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Resveratrol protects mitochondria against oxidative stress through AMPK-mediated GSK3β inhibition downstream of poly(ADP-ribose)polymerase-LKB1 pathway

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Running title: PARP-LKB1-dependent mitochondrial protection

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The abbreviations used are:

AA, arachidonic acid; 3-AB, 3-aminobenzamide; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; CaMKK, calcium/calmodulin-dependent kinase kinase; DCFH-DA, 2',7'-dichlorofluorescin diacetate; GSK3β, glycogen synthase kinase 3β; MMP, mitochondrial membrane potential; MnTBAP, Mn(III) tetrakis 4-benzoic acid porphyrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PAR, poly(ADP-ribose)polymer; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; Rh123, rhodamine 123; ROS, reactive oxygen species; SOD, superoxide dismutase

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ABSTRACT

Arachidonic acid (AA, a proinflammatory fatty acid) in combination with iron promotes excess reactive oxygen species (ROS) production and exerts a deleterious effect on mitochondria. Previously, we showed that activation of AMP-activated protein kinase (AMPK) protects hepatocytes from AA+iron-induced apoptosis. Resveratrol, a polyphenol in grapes, has beneficial effects mediated through SIRT1, LKB1, and AMPK. This study investigated the potential of resveratrol to protect against the mitochondrial impairment induced by AA+iron and the underlying mechanism for this cytoprotection. Resveratrol treatment inhibited apoptosis, ROS production, and GSH depletion elicited by AA+iron in HepG2 cells. Also, resveratrol attenuated superoxide generation in mitochondria and inhibited mitochondrial dysfunction induced by AA+iron. Overall, AMPK activation by resveratrol contributed to cell survival, as supported by the reversal of its restoration of mitochondrial membrane potential by either overexpression of a dominant negative mutant of AMPKα or compound C treatment. Resveratrol increased inhibitory phosphorylation of glycogen synthase kinase-3β (GSK3β) downstream of AMPK, which contributed to mitochondrial protection and cell survival. Likewise, siRNA knockdown of LKB1, an upstream kinase of AMPK, reduced the ability of resveratrol to protect cells from mitochondrial dysfunction. Furthermore, this LKB1-dependent mitochondrial protection resulted from resveratrol's poly(ADP-ribose)polymerase activation, but not SIRT1 activation, as supported by the experiment using 3-aminobenzamide, a poly(ADP-ribose)polymerase inhibitor. Other polyphenols, such as apigenin, genistein, and daidzein, did not activate AMPK, nor did they protect mitochondria against AA+iron. Thus, resveratrol protects cells from AA+iron-induced ROS production and mitochondrial dysfunction through AMPK-mediated inhibitory phosphorylation of GSK3β downstream of poly(ADP-ribose)polymerase-LKB1 pathway.

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INTRODUCTION

Excess oxidative stress causes cell and tissue injury via the modification of membrane phospholipids (Browning and Horton, 2004). The oxidation of fatty acids in phospholipids by reactive oxygen species (ROS) or by the response to proinflammatory cytokines may activate phospholipases (Balboa and Balsinde, 2006). Oxidative modification of fatty acids and phospholipids triggers inflammatory processes and, thus, exerts detrimental effects on cell signaling. In particular, phospholipase A_2 promotes the release of arachidonic acid (AA), a ω-6 polyunsaturated fatty acid, from membrane phospholipids (Balboa and Balsinde, 2006). AA then contributes to further oxidative stress. In addition, AA in the presence of iron (a catalyst of auto-oxidation) leads cells to produce excess ROS and elicits mitochondrial dysfunction (Shin and Kim, 2009). Therefore, the combinatorial treatment of AA and iron exerts a negative effect on cell viability, and this treatment may be used to study potential cytoprotective agents targeting mitochondria against severe oxidative stress.

Several lines of evidence indicate that resveratrol (3,4′,5-trihydroxystilbene), a polyphenolic component in grapes and red wine, has diverse beneficial actions, such as protecting cells and tissues against neurodegeneration, cardiovascular disease, cancer, diabetes, and obesity-related disorders (Baur and Sinclair, 2006), and extending lifespan of organisms (Howitz et al., 2003). This wide range of biological effects might be explained in part by resveratrol's antioxidant properties, including increases in catalase and superoxide dismutase (SOD) activity (Rubiolo et al., 2008). In addition, the antioxidant capacity of resveratrol might be mediated by the induction of phase II enzymes via NF-E2-related factor-2 (e.g., NADPH-quinone oxidoreductase) (Rubiolo et al., 2008). The antioxidant effect of resveratrol has been claimed to be due to the presence of hydroxyl phenolic groups in its chemical structure (Leonard et al., 2003); however, direct scavenging activity cannot account for all cytoprotective efficacy due to its low bioavailability and weak ability to scavenge ROS (Leonard et al., 2003; Sale et al., 2004). More likely, resveratrol may act through specific cell signaling pathways that lead to activation of the defensive and cytoprotective systems.

AMP-activated protein kinase (AMPK, an intracellular sensor of energy status) is activated to reserve cellular energy content, and serves as a key regulator of cell survival or death in response to pathological stress (e.g., oxidative stress, endoplasmic reticulum stress, hypoxia, and osmotic stress) (Hayashi et al., 2000; Terai et al., 2005; Shin and Kim, 2009). This regulatory role is supported by increases in cell viability with treatment by the AMPK activators including 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (Ido et al., 2002). Previous work showed that, through

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AMPK activation, dithiolethiones protect hepatocytes from AA+iron-induced apoptosis (Shin and Kim, 2009). Although the biological effects of resveratrol have been diversely studied, it is not yet clear whether the cytoprotective effect of resveratrol against ROS is mediated by AMPK activation. Moreover, the potential cytoprotective effect of resveratrol against mitochondrial impairment has not been explored.

Studies from several laboratories have shown that the beneficial effects of resveratrol result from the activation of SIRT1, LKB1, and/or AMPK (Howitz et al., 2003; Hou et al., 2008). In mammalian cells, the upstream kinases of AMPK include LKB1, calcium/calmodulin-dependent kinase kinase (CaMKK), and transforming growth factor β-activated kinase-1 (Lage et al., 2008). Recently, several signaling pathways including SIRT1, NO, PKA, and poly(ADP-ribose)polymerase (PARP) were identified as upstream pathways which regulate LKB1 activity (Alessi et al., 2006; Hou et al., 2008; Huang et al., 2009; Vázquez-Chantada et al., 2009). However, it remains to be elucidated which upstream signaling pathway regulates the beneficial effect of resveratrol under conditions of oxidative stress. In addition, it is unclear what molecule or component downstream of AMPK contributes to mitochondrial protection against oxidative stress.

In view of the importance of AMPK in the action of resveratrol, this study investigated whether resveratrol is capable of protecting mitochondria against the severe oxidative stress induced by AA+iron and, if so, whether this compound has the ability to prevent apoptosis. Our work demonstrates, for the first time, that resveratrol protects against AA+iron-induced oxidative stress through the inhibition of mitochondrial impairment and ROS production: this cytoprotective effect is mediated by AMPK-dependent GSK3β serine phosphorylation downstream from LKB1. Moreover, we revealed that this particular cytoprotective effect of resveratrol against oxidative stress is dependent of PARP activation, which leads to LKB1 activation. Additional work compared the effect of other polyphenolic compounds on AMPK and their efficacy on the mitochondrial dysfunction induced by AA and iron.

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MATERIALS AND METHODS

Materials MitoSOX was supplied by Invitrogen (Carlsbad, CA). Anti-procaspase-3, anti-phospho-acetyl-CoA carboxylase (ACC), anti-phospho-AMPK, anti-AMPK, anti-phospho-LKB1, anti-phospho-GSK3β, and anti-GSK3β antibodies were obtained from Cell Signaling Technology (Beverly, MA). Antibodies directed against PARP, Bcl-xL, Bcl-2, and LKB1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-poly(ADP-ribose) (PAR) antibody was supplied by BD Bioscience (San Jose, CA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were purchased from Zymed Laboratories (San Francisco, CA). Mn(III) tetrakis 4-benzoic acid porphyrin (MnTBAP), SB216763, L-nitro-arginine-methyl-ester (L-NAME), and compound C were obtained from Calbiochem (Darmstadt, Germany). DeadEnd™ Colorimetric TUNEL System was supplied from Promega (Madison, WI). Resveratrol, AA, ferric nitrate, nitrilotriacetic acid, AICAR, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), rhodamine123 (Rh123), propodium iodide (PI), 2',7'-dichlorofluorescin diacetate (DCFH-DA), anti-β-actin antibody, trolox, 3-aminobenzamide (3-AB), and other reagents were purchased from Sigma (St. Louis, MO). The solution of iron-nitrilotriacetic acid complex was prepared as described previously (Shin and Kim, 2009).

Cell culture and treatment HepG2 cells, a human hepatocyte-derived cell line, were obtained from ATCC (Rockville, MD) and maintained 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 humidified atmosphere with 5% CO_2. For all experiments, cells (1×10^6) were plated in 10-cm (diameter) plastic dishes for 2–3 days (i.e. 80% confluency) and serum-starved for 24 h. Cells were incubated with 10 μM AA for 12 h, followed by additional exposure to 5 μM iron for the time period indicated in the results section or figure legends. To assess the effects of resveratrol, the cells were treated with 3–60 μM resveratrol for 1 h prior to incubation with AA+iron.

MTT assay To measure cytotoxicity, HepG2 cells were plated at a density of 5×10^4 cells per well in a 96-well plate. After treatment, viable cells were stained with MTT (0.25 mg/ml, 4 h). The media was then removed, and formazan crystals produced in the wells were dissolved with the addition of 200 μl dimethylsulfoxide. Absorbance at 540 nm was measured using an ELISA microplate reader (Tecan, Research Triangle Park, NC). Cell viability was defined relative to

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untreated control [i.e. viability (% control) = 100 × (absorbance of treated sample)/(absorbance of control)].

TUNEL assay TUNEL assay was performed using the DeadEnd™ Colorimetric TUNEL System, according to the manufacturer's instruction. HepG2 cells were fixed with 10% buffered formalin in PBS at room temperature for 30 min and were permeabilized with 0.2% Triton X-100 for 5 min. After washing with PBS, each sample was incubated with biotinylated nucleotide and terminal deoxynucleotidyltransferase in 100 μl of equilibration buffer at 37°C for 1 h. The reaction was stopped by immersing the samples in 2× saline sodium citrate buffer for 15 min. Endogenous peroxidases were blocked by immersing the samples in 0.3% H_2O_2 for 5 min. The samples were treated with 100 μl of horseradish peroxidase-labeled streptavidin solution (1:500) and incubated for 30 min. Finally, the samples were developed using the diaminobenzidine substrate, chromogen, H_2O_2 and diaminobenzidine for 10 min. The samples were washed and examined under light microscope (200×). The counting of TUNEL-positive cells was repeated three times, and the percentage from each counting was calculated.

Immunoblot analysis Cell lysates were prepared according to previously published methods (Shin and Kim, 2009). Briefly, the cells were centrifuged at 3,000*g* for 3 min and allowed to expand osmotically to the point of lysis after the addition of lysis buffer. Lysates were centrifuged at 10,000*g* for 10 min to obtain supernatants and were stored at −70°C until use. Immunoblot analysis was performed according to previously published procedures (Shin and Kim, 2009). Protein bands of interest were developed using an ECL chemiluminescence system (Amersham, Buckinghamshire, UK). Equal protein loading was verified by immunoblotting for β-actin.

Determination of reduced GSH and iron Reduced GSH in the cells was quantified using a commercial GSH determination kit (Oxis International, Portland, OR, USA). Briefly, the GSH-400 method was a two-step chemical reaction. The first step led to the formation of substitution products (thioethers) between 4-chloro-1-methyl-7-trifluromethyl-quinolinum methylsulfate and all mercaptans present in the sample. The second step included β-elimination reaction under alkaline conditions. This reaction was mediated by 30% NaOH which specifically transformed the substituted product (thioether) obtained with GSH into a chromophoric thione. Analyses of total iron in the cells were performed on an ICP-AES Optima 4300DV (Perkin-Elmer, Waltham, MA).

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Flow cytometric analysis of mitochondrial membrane potential (MMP) MMP was measured with Rh123, a membrane-permeable cationic fluorescent dye. Cells were treated according to the individual experiment, were stained with 0.05 μg/ml Rh123 for 1 h, and were harvested by trypsinization. After washing with PBS containing 1% FBS, cells were stained with 0.25 μg PI. The change in MMP was monitored using a BD FACSCalibur flow cytometer (San Jose, CA). In each analysis, 15,000 events were recorded.

Measurement of H_2O_2 production DCFH-DA is a cell-permeable non-fluorescent probe that is cleaved by intracellular esterases and is turned into the fluorescent dichlorofluorescein upon reaction with H_2O_2 and reactive nitrogen species. H_2O_2 generation was determined by the concomitant increase in dichlorofluorescein fluorescence. After treatment, cells were stained with 10 μM DCFH-DA for 1 h at 37°C. Fluorescence intensity in the cells was measured using FACS. In each analysis, 10,000 events were recorded.

Measurement of mitochondrial ROS MitoSOX is a live-cell-permeable and mitochondrial localizing superoxide indicator. After treatment of HepG2 cells with AA+iron, the cells were stained with 5 μM MitoSOX for 10 min at 37°C. Fluorescence intensity in the cells was measured using FACS. In each analysis, 10,000 events were recorded.

Recombinant adenoviral DN-AMPK construct and plasmid transfection A plasmid encoding a dominant negative mutant of AMPKα (D157A; DN-AMPK) was kindly provided by Dr. J. Ha (Kyunghee University, Korea). To generate a recombinant adenovirus expressing DN-AMPK, the construct was subcloned into the *attL*-containing shuttle plasmid, pENTR-BHRNX (Newgex, Seoul, KOREA). Recombinant adenoviral DN-AMPKα was constructed and generated by using the pAd/CMV/V5-DEST gateway plasmid. HepG2 cells were infected with adenovirus diluted in DMEM containing 10% FBS at a multiplicity of infection (MOI) of 50 and incubated for 12 h. After removal of the viral suspension, cells were further incubated with DMEM containing 10% FBS for 2 days and then were treated with the indicated reagent. Adenovirus that expresses LacZ (Ad-LacZ) was used as an infection control. Efficiency of infection was consistently >90% with this method.

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The construct encoding for a kinase mutant (KM) form of GSK3β was kindly provided by Dr. J. R. Woodgett (Samuel Lunenfeld Research Institute, Ontario, Canada). Cells were transfected with the plasmids by using lipofectamine^{2000} (Invitrogen, Carlsbad, CA). The empty plasmid, pCDNA3.1 was used for the mock transfection.

siRNA knockdown To knockdown LKB1, cells were transfected with either a siRNA directed against human LKB1 (Santa Cruz Biotechnology, Santa Cruz, CA) or a nontargeting control siRNA (100 pmol/ml) by using Lipofectamine 2000 according to the manufacturer's instructions. After transfection for 24 h, cells were exposed to AA with or without resveratrol for 12 h, followed by treatment with 5 μM iron. The resultant samples were analyzed by FACS. LKB1 knockdown was confirmed by immunoblot analysis.

Data analysis Scanning densitometry was performed with an Image Scan & Analysis System (Alpha-Innotech Corporation, San Leandro, CA). One way analysis of variance (ANOVA) procedures were used to assess significant differences among treatment groups. For each treatment showing a statistically significant effect, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $p<0.05$ or $p<0.01$.

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RESULTS

Resveratrol inhibition of AA+iron-induced apoptosis

A previous study from this laboratory has shown that treatment of AA (10 μM) and iron (5 μM) elicits synergized toxicity in HepG2 cells (Shin and Kim, 2009). First, we examined the effect of resveratrol on cytotoxicity induced by AA+iron. The MTT assay for cell viability indicated that resveratrol pretreatment (10–60 μM) significantly protected cells from the injury induced by AA+iron. Maximal cytoprotective effect was observed at 30 μM (Fig. 1A). Given this result, 30 μM of resveratrol was used in subsequent experiments. Morphological examination by light microscope (Fig. 1A) and TUNEL assay (Fig. 1B) confirmed the protective effect of resveratrol against the challenge of AA+iron. Resveratrol treatment caused no change in iron contents in cells treated with AA+iron (data not shown), excluding the possibility that resveratrol interferes with iron absorption. In the cells treated with AA+iron, the levels of PARP, procaspase-3, Bcl-xL, and Bcl-2 levels were lower. Resveratrol treatment completely prevented alterations in the levels of proteins associated with apoptosis (Fig. 1C). Overall, resveratrol treatment enables cells to protect against the synergized toxic effects of AA+iron.

GSH restoration and abrogation of ROS

Since GSH depletion increases the susceptibility of cells to ROS-induced toxicity (Kode et al., 2008), GSH content was determined in the cells treated with AA+iron in combination with resveratrol to test whether resveratrol helps maintain redox-homeostasis. AA+iron treatment markedly decreased levels of GSH. However, the GSH content was maintained in cells exposed to AA+iron following resveratrol treatment (Fig. 2A). In addition, a flow cytometric assay using DCFH-DA indicated that resveratrol treatment effectively abrogated increases in H_2O_2 production caused by AA+iron (Fig. 2B). Moreover, trolox (vitamin E analogue, 100 μM) treatment for 14 h completely attenuated ROS production, confirming that oxidant species were involved in the process. Both the maintenance of GSH content and the decrease in H_2O_2 production by resveratrol support the hypothesis that the cytoprotective effect of resveratrol results from its antioxidative capacity.

Inhibition of mitochondrial dysfunction

AA represses mitochondrial respiratory activity by selective inhibition of complex I and III, thereby promoting ROS generation (Cocco et al., 1999). Also, AA treatment may lead to cell death through mitochondrial permeability transition in hepatocytes such as HepG2 and MH1C1 cells

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(Scorrano et al., 2001; Shin and Kim, 2009). To examine the effect of resveratrol on mitochondria, mitochondrial ROS production was analyzed using MitoSOX, a live-cell-permeable and mitochondrial localizing superoxide indicator. AA+iron treatment markedly increased mitochondrial MitoSOX fluorescence, whereas resveratrol treatment (14 h) completely abolished superoxide production in mitochondria (Fig. 3A). Moreover, MnTBAP (novel superoxide dismutase mimetic, 20 μM, for 14 h) enabled cells to scavenge the mitochondrial ROS generated by AA+iron treatment, providing further evidence that the ROS included superoxide (Fig. 3A). To correlate AA-induced apoptosis with alteration in mitochondrial function, MMP was measured using FACS after staining of the cells with Rh123. Rh123 as a membrane-permeable cationic fluorescent is used as a sensitive probe of mitochondrial membrane potential in populations of apoptotic cells because this agent binds to the mitochondrial membranes (Kwon et al, 2009). Low staining intensity of Rh123 represents mitochondrial damage and dysfunction. AA+iron treatment increased the population of Rh123-negative and PI-negative cells (lower left quadrant), which represents viable cells with mitochondria damage (Fig. 3B). The fraction of apoptotic cells in the Rh123-negative and PI-positive field (upper left quadrant) was also increased (Fig. 3B). Therefore, AA+iron treatment induces mitochondrial dysfunction, which consequently leads to cell death. Resveratrol inhibited the change in MMP induced by AA+iron, supporting our conclusion that the cytoprotective effect of resveratrol is associated with protection of mitochondria.

The role of AMPK activation in mitochondrial function

Since resveratrol lowered the contents of hepatic lipids in mice through AMPK activation (Baur et al., 2006; Zang et al., 2006), the effects of resveratrol on the time-responses of ACC and AMPK phosphorylations were measured. Resveratrol treatment increased the phosphorylation of ACC in HepG2 cells, which represents cellular AMPK activity (Fig. 4A). To assess the role of resveratrol's activation of AMPK in protecting mitochondria and cells, the AMPK-inhibitory effect on rhodamine-negative cell populations was then measured. The recovery of rhodamine-positive cells elicited by resveratrol was significantly reversed by DN-AMPKα overexpression (Fig. 4B). Consistently, the beneficial effect of resveratrol on mitochondria was antagonized by simultaneous treatment with compound C (an AMPK inhibitor) (Fig. 4C). DN-AMPKα overexpression and the inhibitory effect of compound C for AMPK were previously confirmed by the decreased ACC phosphorylation (Kwon et al., 2009). Therefore, the protection of mitochondria and cells by resveratrol might be associated at least in part with AMPK activation.

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Resveratrol increases inhibitory phosphorylation of GSK3β, which may be associated with cell survival (Xi et al., 2009). As an effort to identify downstream molecule of AMPK, we next examined the effect of AMPK on GSK3β phosphorylation and its role in mitochondrial protection. Resveratrol treatment (1 or 3 h) increased the phosphorylation of GSK3β at serine 9 residue, and this increase was prevented by compound C pretreatment (Fig. 4D). Moreover, either SB216763 (a GSK3β inhibitor) treatment or GSK3β-KM (a kinase mutant form of GSK3β) overexpression similarly inhibited the changes in MMP or cell death induced by AA+iron (Fig. 4E). Overall, our results demonstrate that the cytoprotective effect of resveratrol may be associated with inhibitory phosphorylation of GSK3β downstream of AMPK.

The role of LKB1 activation in mitochondrial protection

To identify the upstream signal of AMPK activation by resveratrol, the time-course effect of resveratrol on LKB1 phosphorylation was assessed. Resveratrol treatment resulted in a notable increase in the phosphorylation of LKB1 (Fig. 5A). To test the role of LKB1 in protecting mitochondrial function against AA+iron, the effect of siRNA knockdown of LKB1 on MMP change was determined. The protective effect of resveratrol against mitochondrial dysfunction elicited by AA+iron was reversed by siRNA knockdown of LKB1 (Fig. 5B). In contrast, treatment with STO-609 (1 μg/ml), an inhibitor of CaMKK, failed to antagonize the beneficial effect of resveratrol as indicated by no change in MMP (Fig. 5C). So, the cytoprotective effect of resveratrol against mitochondrial dysfunction may depend on LKB1, but not CaMKK.

The effect of PARP activation on mitochondrial function

It has been shown that several signaling pathways such as SIRT1, NO, and PARP may regulate LKB1 (Alessi et al., 2006; Hou et al., 2008; Huang et al., 2009; Vázquez-Chantada et al., 2009). Pharmacological inhibitors were used to assess the upstream signaling of LKB1 phosphorylation by resveratrol. Treatment with either nicotinamide (10 mM, 14 h) or sirtinol (10 μM, 14 h), which has been shown to inhibit SIRT1 activity (Dasgupta and Milbrandt, 2007), failed to change the protective effect of resveratrol on MMP in cells treated with AA+iron (Fig. 6A). Similarly, this protective effect of resveratrol was not reversed by L-NAME (a NOS inhibitor) pretreatment. In contrast, treatment with 3-AB (a PARP inhibitor) significantly reversed the recovery of rhodamine-positive cells elicited by resveratrol (Fig. 6A). Consistent with this result, resveratrol increased PARP activity, as supported

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by increases in PAR production (Fig. 6B). Moreover, 3-AB treatment completely inhibited LKB1 phosphorylation induced by resveratrol (Fig. 6C). Nicotinamide, sirtinol, or L-NAME failed to reverse the LKB1 phosphorylation (data not shown). These results showed that LKB1-dependent mitochondrial protection by resveratrol might be associated with PARP activation.

The effects of other polyphenols on AMPK and mitochondrial protection

Given the current finding that resveratrol increased cell viability through LKB1-AMPK-dependent protection of mitochondrial function, other polyphenols including apigenin, genistein, and daidzein (Fig. 7A) were employed in an effort to find relationship between AMPK activation and mitochondrial protective effects. All of the polyphenols used except resveratrol had no AMPK-activating effect (Fig. 7B). Moreover, the polyphenolic compounds failed to enable cells to restore MMP in response to a challenge by AA+iron (Fig. 7C). All of these results demonstrate that resveratrol has a cytoprotective effect against severe oxidative stress induced by AA+iron and the ability to protect mitochondria, mediated through LKB1-dependent AMPK activation.

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DISCUSSION

Excess iron deposition causes injury to and dysfunction of organs including liver. Hepatic iron accumulation may result from increases in plasma iron due to chronic alcoholics, chronic hepatitis, liver cirrhosis, hemolysis, inflammatory syndrome, or diabetes. Acquired iron-overload disease is characterized by macrophage activation (Fleming and Bacon, 2005). With time, iron is redistributed toward surrounding hepatocytes. Moreover, the production of pro-inflammatory mediators including AA is increased under iron-overload conditions (Galaris and Pantopoulos, 2008). Indeed, we found an increase in plasma AA level after iron deposition in an *in vivo* model, in which phenylhydrazine was used as a hemolytic agent that indirectly creates hepatic iron overload and promotes oxidative damage (Ferrali et al., 1997; Kim WY et al, FRBM, pending revision). AA treatment alone promotes ROS production, causes MMP changes, and thereby leading to cell death (Kwon et al., 2009). Excess iron catalyzes the release of AA by modifying membrane phospholipids (Tadolini and Hakim, 1996). Moreover, AA synergizes the ability of iron to promote oxidative stress and cytotoxicity (Shin and Kim, 2009), which reflects the clinical situations accompanying iron overload. Hence, the combinatorial treatment model may be useful for the study of drug candidates active in the prevention and/or treatment of tissue injury caused by iron accumulation.

The mitochondrion serves as the organelle determining a critical point of the apoptotic process because it functions as a stress sensor (Browning and Horton, 2004). Within the mitochondrion, the respiratory chain is one of the main sites of ROS production (Browning and Horton, 2004). The AA generated from cell membranes undergoing oxidative stress exerts a direct effect on mitochondria and promotes ROS production (Cocco et al., 1999; Scorrano et al., 2001). Similarly, treatment of cells with AA+iron promotes ROS production (superoxide) in mitochondria, as shown by the MitoSOX results. Excess production of ROS in mitochondria may cause apoptosis: combinatorial treatment of AA and iron promotes cell death, as well as the induction of ROS production and mitochondrial dysfunction. The prevention of AA+iron-induced mitochondrial dysfunction by cyclosporin A, an inhibitor of permeability transition pore formation, further suggests that mitochondrial dysfunction and the consequent ROS overproduction might be responsible for cell death (Shin and Kim, 2009). Since PEG-SOD, PEG-catalase, trolox or N-acetyl-L-cysteine prevented the ability of AA+iron to induce both ROS and apoptosis, this evidence provides additional support for the important role of ROS in mitochondrial dysfunction and apoptosis (Shin and Kim, 2009).

Resveratrol, a natural polyphenolic compound, has the capacity to inhibit both mitochondrial

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ROS production and permeability transition, thereby protecting the key intracellular organelle against the oxidative stress promoted by AA+iron. In addition, resveratrol decreases cellular ROS and thus maintains GSH content in cells: these results are consistent with previous findings of resveratrol's potent antioxidant and anti-inflammatory activity in various organs (Baur and Sinclair, 2006). Our work reveals that the mitochondrial protection and the antioxidant effect of resveratrol increases the viability of hepatocytes against AA+iron. Moreover, resveratrol has a cytoprotective effect against iron-catalyzed oxidative burst through mitochondrial protection, as illustrated by the inhibition of apoptosis and alterations in apoptotic markers.

AMPK is a heterotrimeric protein consisting of one catalytic subunit (α) and two non-catalytic subunits (β and γ). When AMP binds to the γ-subunit, AMPK activation is promoted by stimulating phosphorylation at the threonine residue within the kinase domain. AMPK monitors cellular energy status by responding to changes in the AMP/ATP ratio. Treatment with an AMPK activator increases the expression of peroxisome proliferators-activated response-coactivator-1α and MnSOD mRNAs, which may inhibit ROS production in mitochondria (Kukidome et al., 2006). Previous work in our laboratory showed that 1,2-dithiole-3-thiones protect cells from AA+iron-induced ROS production and mitochondrial dysfunction via AMPK activation (Shin and Kim, 2009). Hence, AMPK is a potential target that protects cells from mitochondrial injury. In this study, the cytoprotective effect of resveratrol against AA+iron depended on AMPK activation, as evidenced by the antagonism of AMPKα overexpression or compound C treatment on the capacity of resveratrol to recover MMP against AA+iron. The crucial role of AMPK in protecting cells and mitochondria was verified since AICAR exhibited a similar protective effect (Shin and Kim, 2009).

Oxidative stress activates GSK3β and leads GSK3β to translocate into the mitochondria. Activated GSK3β in mitochondria then binds to and phosphorylates the components of mitochondrial membrane pore (e.g. VDAC and ANT), and thereby induces MMP transition. Thus, GSK3 acts as the pivotal kinase for the regulation of MMP transition (Juhaszova et al., 2004; Xi et al., 2009). In the present study, resveratrol promoted inhibitory phosphorylation of GSK3β at serine 9. Another important finding of this study is that resveratrol-induced activation of AMPK is responsible for this inhibitory phosphorylation. Our data is in line with the finding that AICAR treatment decreases GSK3 activity indirectly through Raf1/ERK MAPKs/p90RSK (Wang et al., 2008). The results of this study demonstrate that the AMPK-dependent mitochondrial protection of resveratrol against oxidative stress may be associated with the downstream inhibitory

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phosphorylation of GSK3β.

Although the mechanism of resveratrol's cytoprotection involves AMPK activation, resveratrol does not directly activate AMPK *in vitro* (Baur et al., 2006). Therefore, proteins and/or components that lie upstream of AMPK might be the target(s) of resveratrol. Currently, LKB1 and CaMKK are the known upstream kinases of AMPK (Lage et al., 2008). Our results demonstrate that resveratrol activates LKB1, which promotes mitochondrial protection in HepG2 cells. By contrast, CaMKK inhibition fails to reverse this beneficial effect. Hence, resveratrol activates AMPK specifically through LKB1, but not CaMKK, which parallels the previous finding that LKB1 was responsible for inhibiting apoptosis in MEF cells under the condition of energy stress (Shaw et al., 2004). The upstream signaling pathways that activate LKB1 might include SIRT1, NOS, and protein kinase A (Alessi et al., 2006; Hou et al., 2008; Vázquez-Chantada et al., 2009). Recently, PARP was found as another possible signaling pathway of LKB1 activation (Huang et al., 2009). PARP belongs to a group of nuclear enzymes that play a critical role in DNA damage repair through PAR production. It has been known that PAR formation is an energetically expensive process, causing failure in cellular ATP production, rapid depletion of NAD^+, and eventually cell death (Huang et al., 2009). On the contrary, PARP plays a protective role against ROS-induced cell death through LKB1-AMPK-mediated autophagy activation (Huang et al., 2009). In the present study, we showed for the first time that resveratrol activated PARP, and which led to phosphorylate LKB1 for mitochondrial protection.

Resveratrol activates SIRT1 and its target peroxisome proliferators-activated response-coactivator-1α, which increases mitochondrial number and function (Lagouge et al., 2006). SIRT1 acts as a NAD^+-dependent deacetylase for numerous protein targets, thereby modulating cell viability (Vaziri et al., 2001). Also, SIRT1 is involved in regulating lipid metabolism through LKB1-dependent AMPK activation (Hou et al., 2008). In the present study, SIRT1 activation by resveratrol was not responsible for mitochondrial protection, consistent with the observation that resveratrol stimulates AMPK in neurons via LKB1, but not SIRT1 (Dasgupta and Milbrandt, 2007). By the same token, the neuroprotective effect of resveratrol was independent of SIRT1 activity (Alvira et al., 2007).

Phenolic compounds — mostly derivatives and/or isomers of flavones, isoflavones, flavonols, catechins, tocopherols, and phenolic acids — are abundant in fruits, vegetables, tea, and wine. Growing evidences support a role for polyphenols in preventing cardiovascular disease, cancer, neurodegenerative disease, or diabetes (Mahn et al., 2005; Rahman et al., 2006; Zang et al., 2006). In

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particular, dietary soy isoflavones, such as genistein and daidzein, increase antioxidant and eNOS gene expression and improve endothelial function (Mahn et al., 2005). Our work utilized cytoprotective polyphenols (i.e., apigenin, genistein, and daidzein) to further examine the role of AMPK in protecting mitochondria. None of these polyphenols activated AMPK, nor enhanced mitochondrial function, supporting our hypothesis that AMPK activation contributes to mitochondrial protection. Consistently, apigenin did not increase AMPK and ACC phosphorylations in HepG2 cells (Zang et al., 2006). Genistein protects cells at 0.1–40 μM (Kajta et al., 2007; Kim et al., 2007), but the compound activates AMPK in adipocytes at 50–200 μM (Hwang et al., 2005). Hence, its beneficial effect may not result from AMPK activation.

Demonstrated for the first time, resveratrol protects cells from AA+iron-induced ROS production and mitochondrial dysfunction; a cytoprotective effect may be mediated by AMPK-mediated inhibitory phosphorylation of GSK3β downstream of poly(ADP-ribose)polymerase-LKB1 pathway (Fig. 8). Thus, resveratrol has the potential to pharmacologically defend mitochondria against an iron-catalyzed burst of oxidative stress through the AMPK pathway. Resveratrol scavenges ROS *in vitro*, which may be mediated by the phenolic hydroxyl groups in its molecular structure (Leonard et al., 2003). Although the cytoprotective effect of resveratrol possibly results from ROS scavenging, a relatively high concentration is required for radical scavenging (Leonard et al., 2003). Therefore, the radical scavenging effect is less likely to contribute to resveratrol's beneficial action. More likely, the antioxidant effect of resveratrol might relate to the activation of a specific signaling pathway that enables cells to promote the defense system. This concept parallels findings that resveratrol increases GSH synthesis (Kode et al., 2007) and activates antioxidant enzymes, catalase, SOD, and NADPH-quinone oxidoreductase (Rubiolo et al., 2008).

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Inhibition of AA+iron-induced cell death by resveratrol

A) The effect of resveratrol on cell viability. Light micrographs show the morphology of the cells incubated with 3–60 μM resveratrol for 1 h and continuously treated with 10 μM AA for 12 h, followed by exposure to 5 μM iron for 6 h (200×). The dose-response effect of resveratrol on cell viability was assessed using MTT assays. Data represent the mean ± S.E. of four separate experiments. For graphs in A and B, the statistical significance of differences between treatments and either the vehicle-treated control (**p<0.01) or cells treated with AA+iron ($^{\#\#}p$<0.01) was determined.

B) TUNEL assay. Cells were treated with 30 μM resveratrol for 1 h, followed by the addition of 10 μM AA for 12 h, and finally treated with 5 μM iron for 6 h. The percentage of TUNEL-positive cells (dark brown staining) was quantified. Data represent the mean ± S.E. of four separate experiments.

C) Immunoblots for apoptotic proteins. Proteins were immunoblotted from the lysates of cells incubated with 30 μM resveratrol for 1 h, continuously treated with 10 μM AA for 12 h, and then exposed to 5 μM iron for 1 h. Equal protein loading was verified by β-actin immunoblotting. Results were confirmed by four separate experiments.

Fig. 2. Inhibition of decrease in GSH content and ROS production by resveratrol

A) GSH content. The GSH content was assessed in HepG2 cells that had been treated as described in the Fig. 1C legend. Data represent the mean ± S.E. of four separate experiments. The statistical significance of differences between treatments and either the vehicle-treated control (**p<0.01) or cells treated with AA+iron was determined.

B) ROS production. Cells were incubated with resveratrol for 1 h, followed by the addition of AA (12 h)+iron (1 h). Results were confirmed by repeated experiments. Resveratrol or trolox treatment attenuated AA+iron-induced ROS production, as shown by the decrease in DCF fluorescence.

Fig. 3. Abrogation of AA+iron-induced mitochondrial ROS production and dysfunction by resveratrol

A) ROS production in mitochondria. Cells were incubated with 30 μM resveratrol for 1 h, treated with 10 μM AA (12 h) followed by incubation with 5 μM iron for 1 h, and then stained with MitoSOX. Increase in MitoSOX fluorescence indicates the production of mitochondrial superoxide. Treatment with either resveratrol or MnTBAP attenuated the ability of AA+iron to induce ROS

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production in mitochondria, as shown by decrease in MitoSOX fluorescence.

B) Mitochondrial membrane permeability (MMP). Cells were treated with 30 μM resveratrol for 1 h, followed by the addition of AA (12 h)+iron (1 h). After staining with Rh123, the cells were harvested, and stained with propidium iodide (PI). Treatment of AA+iron increased both the subpopulation of Rh123-negative and PI-negative cells (lower left quadrant), which represents viable cells with mitochondrial damage, and the fraction of apoptotic cells in the Rh123-negative and PI-positive field (upper left quadrant). Data represent the mean ± S.E. of four replicates. The statistical significance of differences between treatments and either the vehicle-treated control (**p<0.01) or cells treated with AA+iron was determined.

Fig. 4. AMPK-mediated inhibitory phosphorylation of GSK3β by resveratrol

A) AMPK activation by resveratrol. Immunoblot analyses were performed on lysates of cells that had been treated with resveratrol for the indicated time period. Results were confirmed by three separate experiments. Relative phosphorylated ACC band intensities of immunoblot data were quantified (right). The statistical significance of differences between treatments and the vehicle-treated control was determined (*p<0.05, **p<0.01).

B) Reversal by Ad-DN-AMPKα of the ability of resveratrol to restore MMP. After Ad-LacZ or Ad-DN-AMPKα infection (48 h), HepG2 cells were incubated with resveratrol for 1 h and continuously exposed to AA (12 h)+iron (1 h). MMP was analyzed as described in the legend to Fig. 3B. Data represent the mean ± S.E. of four replicates. The statistical significance of differences between treatments and either the vehicle-treated control (**p<0.01), cells treated with AA+iron ($^{\#\#}p$<0.01), or cells treated with AA+iron after Ad-DN-AMPK infection (N.S., no significance) was determined. Immunoblot analyses for HA confirmed DN-AMPKα overexpression (inset).

C) Reversal by compound C of resveratrol's restoration of MMP. After compound C treatment (3 μM, 30 min), cells were incubated with resveratrol for 30 min, followed by the addition of AA (12 h)+iron (1 h). Data represent the mean ± S.E. of four replicates. The statistical significance of differences between treatments and either the vehicle-treated control (**p<0.01), cells treated with AA+iron ($^{\#\#}p$<0.01), or cells treated with AA+iron after compound C treatment (N.S., no significance) was determined.

D) AMPK-dependent inhibitory phosphorylation of GSK3β by resveratrol. Immunoblot analyses against GSK3β phosphorylated at serine 9 were performed on lysates of cells that had been treated

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with resveratrol for the indicated time period. Cells were treated with compound C as described in panel B), and then were exposed to resveratrol for 3 h. Equal protein loading was verified by immunoblotting for GSK3β. Results were confirmed by repeated experiments.

E) The effect of GSK3β inhibition on MMP or cell viability. HepG2 cells were incubated with AA (12 h)+iron (1 h) following SB216763 treatment (10 μM) for 1 h or GSK3β-KM overexpression. MMP was analyzed as described in the legend to Fig. 3B, whereas cell viability was analyzed by MTT assay.

Fig. 5. The role of LKB1 activation by resveratrol in mitochondrial function

A) Phosphorylation of LKB1. Immunoblot analyses were performed on the lysates of cells that had been treated with resveratrol for the indicated time period. Results were confirmed by three replicates. Relative phosphorylated LKB1 band intensities of immunoblot data were quantified (right). The statistical significance of differences between treatments and the vehicle-treated control (**p<0.01) was determined.

B) Reversal by LKB1 knockdown of resveratrol's effect to restore MMP. HepG2 cells were transfected with siRNAs directed against LKB1 or nontargeting control siRNA, continuously incubated with resveratrol for 1 h and then exposed to AA+iron. MMP was analyzed as described in the legend to Fig. 3B. Immunoblot analyses for LKB1 confirmed specific knockdown of LKB1 (inset). Data represent the mean ± S.E. of four replicates. The statistical significance of differences between treatments and either the vehicle-treated control (**p<0.01), cells treated with AA+iron ($^{\#\#}p$<0.01), or cells treated with AA+iron after siLKB1 transfection (N.S., no significance) was determined.

C) The effect of a CaMKK inhibitor on the recovery of MMP by resveratrol. After STO-609 treatment (1 μg/ml, for 30 min), cells were incubated with resveratrol for 30 min, followed by the addition of AA+iron. Results were confirmed by three replicates. MMP was analyzed as described in the legend to Fig. 3B. Data represent the mean ± S.E. of four replicates. The statistical significance of differences between treatments and either the respective vehicle-treated control (**p<0.01) or cells treated with respective AA+iron ($^{\#\#}p$<0.01) was determined.

Fig. 6. Resveratrol activation of PARP and its role in LKB1 phosphorylation

A) Effects of pharmacological inhibitors on the ability of resveratrol to restore MMP. HepG2 cells

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were incubated with resveratrol for 30 min after treatment with 10 mM nicotinamide, 10 μM sirtinol, 10 μM L-NAME, or 10 mM 3-AB for 30 min, and the cells were then continuously exposed to AA (12 h)+iron (1 h). Mitochondrial membrane potential was analyzed as described in the legend to Fig. 3B. Data represent the mean ± S.E. of four replicates. The statistical significance of differences between treatments and either the respective vehicle-treated control (*p<0.05, **p<0.01) or cells treated with respective AA+iron ($^{\#}p$<0.05, $^{\#\#}p$<0.01) was determined.

B) Increase in PAR production by resveratrol. Immunoblot analyses were performed on the lysates of cells that had been treated with 30 μM resveratrol for the indicated time period. Results were confirmed by repeated experiments.

C) Inhibition of resveratrol-induced LKB1 phosphorylation by PARP inhibitor. Cells were pretreated with 3-AB (10 mM, 1 h) and then continuously exposed with 30 μM resveratrol for 1 h. Results were confirmed by three separate experiments. The statistical significance of differences between treatments and either the vehicle-treated control (**p<0.01) or cells treated with resveratrol ($^{\#\#}p$<0.01) was determined.

Fig. 7. The effects of polyphenols on mitochondrial function

A) Bond-line chemical structures of polyphenols.

B) The effects of other polyphenols on AMPK activation. Immunoblot analyses were performed in the lysates of HepG2 cells that had been treated with 30 μM resveratrol, genistein, and daidzein or 10 μM apigenin for 3 h. Results were confirmed by three replicates. Relative phosphorylated ACC band intensities of immunoblot data were quantified (bottom). The statistical significance of differences between treatments and the vehicle-treated control was determined (**p<0.01).

C) Changes in MMP. Cells were treated with resveratrol, apigenin, genistein, or daidzein for 1 h, followed by additional incubation with AA (12 h)+iron (1 h). Because apigenin at the concentration of 30 μM increased the population of Rh123-negative cells (i.e., possible cytotoxicity), 10 μM apigenin was used in this experiment. Results were confirmed by three replicates. MMP was analyzed as described in the legend to Fig. 3B. The statistical significance of differences between treatments and either the vehicle-treated control (**p<0.01) or cells treated with AA+iron ($^{\#\#}p$<0.01) was determined.

Fig. 8. A proposed signaling pathway by which resveratrol inhibits mitochondrial dysfunction

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and cell death against oxidative stress.

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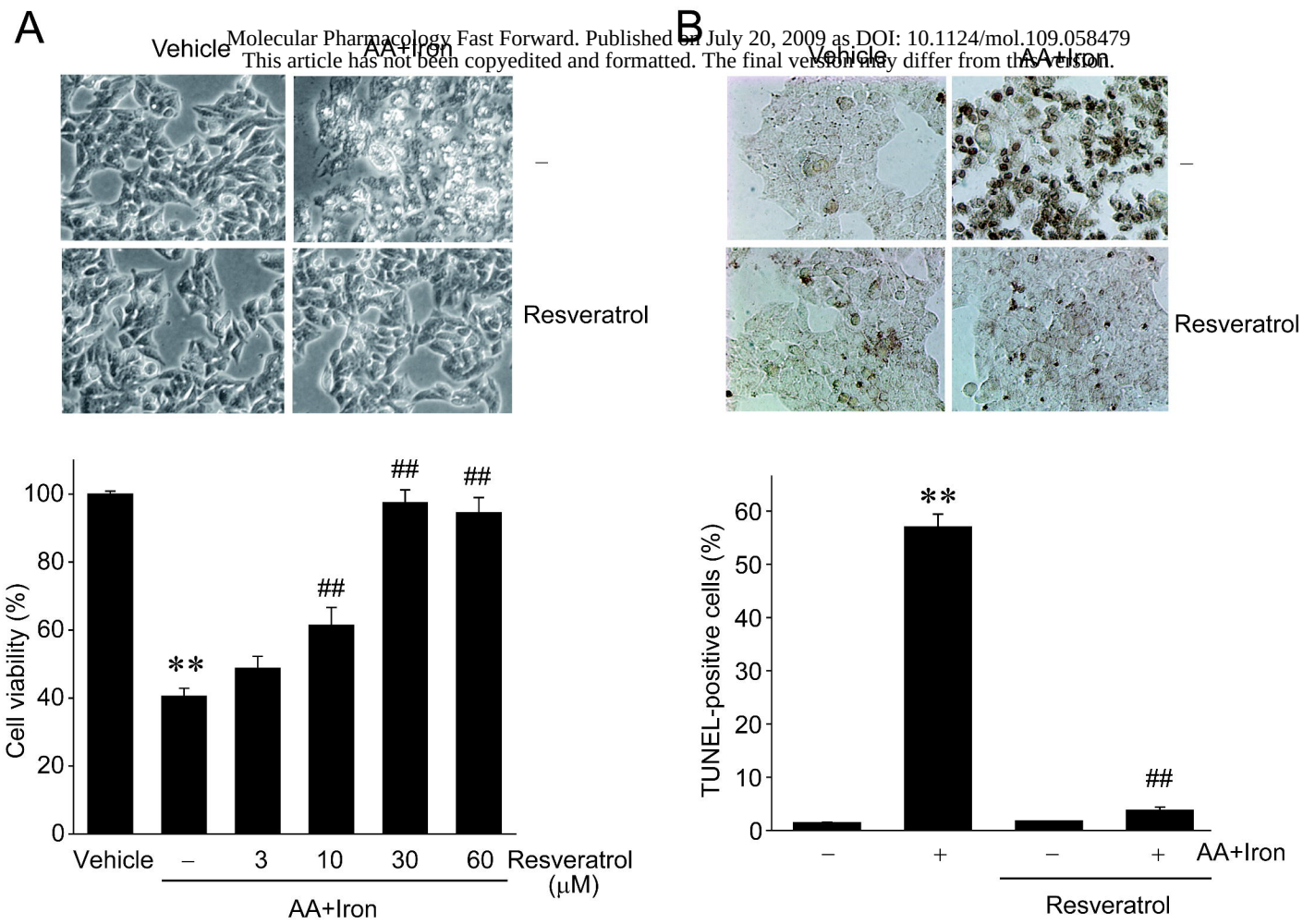

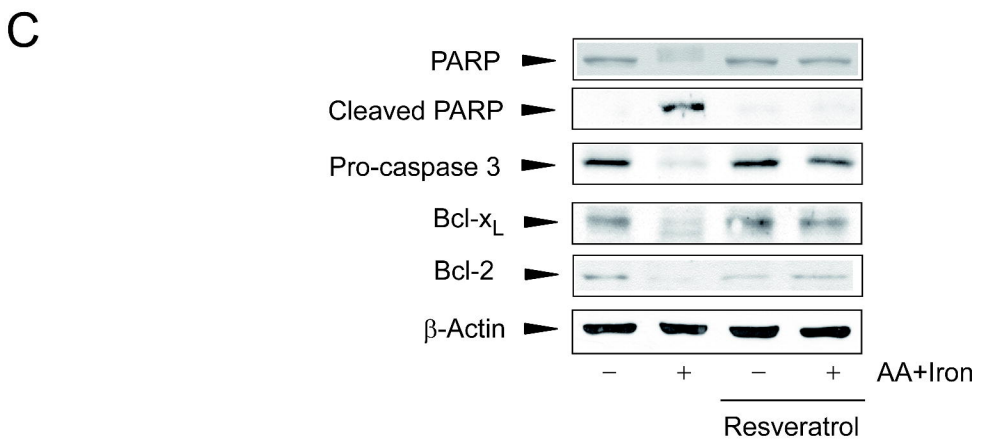

Fig 1

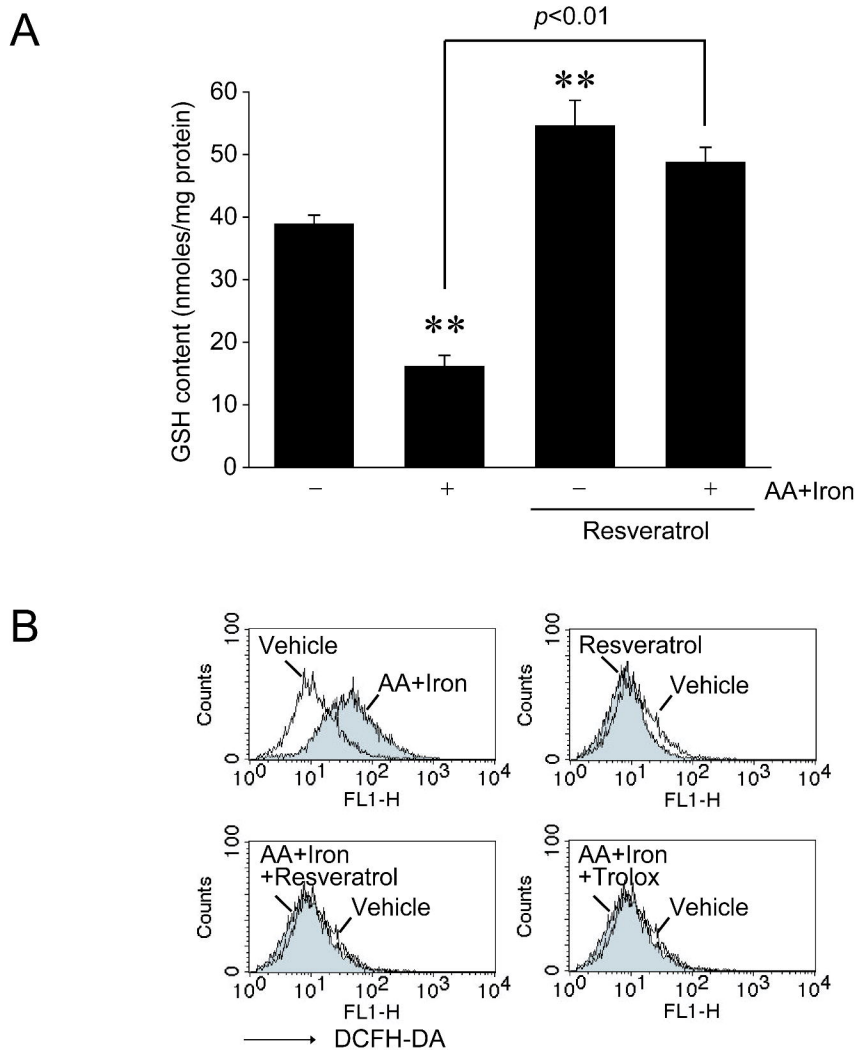

Fig 2

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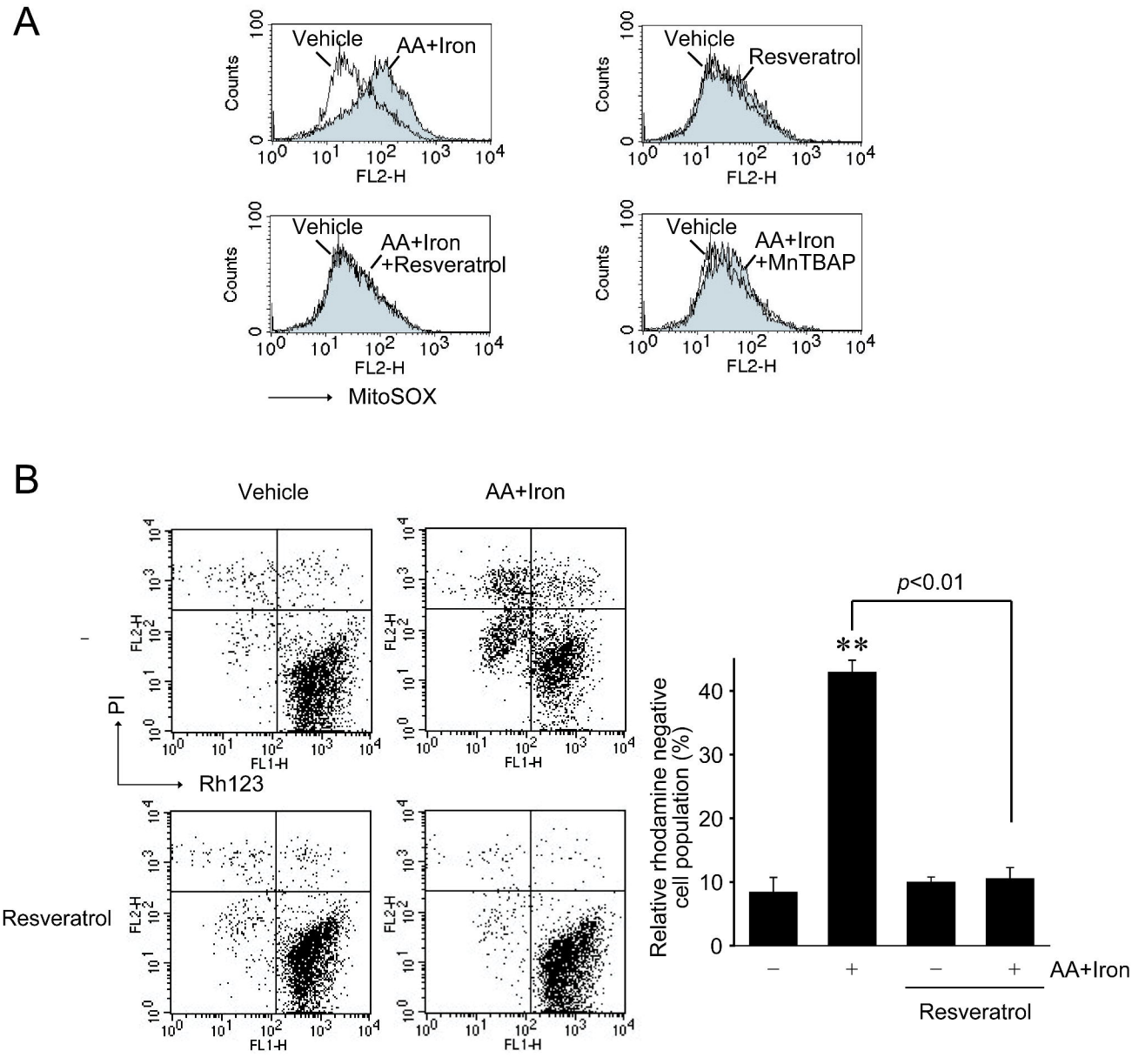

Fig 3

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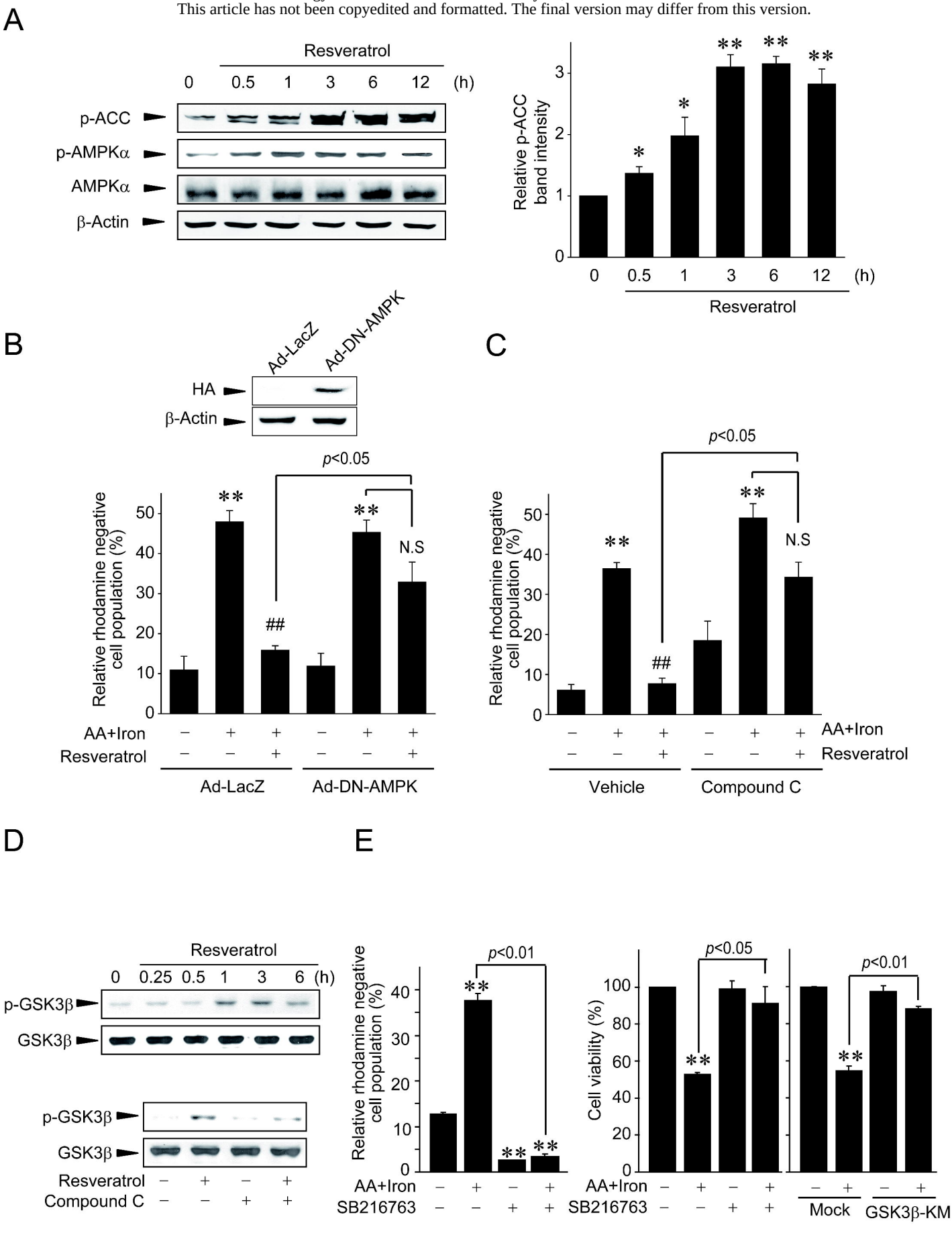

Fig 4

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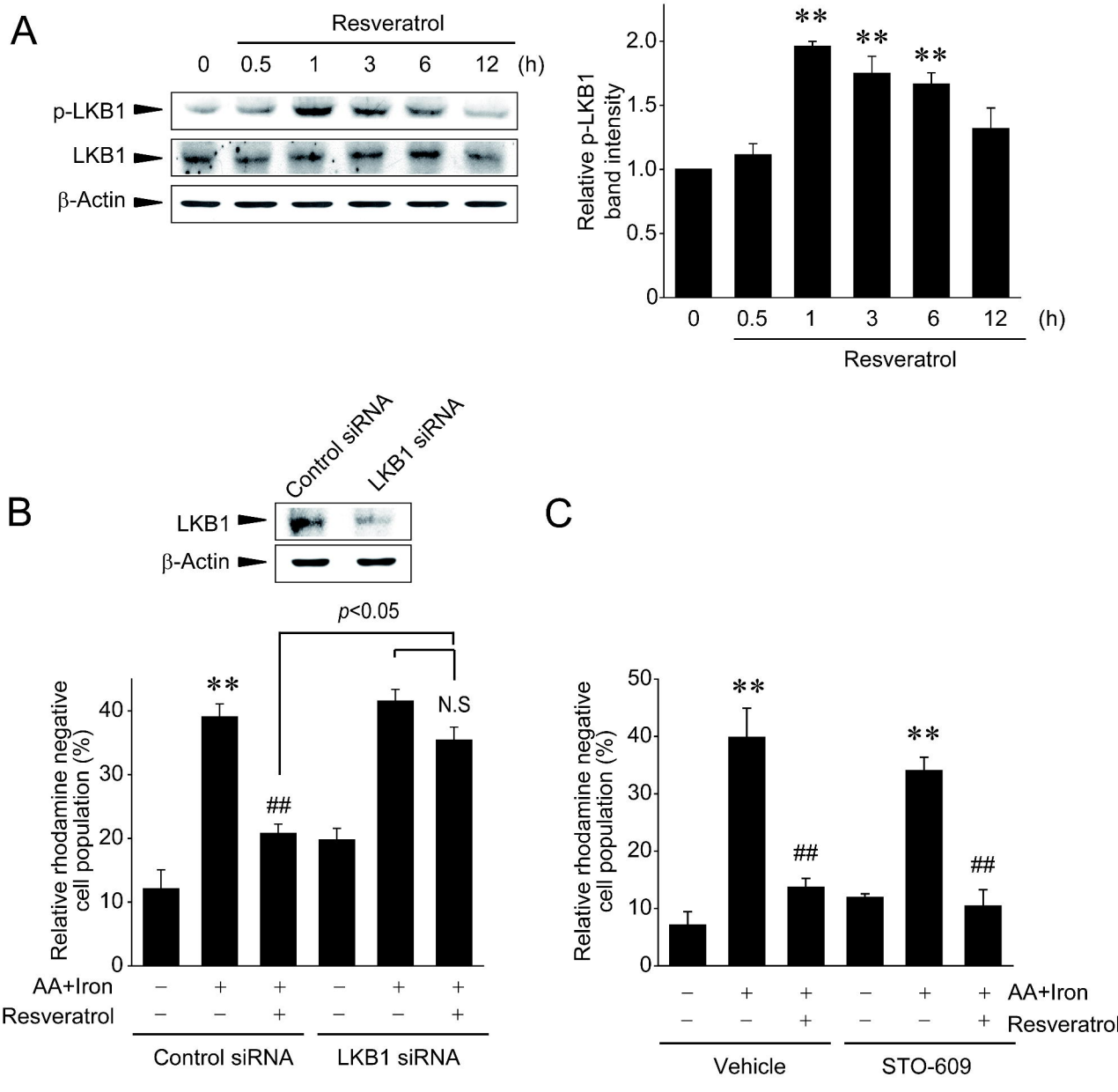

Fig 5

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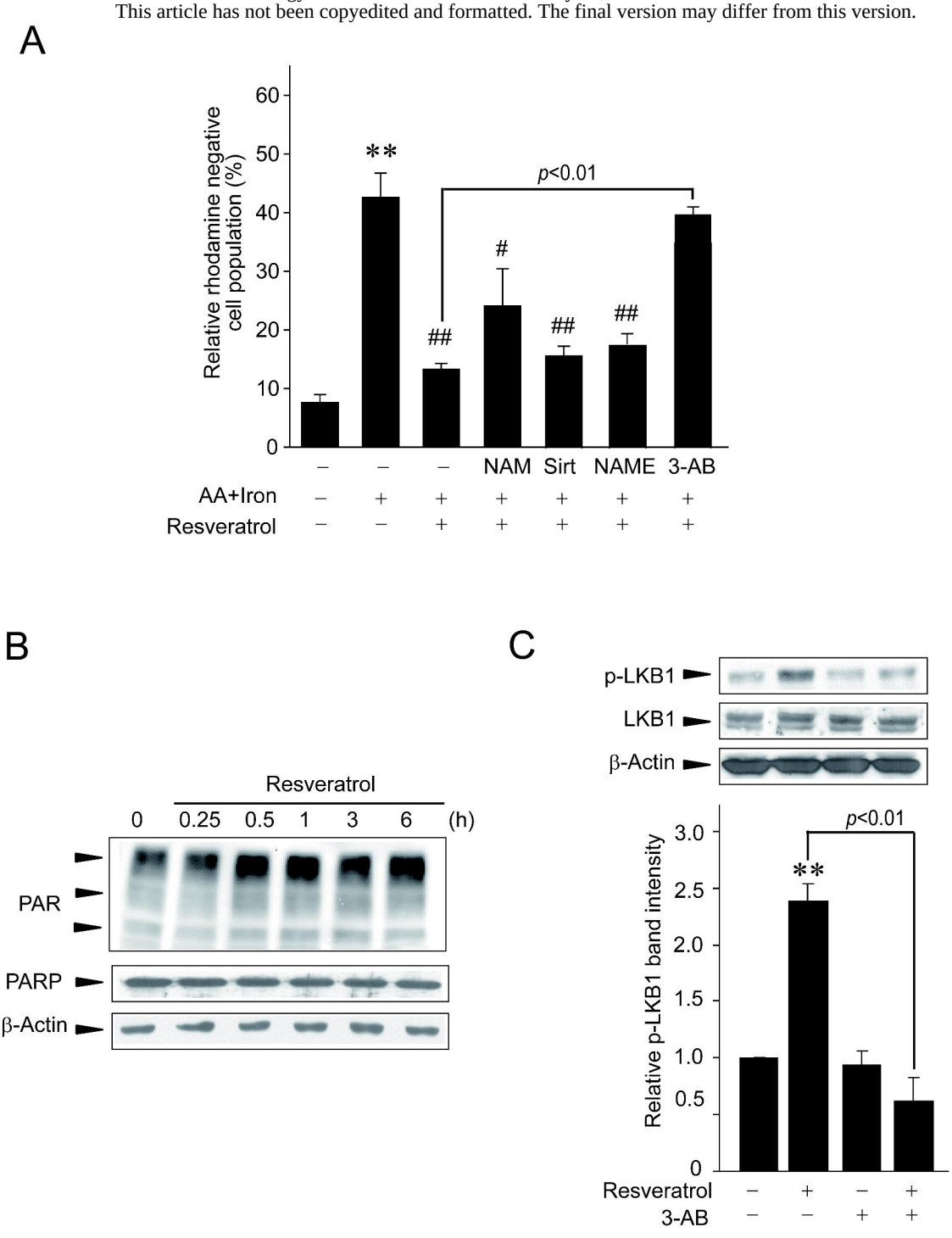

Fig 6

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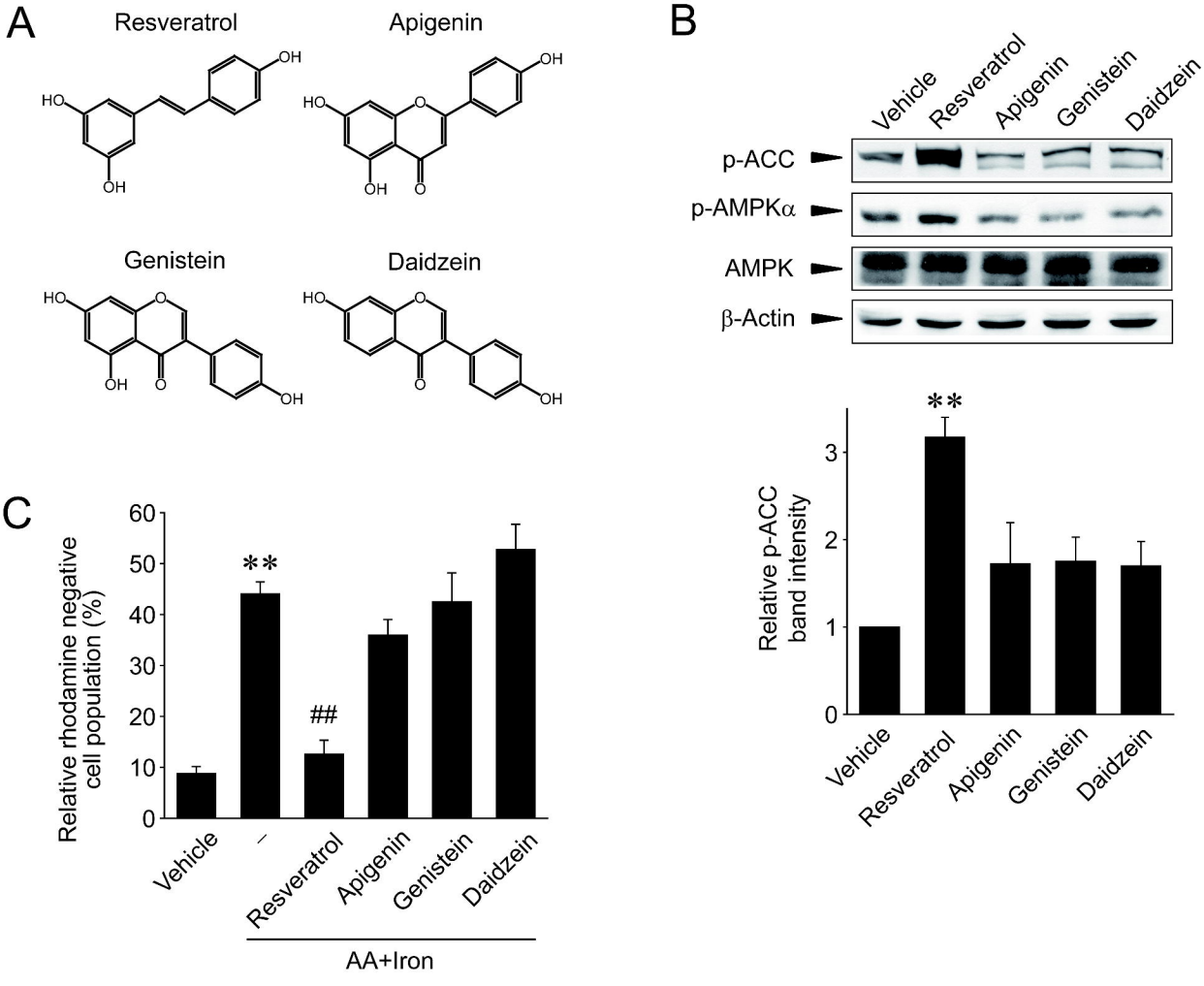

Fig 7

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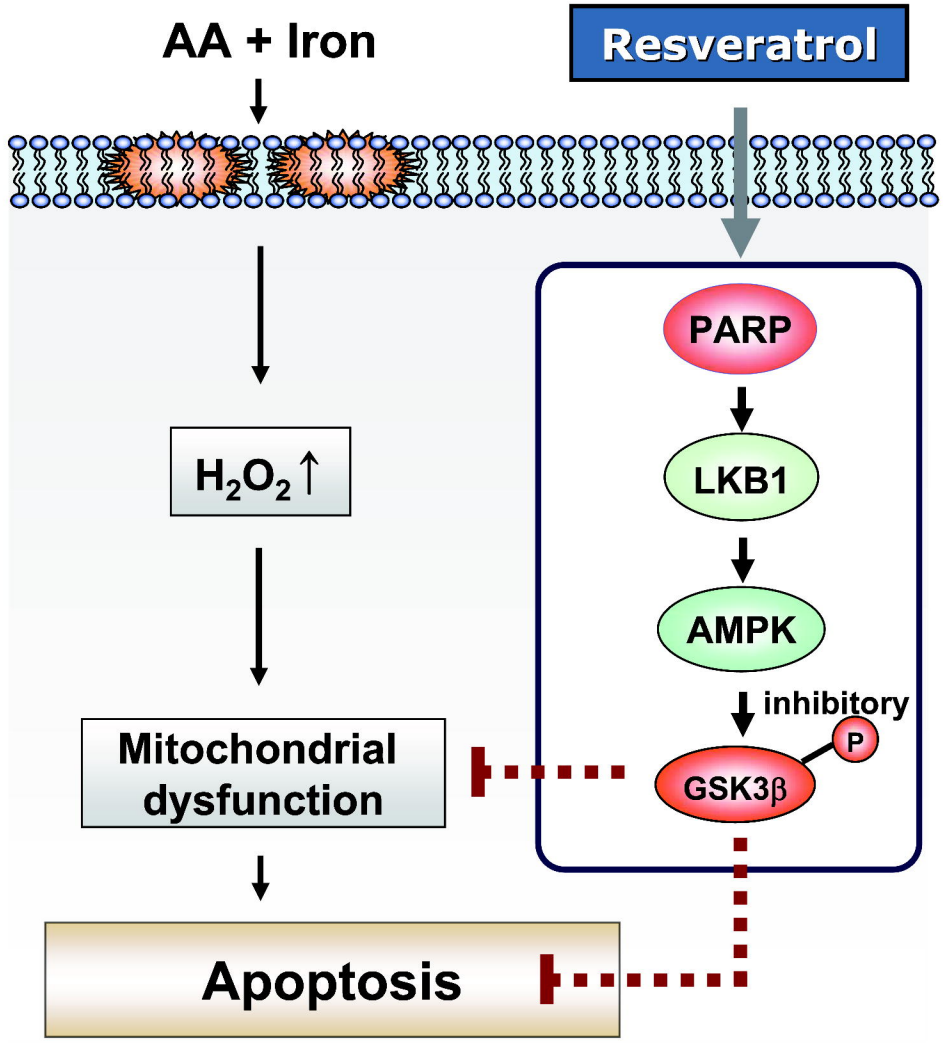

Fig 8