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Effect of Resveratrol on High Glucose-Induced Stress in Human Leukemia K562 Cells

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Abstract Hyperglycemia, a symptom of diabetes mellitus, induces hyperosmotic responses, including apoptosis, in vascular endothelial cells and leukocytes. Hyperosmotic shock elicits a stress response in mammalian cells, often leading to apoptotic cell death. In a previous report, we showed that hyperosmotic shock induced apoptosis in various mammalian cells. Importantly, apoptotic biochemical changes (i.e., caspase-3 activation and DNA fragmentation) were blocked by antioxidant pretreatment during hyperosmotic shock-induced cell death. In the present study, we report that resveratrol, a phytoalexin present in grapes with known antioxidant and anti-inflammatory properties, attenuates high glucose-induced apoptotic changes, including c-Jun N-terminal kinase (JNK) activation and caspase-3 activation in human leukemia K562 cells. Experiments with the cell permeable dye, 2′,7′-dichlorofluorescein diacetate (DCF-DA), an indicator of reactive oxygen species (ROS) generation, revealed that high glucose treatment directly increased intracellular oxidative stress, which was attenuated by resveratrol. In addition, high glucose-treated K562 cells displayed a lower degree of attachment to collagen, the major component of vessel wall subendothelium. In contrast, cells pretreated with resveratrol followed by high glucose exhibited higher affinity for collagen. The results of this report collectively imply the involvement of oxidative stress in high glucose-induced apoptosis and alterations in attachment ability. Moreover, resveratrol blocks these events by virtue of its antioxidant property. J. Cell. Biochem. 94: 1267–1279, 2005.

Key words: resveratrol; high glucose; apoptosis; ROS; osmotic shock

Diabetes mellitus (DM) patients suffer from infections more often than normal individuals [Deresinski, 1995]. For example, in a study of hospital patients, two-thirds of the bacteria were identified in patients with DM, and one-third in those without DM [Carton et al., 1992]. Infections have a complicated course in DM patients compared to non-diabetic patients [Deresinski, 1995]. The pathogenetic mechanisms responsible for high infection rates in DM patients, and the issue of whether the increased infection rate in these patients is due to immunity defects, remain to be elucidated [Geerlings and Hoepelman, 1999]. Recent reports show that DM patients often display hyperglycemia [Wahl et al., 1998], inducing a hyperosmotic environment in blood. In addition, high glucose-induced cell apoptosis is mediated by oxidative stress and c-Jun N-terminal kinase (JNK) activation [Ho et al., 2000]. However, the mechanisms of immunity dysfunction and leukocyte injury by hyperosmotic stress in hyperglycemia are currently unclear, and require further investigation.

Cell–cell or cell-extracellular matrix (ECM) interactions may be affected by various stress conditions. Cell–ECM interactions play an important role in many cell events, including regulation of gene activity and immunity [Albelda and Buck, 1990; Springer, 1994]. Carbohydrate recognition functions in the interaction of leukocytes with endothelial cells of blood vessels, such as during inflammatory processes [Sharon and Lis, 1993]. Leukocytes roll along the walls of blood vessels. During inflammation, leukocytes attach to blood vessel walls in the affected region, and migrate to the inflammation site [Springer, 1994]. Specific

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families of adhesion molecules mediate the leukocyte binding steps in this process. Leukocyte tethering and rolling are the first steps in the inflammation response, and are predominately mediated by the selectin family (cell surface glycoproteins) of adhesion molecules [Springer, 1994; Vestweber and Blanks, 1999]. Various stress-causing agents often cause blood cell injury in the circulation, including physiological ROS producers [Menon and Sudhakaran, 1995].

High glucose treatment induces a hyperosmotic shock environment, and consequently, cell apoptosis, and injury. Osmotic stress triggers various cell responses, including apoptosis [Qin et al., 1997]. A fundamental property of the cells that form body tissue is the maintenance of homeostasis. Upon exposure of cells to anisotonic media, a stress response that maintains cellular volume arises immediately. Cells deal with this challenge via alterations in plasma membrane ion transport and accumulation of non-polar solutes [Paredes et al., 1992; Dall'Asta et al., 1994]. Although it is evident that increased phosphorylation of several proteins and activation of several protein kinases is induced by osmotic stress [Tilly et al., 1993; Galcheva-Gargova et al., 1994; Han et al., 1994; Matsuda et al., 1995], the signal transduction pathway responsible for this cellular change during this type of stress in mammalian cells is yet to be clarified. In a previous study, we demonstrated that hyperosmotic shock-induced cell apoptosis and the accompanying biochemical changes (i.e., activation of caspase-3 and DNA fragmentation) in cells are blocked by antioxidants, such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E). However, further experiments are required to establish the mechanisms of signal transduction and regulation in high glucose/hyperosmotic shock-induced apoptosis.

Reactive oxygen species (ROS) are oxygen-containing molecules with either unpaired electrons or the ability to abstract electrons from other molecules. Numerous chemical and physical treatments capable of inducing apoptosis stimulate oxidative stress via generation of ROS in cells [Halliwell and Gutteridge, 1990], suggesting a close relationship between oxidative stress and apoptosis. Direct evidence establishing oxidative stress as a mediator of apoptosis is established from the observation that oxidants such as H_2O_2 trigger apoptosis, whereas antioxidants block this effect [Buttke and Sandstrom, 1994].

Apoptosis, widely observed in different cells of various organisms, is a unique morphological pattern of cell death characterized by chromatin condensation, membrane blebbing, and cell fragmentation [Kerr et al., 1972]. Internucleosomal DNA cleavage is the most prominent event in the early stages of apoptosis, and thus widely used as a biochemical marker of this cell death process [Wyllie, 1980]. The concept of chemoprevention is increasingly attractive as a novel strategy against cancer. The anti-cancer effects of numerous chemotherapy compounds are attributed to their apoptotic induction ability [Hong and Sporn, 1997; Kelloff et al., 2000; Sporn and Suh, 2000]. There is considerable evidence that a family of cysteine proteases, termed caspases, plays an important role in apoptotic execution [Martins and Earnshaw, 1997; Nicholson and Thornberry, 1997]. However, the precise molecular mechanisms of the apoptotic process are yet to be clearly defined.

Protein phosphorylation is involved in regulating apoptosis. Alterations in the activities of several protein kinases are evident during apoptosis in a variety of cell types [Anderson, 1997]. It appears that c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) serves as a key component in regulating entry into apoptosis in several cell types under specific circumstances [Xia et al., 1995; Verheij et al., 1996]. Activation of JNK by UV irradiation may be prevented by the antioxidant, *N*-acetylcysteine [Alder et al., 1995; Tao et al., 1996], suggesting that JNK activity is stimulated by oxidative stress. Using dominant interfering mutants of c-Jun or SAPK/extracellular-signal-regulated kinase kinase 1 (SEK-1) as tools, it was shown that activation of the JNK/SAPK pathway is required for stress-induced apoptosis in U937 leukemia cells, and growth factor withdrawal-triggered apoptosis in PC-12 pheochromocytoma cells [Xia et al., 1995; Verheij et al., 1996]. However, additional experiments are necessary to clarify the role of JNK/SAPK in apoptosis signaling.

Resveratrol, a member of the family of phytoalexins found in grapes and other dietary plants, inhibits tumor initiation and progression [Jang et al., 1997; Mgbonyebi et al., 1998; Huang et al., 1999]. Resveratrol exhibits a wide range of pharmacological effects, including the

prevention of heart disorders, blocking of lipoprotein oxidation, and inhibition of platelet aggregation [Frankel et al., 1993; Pace-Asciak et al., 1996; Gusman et al., 2001]. The anti-tumor properties of resveratrol are attributed to its antioxidant activity and inhibition of cyclooxygenase 1 and 2 activation [Jang et al., 1997; Subbaramaiah et al., 1998]. In addition, this anti-tumor activity may be due to its ability to cause cell cycle arrest and induce apoptosis [Clement et al., 1998; Hsieh and Wu, 1999; Surh et al., 1999; Ahmad et al., 2001]. Although multiple biological functions of resveratrol have been described, the precise molecular mechanisms underlying its actions are currently unknown. Here, we show that resveratrol could block high glucose-induced apoptosis and injury in human leukemia K562 cells.

EXPERIMENTAL PROCEDURES

Materials

The polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). RPMI medium, resveratrol, 2′,7′-dichlorofluorescein diacetate (DCF-DA), and goat anti-rabbit immunoglobulin G (IgG) antibodies conjugated with alkaline phosphatase were purchased from Sigma (St. Louis, MO). Anti-JNK1 (C17), anti-p-JNK (G-7), and anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PARP antibody was purchased from Cell Signaling Technology (Beverly, MA). Z-DEVD-AFC was obtained from Calbiochem (La Jolla, CA). CDP-Star™ chemiluminescent substrate for alkaline phosphatase was acquired from Boehringer Mannheim (Mannheim, Germany).

Cell Culture and High Glucose Treatment

Human leukemia K562 cells were cultured at 37°C in 95% air/5% CO_2 and water-saturated atmosphere in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. One day before the experiments, cells ($\sim 5-6 \times 10^6$) were plated on 100-mm culture dishes. High glucose treatment was performed by incubating cells either in medium containing 5 mM glucose (normal or control; 300 mosmol/kg) or control media containing various concentrations of glucose (5–30 mM) (305–330 mosmol/kg) for the indicated times. Cells were washed twice with ice-cold PBS, and lysed in 600 μl of solution (20 mM Tris-HCl at pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 20 mM sodium pyrophosphate, and 1 mM sodium orthovanadate) on ice for 10 min. Cell lysates were collected and sonicated on ice for 3×10 s, followed by centrifugation at 15,000*g* for 20 min at 4°C. The resulting supernatant fractions were used as cell extracts.

Immunoblots

Immunoblotting was performed essentially as described previously [Chan and Yu, 2000]. Proteins were transferred from SDS–PAGE gels to PVDF membranes, and probed with antibodies against JNK1(C17) (0.2 μg/ml), poly (ADP-ribose) polymerase (PARP) (0.2 μg/ml), β-actin (0.2 μg/ml), or phospho-JNK (p-JNK) (0.2 μg/ml). Proteins of interest were detected using goat anti-rabbit IgG antibody conjugated with alkaline phosphatase and CDP-Star™, according to the manufacturer's protocol.

Apoptosis Assay

Oligonucleosomal DNA fragmentation in apoptotic cells was measured using the Cell Death Detection ELISA^{plus} kit, according to the manufacturer's instructions (Roche Molecular Biochemicals). Cells (1×10^5) were treated with high glucose or left untreated for 24 h at 37°C. Spectrophotometric data were obtained using an ELISA reader at a wavelength of 405 nm.

In addition, apoptosis was assayed by staining with propidium iodide and Hoechst 33342. Specifically, cells were incubated with propidium iodide (1 μg/ml) and Hoechst 33342 (2 μg/ml) at room temperature for 10 min in the dark. The percentage of apoptotic cells was determined using plasma membrane impermeable to propidium iodide (propidium iodide-negative cells) and the condensed/fragmented nuclei stained with Hoechst 33342 were examined with a fluorescent microscope. In each experiment, 7–10 independent fields (~600–1,000 nuclei in total) were counted per condition.

Caspase-3 Activity Assay

Caspase-3 activity was measured using the fluorogenic substrate, Z-DEVD-AFC. Cell lysates (100 μg) were incubated in 250 μl caspase assay buffer (25 mM HEPES at pH 7.5,

0.1% CHAPS, 10 mM dithiothreitol, 100 U/ml aprotinin) containing 0.1 mM Z-DEVD-AFC for 3 h at 37°C. Ice-cold caspase assay buffer (1.25 ml) was added to the mixture, and the relative caspase-3 activity determined using a fluorescence spectrophotometer (Hitachi, F-2000; excitation at 400 nm, emission at 505 nm).

ROS Assay

ROS arbitrary units were measured using 2′-7′-dichlorodihydrofluorescein diacetate (DCFDA) dye. Cells (1.0×10^6) were incubated in 50 μl PBS containing 20 μM DCFDA for 1 h at 37°C, and relative ROS units determined using a fluorescence ELISA reader (excitation at 485 nm, emission at 530 nm). An aliquot of the cell suspension was lysed, and protein concentration determined. Results are expressed as arbitrary absorbance units/mg protein.

Analysis of Attachment Ability Changes in K562 Cells

K562 cells were treated with various concentrations of glucose for 24 h or left untreated. Cells were allowed to adhere to collagen-coated petridishes (50 μg/ml) by incubation in standard control medium at 37°C for 90 min. Cell attachment was quantitatively evaluated using microscopy after staining with trypan blue.

Inhibition of JNK1 by Antisense Oligonucleotides

JNK1 sense (5′-ATC ATG AGC AGA AGC AAG CGT GAC-3′) and antisense (5′-GTC ACG CTT GCT TCT GCT CAT GAT-3′) oligonucleotides were obtained from Life Technologies (Grand Island, NY). Oligonucleotides were synthesized under phosphorothioate-modified conditions and purified by HPLC. The sequences represent the amino acid codons +1 to −7 of human JNK1. Next, oligonucleotides were dissolved in 30 mM HEPES buffer (pH 7.0), and transfected into cells using lipofectAMINE4 (Life Technologies). K562 cells grown in 60-mm culture dishes were incubated at 37°C in 1 ml of Opti-MEM I medium (Life Technologies; modified Eagle's minimum essential medium buffered with HEPES and sodium bicarbonate), containing lipofectAMINE™ (12 μg) and oligonucleotides (70 μM) for 72 h. Cells were subjected to high glucose treatment and extracts analyzed.

Statistics

Data were analyzed using one-way ANOVA, and differences were evaluated using a two tailed Student's *t*-test and analysis of variance. A P value < 0.05 was considered significant.

Analytical Methods

Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Quantification of the relative intensity of signals detected from immunoblots and autoradiograms was performed by densitometric scanning using a Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

RESULTS

High Glucose Induces Apoptosis in K562 Cells

To establish the effect of high glucose, K562 cells were incubated with various doses of glucose, following which cell viability was measured. Cell viability decreased with increasing concentrations of glucose up to 25 mM in a dose-dependent manner (Fig. 1A,B). ELISA of the TUNEL assay was employed to quantitatively determine the level of histone-associated oligonucleosome DNA fragments, a biochemical event of apoptosis. High glucose concentrations increased the apoptosis-associated parameter in a dose-dependent manner (Fig. 1C). Cells were further analyzed by staining with propidium iodide and Hoechst 33342 to measure the percentage of apoptotic cells. Notably, the apoptotic percentage was increased, following treatment with various concentrations of glucose (Fig. 1D). Further experiments disclosed that high glucose induced a time-dependent increase in cell apoptosis (Fig. 1E).

Resveratrol Inhibits High Glucose-Induced Cell Apoptosis, JNK and Caspase-3 Activation in K562 Cells

Next, we examined the effect of resveratrol on high glucose-induced apoptosis. K562 cells were incubated with various doses of resveratrol or treated with 25 mM glucose after preincubation with resveratrol, and cell viability was determined. Resveratrol alone (up to 20 μM) had no effect on viability (Fig. 2A,B). However, the compound inhibited glucose-induced cell apoptosis in a dose-dependent manner (Fig. 2A–C). Activation of the JNK pathway is essential

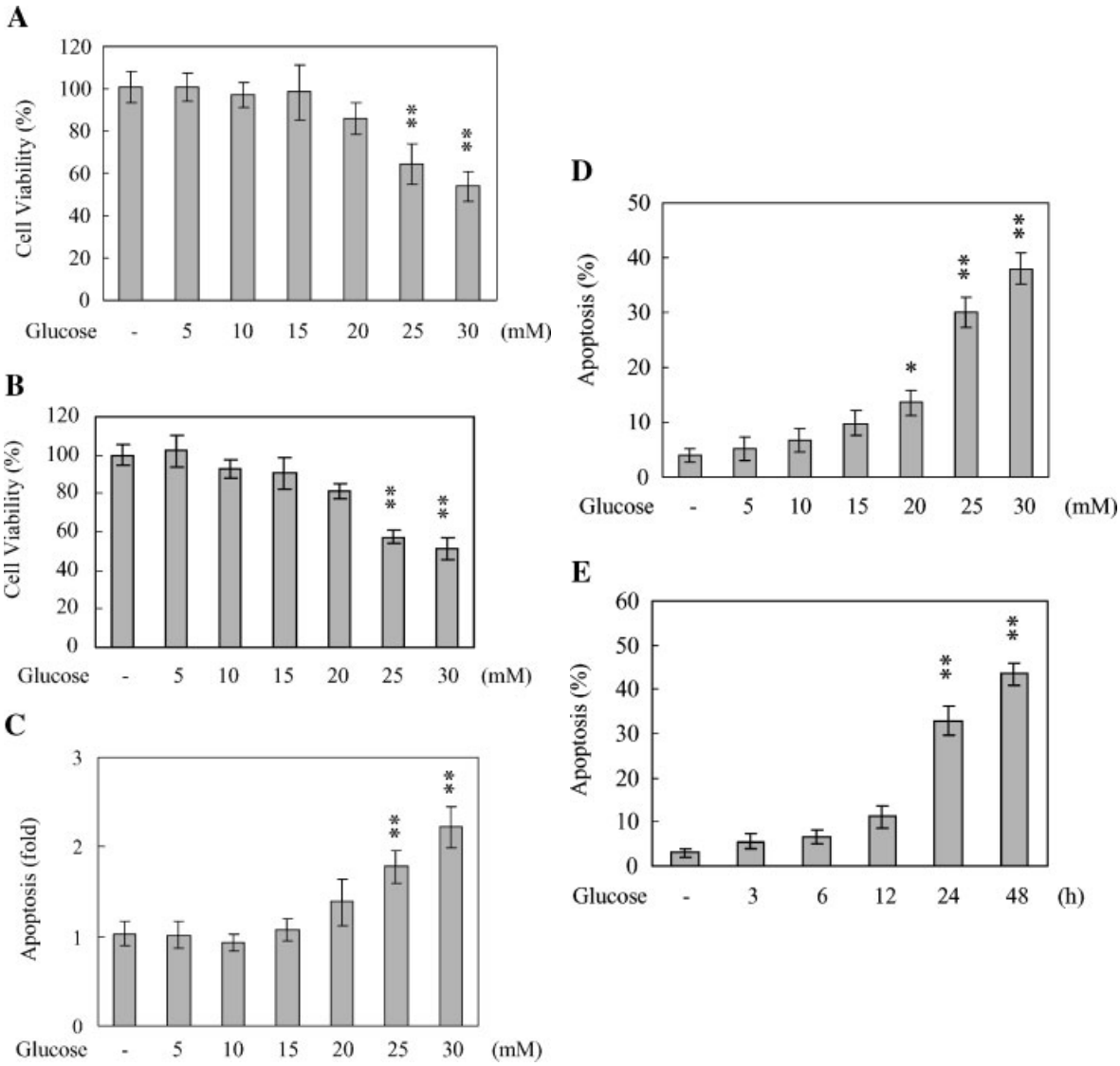

Fig. 1. Effects of high glucose on K562 cells. **A–D**: K562 cells were incubated with or without various concentrations of glucose at 37°C for 24 h. Cell viability was determined using the MTT assay (A) and trypan blue exclusion assay (B), and apoptosis was detected with the Cell Death Detection ELISA kit (C) and staining with propidium iodide and Hoechst 33342 (D), as described in Materials and Methods. **E**: K562 cells were incubated with culture media containing 25 mM glucose for the indicated times or left untreated. Apoptosis was evaluated using propidium iodide and Hoechst 33342 staining. Values are presented as means ± SE. $*P < 0.05$ and $**P < 0.01$ versus control (without glucose treatment) group.

for apoptotic induction in some cell types [Xia et al., 1995; Verheij et al., 1996]. Previously, we demonstrated that UV irradiation-induced apoptosis and caspase-3 activation in A431 cells are mediated by JNK activity [Chan et al., 2003]. In this report, we further examined the effect of resveratrol on high glucose-induced JNK activity. Resveratrol inhibited high glucose-induced JNK activation in a dose-dependent manner (Fig. 3A). Specifically, pre-treatment with 20 μM resveratrol induced a ~70% reduction in activity, but had little effect on JNK protein levels (Fig. 3A). Caspase-3, a family of cysteine proteases, plays an important role in apoptotic processes [Martins and Earnshaw, 1997; Nicholson and Thornberry, 1997]. In the presence of 20 μM resveratrol, activation of caspase-3 and cleavage of PARP by high glucose were significantly attenuated. (Fig. 3B,C).

Resveratrol Prevents High Glucose-Induced ROS Formation and Apoptotic Biochemical Changes in K562 Cells

A number of studies show that high glucose stimulates oxidative stress in cells, and that

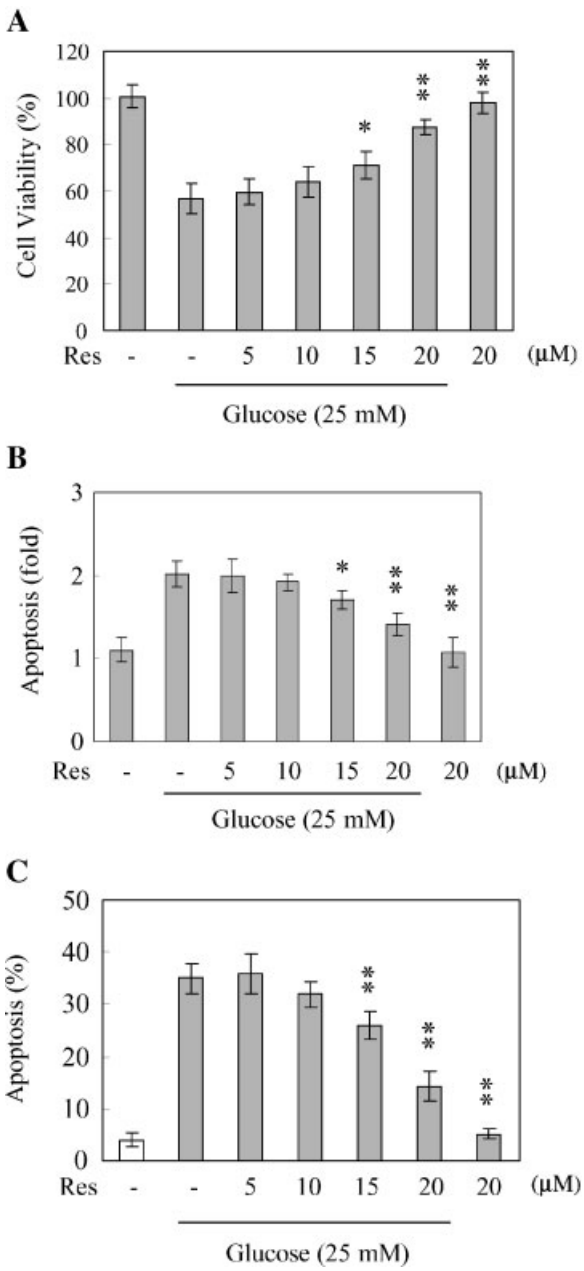

Fig. 2. Effects of resveratrol on high glucose-induced apoptosis. K562 cells were incubated with or without various concentrations of resveratrol (Res) for 1 h, followed by culture media containing 25 mM glucose for 24 h. Cell viability determined by the trypan blue exclusion assay (**A**) and apoptosis detected with the ELISA kit (**B**) and staining with propidium iodide and Hoechst 33342 (**C**), as described in Materials and Methods. Values are presented as means ± SE. $*P < 0.05$ and $**P < 0.01$ versus the value of 'high glucose only' treatment group.

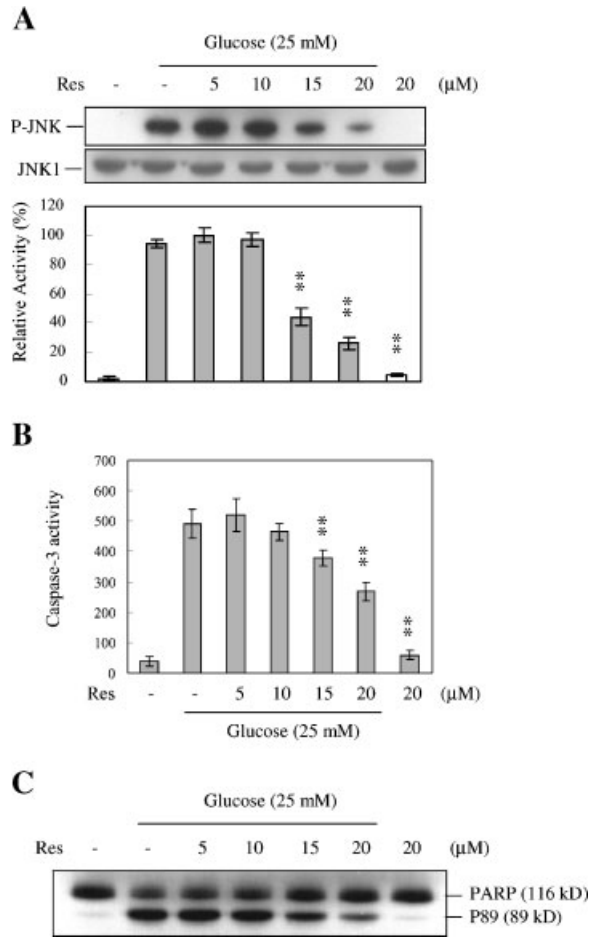

Fig. 3. Resveratrol inhibits high glucose-induced JNK and caspase-3 activation. K562 cells were incubated with or without various concentrations of resveratrol (Res) for 1 h and in culture media containing 25 mM glucose for 24 h. **A**, **upper and middle panel**: Cell extracts (40 μg) were resolved by 10% SDS–PAGE, electroblotted onto PVDF membranes, and probed with an anti-p-JNK antibody. The middle panel shows an immunoblot analysis of total JNK1 protein from 40 μg cell extracts at each dose. **A, lower panel**: The signal intensity on the immunoblot of P-JNK was quantitated using a densitometer and the maximum signal was assigned a value of 100%. **B**: Cell extracts (100 μg) were analyzed for caspase-3 activity using Z-DEVD-AFC as the substrate. **C**: Cell extracts (40 μg) were resolved by 10% SDS–PAGE, electroblotted onto PVDF membranes, and probed with an anti-PARP antibody. Values are presented as means ± SE. $**P < 0.01$ versus the value of the 'high glucose only' treatment group.

ROS are effective apoptotic inducers [Tanaka et al., 1999; von Harsdorf et al., 1999; Frustaci et al., 2000; Quagliaro et al., 2003]. Accordingly, we examined whether ROS formation occurs in high glucose-treated K562 cells, and analyzed the effects of resveratrol on ROS formation using DCF-DA as the detection reagent [LeBel et al., 1992]. As shown in Figure 4A, upon treatment of K562 cells with menadione, an established oxidant [Caricchio et al., 1999], the magnitude of intracellular ROS content was markedly increased, as assessed by the intensity of DCF-DA fluorescence, confirming the

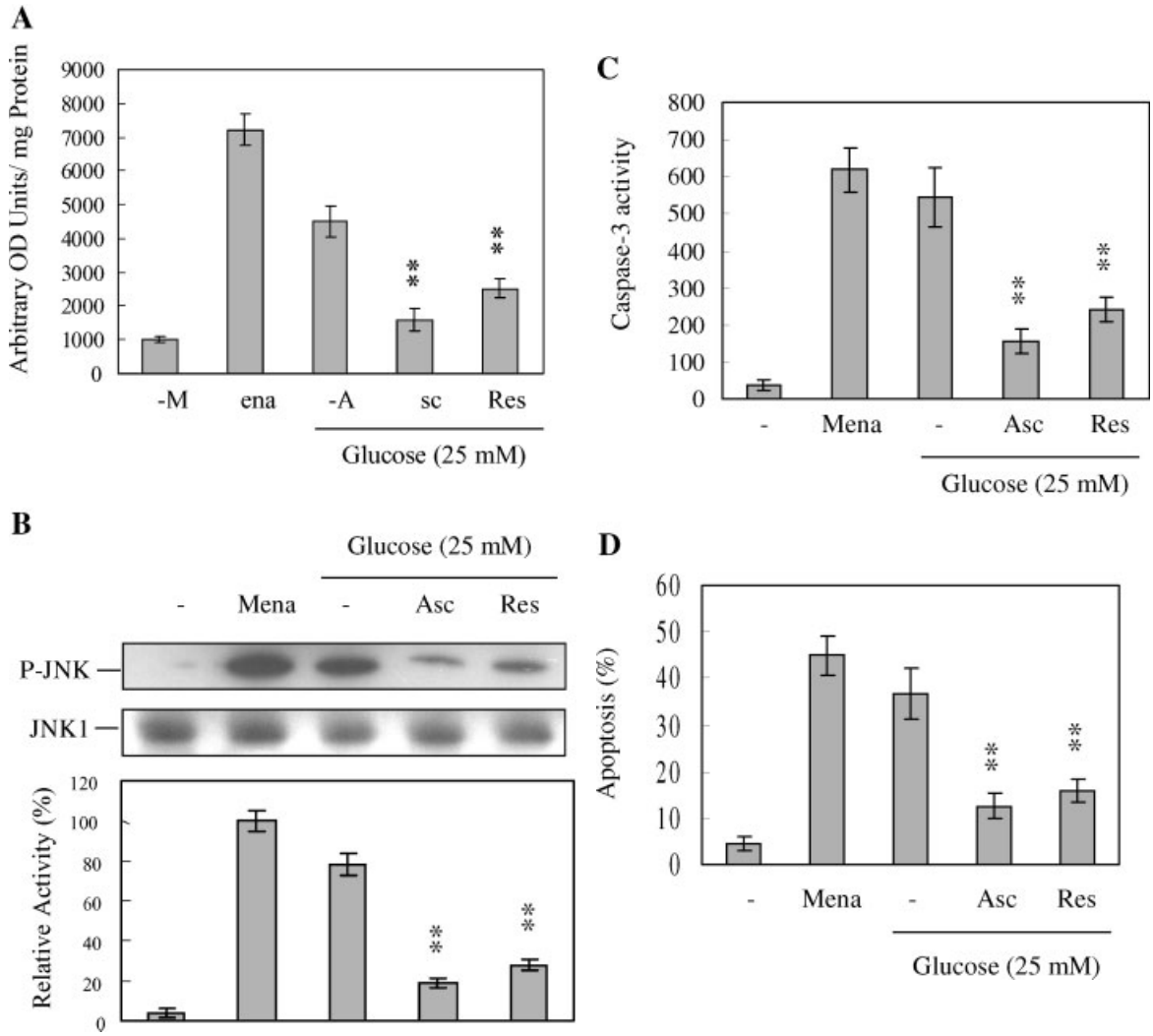

Fig. 4. Resveratrol attenuates high glucose-induced oxidative stress. K562 cells were either (a) left untreated or treated with 50 μM menadione (Mena), (b) incubated in culture media containing 25 mM glucose for 24 h, or (c) pretreated with ascorbic acid (vitamin C; Asc) (300 μM) or resveratrol (Res) (20 μM) for 1 h, followed by treatment with 25 mM glucose for 24 h. **A**: ROS generation was assayed using 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), and expressed as absorbance/mg of protein. Cell extracts were prepared and analyzed for JNK activation (**B**), caspase-3 activation (**C**), and apoptosis (**D**), as described in the legends to Figures 1 and 3. Values are presented as means ± SE. $^{**}P < 0.01$ versus the value of the 'high glucose only' treatment group.

efficacy of this assay system in the quantitative measurement of intracellular ROS. Using this assay system, the relative intracellular ROS content was measured in K562 cells during high glucose treatment, both with and without resveratrol pretreatment. High glucose increased the intracellular ROS content in K562 cells, and pretreatment with ascorbic acid (vitamin C), a well-known ROS scavenger, and resveratrol significantly attenuated this increase (Fig. 4A). Interestingly, inhibition of ROS generation by ascorbic acid and resveratrol resulted in significant reduction of the apoptotic biochemical changes (i.e., JNK activation, caspase-3 activation, and DNA fragmentation) in high glucose-treated K562 cells (Fig. 4B,D). The results collectively demonstrate that ROS is an essential regulator for high glucose-induced apoptosis in K562 cells.

Resveratrol Prevents the Decrease in Attachment Ability of K562 Cells With Collagen I Induced by High Glucose

The number of K562 cells attaching to a collagen I-coated petridishes decreased with increasing doses of glucose. Attachment of K562

cells to collagen I following high glucose pretreatment was significantly lower than that of untreated control cells (Fig. 5A). We observed a time-dependent decrease in attachment, following treatment with 25 mM glucose (Fig. 5B). Using propidium iodide and Hoechst 33342 staining to measure the apoptosis of the attached and non-attached cells, we showed that petridish-binding cells were all not apoptotic in the control and glucose-treated groups (Fig. 5C). All apoptotic cells were measured in the non-attached (suspension) fraction (Fig. 5C). These data support the possibility that high glucose decreases the attachment ability of K562 cells by inducing apoptotic injury. To further investigate the effects of resveratrol and ascorbic acid (vitamin C) on the observed decrease in attachment ability, cells were pretreated with the above compounds, followed by high glucose. Higher levels of attachment to collagen I were observed following resveratrol or ascorbic acid pretreatment, compared to control cells treated with high concentrations of glucose only (Fig. 5D). Based on the data, we propose that the hyperosmotic shock effects induced by high glucose inhibit the binding of K562 cells to collagen I, which is reversed in the presence of resveratrol. Interestingly, ascorbic acid (a well-known antioxidant) also attenuated the effect of high glucose-induced attachment changes (Fig. 5D). These results imply that resveratrol prevents high glucose-induced attachment changes by virtue of its ability to act as a ROS scavenger.

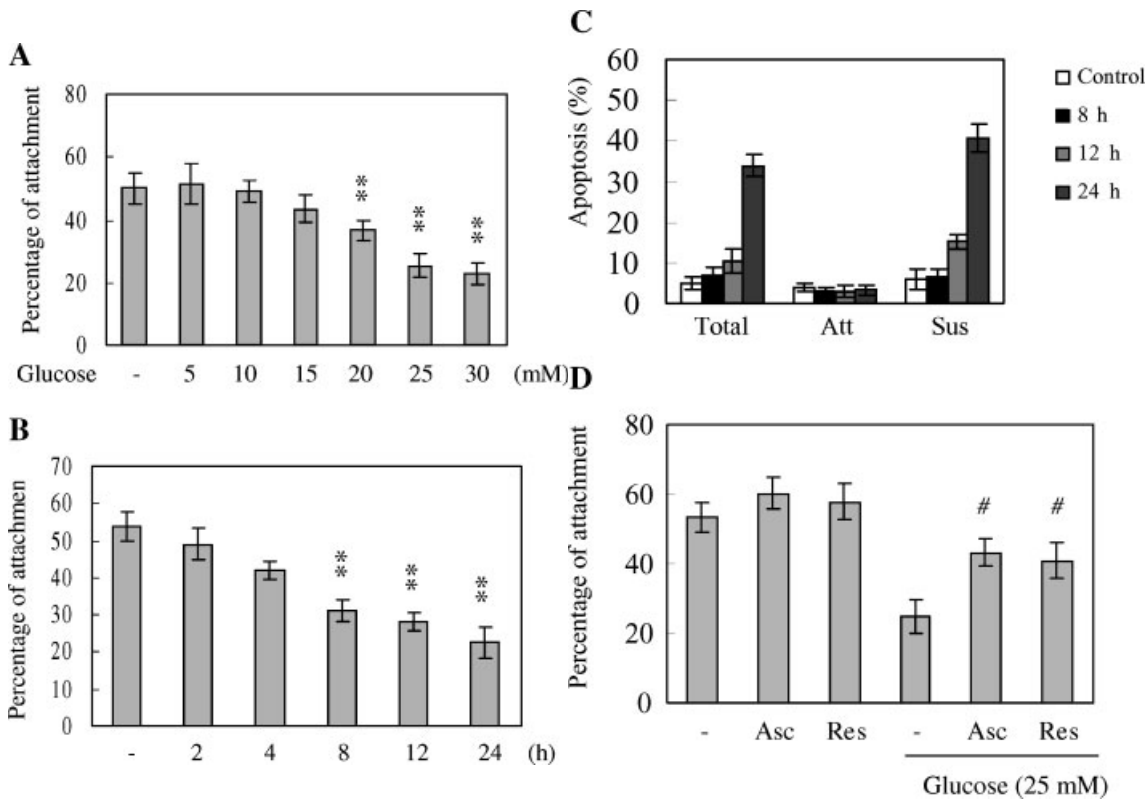

Fig. 5. Analysis of attachment ability changes to determine the effect of resveratrol on high glucose-treated K562 cells. **A**: K562 cells were incubated with or without various concentrations of glucose at 37°C for 24 h. Cells were allowed to adhere to collagen-coated petridishes by incubation at 37°C for 90 min. Cell attachment was quantitatively evaluated using microscopy after staining with trypan blue. **B**: K562 cells were incubated with or without culture media containing 25 mM glucose for the indicated times, and the number of attached cells was counted. **C**: K562 cells were incubated with 25 mM glucose at 37°C for 8–24 h or left untreated. Cells were allowed to adhere to collagen-coated petridishes by incubation at 37°C for 90 min. Apoptosis was measured by staining total, petridish-attachment (Att), and petridish-non-attachment (suspension; Sus) cells with propidium iodide and Hoechst 33342. **D**: K562 cells were incubated with or without 300 μM ascorbic acid (vitamin C; Asc) or 20 μM resveratrol (Res) for 1 h, incubated in culture medium containing 25 mM glucose for 24 h, and the number of attached cells was counted. Cell attachment was expressed as a percentage of total cells. Values are presented as means ± SE. $^{**}P < 0.01$ versus the value of the control (glucose untreated) group. $^{\#}P < 0.01$ versus the value of the 'high glucose only' treatment group.

Resveratrol Inhibits JNK Activation Required for Caspase-3 Activation During High Glucose-Induced Apoptosis

To determine the relationship between JNK and caspase-3 activities during apoptosis, we examined the effect of pretreatment with JNK1 antisense oligonucleotides (to inhibit de novo synthesis) on K562 cells subjected to high concentrations of glucose. After pretreatment with antisense oligonucleotides, the amount of JNK was reduced to approximately 50% that in the control cells (Fig. 6A). The JNK activity of cells pretreated with JNK1 antisense oligonucleotides was also reduced to ~50% that in control cells treated with 25 mM glucose (Fig. 6B). Significantly, the inhibition of JNK synthesis by the antisense oligonucleotide led to a reduction in caspase-3 activation and cell apoptosis (Fig. 6C,D). Using SP600125, an inhibitor specific for JNK [Bennett et al., 2001], we additionally demonstrated that high glucose-induced caspase-3 activation and apoptosis in K562 cells is mediated by JNK activity (Fig. 6E,G). These findings indicate that JNK is an important regulator of caspase-3 activation and subsequent apoptotic biochemical changes during high glucose-induced apoptosis.

DISCUSSION

Earlier studies have demonstrated that high glucose increases ROS generation in human aortic endothelial cells [Cosentino et al., 1997], and additionally triggers apoptosis in HUVEC cells [Baumgartner-Parzer et al., 1995]. In this study, we have shown that high glucose directly evokes ROS generation in K562 cells (Fig. 4). An association between ROS generation and high glucose concentrations is possible, but requires further investigation.

The inhibitory effect of resveratrol on apoptotic biochemical changes triggered by several stimuli is attributed to its antioxidant properties [Jang and Surh, 2001]. Oxidative stress is a recognized stimulator of cell responses, such as apoptosis. Antioxidants protect cells against apoptosis induced by various stimuli that exert both direct and indirect oxidant effects [Buttke and Sandstrom, 1994; Slater et al., 1995]. The antioxidant and anti-inflammatory properties of resveratrol may be due to cyclooxygenase inhibition [Jang et al., 1997]. A recent report shows that resveratrol exerts a powerful antioxidant effect on multiple ROS (such as O_2^- and H_2O_2) production in macrophage cells subjected to lipopolysaccharide (LPS)- or phorbol ester (PMA) treatment. O_2^- production by LPS- or PMA-treated phagocytic cells occurs via the NADPH oxidase pathway [Martinez and Moreno, 2000]. Other experiments show that α-tocopherol and another natural antioxidant inhibit O_2^- production by blocking NADPH oxidase [Cachia et al., 1998]. Thus, it is possible that the mechanisms affecting NADPH oxidase activation are additionally involved in the effects of resveratrol on ROS production. Here we show that resveratrol attenuates high glucose-induced intracellular ROS formation, supporting the hypothesis that the compound suppresses apoptosis by inhibiting NADPH oxidase activation and consequently reducing the level of ROS that forms after high glucose treatment (Fig. 4).

The ability of K562 cells to attach to collagen decreased under high glucose conditions (Fig. 5), possibly due to an increase in ROS formation. Previous studies report that ROS attack the polyunsaturated fatty acid component of the membranes of polymorphonuclear leukocyte cells, leading an alteration in membrane composition [Chari and Muddeshwar, 1991]. Based on the data obtained, we suggest that the antioxidants, ascorbic acid and resveratrol, actively scavenge ROS formed by high glucose, thus restoring the attachment of K562 to collagen I (Fig. 5). The alterations in attachment activity may interfere with the transendothelial migration of leukocytes that further affect immune and inflammatory responses. Thus, resveratrol may aid in decreasing the toxic effect of high glucose in DM due to its antioxidant property.

Previous studies show that resveratrol can both stimulate and inhibit apoptotic signaling [Jang and Surh, 2001; Nicolini et al., 2001; Fulda and Debatin, 2004; Su et al., 2004]. The results of this report show that resveratrol (at doses greater than 20 μM) blocks high glucose-induced apoptotic biochemical changes in K562 cells (Figs. 2 and 3). Taken together, these observations imply that the cell type specificity and action of resveratrol depend on the treatment protocol (i.e., treatment period and dosage of resveratrol). Clearly the molecular mechanism of action of resveratrol on cell apoptosis requires further investigation.

JNK functions in many cell responses, including entry into apoptosis. Using antisense

oligonucleotides of JNK to inhibit de novo protein synthesis and JNK specific inhibitor (SP600125) to inhibit JNK activation, we demonstrated that high glucose-induced caspase-3 activation and apoptosis in K562 cells are mediated by JNK activity (Fig. 6). These observations, together with the findings that ROS generation and JNK activation triggered by

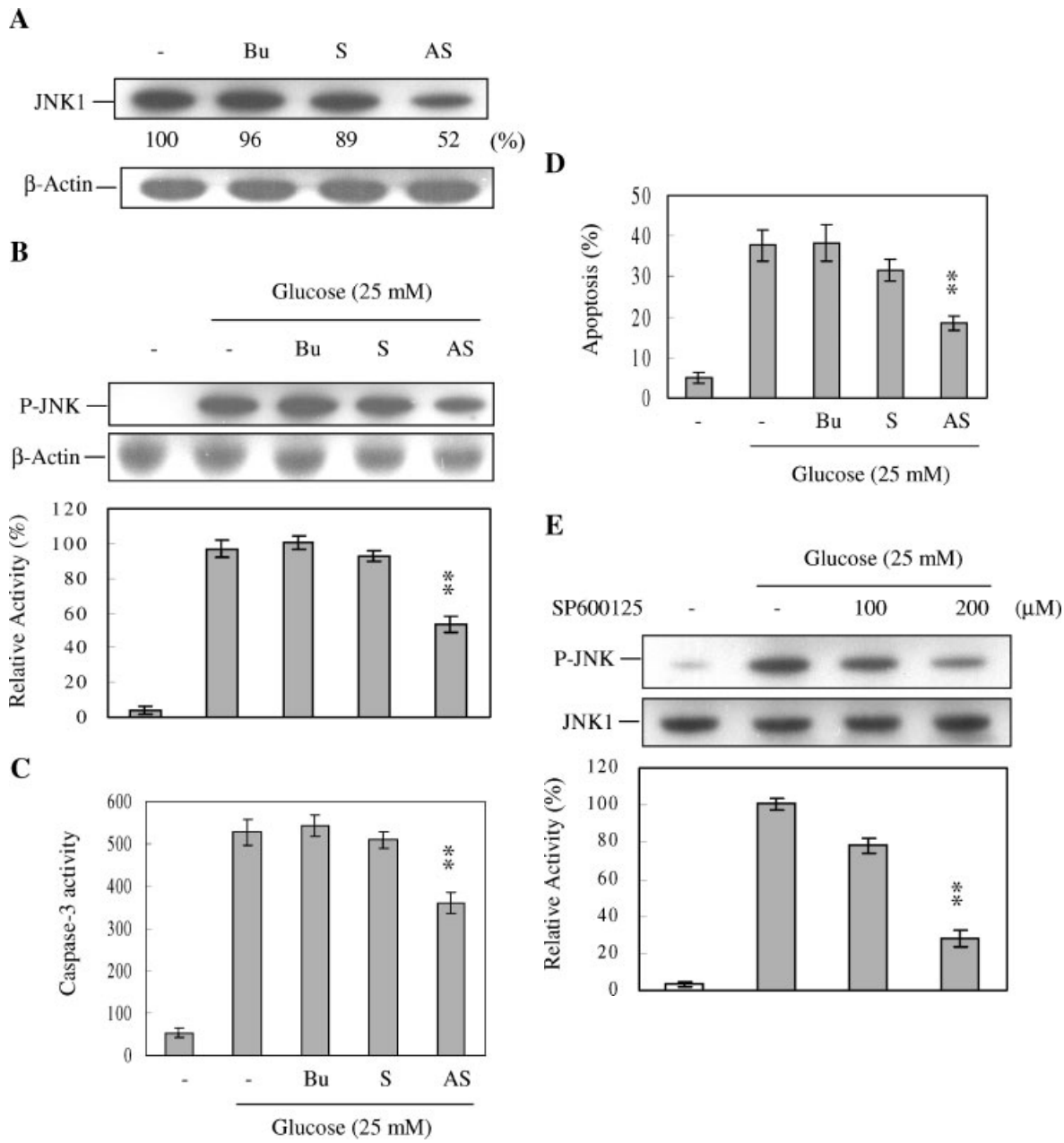

Fig. 6. Effect of an antisense oligonucleotide against JNK1 on high glucose-induced apoptotic biochemical changes and ROS generation. K562 cells were incubated with HEPES buffer alone (Bu) or 70 μM JNK1 sense (S) or antisense (AS) oligonucleotides in the presence of lipofetamine for 72 h. **A**: Cell extracts (40 μg) were resolved on 10% SDS gels and electroblotted onto PVDF membranes. Membranes were probed with anti-JNK1 (C17) and anti-β-actin antibodies. **B–E**: Cells were subjected to 25 mM glucose for 24 h. Cell extracts (40 μg) were analyzed by immunoblotting with anti-p-JNK and anti-β-actin antibodies (B). Cell extracts (100 μg) were analyzed for caspase-3 activity using Z-DEVD-AFC as the substrate (C). Apoptosis was measured using propidium iodide and Hoechst 33342 staining (D). E–G: K562 cells were preincubated with various concentrations of SP600125 at 37°C for 1 h, and exposed to high glucose (25 mM). Cell extracts (40 μg) were analyzed for JNK activation (E), caspase-3 activity (**F**), and apoptosis (**G**). Values are presented as means ± SE. $^{**}P < 0.01$ versus the value of the 'high glucose only' treatment group.

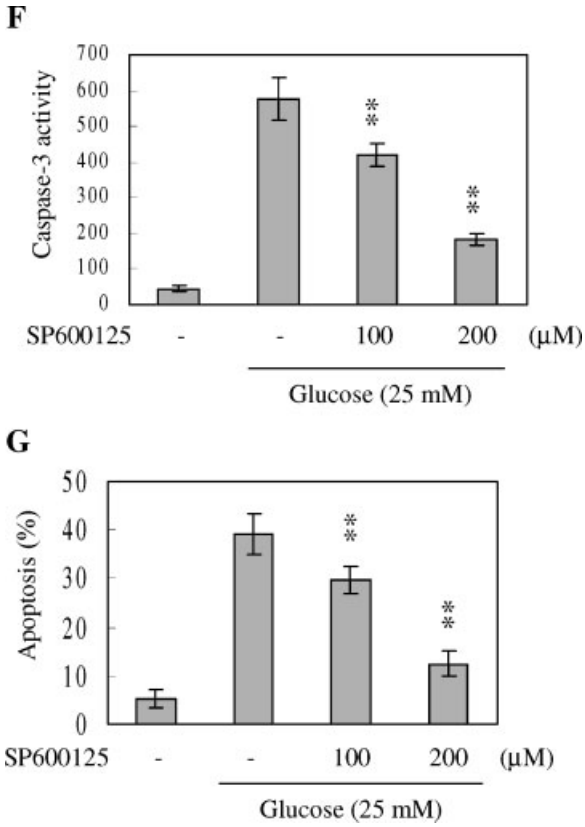

Fig. 6. (*Continued*)

high glucose can be blocked by resveratrol, support the hypothesis that this compound inhibits high glucose-induced apoptotic biochemical changes by blocking ROS formation and JNK activation, both of which are important triggers for subsequent apoptotic biochemical changes.

In summary, this study shows that high glucose causes various apoptotic biochemical changes in K562 cells. Resveratrol effectively blocks high glucose-induced biochemical changes, including ROS generation, JNK activation, caspase-3 activation, and DNA fragmentation. In addition, the ability of K562 cells to attach to collagen I is reduced in the presence of high glucose, which is reversed following pretreatment with resveratrol. Thus our findings support the possibility that resveratrol aids in decreasing the toxic effect of high glucose in DM patients.

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