)-1,2-dithiole-3-thione (SNU3E) had a minimal effect. SNU1A treatment decreased mitochondrial superoxide production and enabled cells to restore mitochondrial membrane permeability. Oxidative injury caused by arachidonic acid and iron enhanced Fyn phosphorylation at a tyrosine residue, which was decreased by SNU1A treatment. 2,3-Dihydro-*N*,*N*-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1*H*-indol-2-yl)methylene]-1*H*-indole-5-sulfonamide (SU6656), a known Fyn inhibitor, had a similar effect. Fyn inhibition contributed to protecting mitochondria from injury through AMP-activated protein kinase (AMPK), as supported by reversal of this effect with Fyn overexpression. Consistently, Fyn overexpression attenuated AMPK activation by SNU1A, which strengthens the inhibitory role of Fyn in AMPK activity. CDTs had antioxidant effects, as shown by increases in GSH contents and inhibition of H_2O_2 production. They also had the ability to activate nuclear factor E2–related factor 2 (Nrf2), a key antioxidant transcription factor. Fyn overexpression decreased the Nrf2 activation induced by SNU1A. Our results demonstrate that CDTs exert cytoprotective effects by protecting mitochondria and increasing the cellular antioxidant capacity, which may result not only from Fyn inhibition leading to AMPK activation but also from Nrf2 activation.

Introduction

4-Methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione (oltipraz), a prototypical 1,2-dithiole-3-thione, has been comprehensively investigated as a chemopreventive agent against cancer (Bolton et al., 1993; Jacobson et al., 1997; Wang et al., 1999; Kang et al., 2003). Oltipraz treatment induces phase II enzymes, including glutathione transferase, UDP-glucuronyltransferase, and heme oxygenase 1, which contributes to inhibition of the formation of carcinogen-DNA adducts. However, it failed to prevent oxidative DNA damage in healthy individuals (Glintborg et al., 2006). Moreover, the biotransformation of oltipraz causes variability in the pharmacokinetic profile in humans (Kim et al., 2010). The efficacy and/or metabolic stability of dithiolethiones may be increased through specific design; efforts were made to diversify candidates with the core structure of 1,2-dithiole-3-thione, some of which had the ability to decrease reactive oxygen species (ROS) levels in cells (Shin and Kim, 2009).

As the basis of cell metabolism, mitochondria maintain the equilibrium between basal and excess levels of ROS. Under pathological conditions, the mitochondrial respiratory chain

This work was supported by the National Research Foundation of Korea, with grants funded by the Ministry of Education, Science, and Technology [Grant 2011-0001204] and by the World Class University Project [Grant R32-2011-000-10098-0].

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

http://dx.doi.org/10.1124/mol.111.077149.

ABBREVIATIONS: ROS, reactive oxygen species; AA, arachidonic acid; Ad, adenoviral; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CDT, cycloalkane-fused dithiolethione; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; FACS, fluorescence-activated cell sorting; GSK3β, glycogen synthase kinase 3β; LKB1, liver kinase B1; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Nrf2, nuclear factor E2–related factor 2; Rh123, Rhodamine 123; ARE, antioxidant-response element; SNU1A, 4,5,6,7-tetrahydrobenzo-1,2-dithiole-3-thione; SNU2A, 5,6-dihydro-4*H*-cyclopenta-1,2-dithiole-3-thione; SNU3A, 5-methyl-1,2-dithiole-3-thione; SNU3E, 5-(quinolin-2-yl)-1,2-dithiole-3-thione; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DN, dominant-negative; SU6656, 2,3-dihydro-*N*,*N*-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1*H*-indol-2-yl)methylene]-1*H*-indole-5-sulfonamide.

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chiefly produces ROS (Browning and Horton, 2004). Because mitochondria function as energy sensors, dysfunction of the organelles determines the fate of cells (cell survival or death) (Zamzami and Kroemer, 2001). Mitochondrial permeability transitions and impairment caused by oxidative stress or other stimuli promote the process of apoptosis. Conversely, preservation of mitochondrial function is important in protecting cells or organs from toxic stimuli. Therefore, it is possible that agents with the ability to prevent mitochondrial dysfunction would have antioxidant and/or cytoprotective effects.

Members of the Fyn/Src kinase family have emerged as the major regulators of various pathophysiological processes (Büchner et al., 2010). Several growth factors activate Fyn through the phosphatidylinositol 3-kinase/Akt pathway (Cui et al., 2005). In skeletal muscle and adipose tissue, Fyn inhibition may contribute to the regulation of energy metabolism through LKB1 because Fyn phosphorylates tyrosine residues (Tyr261 and Tyr365) in LKB1 in the nucleus, which prevents its cytoplasmic translocation for inactivation (Yamada et al., 2010). Certain chemopreventive agents activate AMPK through LKB1 (Hezel and Bardeesy, 2008). The agents that activate AMPK may enhance antioxidant capacity in cells by increasing nuclear factor E2–related factor 2 (Nrf2) activity (Liu et al., 2011). Glycogen synthase kinase 3β (GSK3β), a kinase inhibited by AMPK, negatively phosphorylates Nrf2, thereby repressing the antioxidative genes (Salazar et al., 2006). Therefore, Fyn is likely to affect not only AMPK but also Nrf2. However, the effects of Fyn modulation by chemical means on mitochondria and antioxidant capacity remain unclear.

This study investigated whether cycloalkane-fused dithiolethiones (CDTs) or other congeners have the potential to enhance cellular antioxidant capacity in association with Fyn inhibition and, if so, what the underlying molecular basis is. In this study, we used a series of novel synthetic dithiolethione derivatives to determine the effects on antioxidant capacity and cell viability with oxidative injury elicited by arachidonic acid (AA) and iron. We were interested in identifying the effect of Fyn inhibition by the agents on the activation of AMPK and the role of Fyn inhibition in mitochondria protection. In our findings, the ability of CDTs to impede mitochondrial permeability transition pore-opening helped protect hepatocytes from oxidative injury. In addition, we examined the effects of CDTs on the activity of Nrf2.

Materials and Methods

Materials. A solution of iron-nitrilotriacetic acid complex was prepared as described previously (Kim et al., 2009). MitoSOX was purchased from Invitrogen (Carlsbad, CA). Anti-phospho-Src, anti-phospho-acetyl-CoA carboxylase (ACC), anti-phospho-AMPKα, anti-phospho-GSK3β, and anti-GSK3β antibodies were supplied by Cell Signaling Technology (Danvers, MA). Antibodies directed against AMPK were provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were purchased from Zymed Laboratories (South San Francisco, CA). Anti-Fyn antibody and the GSH assay kit were obtained from OXIS Research, Inc. (Portland, OR). AA was purchased from Calbiochem (San Diego, CA). Rhodamine 123 (Rh123), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), compound C, anti-β-actin antibody, and other reagents were supplied by Sigma-Aldrich (St. Louis, MO).

Chemical Synthesis. Oltipraz and its cycloalkane derivatives (Fig. 1) were provided by CJ Central Laboratories (Ichon, Korea).

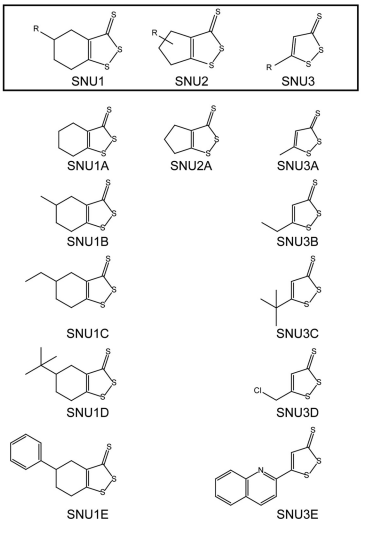
Cell Culture and Treatment. HepG2 cells, a human hepatocyte-derived cell line, were purchased from American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin, at 37°C in a humidified atmosphere with 5% CO_2. All experiments were performed by using cells with passage numbers of less than 15. For all experiments, cells (10^6) were seeded in 10-cm^2 plastic dishes for 2 to 3 days (i.e., 80% confluence) and serum-starved for 24 h. Cells were treated with 10 μM AA for 12 h, washed with minimal essential medium, and then continuously incubated with AA plus iron-nitrilotriacetic acid complex (iron; 5 μM) for the indicated time period. The cells were treated with 1 to 10 μM concentrations of each agent of interest for 1 h, followed by continuous incubation with AA and iron.

Cell-Cycle Analysis. Cell-cycle analysis was performed by using propidium iodide, as described previously (Shin and Kim, 2009). The fluorescence intensity of cells stained with propidium iodide was monitored by using a BD Biosciences FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

MTT Assay. The MTT assay was performed according to previously published methods (Shin and Kim, 2009). Absorbance was detected at 540 nm by using an enzyme-linked immunosorbent assay microplate reader (Tecan, Durham, NC). Cell viability was calculated relative to untreated control samples: i.e., viability (% of control) = 100 × (absorbance of treated sample/absorbance of control sample) (Kim et al., 2009).

Immunoblot Analysis. Cell lysates were prepared according to previously published methods (Shin and Kim, 2009). Briefly, cells were centrifuged at 3000*g* for 3 min and were subjected to osmotic expansion to the point of lysis after the addition of lysis buffer containing 10 mM Tris-HCl, pH 7.1, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, supplemented with a protease inhibitor cocktail (Calbiochem). The lysates were centrifuged at 10,000*g* for 10 min to yield supernatants, which were stored at −70°C until use. Immunoblot analysis was performed according to previously published procedures (Shin and Kim, 2009).

A



B

Compounds	R	ED_{50} (μM)
SNU1A	H	2.98±0.77
SNU1B	Methyl	3.86±1.46
SNU1C	Ethyl	1.72±1.18
SNU1D	*t*-Buthyl	3.78±1.50
SNU1E	Phenyl	3.00±1.27
SNU2A	H	3.20±0.77
SNU3A	Methyl	10.99±1.80
SNU3B	Ethyl	6.71±1.22
SNU3C	*t*-Buthyl	9.87±1.52
SNU3D	Chloromethyl	>50
SNU3E	2-Quinolyl	>50

Fig. 1. Effects of dithiolethiones on the viability of HepG2 cells. A, chemical structures of dithiolethione derivatives. B, ED_{50} values of dithiolethiones for cell viability. HepG2 cells were treated with 0.3 to 30 μM dithiolethione congeners for 1 h and were continuously incubated with 10 μM AA for the next 12 h. After washing, the cells were challenged with 5 μM iron for 6 h, with or without each agent. Cell survival was measured by using MTT assays. Data represent the mean ± S.E.M. of six replicates.

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Protein bands of interest were detected by using an enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Equal loading of proteins was verified with immunoblotting for β-actin. Scanning densitometry was performed with an image scan and analysis system (Alpha Innotech, San Leandro, CA).

Flow Cytometric Analysis of Mitochondrial Membrane Potential. The mitochondrial membrane potential (MMP) was measured by using Rh123, as reported previously (Shin and Kim, 2009). The cells in the M1 fraction were measured by using a BD Biosciences FACSCalibur flow cytometer.

Measurement of H_2O_2 Production. DCFH-DA, a cell-permeable nonfluorescent probe, is converted by intracellular esterases to fluorescent dichlorofluorescein through reaction with H_2O_2, as described previously (Shin and Kim, 2009). The level of H_2O_2 generation was determined on the basis of the concomitant increase in dichlorofluorescein fluorescence, which was measured through FACS.

Measurement of Mitochondrial ROS Levels. Mitochondrial ROS levels were monitored in HepG2 cells that had been loaded with 5 μM MitoSOX (Invitrogen, Carlsbad, CA), a mitochondrial superoxide indicator, for 10 min, as described previously (Choi et al., 2010). The fluorescence intensity in the cells was detected through FACS.

Determination of Reduced GSH Content. Reduced GSH in the cells was quantified by using a commercial GSH determination kit (GSH-400; OXIS Research, Inc.), as described previously (Kay et al., 2011).

Recombinant Adenoviral DN-AMPK Construct. A plasmid encoding dominant-negative (DN) AMPK, which was provided by Dr. J. Ha (Kyung Hee University, Seoul, Korea), was used for the preparation of its adenoviral construct. HepG2 cells were infected with adenoviral DN-AMPK diluted in DMEM containing 10% FBS, at a multiplicity of infection of 50, and were incubated for 12 h. After removal of the viral suspension, the cells were incubated for 2 days with DMEM containing 10% FBS and were treated with the indicated agent, as described previously (Choi et al., 2010). Adenovirus expressing LacZ (Ad-LacZ) was used as an infection control.

Transient Transfection and Luciferase Reporter Assay. Cells were plated at a density of 10^6 cells per well in six-well dishes and were transfected the following day, according to previously published methods (Kay et al., 2011). Briefly, the cells were incubated for 3 h with 1 μg of NQO1-ARE reporter plasmid and 3 μl of FuGENE HD reagent (Roche Diagnostics, Indianapolis, IN) in 1 ml of antibiotic-free minimal essential medium. The ARE-luciferase reporter was provided by N. Wakabayashi (Johns Hopkins University, Baltimore, MD). The cells were incubated in serum-free medium for 6 h and were exposed to medium containing FBS with or without the agent of interest. Luciferase activities were measured by using a dual-luciferase assay system (Promega, Madison, WI).

Preparation of Nuclear Extracts. Nuclear extracts was prepared according to a previously published method, as described previously in detail (Kay et al., 2011). The nuclear fractions were stored at −70°C until use.

Immunoprecipitation Assays. To measure Fyn phosphorylation, cell lysates were incubated with anti-Fyn antibody overnight at 4°C. The antigen-antibody complexes were immunoprecipitated after incubation with protein G-agarose for 2 h. Immune complexes were dissolved in 2× Laemmli buffer. Protein samples were resolved and immunoblotted with anti-Src antibody.

Plasmid Transfection. Plasmids encoding wild-type and dominant-negative mutant forms of Fyn were obtained from Addgene (Cambridge, MA). Cells were transfected with the plasmids (1 μg) by using FuGENE HD reagent (Roche Diagnostics). The empty plasmid, pcDNA3.1, was used for mock transfection.

In Vitro Kinase Assay. After preincubation of recombinant human Fyn (Millipore, Billerica, MA) in Tris-HCl buffer (50 mM, pH 7.5) containing KVEKIGEGTYGVVYK (Cdc2 peptide, 250 μM), 0.1 mM EGTA, 0.1 mM Na_3VO_4, and 10 mM magnesium acetate, the reaction was initiated with the addition of a mixture containing 70 μM Mg^{2+}ATP and [γ-^{32}P]ATP (specific activity, ~500 cpm/pmol), at room temperature. After incubation for 40 min, the reaction was stopped with the addition of 3% phosphoric acid solution. An aliquot of the reaction mixture (10 μl) was spotted onto a P30 filtermat (PerkinElmer Life and Analytical Sciences, Waltham, MA), which was washed three times with 75 mM phosphoric acid for 5 min and once with methanol before drying and scintillation counting.

Data Analysis. One-way analysis of variance tests were used to assess the significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman-Keuls test was used for comparisons between multiple group means. The data are expressed as mean ± S.E.M. The criterion for statistical significance was set at $p < 0.05$ or $p < 0.01$.

Results

Increases in Cell Survival after Oxidative Injury. Among the newly synthesized dithiolethiones, 11 CDTs and other 1,2-dithiole-3-thiones were selected for comparison of their effects on cell survival in vitro (Fig. 1A); we determined their effects on the viability of HepG2 cells with oxidative injury elicited by AA and iron, by using MTT assays. The ED_{50} values for cell survival were in the range of 0.5 to 10 μM (Fig. 1B). Among those representing each basic core structure, 4,5,6,7-tetrahydrobenzo-1,2-dithiole-3-thione (SNU1A) and 5,6-dihydro-4*H*-cyclopenta-1,2-dithiole-3-thione (SNU2A) were the most effective, whereas others showed lesser or minimal effects (Fig. 1B). A bulky substitution at the 5-position of 1,2-dithiole-3-thione resulted in complete loss of activity. In subsequent experiments, we compared the effects of SNU1A, SNU2A, 5-methyl-1,2-dithiole-3-thione (SNU3A), and 5-(quinolin-2-yl)-1,2-dithiole-3-thione (SNU3E). SNU3E was used as the least-active control compound (Fig. 2A).

The dose-response relationships for effects are depicted in Fig. 2A. SNU1A, SNU2A, SNU3A, and SNU3E had no cytotoxicity (Fig. 2A, inset). Morphological examination with light microscopy confirmed that SNU1A treatment had an obvious protective effect against injury (Fig. 2B). The agent alone showed no toxicity. To confirm an increase in cell viability with the agents, levels of apoptotic marker proteins were measured in the lysates. AA plus iron decreased the level of poly(ADP-ribose)polymerase but increased that of cleaved caspase-3, which indicates apoptosis (Fig. 2C). Treatment with SNU1A, SNU2A, or SNU3A protected cells from apoptosis (Fig. 2C). SNU3E had no effect. Our results showed that CDTs, including SNU1A, SNU2A, and SNU3A, have the ability to rescue cells from oxidative stress elicited by AA and iron.

Mitochondrial Protective Effects. AA treatment causes dysfunction of MMP through impairment of mitochondrial respiratory activity (i.e., complex I and III binding) (Cocco et al., 1999). Excess iron accumulation and the resultant ROS production impair mitochondrial function (George et al., 1998; Kumar and Bandyopadhyay, 2005). MMP was measured with FACS analysis after staining of HepG2 cells with Rh123 (a probe for MMP). Treatment of the cells with AA plus iron decreased the proportion of Rh123-positive cells and caused an increase in the M1 fraction (i.e., the fraction with low Rh123 fluorescence intensity) (Fig. 3A). SNU1A, SNU2A, and SNU3A effectively abolished the mitochondrial dysfunction, whereas SNU3E failed to do so. Because mito-

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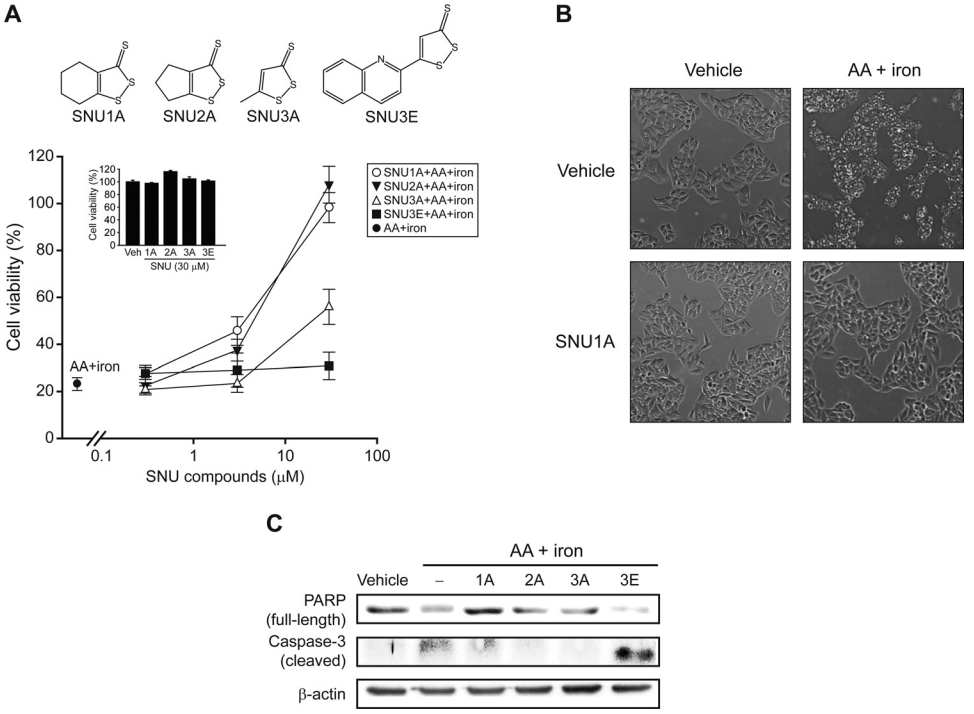

Fig. 2. Inhibition of apoptotic injury by dithiolethiones. A, MTT assays. HepG2 cells were treated with 0.3 to 30 μM concentrations of each dithiolethione congener (top) for 1 h and were continuously incubated with 10 μM AA for the next 12 h. After washing, the cells were challenged with 5 μM iron for 6 h with the agent of interest. Cells were also incubated for 19 h with each agent alone (inset). Cell viability was measured by using MTT assays. Data represent the mean ± S.E.M. of six replicates. B, representative photographs of cell morphological features. Light microscopy shows the morphological features of cells treated as described above. Original magnification, 200×. Images were inverted for visual clarity. C, immunoblotting assays for proteins associated with apoptosis. Proteins were immunoblotted in the cell lysates. PARP, poly(ADP-ribose)polymerase.

chondrial dysfunction disturbs oxidative phosphorylation and depletes ATP with ROS generation (Shin and Kim, 2009), we assessed whether treatment with the agents inhibited mitochondrial superoxide production by using MitoSOX, a mitochondrial superoxide indicator. MitoSOX fluorescence levels in the mitochondria were remarkably increased with AA plus iron treatment; this was inhibited completely by SNU1A and partially by SNU3A (Fig. 3B). SNU1A and SNU2A most effectively protected the mitochondria from oxidative injury and inhibited superoxide overproduction. In addition, AA plus iron treatment arrested cell-cycle progression in the sub-G_1 phase, which was relieved by SNU1A but not by SNU3E (Fig. 3C). Each of these agents alone did not change the cell-cycle progression (data not shown).

AMPK-Dependent Mitochondrial Protection. AMPK plays a role in sensing intracellular energy status and is activated by various stresses, such as an increased AMP/ATP ratio. In HepG2 cells, each agent showed the ability to activate AMPK, as evidenced by an increase in the phosphorylation of AMPK or its downstream enzyme ACC (Fig. 4A). Among the agents, SNU1A was the most efficacious. GSK3β, a serine/threonine kinase that is constitutively active in the normal state, regulates cell viability against oxidative injury by promoting mitochondrial permeability transition pore-opening (Kockeritz et al., 2006). It was reported that resveratrol inhibits GSK3β through phosphorylation as a downstream substrate of AMPK (Shin et al., 2009). We examined the effects of CDTs on the serine phosphorylation of GSK3β. As expected, treatment with each agent increased the phosphorylation of GSK3β (Fig. 4B); SNU1A was the most effective. Ad-DN-AMPK infection decreased the ability of SNU1A to inhibit MMP transition (Fig. 4C). In addition, it decreased the basal phosphorylation of ACC and GSK3β, as well as their SNU1A-inducible phosphorylation (Fig. 4D). All of these results demonstrate that CDTs have the ability to activate AMPK, which may be responsible for the inhibition of GSK3β for mitochondrial protection.

Role of Fyn Inhibition in Mitochondrial Protection. In an effort to find the underlying basis for the antioxidant effect, we determined whether CDTs affect the activity of Fyn by monitoring its tyrosine phosphorylation. One active phosphorylation site in Fyn at a tyrosine residue (Tyr420) has been identified (Pariser et al., 2005). Exposure of HepG2 cells to AA plus iron caused an increase in the phosphorylation of Fyn, which was attenuated by SNU1A, SNU2A, and SNU3A (Fig. 5A). SNU3E showed a weaker effect. SU6656, a known Fyn inhibitor, also inhibited the phosphorylation of Fyn. Moreover, SNU1A inhibited the basal activity of Fyn, as did SU6656 (Fig. 5B). Next, we assessed whether the CDTs had direct inhibitory effects on Fyn by using an in vitro kinase assay; unlike SU6656, they failed to inhibit Fyn directly (Fig. 5C), which suggests that there may exist an upstream regulator of Fyn that is affected by the agents. To test the role of Fyn inhibition by SNU1A in protecting mitochondria from oxidative injury, the effect of Fyn overexpression on the MMP change was measured after Rh123 staining. Fyn overexpression reversed the protective effect of SNU1A against mitochondrial injury (Fig. 5D). The CDTs had the ability to inhibit Fyn phosphorylation, which might be associated with their mitochondrial protective effects. For a deeper understanding of the mechanism underlying the antioxidant effect, we determined whether the agents activated AMPK through Fyn inhibition. Enforced expression of Fyn decreased the phosphorylation of AMPKα or ACC increased by SNU1A (Fig. 5E), which suggests that Fyn inhibition by the agents contributes to activation of AMPK.

Enhancement of Antioxidant Capacity. Next, we analyzed the level of reduced GSH in HepG2 cells treated with AA plus iron in combination with the agents. The level of GSH was decreased significantly by AA plus iron treatment, which was prevented by simultaneous treatment with SNU1A, SNU2A, or SNU3A (Fig. 6A, left). As expected, SNU3E had no effect. We also found that each CDT treatment alone increased GSH levels (Fig. 6A, right). To confirm

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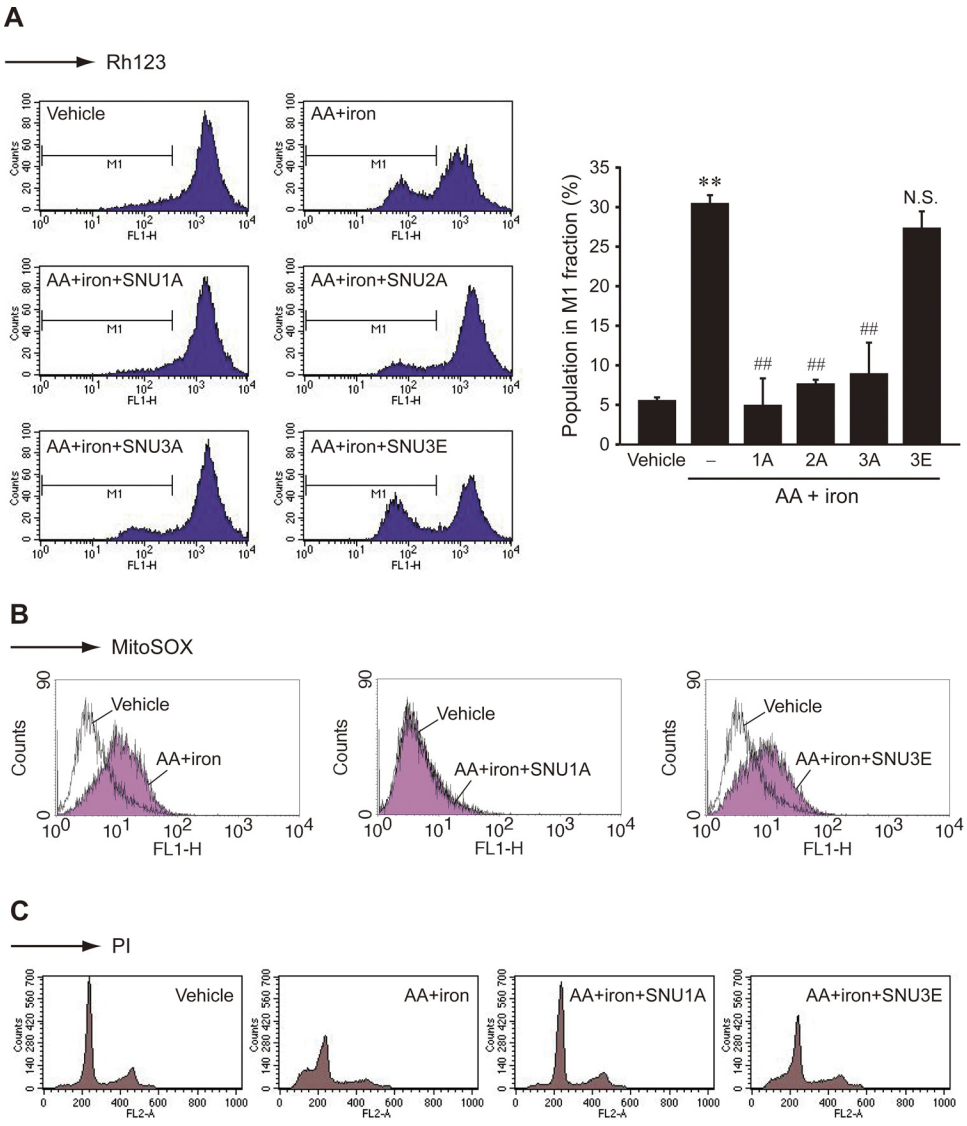

Fig. 3. Prevention of mitochondrial injury by dithiolethiones. A, MMP. HepG2 cells were challenged with 30 μM concentrations of each agent for 1 h, followed by incubation with AA (12 h) and iron (6 h). Cells were collected after Rh123 staining. The M1 fraction indicates cells with low-intensity Rh123 fluorescence. The percentage of the M1 population was quantified. Data represent the mean ± S.E.M. of three separate experiments. The statistical significance of differences between treated cells and either vehicle-treated control cells (**, $p < 0.01$) or cells treated with AA plus iron (##, $p < 0.01$) was determined. N.S., not significant. B, superoxide production in mitochondria. Cells were exposed to 30 μM concentrations of each dithiolethione congener for 1 h and then to 10 μM AA for 12 h, followed by incubation with 5 μM iron for 6 h, and cells were stained with MitoSOX. Increases in fluorescence indicate mitochondrial superoxide production. Results were confirmed with repeated experiments. C, analyses of cell-cycle progression with flow cytometry. Cells were treated as described above. PI, propidium iodide.

the antioxidant effect, the extent of intracellular H_2O_2 production was measured with a flow cytometric assay using DCFH-DA, a specific marker for H_2O_2. Treatment with SNU1A, SNU2A, or SNU3A (30 μM each) abrogated the increase in H_2O_2 production caused by AA plus iron (Fig. 6B). SNU3E had no effect. Our findings also indicated that the combination of Fyn overexpression and compound C (an AMPK inhibitor) treatment significantly decreased the increase in GSH content produced by SNU1A (Fig. 6C), which confirms the regulatory effect of the kinases on the antioxidant effect.

Nrf2-Antioxidant Response Element Activation. In a continuing effort to determine the molecular basis for the cytoprotective effect, we examined the level of Nrf2, a critical transcription factor responsible for antioxidant gene expression. Prooxidants or low levels of ROS may activate Nrf2 (Nguyen et al., 2004). Among the agents examined, only SNU2A showed a slight increase in ROS production when used alone, which indicated that it has a prooxidant effect (Fig. 7A). Consistently, SNU2A increased the level of Nrf2 most effectively, as shown by increases in Nrf2 band intensity and ARE-driven reporter activity (Fig. 7, B and C). SNU1A and SNU3A showed no prooxidant effect but increased Nrf2 levels. SNU3E had no effect. Ad-DN-AMPK infection prevented the increases in Nrf2 levels and NQO1-ARE gene transactivation produced by SNU1A (Fig. 7D), which supports the role of AMPK in Nrf2 activation. In an effort to link Fyn and Nrf2, we monitored the effect of Fyn overexpression on the increase in Nrf2 levels produced by SNU1A; Fyn overexpression prevented both the accumulation of Nrf2 and the induction of the ARE reporter gene (Fig. 7E). These results provide evidence that the ability of SNU1A to increase Nrf2 activity may result at least in part from Fyn inhibition leading to AMPK activation.

Discussion

Oxidative stress induces mitochondrial permeability transition and alters the integrity of membrane phospholipids, including AA. The oxidation of fatty acids and phospholipids activates phospholipase. AA, a ω-6 proinflammatory fatty acid originating from cell membranes, stimulates ROS production, augmenting lipid peroxidation. In fact, increases in the ω-6/ω-3 fatty acid ratio were detected in patients with cancer, cardiovascular disease, or hepatitis (Araya et al., 2004; Dwyer et al., 2004; Simopoulos, 2006). Oxidative stress

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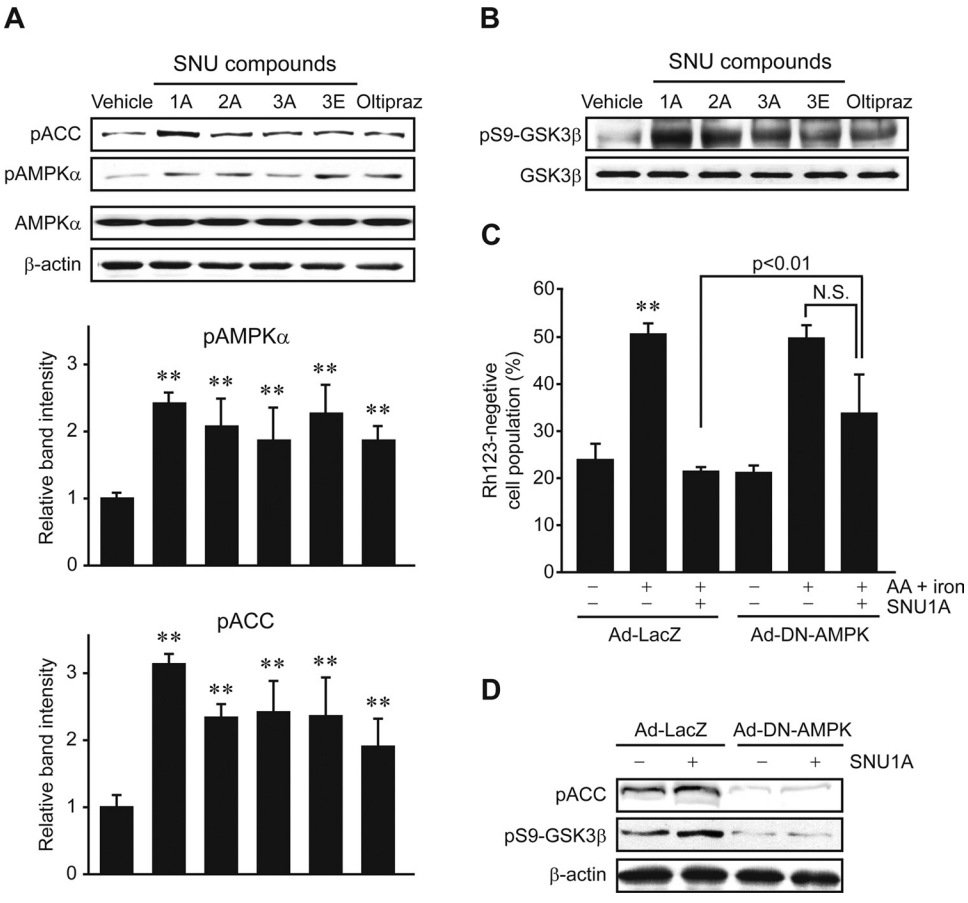

Fig. 4. Role of AMPK activation by CDTs in mitochondrial membrane permeability. A, AMPK activation. Immunoblot analyses were performed with lysates of cells treated with 30 μM concentrations of each agent for 1 h. Oltipraz (30 μM) was used as a positive control. pAMPKα, phosphorylated AMPKα; pACC, phosphorylated ACC. B, inhibitory phosphorylation of GSK3β. C, reduced mitochondrial protective effect with DN-AMPK. After infection with adenoviral DN-AMPK for 12 h, HepG2 cells were treated with SNU1A (10 μM) for 1 h and were continuously exposed to AA plus iron. The Rh123-negative cell population was measured in the cells. For A and C, data represent the mean ± S.E.M. of four separate experiments. **, $p < 0.01$, significant difference, compared with respective vehicle-treated control cells. N.S., not significant. D, immunoblotting assays for phosphorylated ACC or phosphorylated GSK3β. Immunoblotting assays were performed with lysates of HepG2 cells treated with SNU1A for 12 h after infection with Ad-LacZ or Ad-DN-AMPK.

elicited by AA may have detrimental effects on mitochondria (Cocco et al., 1999; Scorrano et al., 2001); it also increases the levels of proapoptotic ceramides and calcium uptake into mitochondria. Iron causes organ damage and dysfunction. In particular, the liver is susceptible to excess iron because it is the main organ for storage (McLaren et al., 1995). In an early pathological process, excess iron accumulation in nonparenchymal cells and redistribution toward surrounding cells enhance oxidative stress. Therefore, the presence of iron synergizes the deleterious effect of AA, provoking apoptosis. In the present study, a series of CDTs and other congeners had the ability to enhance antioxidant capacity and to rescue mitochondria from oxidative injury elicited by AA plus iron. In our findings, the CDTs exhibited greater potencies in protecting cells from oxidative injury, compared with other dithiolethiones, which suggests that they may have beneficial effects in oxidative and inflammatory diseases.

Compounds with the core dithiolethione structure have chemopreventive properties, as shown by the results of animal and clinical studies (Zhang and Munday, 2008; Kensler and Wakabayashi, 2010). Efforts have been made to develop dithiolethione derivatives with better efficacy; aryldithiolethione compounds exert an anticancer effect through histone deacetylation (Tazzari et al., 2010), whereas anethole dithiolethiones may yield chemopreventive effects by inhibiting the aryl hydrocarbon receptor pathway (Bass et al., 2009). Dithiolethiones fused with nonsteroidal anti-inflammatory drugs were studied as antiangiogenic agents (Isenberg et al., 2007). Oltipraz, a prototypical dithiolethione, is oxidized by two major pathways common to various mammals; i.e., oxidative desulfuration of the thione to yield the M1 metabolite and desulfuration, methylation, and molecular rearrangement to yield the M2 metabolite (Bieder et al., 1983; O'Dwyer et al., 1997). Because biotransformation of dithiolethiones causes variability in the pharmacokinetic profiles in humans (O'Dwyer et al., 2000), their efficacy and/or metabolic stability may be increased with different ring structures. Cyclized compounds generally show less vulnerability to metabolic assault (Thompson, 2001). Therefore, the CDTs may have an advantage to bypass biotransformation with ring rearrangement.

AMPK regulates energy metabolism and increases cell viability in response to pathological stresses such as endoplasmic reticulum stress, oxidative stress, and osmotic stress (Hayashi et al., 2000; Ido et al., 2002; Terai et al., 2005; Bae et al., 2008; Shin et al., 2009). Oxidative stress increases the activity of GSK3β and facilitates its mitochondrial translocation; activated GSK3β phosphorylates and binds to the elements of mitochondrial membrane pores (e.g., voltage-dependent anion channel and adenine nucleotide translocase), inducing MMP transition (Nishihara et al., 2007; Das et al., 2008). AMPK contributed to protecting mitochondria by inhibiting GSK3β activity (Shin et al., 2009). Oltipraz has the ability to prevent mitochondrial impairment from oxidative stress by activating AMPK (Shin and Kim, 2009). Other agents, such as resveratrol and isoliquiritigenin, also inhibited superoxide production in mitochondria and protected them through GSK3β phosphorylation downstream of AMPK (Shin et al., 2009; Choi et al., 2010). Our present finding that CDTs inhibited GSK3β through AMPK activation corroborated the inhibitory effect of AMPK on GSK3β in the protection of mitochondria.

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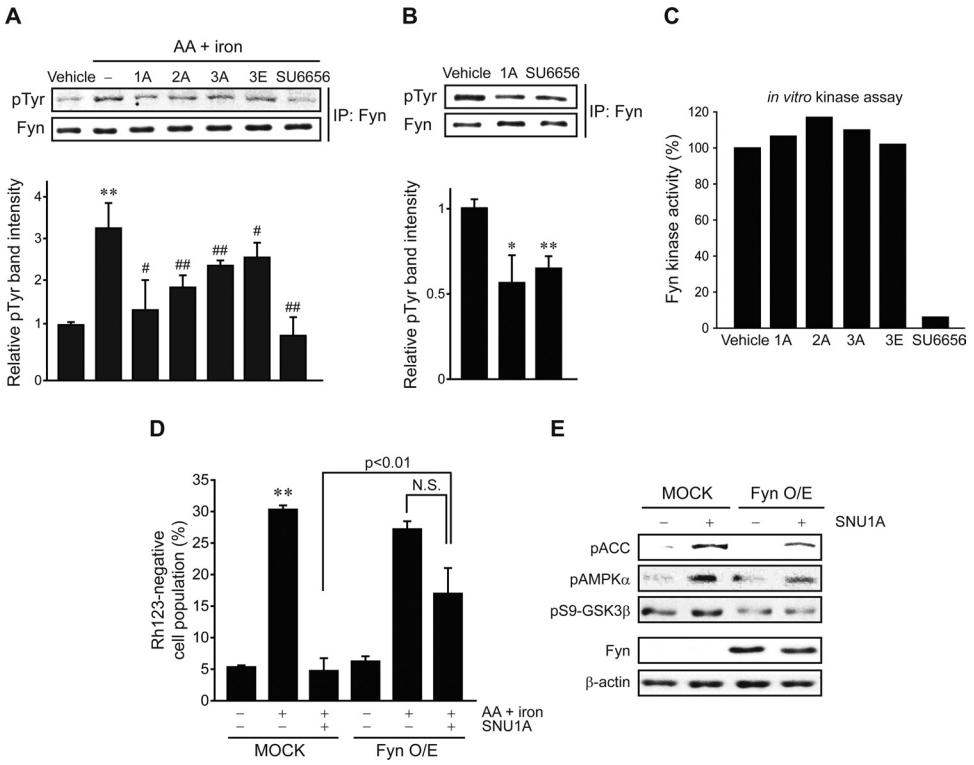

Fig. 5. Fyn inhibition by dithiolethiones for mitochondrial protection. A, inhibition of Fyn phosphorylation. Fyn immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. SU6656, a Fyn inhibitor, was used as a positive control. Cells were treated with each 30 μM dithiolethione congener or 10 μM SU6656 and then were exposed to 10 μM AA for 12 h, followed by incubation with 5 μM iron for 30 min. pTyr, phosphotyrosine; IP, immunoprecipitation. B, inhibition of basal Fyn phosphorylation. Cells were treated with 30 μM SNU1A or 10 μM SU6656 for 1 h. C, in vitro kinase assays. Experimental details were described under *Materials and Methods*. The data represent the means of duplicate determinations. D, reversal with Fyn overexpression of decreases in the Rh123-negative cell population. After Fyn overexpression, HepG2 cells were incubated with SNU1A (10 μM) for 1 h and were continuously exposed to AA (12 h) plus iron (6 h). The Rh123-negative cell population was analyzed as described for Fig. 3A. Data represent the mean ± S.E.M. of three separate experiments. E, reversal with Fyn overexpression of SNU1A effects on AMPK activation. After Fyn transfection, cells were treated with 10 μM SNU1A for 1 h. O/E, overexpression; pACC, phosphorylated ACC; pAMPKα, phosphorylated AMPKα. For A, B, and D, the statistical significance of differences between treated cells and either vehicle-treated control cells (*, $p < 0.05$; **, $p < 0.01$) or cells treated with AA plus iron (#, $p < 0.05$; ##, $p < 0.01$) are noted. N.S., not significant.

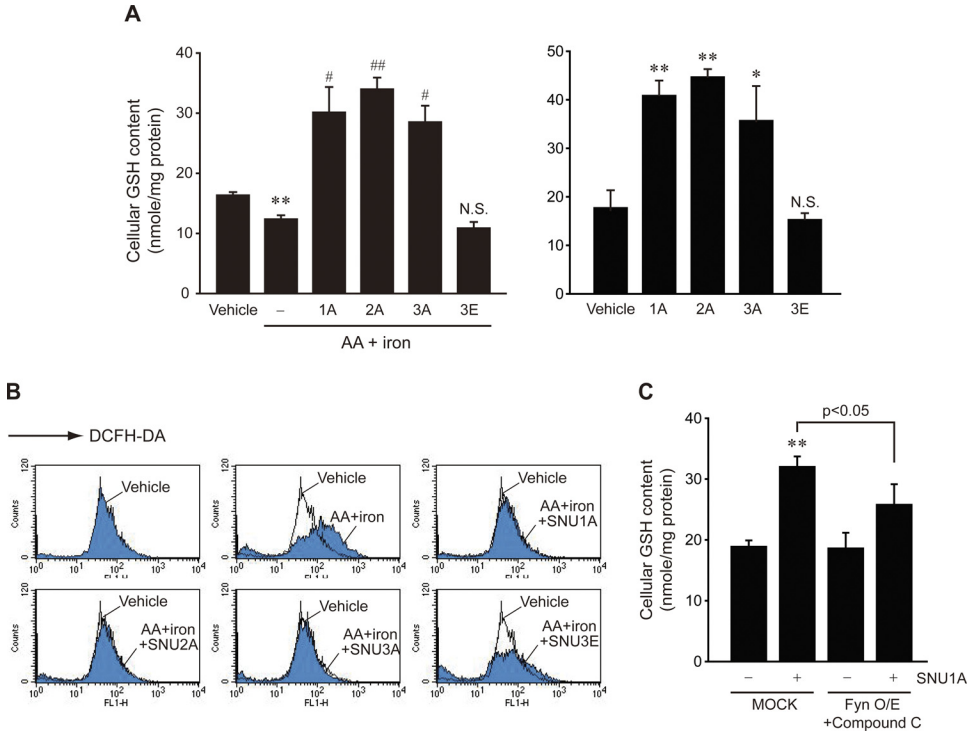

Fig. 6. Enhancement of antioxidant capacity by dithiolethiones. A, cellular GSH contents. The GSH contents were assessed in HepG2 cells treated as described for Fig. 2A (left). Cells were also treated with 30 μM concentrations of each agent alone for 18 h (right). B, dichlorofluorescein fluorescence assays. H_2O_2 production was monitored by using dichlorofluorescein fluorescence. HepG2 cells were treated with 30 μM concentrations of each agent for 1 h and were incubated with AA (12 h) and iron (6 h). Results were confirmed with three separate experiments. C, effects of Fyn overexpression and compound C treatment on the increase in GSH contents. Cells were transfected with mock or Fyn vectors and were continuously exposed to 10 μM SNU1A (18 h), with or without 10 μM compound C pretreatment (1 h). O/E, overexpression. For A and C, data represent the mean ± S.E.M. of three separate experiments. The statistical significance of differences between treated cells and either vehicle-treated control cells (*, $p < 0.05$; **, $p < 0.01$) or cells treated with AA plus iron (#, $p < 0.05$; ##, $p < 0.01$) was determined. N.S., not significant.

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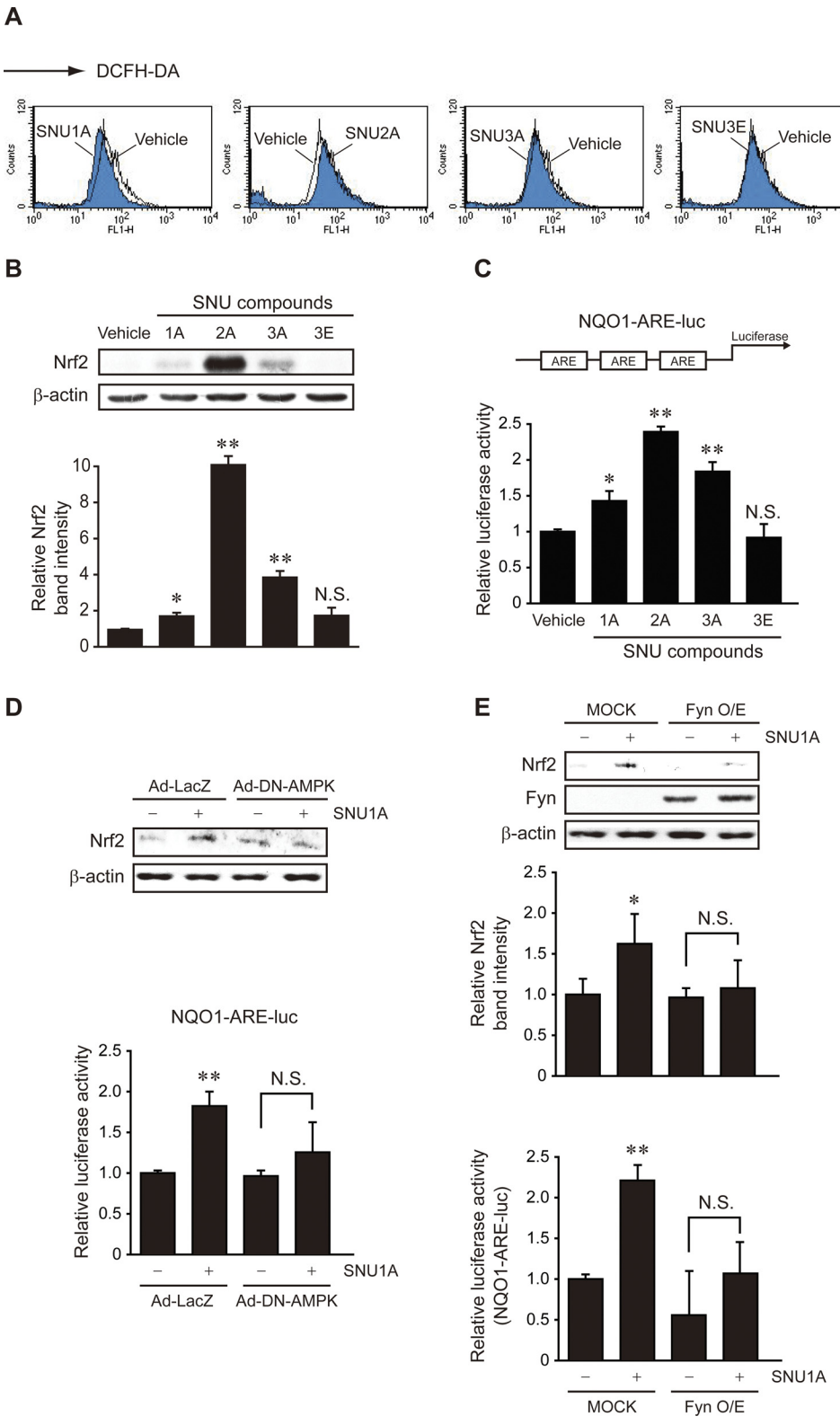

Fig. 7. Nrf2-ARE activation by dithiolethiones. A, dichlorofluorescein fluorescence assays. HepG2 cells were treated with 30 μM concentrations of each agent alone for 6 h. Results were confirmed with three separate experiments. B, immunoblotting assays for Nrf2. Nrf2 was immunoblotted in the lysates of cells treated with each agent for 12 h. The band intensity of Nrf2 relative to that of β-actin was assessed through scanning densitometry of the immunoblots. C, NQO1-ARE reporter gene assays. The luciferase reporter activity was measured in cells that had been treated with SNU compounds for 12 h. D, effects of Ad-DN-AMPK on Nrf2 and ARE reporter activity. HepG2 cells were treated as described for Fig. 4D (top). After adenoviral DN-AMPK infection, cells were treated with SNU1A for 12 h (bottom). E, effects of Fyn overexpression on Nrf2 and ARE reporter activity. Cells were treated as described above. For C, D, and E, values represent the mean ± S.E.M. of five independent experiments. *, $p < 0.05$; **, $p < 0.01$, treatment mean significantly different from vehicle-treated control mean. N.S., not significant.

Fyn, a member of the Src kinase family, is a constitutively expressed, membrane-localized, nonreceptor, tyrosine kinase. In the present study, the new dithiolethiones protected mitochondria by activating AMPK. Our result also shows that Fyn is an upstream molecule that controls AMPK activity, which is consistent with the observation that chemical inhibition of Fyn led to AMPK activation (Bastie et al., 2007). Under conditions of oxidative stress or osmotic shock, Fyn stimulates mitogen-activated protein kinase pathways (Abe and Berk, 1999). In our work, the negative regulation of AMPK by Fyn was inhibited by the CDTs with antioxidant activity. This is in line with the observation that Fyn-knockout altered ROS-induced signaling pathways (Abe and Berk, 1999). Our finding that Fyn overexpression and compound C (AMPK inhibitor) treatment signifi-

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cantly diminished the increase in GSH content produced by SNU1A supports the roles of Fyn inhibition and AMPK activation in its antioxidant activity. Fyn overexpression or AMPK inhibition alone failed to reverse significantly the effect of SNU1A, which suggests that AMPK may affect Fyn activity in a feedback loop.

There are some known regulators of Fyn kinase. Fyn phosphorylation at the Tyr420 residue (a major active phosphorylation site) depends on autophosphorylation. Phosphorylation at the Tyr531 residue by c-Src tyrosine kinase inhibits Fyn, whereas dephosphorylation at the same site by protein tyrosine phosphatase, receptor type, C/CD45 activates the overall activity (Peters et al., 1990; Mustelin et al., 1992). Because Fyn activity is associated with membrane receptor signaling (Parsons and Parsons, 2004; Sandilands et al., 2007; Lu et al., 2009), SNU compounds may modulate the upstream receptor signals (e.g., receptor tyrosine kinase). These possibilities remain to be clarified.

Despite the known effects of Fyn on cell growth and survival, little information had been available regarding its effect on mitochondria. An important finding of our study is the identification of Fyn activation along with mitochondrial dysfunction; activated Fyn seems to cause impairment of mitochondrial function and to facilitate apoptosis. In the present study, the ability of new dithiolethiones to decrease Fyn phosphorylation contributed to the protection of cells from oxidative stress, as supported by the finding that Fyn overexpression decreased the mitochondrial protective effect. Our results match the finding that a deficiency in Fyn decreased caspase activation and DNA fragmentation with proapoptotic stimuli in embryonic fibroblasts and thymocytes (Ricci et al., 2001; Donlin et al., 2002). The observation that SU6656 (a Fyn inhibitor) inhibited GSK3β through phosphorylation (data not shown) supports this hypothesis.

In the current study, CDTs (i.e., SNU1A and SNU2A) inhibited H_2O_2 production. Treatment with each of the agents enabled cells to restore GSH contents. Many AMPK activators may also enhance Nrf2 activity (Kay et al., 2010, 2011). Similarly, AMPK activation increases Nrf2 expression levels (Liu et al., 2011). The possibility that Nrf2 protects mitochondria from oxidative stress irrespective of the level of AMPK phosphorylation is strengthened by our observation that metformin, an AMPK activator that lacks Nrf2-activating effects, failed to alleviate oxidative injury caused by AA plus iron (S. M. Shin and S. G. Kim, unpublished data). Overall, it is highly likely that the antioxidant capacities of CDTs parallel their abilities to activate Nrf2. In the present study, the degree of Nrf2 activation produced by SNU2A was greater than that produced by others, which may be associated with its effect on mitochondrial superoxide production (Fig. 8).

Protein kinase C, Akt, and protein kinase R-like endoplasmic reticulum kinase activate Nrf2 through phosphorylation (Nguyen et al., 2004). In contrast, Nrf2 phosphorylation by GSK3β decreases Nrf2 activity (Salazar et al., 2006). Our results support the notion that Nrf2 activation caused by CDTs may be linked to GSK3β inhibition, as suggested by the finding that DN-AMPK overexpression decreased the ability of SNU1A to enhance Nrf2 activity. However, the degree of Nrf2 activation produced by SNU2A was much greater than that produced by other agents, despite their comparable inhibitory effects on Fyn. SNU2A has a prooxidant effect, which may account for its greater activation of Nrf2, because prooxidants and low levels of ROS activate Nrf2 through Keap1 oxidation (Nguyen et al., 2004). This also may account for the discrepancy between its AMPK activation and cell survival effects.

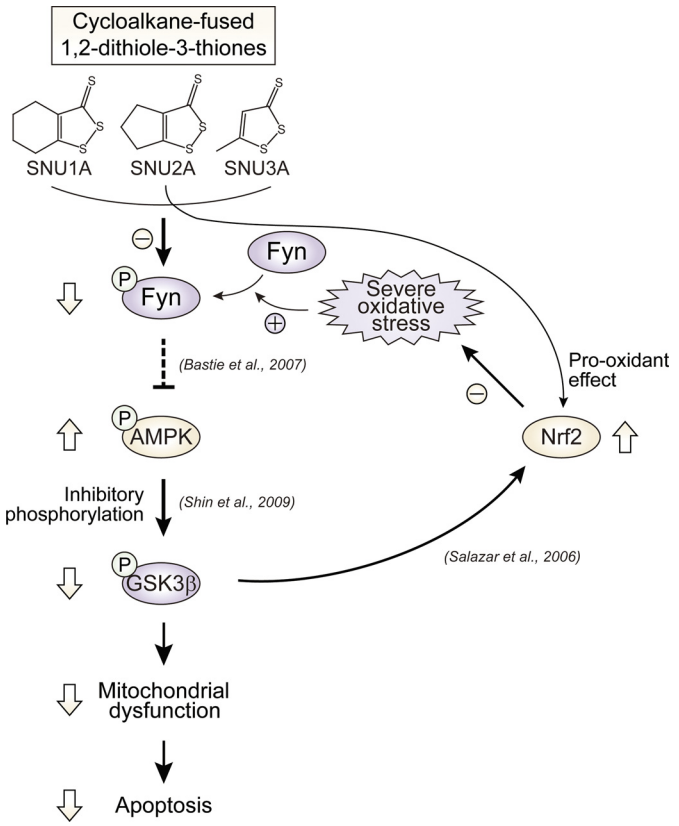

Fig. 8. Schematic diagram illustrating the proposed mechanism through which cycloalkane-fused dithiolethiones protect cells from oxidative injury.

We found CDTs to be cytoprotective agents against oxidative stress, and their beneficial effects may result from improvement of mitochondrial function through Fyn inhibition, which leads to AMPK activation, and GSK3β inhibition, which leads to Nrf2 activation (Fig. 8). Some of the agents have additional stimulating effects on Nrf2, which further increases antioxidant capacity. Because the new dithiolethiones have the capability to protect cells from oxidative injury, they might be used as preventive and/or therapeutic candidates for chronic inflammatory illnesses.

Authorship Contributions

Participated in research design: Koo, W. H. Lee, and Kim.
Conducted experiments: Koo and C. G. Lee.
Contributed new reagents or analytic tools: Koo, W. H. Lee, and Kim.
Performed data analysis: Koo and Kim.
Wrote or contributed to the writing of the manuscript: Koo, W. H. Lee, and Kim.

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