Resveratrol induces FasL-related apoptosis through Cdc42 activation of ASK1/JNK-dependent signaling pathway in human leukemia HL-60 cells

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Trans-resveratrol, a phytoalexin found at high levels in grapes and in grape products such as red wine, has been shown to prevent carcinogenesis or antitumor growth in murine models. Here we dissect the detailed signaling pathway involved in resveratrol-induced apoptosis. Our data showed that treatment with resveratrol-induced activation of apoptosis signal-regulating kinase 1α, a mitogen-activated protein kinase kinase kinase, in turn, activated the downstream kinases c-Jun N-terminal kinase and p38 mitogen-activated protein kinase, but not extracellular signal-regulated kinase. Transfection with a dominant-negative c-Jun N-terminal kinase expression vector reduced FasL expression and DNA fragmentation induced by resveratrol. However, inhibition of p38 mitogen-activated protein kinase activity by treatment with SB203580 (p38 mitogen-activated protein kinase specific inhibitor) or expression of mutant p38 mitogen-activated protein kinase expression vector did not alter the apoptosis and FasL expression in response to resveratrol. Furthermore, genetic inhibition of apoptosis signal-regulating kinase 1α signaling inhibited not only the activation of c-Jun N-terminal kinase, but also the expression of FasL and apoptosis. Similarly, over-expression of wild-type apoptosis signal-regulating kinase 1 strengthened the resveratrol-induced c-Jun N-terminal kinase activation, FasL expression and subsequent apoptosis. These results suggest the possible involvement of apoptosis signal-regulating kinase 1α/c-Jun N-terminal kinase signaling in the regulation of FasL expression and subsequent apoptosis induced by resveratrol in HL-60 cells. Resveratrol also activated the small GTP-binding protein Cdc42, rather than other members such as RhoA or Rac1. Expression of a mutant Cdc42 (N17 Cdc42) dramatically reduced resveratrol-induced c-Jun N-terminal kinase activity, FasL expression and apoptotic cell death. These results showed that resveratrol-induced apoptosis through the Cdc42/apoptosis signal-regulating kinase 1α/c-Jun N-terminal kinase/FasL signaling cascade in HL-60 cells.

Introduction

A new dimension in the management of neoplasia was the increasing awareness that chemoprevention, which refers to the administration of chemical agents to prevent the events associated with carcinogenesis (1) and this could be the most direct way to reduce mortality and morbidity. A large number of chemopreventive and chemotherapeutic agents have been discovered from natural products and these provide a promising strategy to fight against cancer by inducing apoptosis in malignant cells (2,3). Resveratrol (trans-3,4',5-trihydroxystilbene) is a phytoalexin found in grapes, fruits, red wines and root extracts of the weed Polygonum cuspidatum and has been an important constituent of Japanese and Chinese folk medicine; it has become one of the most promising chemopreventive agents against cancer (4,5). Resveratrol had been shown to exhibit cancer chemopreventive effects in different systems based on its striking inhibition of a variety of diverse cellular events associated with tumor initiation, promotion and progression (6). In addition, resveratrol also suppressed the expression of inducible nitric oxide synthetase and cyclooxygenase-2 and this was likely to contribute to both its anti-inflammatory and anti-angiogenic mechanisms (7–9). The antitumor activity of resveratrol has been indicated by its apoptosis-inducing ability in numerous cell types (4–6). Resveratrol induces apoptosis in human leukemia cells as demonstrated by DNA fragmentation, an increased proportion of sub-diploids in the cell population, and a dose-dependent increase in cleavage of the caspase substrate poly (ADP-ribose) polymerase (PARP) (4). Recent evidence emphasized the importance of up-regulating the Fas/FasL (CD95/CD95L) system for the control of apoptosis and a number of cytotoxic drugs up-regulate such expression leading to Fas/FasL-mediated signal transduction, activation of caspases, and ultimately, cell death (10). Induction of the Fas/FasL system was also shown to be a critical mechanism of resveratrol-induced cell death in HL-60 cells, as well as T47D breast carcinoma cells (4). Despite these important advances, the molecular mechanism(s) by which resveratrol exerts its anticancer effect has not yet been elucidated.

Response to numerous types of extracellular signals were mediated by mitogen-activating protein kinases (MAPKs), which were members of the serine/threonine kinase family. Several studies revealed that c-Jun N-terminal kinase (JNK)1/2/stress-activated protein kinase (SAPK) and/or p38 MAP kinase activation were involved in apoptosis induced by a variety of different stimuli, such as γ-radiation-induced apoptosis in Jurkat T-cells (11), glutamate-induced apoptosis in rat cerebellar granule cells (12), mlgM-induced apoptosis in human B

Abbreviations: ASK1, apoptosis signal-regulating kinase 1; DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; MAPKs, mitogen-activating protein kinases; PBS, phosphate-buffered saline; SAPK, stress-activated protein kinase; SDS-PAGE, sodium dodecyl-sulfate-polyacrylamide gel electrophoresis; TNF, tumor necrosis factor.
lymphocytes (13) or apoptosis induced by genotoxic stresses, such as ultraviolet, X-ray, H₂O₂, heat shock (14–18) and cell surface receptor Fas (19,20). Various well-known chemotherapeutic drugs, such as Adriamycin, vinblastine, VP-16 and CPT were also capable of activating JNK. These drugs were critical in triggering apoptosis in different cell lines (21–24). This suggests that the JNK signaling cascade may be the dominant participant in apoptosis. Apoptosis signal-regulating kinase 1 (ASK1), a member of MAPK kinase kinase group, was reported to activate two different subgroups of MAPK kinase, SEK1/MKK4 and MKK3/MKK6, which in turn, activate JNK/SAPK and p38 MAPK, respectively (25). Over-expression of ASK1 in epithelial cells cultured in a low serum environment-induced apoptosis. In ovarian cancer cells, expression of a dominant-negative mutant of ASK1 or JNK1 inhibited microtubule-interfering agent-induced apoptosis, suggesting that ASK1 plays a role in the mechanism of stress-induced apoptosis (26,27). ASK1 was activated upon treatment with tumor necrosis factor (TNF)-α or agonistic anti-Fas antibody, and a dominant-negative mutant of ASK1 reduced TNF-α and Fas-induced JNK activation and apoptosis, suggesting that ASK1 was a pivotal component in cytokine-induced apoptosis (28,29).

Thus, we examined the signaling pathway involved in resveratrol-induced apoptosis in human leukemic HL-60 cells. Our data showed that resveratrol induced the activation of the small GTP-binding protein, Cdc42 and, in turn, the apoptotic signaling pathway ASK1/JNK1/FasL.

Materials and methods

Cell culture and chemicals
HL-60 cells, a human promyelocytic leukemia cell line, were obtained from the American Type Culture Collection. Cells were maintained in a humidified 5% CO₂ atmosphere and cultured in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. Resveratrol, propidium iodide and cell transfection and establishing stable expressing cell lines

Transfection was created by electroporation (model T800, BTX, San Diego, CA). Briefly, cells were plated in 10-cm dishes, serum-starved for 12 h, and lysed by freeze-thawing in ice cold 400 μl of lysis buffer [50 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin] and centrifuged at 14 500 r.p.m. for 20 min at 4°C. The cell lysate was mixed with 10 μg of bacterially expressed GST-rhotekin (murine amino acids 252–258) and GST-PAK (rat PAK amino acids 186–194) was used as the substrate for JNK1 and GST-ATF-2 was used as the substrate for p38. Rho family pull-down assay

The Rho family pull-down assay was performed as described by Ren et al. (31) and Z sondag et al. (32). Briefly, cells were plated in 10-cm dishes, serum-starved for 12 h, and lysed in 50 mM Tris-Cl, pH 7.2, 500 mM NaCl, 1% (v/v) Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM diithiothreitol (DTT), 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. The soluble extracts were prepared by centrifugation at 14 500 r.p.m. for 15 min at 4°C. Following normalization of the protein concentration, equal amounts of protein were incubated with protein A-Sepharose and anti-JNK1 or anti-p38 antibody for 3 h at 4°C. The immune complexes were washed twice with ice cold PBS and lyzed in buffer containing 20 mM HEPES (pH 7.4), 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM dithiothreitol (DTT), 100 μM sodium fluoride, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. Detection was performed using ECL western blotting reagent and chemiluminescence exposed onto Kodak X-Omat film.

Immunoprecipitate and kinase activity assays

Immunoprecipitates containing ASK1 were analyzed by western blotting with anti-RhoA, anti-Rac1 and anti-Cdc42 antibodies.

Western blot analysis

Western blotting was performed according to a previously described method (24). Briefly, cell lysates were prepared, electrophoresed and then immunoblotted with anti-phospho-JNK1, anti-phospho-p38 anti-phospho-extracellular signal-regulated kinase (ERK)/1,2, anti-JNK1, anti-p38, anti-ERK1, anti-CDC95 and anti-CDC95 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed using ECL western blotting reagent and chemiluminescence exposed onto Kodak X-Omat film.

Immunofluorescence for CD95L

Immunofluorescence staining for CD95L was performed as described previously. Immunofluorescence for CD95L and anti-CD95 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed using ECL western blotting reagent and chemiluminescence exposed onto Kodak X-Omat film.

Immunoprecipitation and kinase activity assays

Cell lysates were resolved by SDS polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. Glutathione S-transferase (GST)-c-jun (amino acids 1–169) was used as the substrate for JNK1 and GST-ATF-2 was used as the substrate for p38. Western blot analysis

Western blotting was performed according to a previously described method (24). Briefly, cell lysates were prepared, electrophoresed and then immunoblotted with anti-phospho-JNK1, anti-phospho-p38 anti-phospho-extracellular signal-regulated kinase (ERK)/1,2, anti-JNK1, anti-p38, anti-ERK1, anti-CDC95 and anti-CDC95 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed using ECL western blotting reagent and chemiluminescence exposed onto Kodak X-Omat film.

DNA fragmentation assay

Cells were collected, washed with PBS twice and then lysed in 100 μl of lysis buffer [50 mM Tris (pH 8.0), 10 mM EDTA, 0.5% sodium laurel sarcosinate and 1 mg/ml proteinase K] for 3 h at 56°C and treated with 0.5 mg/ml RNase A for an additional 1 h at 56°C. DNA was extracted by the phenol/chloroform/ isomyl alcohol (25/24/1) method before loading. Loading buffer [50 mM Tris, 10 mM EDTA, 1%, (v/v) low melting point agarose (DTT), 10 mM blue] and samples were loaded onto a pre-solidified, 2% (v/v) agarose gel containing 0.1 μg/ml ethidium bromide. Agarose gels were electrophoresed at 50 V for 90 min in TBE buffer. Gels were observed and photographed under ultraviolet light.

Separation of particulate and cytosolic fractions

Cells were plated in 10-cm dishes, serum-starved for 12 h, and lysed by freeze-thawing in ice cold 400 μl of lysis buffer [50 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin] and centrifuged at 14 500 r.p.m. for 20 min at 4°C, and the supernatant was collected as the cytosolic fraction. Pellets were re-suspended, and membrane proteins were lysed in 250 μl of lysis buffer containing 2% Triton X-100. The homogenate was added to 1% Triton X-100, 5 mM MgCl₂, 1 mM DTT and 0.1% Triton X-100, following which, they were re-suspended in 20 μl of kinase assay buffer containing 5 μCi of [γ-32P]ATP, 30 μM cold ATP and 2 μg of substrate and incubated for 20 min at 30°C. Reactions were terminated by the addition of the sodium dodecylsulfate (SDS) sample buffer and boiling for 5 min. The phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. Glutathione S-transferase (GST)-c-jun (amino acids 1–169) was used as the substrate for JNK1 and GST-ATF-2 was used as the substrate for p38.
centrifuged at 100,000 r.p.m. for 30 min at 4°C. The supernatant was collected and is referred to here as the particulate fraction. Cytosolic and particulate fraction proteins were separated by SDS-PAGE.

Results

Resveratrol increases FasL expression and MAPKs activity in HL-60 cells

It has been shown that resveratrol induces apoptosis via the activation of the Fas/FasL-dependent pathway in HL-60 cells, but the mechanism(s) are not clear (4). To elucidate the mechanism(s) of Fas/FasL induction in response to resveratrol, we examined the MAP kinases, which play a critical role in the apoptosis-related signaling pathway. Time-dependent studies revealed that resveratrol enhanced FasL expression, but not Fas in HL-60 cells and this was detectable at 1 h as well as being sustained to 24 h (Figure 1A). Incubation of HL-60 cells with resveratrol (20 μM) led to phosphorylation of JNK up to 24 h (8.6-fold induction compared with control) (Figure 1B, upper panel). Activation of JNK was detected at 1 h and increased for up to 24 h upon resveratrol treatment. Resveratrol also induced phosphorylation of p38, and phosphorylated p38 MAPK remained elevated for up to 24 h (Figure 1B) (4.1-fold induction compared with control). Western blot analysis showed that the activation of JNK and p38 was not due to enhanced expression of the JNK and p38 proteins (Figure 1B). However, under the same treatment, we did not detect any significant difference in ERK1/2 activation, with or without resveratrol treatment (Figure 1B, lower panel). These observations suggest that JNK and p38, but not ERK1/2, are persistently activated during resveratrol-induced FasL expression.

JNK kinase activity is required for resveratrol-induced FasL expression and subsequent apoptosis

To analyze the involvement of JNK and p38 MAPK in FasL induction by resveratrol, we used a genetic approach to inhibit JNK activity by establishing a HL-60 cell line constitutively expressing a dominant-negative JNK (HL-60/DN-JNK). Resveratrol-induced JNK activation was effectively blocked in HL-60/DN-JNK cells, but not in the control vector expressing cells (HL-60/vector), according to immunocomplex kinase assay results (Figure 2A). Blocking the JNK pathway by DN-JNK significantly diminished not only the resveratrol-induced up-regulation of FasL (~75% less), but also DNA fragmentation (Figure 2B). To confirm the above observations, we used another well-established method to quantify apoptosis. Detection of the number of hypodiploid cells (apoptotic cells), which are stained less intensely with propidium iodide, showed...
can be unequivocally measured from the peak in the flow cytometry sub-G1 region. Figure 2C indicates that treatment with resveratrol for 24 h induced dramatic apoptotic cell death in vector control cells and the dominant-negative mutant of JNK (DN-JNK) reduced 50% apoptosis compared with control cells (resveratrol-induced apoptotic cells was from 53% in control cells to 26% in HL-60/DN-JNK cells). These results suggest that the JNK kinase cascade is required for resveratrol-mediated FasL expression and apoptotic cell death for human leukemic HL-60 cells.

Activation of p38 MAPK is involved in Fas/FasL signaling and cell death (33–35) but the role of p38 MAPK in resveratrol-induced apoptotic cell death in human leukemic HL-60 cells is not clear. To address this issue, HL-60 cells were pretreated with the specific p38 MAPK inhibitor SB203580 with or without resveratrol, and then FasL expression and apoptotic cell numbers were analyzed by flow cytometry. Figure 3A reveals that 20 μM SB203580 significantly reduced the increased levels of phosphorylated p38 MAPK induced by resveratrol. However, there was no significant difference in FasL protein expression (Figure 3A, lower panel) and subsequent apoptosis (Figure 3B) in the same treatment. To further confirm this observation, we used another approach to test the role of p38 MAPK by establishing a HL-60 cell line expressing dominant-negative p38 (p38AF). These results support those obtained previously and resveratrol-induced up-regulation of FasL protein (Figure 3C, upper panel) and the number of apoptotic cells (Figure 3D) were not changed by expression of p38AF. However, the failure to decrease resveratrol-induced FasL expression and apoptosis by p38AF was not due to a low level of inhibition efficiency, because resveratrol-induced activation of p38 MAPK was dramatically reduced in p38AF expressed cells as measured by an immunocomplex kinase assay (Figure 3C, lower panel). These results suggest the possible involvement of JNK, but not p38 MAPK, in the regulation of FasL expression and apoptosis in leukemic cancer cells induced by resveratrol.

ASK1 acts as an up-stream regulator of resveratrol-induced apoptosis

ASK1 is necessary for cytotoxic stress-induced apoptosis and relays its signals to stress-activated MAPKs such as JNK and p38 (36,37). To examine the role of ASK1 in resveratrol-induced apoptotic process, the kinase-inactive ASK1 mutant (ASK1-KM) was transfected into HL-60 cells (HL-60/ASK1-KM) and the JNK activity was assessed. Data from the immune complex kinase assay confirmed that over-expression of ASK1-KM significantly decreased resveratrol-induced JNK activation and FasL expression (Figure 4A). Similarly, HL-60/ASK1-KM cells exhibited more resistance to resveratrol-induced DNA fragmented hypodiploid cells than the HL-60/vector control cells (Figure 4B). Furthermore, expression of wild-type ASK1 (ASK1-WT) strengthened resveratrol-induced JNK activation and FasL expression (Figure 4C). The increase in apoptotic cells was also enhanced by 45%.
under the expression of the ASK1-WT in response to resveratrol for 12 h (Figure 4D). Together, these findings provide realistic evidence that ASK1 was involved in resveratrol-induced JNK activation, FasL expression and subsequent apoptosis.

**Cdc42 is involved in resveratrol-induced signaling cascade and apoptosis**

Many researchers have reported that MAP kinase pathways are activated via small GTP-binding proteins in response to different stimulations (38,39). Because of these previous studies, we attempted to demonstrate whether the Rho family of small GTP-binding proteins was involved in resveratrol-induced apoptotic signaling in HL-60 cells. The effects on Rho family protein activity were assayed using GST pull-down assays after treatment with resveratrol for indicated times. Our results show that resveratrol activated Cdc42, rather than the two other Rho family small GTPases, RhoA and Rac1 in HL-60 cells in a time-dependent manner (Figure 5A, upper panel).

Resveratrol-induced Cdc42 activation was noticed at 30 min and peaked at 60 min. As shown in Figure 5A (lower panel), Cdc42 was translocated from the cytosol to the membrane and translocation of Cdc42 was initially detected at 5 min, became evident at 30 min and maximal at 60 min after stimulation of HL-60 with resveratrol. To further confirm that activated Cdc42 played a critical role in resveratrol-induced apoptosis in HL-60 cells, we used a series of dominant-negative mutants of the various proteins involved. Transient transfection with the dominant-negative Cdc42 expression vector (N17Cdc42) dramatically decreased resveratrol-induced apoptosis (Figure 5B, upper panel). Transient expression of dominant-negative RhoA or Rac1 (N19RhoA or N17Rac1) did not reduce the number of apoptotic cells in response to resveratrol (Figure 5B, upper panel). However, the failure of inhibition of resveratrol-induced apoptosis by N19RhoA and N17Rac1 was not due to the under-expression of these two mutant constructs, because the expression level of these three myc-tagged dominant-negative mutants were similar by detection of
the expression of myc protein by western blot (Figure 5B, lower panel). More importantly, inhibition of Cdc42 reduced JNK kinase activity and also the expression of FasL and the levels that result were similar to those detected before stimulation with resveratrol (Figure 5C). This suggests that Cdc42 controls resveratrol-induced activation of JNK kinase, FasL expression and subsequent apoptosis. To the best of our knowledge, this is the first time that Cdc42 has been shown to play a role in resveratrol-induced apoptosis in HL-60 cells and we have shown that the expression of FasL and subsequent apoptosis are regulated via Cdc42/ASK1/JNK signaling cascade.
Discussion

Recent reports have shown that resveratrol was a potent cancer chemopreventive agent in assays representing the three major stages of carcinogenesis (4). Resveratrol is a potent chemoprevention agent that prevents, inhibits or reverses carcinogenesis by either reducing angiogenesis or inducing cancer cell apoptosis (40–42). In a recent study, we indicated that resveratrol inhibits VEGF-induced angiogenesis through interruption of Src-dependent VE-cadherin phosphorylation (43). Although treatment with resveratrol is an appropriate strategy for chemoprevention, the mechanism is, as yet, unclear. In this study we verified that the activation of a novel signaling pathway from the Cdc42 small GTP-binding protein to subsequent ASK1-mediated JNK activation is a potential requirement for resveratrol-induced elevation of FasL expression and apoptosis in human leukemic cancer cells. Activation of this cascade seems to be necessary for resveratrol-induced cell death, since genetic inhibition of Cdc42, JNK or ASK1 prevents the induction of apoptosis.

Fas is a well characterized member of the death receptor family. Engagement of Fas by a Fas-ligand led to the formation of a protein complex known as death-inducing signaling complex and permit acute execution of apoptosis by caspase-8 activation (44). The Fas-binding protein, Daxx, binds to the N-terminal region of ASK1 and, thereby, activated JNK, which may sensitize the cell to apoptosis (29). The results of our study showed that a signaling cascade of Cdc42/ASK1/JNK/FasL in response to resveratrol-induced apoptosis. Therefore, we suggest that resveratrol-induced FasL expression through the Cdc42/ASK1/JNK signaling pathway and increased FasL may activate the same pathway, enhancing FasL expression and/or inducing apoptosis. This accumulates more evidence to support such an auto-enhancement hypothesis.

In the present study, inhibition of FasL expression by genetic inhibition of JNK, but not p38, indicates that resveratrol-induced activation of these two kinases may have different effects on FasL expression and apoptosis. Other studies (45,46) have shown that a single inhibition of JNK or p38 MAPK does not prevent programmed cell death induced by some stresses, such as Fas or ceramide. Nonetheless, combined inhibition of JNK and p38 MAPK significantly reduced apoptosis; the activation of both kinases seems to be required for induction of apoptosis in Jurkat cells (11). Likewise, JNK activation was essential for the induction of apoptosis after ionizing radiation, ultraviolet light exposure, heat shock, TNFα or H2O2 (14–18). In some cell types, blockage of JNK activity might be sufficient to prevent apoptosis by ceramide, Fas or stress, whereas in other cells, e.g. Jurkat cells, inhibition of both JNK and p38 MAPK was required to prevent apoptosis. JNK and p38 MAPKs did not seem to be equally important in
all forms of apoptosis, since inhibition of both kinases did not prevent apoptosis in Jurkat cells incubated with thapsigargin, a microsomal Ca\(^{2+}\) ATPase inhibitor. Resveratrol also induced apoptosis through these two kinases in different cell types (4,40,41). However, in this study, JNK-DN-transfected HL-60 cells were almost completely resistant to induction of FasL and apoptosis by resveratrol (Figure 2). In contrast, inhibition of resveratrol-induced p38 MAPK activity by treatment with SB203580 or transfection with the mutant p38 expression vector (p38AF) did not decrease the induction of FasL (Figure 3A and C), but slightly increased apoptosis in the presence of resveratrol (Figure 3B). According to the above observation, we presume that resveratrol-induced FasL expression is p38-independent and the slight difference in apoptosis may be due to inhibition of p38-dependent survival signals, such as those from ERK1/2 (47–49). The ASK1/p38 MAPK signal cascade is also reported to be involved in the differentiation and survival of other cells (50). Based on our observations, we suggest that resveratrol-induced FasL expression and apoptosis are mediated through the JNK pathway, rather than the p38 MAPK pathway.

Over-expression of wild-type or constitutively active ASK1 induced apoptosis in various cells through mitochondria-dependent caspase activation (51,52), and ASK1 was required for apoptosis induced by oxidative stress, TNF and endoplasmic reticulum stress (26,28,53). Several lines of evidence suggest that ASK1 has diverse functions in deciding cell fate, such as differentiation and survival. We showed that resveratrol activated ASK1/JNK signaling and this activation was necessary for FasL induction and apoptotic cell death (Figure 4). On the other hand, resveratrol-activated ASK1 also induced p38 MAPK activity in HL-60 cells (data not shown), but there was no significant difference in the resveratrol-induced apoptosis with or without inhibition of p38 MAPK (Figure 3). Thus, we suggest that resveratrol-activated ASK1 triggers phosphorylation of the downstream MAPK members, JNK and p38 MAPK, in individual ways. Resveratrol-induced ASK1/JNK and ASK1/p38 MAPK pathways also had different effects on FasL expression and apoptosis. We propose that these differences might be due to the different transcription factors activated by JNK and p38 MAPK, inducing expression of different genes. Our data confirm that ASK1 appears to be a pivotal component not only in stress-induced cell death, but also in a broad range of biological activities.

Small GTP-binding proteins of the Rho family have been traditionally linked to the reorganization of the actin-based cytoskeleton (54). More recent evidence has revealed that Rho-related GTPase can also regulate gene expression often through the activation of kinase cascades leading to enhanced activity of SAPKs, including JNK and p38 MAP kinase (38,39,55). The ability to stimulate SAPK also suggests that these Rho GTP-binding proteins can promote the initiation of the apoptotic programs mediated by JNK and p38, thus resulting in cell death. To the best of our knowledge, there are currently no reports on Rho family small GTP-binding protein being involved in resveratrol-induced apoptosis. In the present study, we showed that resveratrol activated Cdc42 but not other Rho family members (Figure 5A). Inhibition of Cdc42 also significantly reduced resveratrol-induced apoptosis in HL-60 cells (Figure 5B). Supportively, previous studies have reported that expression of the active form of Cdc42 in Jurkat T lymphocytes (56) as well as in rat sympathetic neurons (57) induce an apoptotic response. Cdc42 regulates signaling by binding to downstream effector proteins (58) mainly through its CRIB domain (59). Reports have shown that Cdc42 and its downstream effectors, PAKs and JNK/SAPK, are involved in stress-activated apoptosis (60). In the present study, ASK1 may be the novel potent downstream events or the phosphorylation targets of Cdc42 that promote resveratrol-induced apoptosis. Cdc42 has been reported to recruit PAK1 and phosphorylates JNK and thus induce subsequent apoptosis (60). In this study, the questions of whether Cdc42 can directly interact with ASK1 or JNK molecules need further investigation.

Other naturally occurring polyphenol agents, such as garcinol, induced Rho family GTPase-related apoptosis through D4-GDI, a hematopoietic cell-abundant regulator of Rho family GTPases in HL-60 cells (61). Similarly, resveratrol, just like garcinol, has antioxidant and free radical scavenging effects and antibacterial/antifungal activities. In contrast to this, we found that Cdc42 played a crucial role in resveratrol-induced apoptosis in the current study. Because of its critical role as a modulator of Cdc42, resveratrol might, in turn, have a significant impact on the mechanisms that induce cytoskeletal and morphological changes in apoptotic cells.

In conclusion, we present the first evidence demonstrating that the cancer-chemopreventive agent resveratrol induces a novel Cdc42-dependent apoptosis program. We delineated the resveratrol-induced apoptosis-signaling pathway in which Cdc42 is initially activated and which, in turn, activated the ASK1/JNK pathway that triggered the expression of FasL and resulted in apoptosis of HL-60 cells (Figure 6).

![Fig. 6. Resveratrol induced apoptosis via the novel signaling cascade Cdc42/ASK1/JNK/FasL. Resveratrol induced Cdc42 expression, and activates ASK1/JNK pathway that triggers the expression of FasL and induces apoptosis of HL-60 cells.](https://academic.oup.com/carcin/article-abstract/26/1/1/2475999)
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