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Role of oxidation-triggered activation of JNK and p38 MAPK in black tea polyphenols induced apoptotic death of A375 cells

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Theaflavins (TF) and thearubigins (TR) are the major polyphenols of black tea. Our previous study revealed that TF- and TR-induced apoptosis of human malignant melanoma cells (A375) is executed via a mitochondria-mediated pathway. In our present study we observed the role of the three most important MAPK (ERK, JNK, and p38) in TF- and TR-induced apoptosis. TF and TR treatment of A375 cells led to sustained activation of JNK and p38 MAPK but not ERK, suggesting that JNK and p38 are the effector molecules in this polyphenol-induced cell death. This idea was further supported by subsequent studies in which JNK and p38 activation was inhibited by specific inhibitors. Significant inhibition was found in TF- and TR-treated A375 cell death pretreated with JNK- or p38-specific inhibitors only. Further, we have found that TF and TR treatment induces a time-dependent increase in intracellular reactive oxygen species generation in A375 cells. Interestingly, treatment with the antioxidant *N*-acetyl cystein inhibits TF- and TR-induced JNK and p38 activation as well as induction of cell death in A375 cells. We also provide evidence demonstrating the critical role of apoptosis signal-regulating kinase 1 in TF- and TR-induced apoptosis in A375 cells. Taken together our results strongly suggest that TF and TR induce apoptotic death of A375 cells through apoptosis signal-regulating kinase 1, MAPK kinase, and the JNK–p38 cascade, which is triggered by *N*-acetyl cystein intracellular oxidative stress. (*Cancer Sci* 2009; 100: 1971–1978)

The water extract of the dry leaves of the plant *Camellia sinensis* is popularly known as tea. According to its processing, tea can be classified into green, oolong, and black tea, among which black tea is the most popular. TF and TR are the two most important and abundant polyphenols of black tea. Black tea has been shown to be potent in inhibiting tumorigenesis in animal models including lung, colon, and skin.[1–3] Some reports provide evidence that black tea and its polyphenols, especially TF, significantly inhibit proliferation and enhance apoptosis in some cancer cells.[4–6]

Apoptosis is a form of programmed cell death in multicellular organisms. The process of apoptosis is controlled mainly by two cascades, namely the kinase cascade[7,8] and the protease cascade.[8,9] The MAPK cascade, one of the very important members of the kinase cascade, plays an important role in apoptosis induction especially when the apoptotic signal is initiated by various types of stresses such as intracellular oxidative stresses.[10] It has been reported that ROS are important mediators of apoptosis.[11] Oxidative stress induces multiple signal transduction pathways, including the MAPK pathway.[12,13] The extent and duration of MAPK activation plays a key role in controlling different cell functions.[14,15]

MAPK are members of a family of serine/threonine kinases that are involved in both the stress response and apoptosis. To date, three MAPK cascades have been extensively characterized: ERK, JNK, and p38. The biological effects of MAPK signaling are mainly executed by phosphorylation of downstream substrates including transcription factors such as c-Jun and c-Fos.[15,16] ERK is predominantly activated by mitogens leading to cell differentiation, growth, and survival.[16,17] On the other hand, JNK and p38 are preferentially activated by oxidative stress and cytokines resulting in inflammation and apoptosis.[7,17]

JNK and p38 MAPK, as well as their upstream kinases such as MKK and MKKK, are the key regulators of stress-activated apoptosis in various cells.[7,18–20] ASK1 is a MKKK family member that is activated in response to various cellular stresses such as ROS, activates stress-activated MAPK such as JNK and p38, and induces apoptosis.[7,18,20,21] Many recent reports indicate that EGCG can produce intracellular ROS in various cell types.[22,23] ROS act as the signaling intermediates and can trigger downstream cellular events, like mitochondrial dysfunction and activation of JNK and p38 MAPK and other signaling pathways that ultimately lead to apoptotic cell death.[11]

Previously we determined that TF and TR induce apoptosis in A375 cells, and the IC_{50} values of both TF and TR are approximately 50 μg/mL at 48 h.[24] Several reports indicate that EGCG can modulate the activation of stress-activated MAPK and apoptosis[25,26] but the effect of black tea polyphenols on the apoptosis-inducing effect of MAPK is hardly reported. In the present study, we have attempted to explore the role of MAPK and oxidative stress in TF- and TR-induced apoptotic death in A375 cells.

Materials and Methods

Reagents. Primary antibodies (JNK1/2, ERK1/2, p-38, ASK1, MKK4, MKK3, p-JNK, p-ERK, p-p38, p-ASK1, p-MKK4, p-MKK3/6, NF-κB [p65], and β-actin) and polyclonal secondary antibody were obtained from Cell Signalling Technology (Danvers, MA, USA). Bromophenol blue, EGTA, NaF, Na_3VO_4, Nonidet P-40 (NP-40), PMSF, aprotinin, leupeptin, pepstatin A, NAC, HEPES, MTT, H_2O_2, molecular-grade BSA, Tween-20, NBT/BCIP, Tris-HCl, and DMSO were procured from Sigma-Aldrich (St Louis, MO, USA). DCFH-DA was from Molecular Probes (Eugene, OR, USA). The nucleus/cytosol fractionation kit was from BioVision (Mountain View, CA, USA). Caspase fluorometric assay kits were obtained from Chemicon International Corporation (Temecula, CA, USA). All MAPK inhibitors were purchased from Calbiochem (La Jolla, CA, USA). The Bio-Rad Protein Assay Kit was from Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture. A375 cells (human malignant melanoma) were purchased from the National Centre for Cell Science (Pune, India) and maintained in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen Corporation, Grand Island, NY, USA). The cells were incubated

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at 37°C in a humidified atmosphere containing 5% CO_2 inside a CO_2 incubator.

Extraction of TF and TR from black tea. Both TF and TR were extracted from the black tea (Tata Tea Ltd., Kolkata, India) as described previously.[24,27,28] Extraction was carried out in the Department of Chemistry of our institute. In short, black tea (10 g) was initially extracted with boiling water (250 mL), and then the aqueous fraction was further extracted with chloroform to remove caffeine and successively with ethyl acetate and *n*-butanol by liquid–liquid partitioning.[29] The ethyl acetate fraction contained TF whereas the *n*-butanol fraction contained TR.[30] The total TF and TR contents of our samples were calculated to be 2.3 and 12.5%, respectively, of the dry weight of the sample.

MTT assay. The effect of TF and TR on the viability of A375 cells in the presence or absence of different inhibitors was determined by MTT assay following the method of Mosmann.[31] Briefly, approximately 5×10^4 cells per well were plated in 96-well plates and treated with TF or TR (0, 25, 50, 75, 100 μg/mL) for 48 h either with or without inhibitors. At the end of the stipulated time the medium was aspirated and MTT (50 μL from 5 mg/mL stock solution in PBS) was added into each well and incubated at 37°C for 2 h. The purple-colored precipitate of formazan was dissolved in 150 μL DMSO. The color absorbance was recorded at 540 nm with a microplate reader with a reference serving as blank. The IC_{50} value was also determined.[24,32]

Preparation of cytosolic and nuclear fractions. A375 cells (5×10^6) were harvested after treatment with TF or TR for 1, 3, 6, 12, and 24 h. Isolation of the nuclear and cytosolic cell fractions was carried out using a nucleus/cytosol fractionation kit according to the manufacturer's protocol.

Measurement of intracellular ROS level. A375 cells were seeded in 90-mm dishes (5×10^7 cells per dish) overnight. Cells were treated with 50 μg/mL TF or TR for 0.5, 1, 3, 6, 12, and 24 h and then collected by trypsinization. The cell suspension (200 μL, containing 2×10^6 cells/mL) was added to PBS (800 μL) and incubated with DCFH-DA (10 μM) for 15 min at 37°C. The production of intracellular DCF-DA due to the oxidation of DCFH-DA by H_2O_2 was measured using a fluoremeter (excitation at 488 nm and emission at 515 nm). Another set of experiments was carried out simultaneously, with the same treatments and conditions, where NAC (5 mM) was added to the cell culture medium 30 min before the addition of TF and TR.

Activity of caspases. Caspase-3 and caspase-9 were assayed using the fluorometric caspase assay kits designed for the respective caspases, as per the manufacturer's protocol. Briefly, A375 cells (2×10^5 per well) were plated in triplicate in a 24-well tissue culture plate and treated with TF or TR (50 μg/mL) in the absence or presence of different MAPK inhibitors for 12 and 24 h. The caspase activity was determined fluorimetrically (excitation at 400 nm and emission at 505 nm).

Western blot analysis. Western blot analysis was done to determine the expression of different proteins. A375 cells were treated with the optimal (50 μg/mL) concentration of TF or TR for 1, 3, 6, 12 and 24 h, or with different concentrations of TF or TR (0, 25, 50, 75, 100 μg/mL) for 6 or 12 h. Cells were harvested, washed with cold PBS (pH 7.4), and lysed with ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 1% NP-40, 1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin, pH 7.4) for 30 min and centrifuged at 12 000 *g* for 30 min at 4°C. Protein was evaluated using the Bio-Rad Protein Assay Kit. Equal amounts of protein from each treatment were subjected to SDS-PAGE. Thereafter, proteins were electrophoretically transferred to nitrocellulose membrane and non-specific sites were blocked with 5% skim milk in 20 mM TBS (pH 7.5) containing 0.1% Tween-20 and reacted with primary polyclonal antibodies (JNK1/2, ERK1/2, p38, ASK1, MKK4, MKK3, p-JNK, p-ERK, p-p38, p-ASK1, p-MKK4, p-MKK3/6, NF-κB [p65], and β-actin) for 4 h at room temperature. After washing with TBS containing 0.1% Tween-20 the membrane was then incubated with alkaline phosphatase-conjugated goat antirabbit secondary antibody. The protein bands were visualized using NBT–BCIP mixture.

Statistical analyses. GraphPad Instat software (Graphpad Software Inc., La Jolla, CA, USA) was used for statistical analyses. All data are expressed as the mean ± SD of three independent experiments. The differences between the control and the experimental groups were determined by one-way analysis of variance (ANOVA) and post tests were done using Dunnett's multiple comparison test to determine the levels of significance. $P < 0.05$ and $P < 0.01$ were considered to be significant.

Results

JNK and p38, but not ERK, phosphorylation was upregulated by TF and TR treatment in A375 cells. MAPK are activated during the course of apoptosis induced by a number of compounds. Hence we monitored the activation of three members of the MAPK family (p38, JNK1/2, and ERK1/2) using western blot analysis with antibodies that recognize the phosphorylated forms of the three kinases. In the time-dependent study, both TF (Fig. 1AI) and TR (Fig. 1AII) treatments induced sustained phosphorylation of JNK and p38 MAPK whereas expression of p-ERK was maintained at a constant level in A375 cells. The expressions of total p38, JNK, and ERK MAPK were unaltered throughout the time course (Fig. 1AI,AII). We also studied the dose-dependent changes of the phosphorylated forms of these MAPK proteins 12 h after TF or TR treatment (Fig. 1BI,BII). The expression of p-p38 and p-JNK was increased dose dependently but p-ERK did not show a dose-dependent increase in expression upon TF (Fig. 1BI) or TR (Fig. 1BII) treatment.

Inhibition of JNK and p38 confers resistance to TF- and TR-induced caspase activation and induction of cell death in A375 cells. To assess the role of the sustained activation of p38 and JNK during TF- and TR-induced apoptosis, we used the specific cell-permeable inhibitors SB203580 for p38 and SP600125 for JNK, and both inhibitors attenuated TF-induced (Fig. 2AI) and TR-induced (Fig. 2AII) cell death significantly. Blocking ERK with the MEK inhibitor PD98059 did not show any change in the rate of cell death induction by TF and TR (Fig. 2AI,AII). The inhibitory effects of SB203580 and SP600125 on TF- and TR-induced caspase-3 (Fig. 2BI) and caspase-9 (Fig. 2BII) activities in A375 cells were also observed. Both of the inhibitors markedly reduced their activities as measured by the fluoremetric process. But PD98059 did not show any significant changes in caspase-3 or caspase-9 activity (Fig. 2BI,BII).

Effect of TF and TR treatment on NF-κB in A375 cells. TF and TR did not alter the expression level of NF-κB as determined by western blot analysis of the nuclear and cytosolic fractions of TF- or TR-treated A375 cells (Fig. 3).

TF and TR induced ROS generation time-dependently. ROS are known to play an important role in the intracellular signaling pathway of apoptosis. We studied intracellular ROS production caused by TF (Fig. 4AI) and TR (Fig. 4AII) treatment at their optimal concentration (50 μg/mL) in A375 cells at different time points. The fluorescence of DCF-DA increased gradually during the first 3 h of incubation and then decreased (Fig. 4AI,AII). Control or vehicle-treated cells (0.05% DMSO) alone did not increase the fluorescence (data not shown). Next, the cells were pretreated with 5 mM NAC for 30 min prior to TF or TR treatment. We observed that the intracellular ROS level decreased upon NAC treatment (Fig. 4AI,AII).

Effect of NAC on TF- and TR-induced cell death induction and caspase-3 activation. We also studied the effects of NAC, a known scavenger of ROS, on the extent of cell death induced by TF and TR in A375 cells. Treatment with NAC (2, 5, and 10 mM) prior to TF and TR treatment in A375 cells showed a

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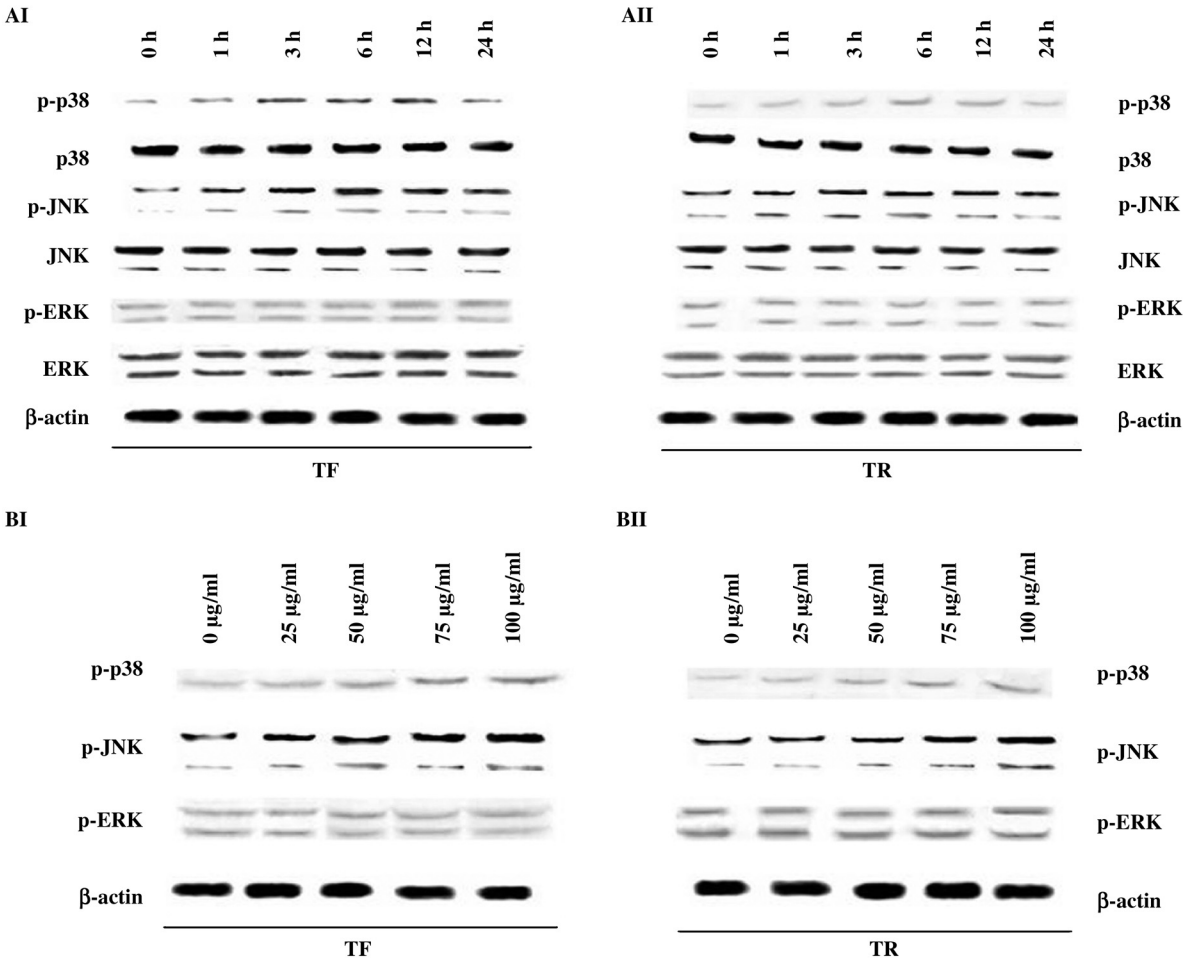

Fig. 1. Time- and dose-dependent effect of theaflavins (TF) and thearubigins (TR) on the expression of MAPK proteins. (AI,AII) Time-dependent study of the effect of (AI) TF or (AII) TR on MAPK proteins. A375 cells (5×10^7 per treatment) were treated with TF or TR at 50 μg/mL concentration and the expression patterns of ERK, phosphorylated (p)-ERK, JNK, p-JNK, p38, and p-p38 were observed at 0, 1, 3, 6, 12, and 24 h of treatment. Protein from the total cell lysate was subjected to SDS-PAGE and western blotting using p-p38, p38, p-JNK, JNK, p-ERK, ERK, and β-actin antibodies. Representative blots from three independent experiments gave identical results. The relative intensity of each band after normalization with the intensity of β-actin in a blot (below each western blot) was measured. (BI,BII) Dose-dependent study of (BI) TF or (BII) TR on MAPK proteins after 12 h of incubation. A375 cells (5×10^7 per treatment) were treated with TF or TR at 0 (control), 25, 50, 75, and 100 μg/mL concentrations and the expression patterns of p-ERK, p-JNK, and p-p38 were observed at 12 h of treatment. Protein from the total cell lysate was subjected to SDS-PAGE and western blotting using p-JNK, p-p38, p-ERK, and β-actin antibodies. Representative blots from three independent experiments gave identical results. The relative intensity of each band was measured after normalization with the intensity of β-actin in a blot (below each western blot).

significant reduction in black tea polyphenol-induced cell death in the cancer cells (Fig. 4B). An inhibitory effect of NAC (2, 5, and 10 mM) on TF- and TR-induced caspase-3 activation was observed when NAC was added to A375 cells 30 min before treatment with TF and TR (Fig. 4C).

Effect of NAC on the TF- and TR-induced activation of p38 and JNK MAPK in A375 cells. Our results showed that phosphorylation of JNK and p38 MAPK with different doses of TF and TR (25, 50, and 75 μg/mL) treatment in A375 cells was clearly reduced when the cells were pretreated with NAC (Fig. 5AI,AII). When we treated A375 cells with H_2O_2 alone as a known ROS inducer, it clearly upregulated the expression of p-JNK and p-p38 in a dose-dependent manner but not p-ERK (Fig. 5B).

Effect of TF and TR treatment on the upstream signaling molecules of JNK and p38 in A375 cells. Our time-dependent study revealed that TF (Fig. 6AI) and TR (Fig. 6AII) treatments upregulated the level of p-MKK3 and 6 and p-MKK4, whereas the non-phospho forms of MKK3 and MKK4 remained unaltered. Our result also indicates that ASK1 is phosphorylated during the early hours of TF and TR treatment (Fig. 6AI,AII). The expression of p-ASK1 was also upregulated upon TF and TR treatment in a dose-dependent manner (Fig. 6B).

Effect of H_2O_2 and NAC on phospho-ASK1 expression in TF- or TR-treated A375 cells. To find out the link between ROS generation and the activation of MAPK, we investigated the effect of H_2O_2 on ASK1 in A375 cells after TF or TR treatment using the specific antibody that recognizes the phosphorylated form of this upstream MKKK. We found that the p-ASK1 level was increased dose dependently upon treatment with H_2O_2, a known ROS inducer (Fig. 6C).

As shown in Figure 6(D), TF- and TR-induced activation of p-ASK1 was effectively inhibited by treatment with NAC at different doses, suggesting that ASK1 is one of the most important upstream molecules that might influence the ROS-dependent upregulation of JNK and p-38 upon TF and TR treatment.

Discussion

The MAPK pathway is an important intracellular signaling cascade in the transduction of apoptotic signals initiated in response to various extracellular stimuli, including environmental stresses.[7,12]

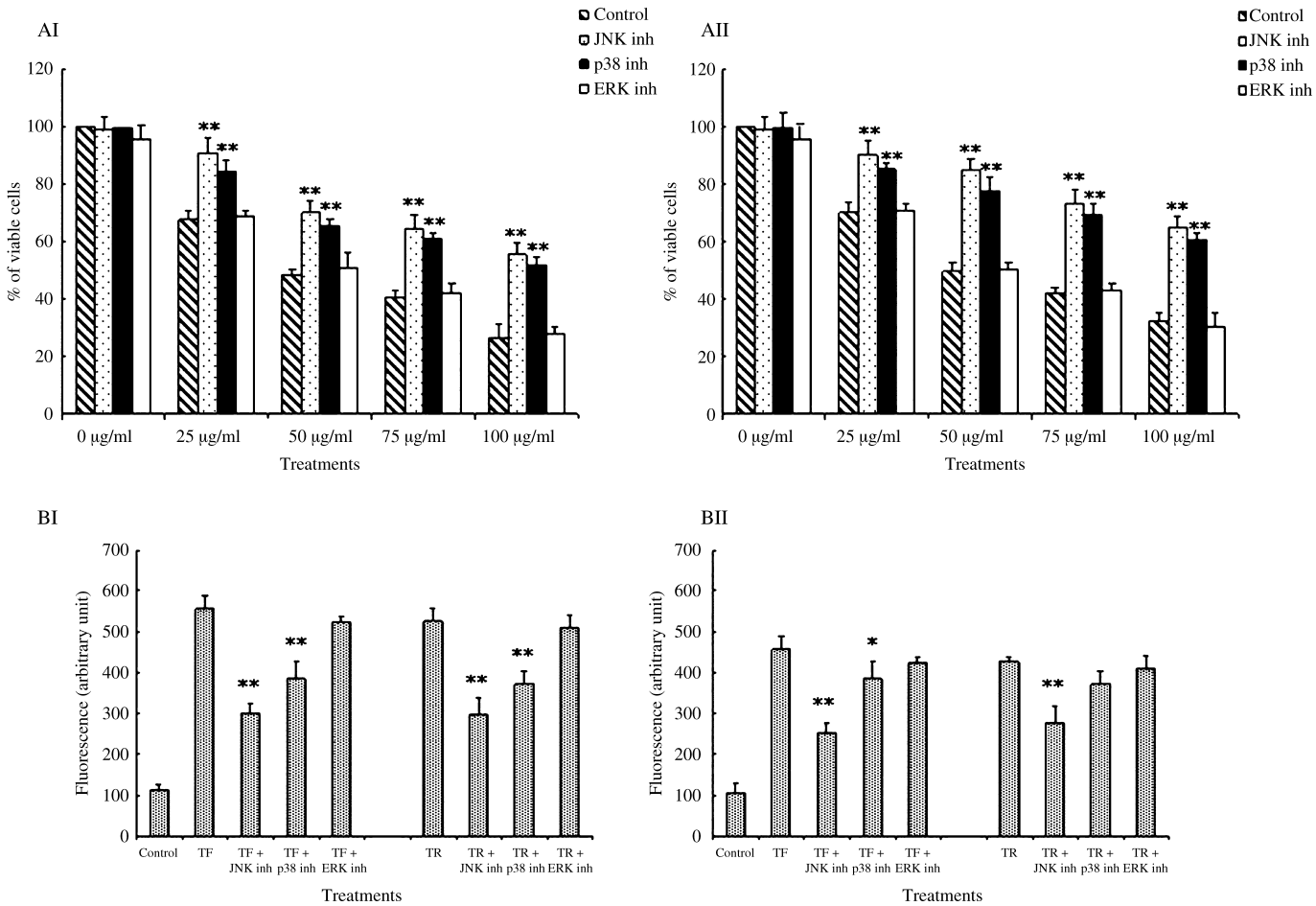

Fig. 2. Effect of theaflavins (TF) and thearubigins (TR) on the viability and activity of caspase-3 and caspase-9 in A375 cells in the presence of MAPK inhibitors. (AI,AII) Effect of (AI) TF and (AII) TR on A375 cell viability in the presence of different MAPK inhibitors. A375 cells (5×10^4 per well) were pretreated with JNK-specific inhibitor, p38-specific inhibitor, or ERK-specific inhibitor for 30 min at 37°C and then treated with (AI) TF or (AII) TR at different concentrations (0, 25, 50, 75, and 100 μg/mL). Then the cells were grown in a 96-well plate for 48 h. The viability of the cells was observed by MTT assay. Each value is expressed as mean ± SD ($n = 3$). *$P < 0.05$ and **$P < 0.01$ are the TF plus inhibitor or TR plus inhibitor groups compared with the TF- or TR-treated groups at different concentrations (0, 25, 50, 75, 100 μg/mL). (BI,BII) Effect of TF and TR on the activity of (BI) caspase-3 and (BII) caspase-9 in A375 cells in the presence of different MAPK inhibitors. Cells were preincubated with JNK-specific inhibitor SP600125 (20 μM), p38-specific inhibitor SB203580 (10 μM), or ERK-specific inhibitor PD98059 (5 μM) for 30 min at 37°C. Then cells were treated with (BI) TF or (BII) TR at 50 μg/mL for 12 h and subjected to measurement of caspase activities. Each value is expressed as mean ± SD ($n = 3$). *$P < 0.05$ and **$P < 0.01$ are the TF plus inhibitor or TR plus inhibitor groups compared with TF- or TR-treated cells.

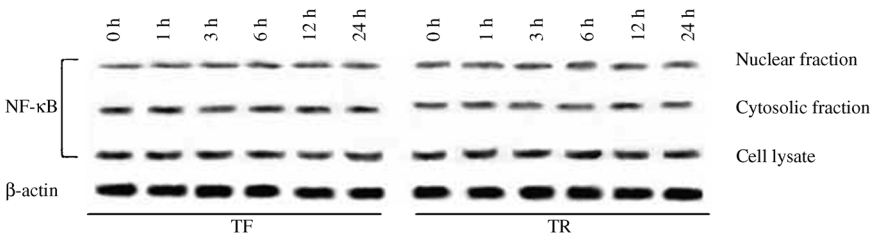

Fig. 3. Effect of theaflavin (TF) or thearubigin (TR) treatment on the expression level of nuclear factor (NF)-κB (p65) in the cytosolic fraction, nuclear fraction, and whole-cell lysate of A375 cells at different time points. A375 cells (5×10^7 per treatment) were treated with TF or TR at 50 μg/mL for 0, 1, 3, 6, 12, and 24 h. Protein from the cytosolic fraction, nuclear fraction, and total-cell lysate was subjected to SDS-PAGE and western blotting using NF-κB (p65) and β-actin antibodies. Representative blots from three independent experiments gave identical results. The relative intensity of each band was measured after normalization with the intensity of β-actin in a blot (given below each western blot).

This pathway is well conserved in cells from yeast to vertebrates and consists of MAPK, MKK, and MKKK.[33,34] MKK, such as MKK3 and 6 or MKK4 and 7, in turn can be activated by several MKKK, including ASK1, ASK2, TGF-β activated kinases 1, and Tumor progression locus 2.[12,13] Of the MKKK molecules, ASK1, is ubiquitously expressed and activates the MKK4/7–JNK and MKK3/6–p38 signaling cascades.[13,18] Overexpression of ASK1 also induces apoptosis in cultured cells,[35] suggesting that ASK1 is a pivotal component in stress or cytokine-induced apoptosis. Three distinct MAPK (ERK, p38, and JNK) have been characterized and reported to be involved in apoptosis in many different paradigms of cellular toxicity.[7]

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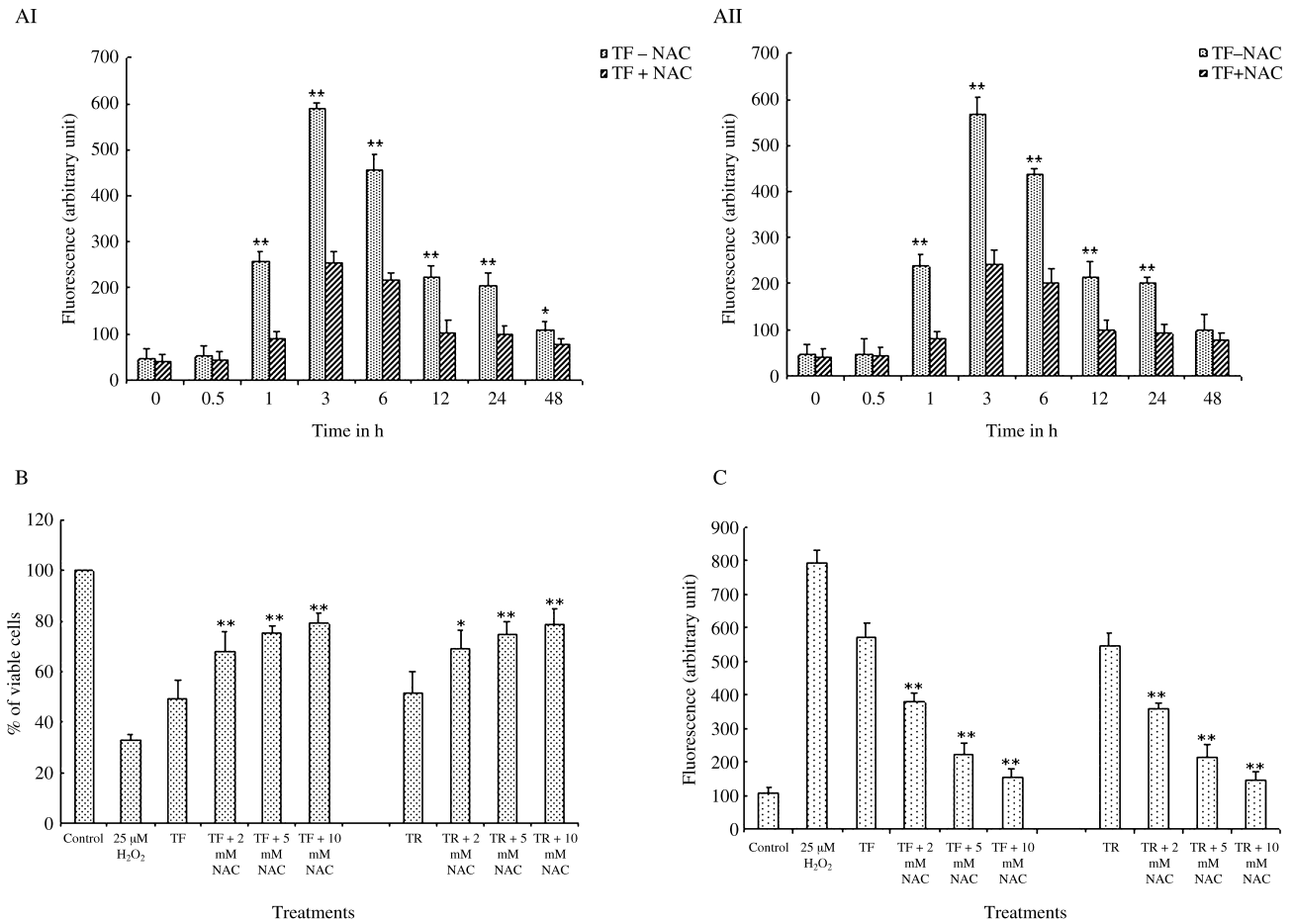

Fig. 4. Effect of theaflavins (TF) and thearubigins (TR) on intracellular reactive oxygen species (ROS) generation, cell viability, and caspase-3 activity in presence of *N*-acetyl cystein (NAC) (ROS inhibitor) in A375 cells. (AI,AII) Time-dependent effect of (AI) TF and (AII) TR on ROS production in A375 cells in the absence or presence of NAC. NAC was added at 5 mM, 30 min prior to the addition of TF and TR. Then the cells were treated with TF or TR at a concentration of 50 μg/mL for 0, 0.5, 1, 3, 6, 12, 24, and 48 h. Each value is expressed as mean ± SD ($n = 3$). *$P < 0.05$ and **$P < 0.01$ are the TF- or TR-treated groups at 0 h of treatment, as compared with the TF- or TR-treated groups at other time points (0.5, 1, 3, 6, 12, 24, 48 h). (B) Effect of TF and TR on A375 cell viability in the presence of NAC at different concentrations. NAC was added at different concentrations (2, 5, 10 mM) to the cell culture medium 30 min before the addition of TF and TR. Cells were then treated with TF or TR at a concentration of 50 μg/mL for 48 h. Each value is expressed as mean ± SD ($n = 3$). *$P < 0.05$ and **$P < 0.01$ are the TF plus different concentrations of NAC or TR plus different concentrations of NAC groups, compared with TF- or TR-treated groups. (C) Effect of TF and TR on caspase-3 activity in the presence of NAC at different concentrations. NAC was added at different concentrations (2, 5, 10 mM) to the cell culture medium 30 min before the addition of TF and TR. Then the cells were treated with TF or TR at a concentration of 50 μg/mL for 12 h. Each value is expressed as mean ± SD ($n = 3$). **$P < 0.01$ is the TF plus different concentrations of NAC or TR plus different concentrations of NAC groups compared with TF- or TR-treated groups.

Previously we have reported that TF and TR can induce apoptosis in A375 cells via a mitochondrial pathway.[24] The objective of our present work was to investigate the involvement of all major MAPK, namely ERK, p38, and JNK, in induction of apoptosis of A375 cells upon TF and TR treatment.

The active form of the MAPK is the phosphorylated form of these proteins.[14] Hence we observed the expression of the phosphorylated forms of these proteins upon TF and TR treatment in A375 cells. Our findings suggest that both p-p38 and p-JNK are upregulated by means of TF and TR treatment in a time- and dose-dependent manner. But the p-ERK and ERK levels were not modulated either time or dose dependently upon treatment with TF or TR, suggesting that ERK might not be affected by this treatment in A375 cells. To validate whether JNK and p38 have any significant role in death induction we used the JNK inhibitor SP600125 (20 μM) and p38 inhibitor SB203580 (10 μM) before treatment with TF or TR and found that the cell viability was increased significantly after 48 h of incubation. This indicates that these two proteins are important in transduction of the death signal upon TF and TR treatment in A375 cells. However, no significant change in the viability of cells was observed in presence of the ERK-specific inhibitor PD98059 (5 μM) along with TF and TR treatment. These findings together suggest that TF and TR induce death of A375 cells via both p38 and JNK MAPK, without involving the ERK pathway.

The death of A375 cells upon TF and TR treatment occurs mainly by mitochondria-mediated apoptosis that involves caspase-9 and caspase-3 activity.[24] Therefore the effect of TF and TR on the activity of caspase-3 and caspase-9 in A375 cells was studied in the presence of different MAPK inhibitors and it was found that both the JNK and p38 inhibitors could reduce the activity of caspases significantly. ERK inhibitor did not alter the activity of the caspases upon TF or TR treatment. This result further supports our previous findings and suggests that JNK and p38, but not ERK, are involved in apoptosis induction of A375 cells by TF and TR via a caspase-mediated pathway.

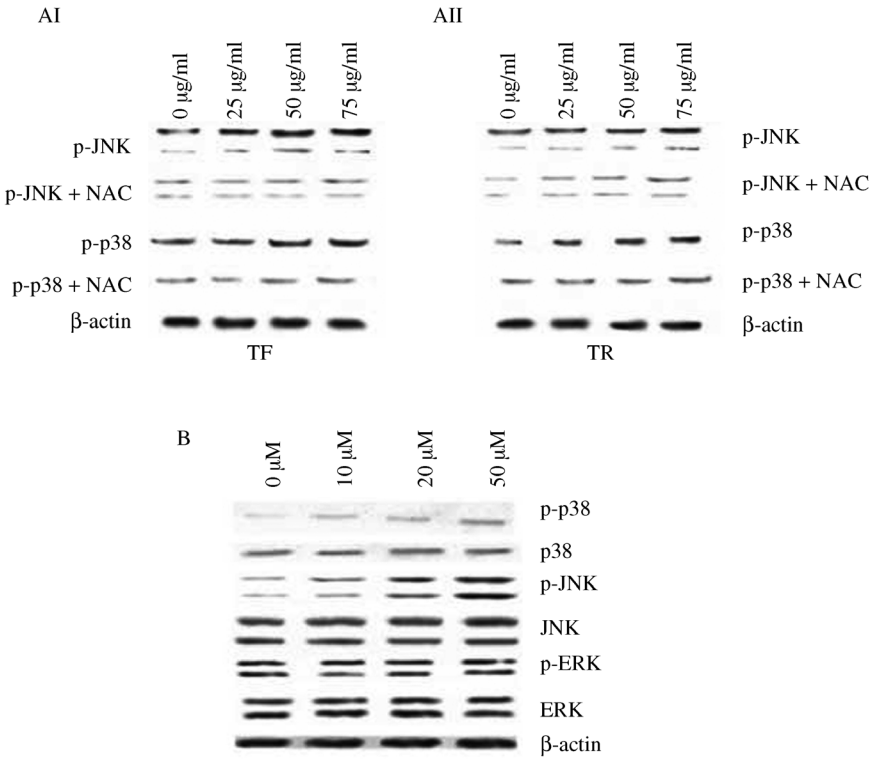

Fig. 5. Effect of *N*-acetyl cystein (NAC) and H_2O_2 on phosphorylated (p)-JNK and p-p38 expression in theaflavin (TF)- or thearubigin (TR)-treated A375 cells. (AI,AII) Effect of NAC on the expression of phosphorylated forms of JNK and p38 after 12 h of (AI) TF or (AII) TR treatment at different concentrations. A375 cells (5×10^7 per treatment) were pretreated with NAC (5 mM) for 30 min at 37°C and then treated with TF or TR at 0 (control), 25, 50 and 75 μg/mL for 12 h. Protein from the total-cell lysate was subjected to SDS-PAGE and western blotting using p-JNK, p-p38, and β-actin antibodies. Representative blots from three independent experiments gave identical results. The relative intensity of each band was measured after normalization with the intensity of β-actin in a blot (below each western blot). (B) Effect of H_2O_2 at different concentrations (0, 10, 20, and 50 μM) on the expression of phosphorylated forms of p38, JNK, and ERK after 12 h of TF or TR treatment. A375 cells (5×10^7 per treatment) were treated with H_2O_2 at different concentrations for 30 min at 37°C and then treated with TF or TR at 50 μg/mL for 12 h. Protein from the total-cell lysate was subjected to SDS-PAGE and western blotting using p-JNK, p-p38, p-ERK, and β-actin antibodies. Representative blots from three independent experiments gave identical results. The relative intensity of each band was measured after normalization with the intensity of β-actin in a blot (below each western blot).

NF-κB is a ubiquitously expressed, inducible transcription factor that has been implicated in ROS signaling and can induce cell death.[36–42] Green tea polyphenols like EGCG can downregulate the NF-κB activity and induce apoptosis.[43] As NF-κB might induce cell death, we studied the expression level of this protein in whole-cell lysate as well as in nuclear and cytoplasmic fractions. However, we did not find any significant change in the occurrence of NF-κB protein in either the nuclear fraction or the cytoplasmic fraction. This observation suggests that NF-κB might not be a target of TF or TR in A375 cells.

In various cells, the apoptosis-triggering effects of ROS were noted.[44,45] A few reports show that green tea polyphenols can induce ROS-dependent apoptosis in certain cancer cells.[22,23] Accordingly we hypothesized that TF and TR might induce ROS in A375 cells. We found that both TF and TR can induce intracellular ROS generation time dependently, thereby supporting our hypothesis. The cell-permeable stable antioxidant NAC inhibited induction of ROS by TF and TR. We also observed enhanced viability of the cells and reduced activity of caspase-3 when the cells were incubated with NAC before the treatment with TF or TR. These observations signify that ROS could be an important factor in inducing caspase activity and apoptotic death of A375 cells upon TF and TR treatment.

There are several reports suggesting that ROS can induce activation of JNK and p-38 MAPK and can also induce apoptosis.[10,21,46] We therefore wanted to know whether activation of these two MAPK and induction of ROS are interlinked. When the A375 cells were pretreated with NAC prior to TF and TR treatment the expression of p-JNK and p-p38 were suppressed. This result suggests that the TF- and TR-induced intracellular ROS might have upregulated the expression of p-JNK and p-p38. For further confirmation, when we treated the cells with different concentrations of H_2O_2, upregulation of p-JNK and p-p38 was observed dose dependently but the p-ERK level was unaltered. These results imply that the activation of JNK as well as p38 and induction of ROS in TF- and TR-treated A375 cells are interlinked. ROS can activate JNK and p38 via different proteins, the most important of which is ASK1. ASK1 is one of the MKKK that are activated by various types of stress such as ROS, tumor necrosis factor α, lipopolysaccharide, endoplasmic reticulum stress, and calcium influx. ASK1 in turn selectively activates the JNK and p38 MAPK pathways.[18,46–48] Oxidative stress is one of the most potent activators of ASK1, which is essential for oxidative stress-induced cell death.[20] ASK1 can induce apoptosis, which also activates the JNK and p38 signaling pathways.[18] As a result we hypothesized that ASK1 and its downstream MKK MKK3 and MKK4 could activate JNK and p38 after TF and TR treatment. Interestingly we found that p-ASK1 was being upregulated time dependently upon TF and TR treatment prior to JNK and p38. MKK3 and MKK4 showed almost similar patterns of activation in a time-dependent manner upon TF or TR treatment suggesting that ASK1, MKK3, and MKK4 are the upstream molecules that might play important roles in activation of JNK and p38 due to TF or TR treatment in A375 cells. Pretreatment of cells with NAC significantly inhibited the upregulation of p-ASK1 upon TF or TR treatment as well. These observations further confirm our hypothesis that in A375 cells ASK1 is upregulated by intracellular ROS when treated with TF and TR, and subsequently this protein upregulates the stress-activated MAPK together.

In conclusion, our results strongly suggest that both TF and TR can induce JNK- and p38-dependent apoptotic cell death in A375 cells and ROS might be one of the most important upstream substances that triggers this apoptotic stimulus. ROS-mediated activation of MAPK is controlled or maintained by ASK1 protein. So ASK1 is one of the key regulator proteins in TF- and TR-induced apoptotic death in A375 cells. These findings provide a rationale to explore the role of TF and TR as chemopreventive and perhaps as chemotherapeutic agents and may add some information to the existing knowledge that black tea is beneficial for health and prevents various diseases, including cancer.

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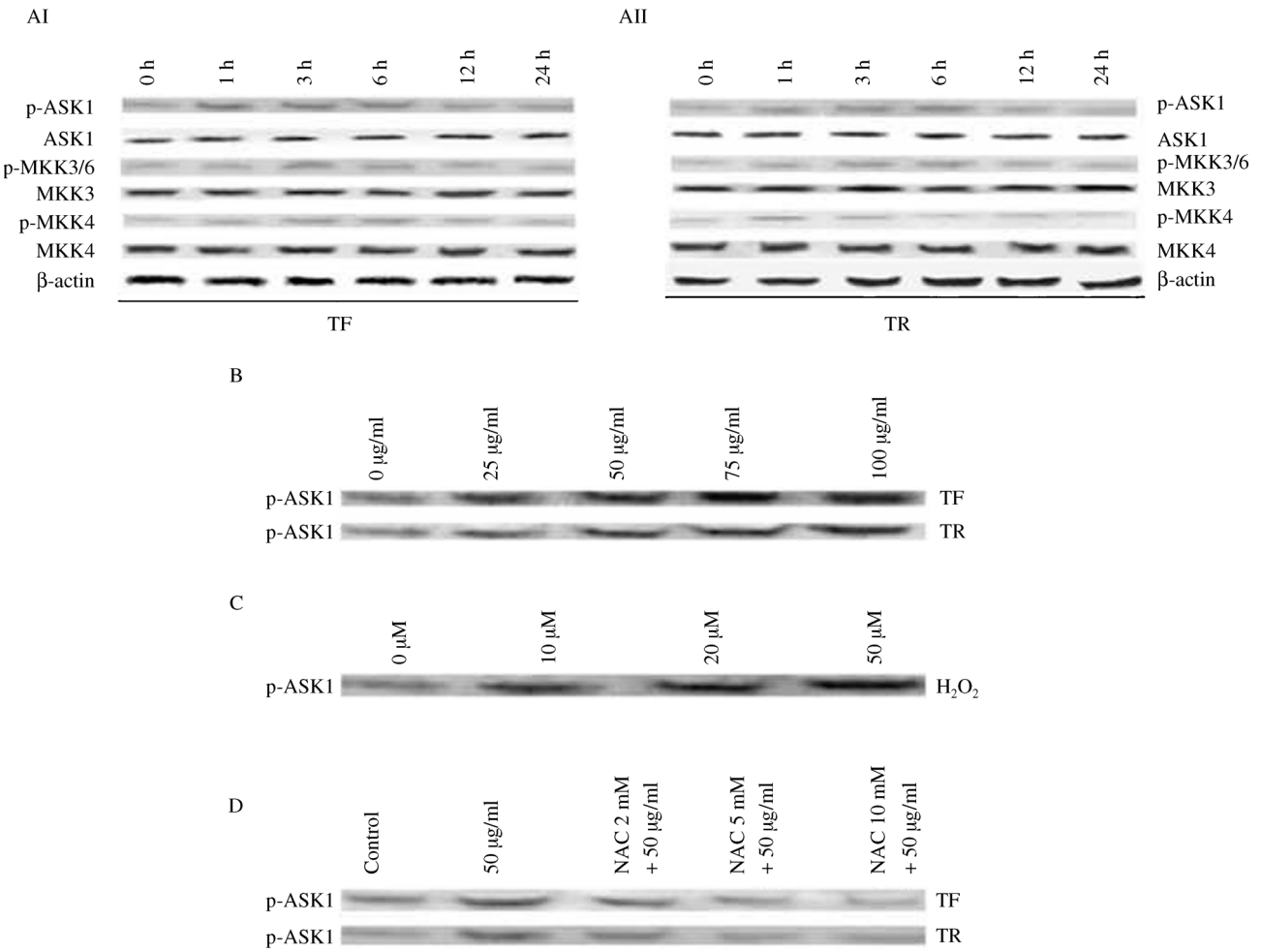

Fig. 6. Study of the expression patterns of molecules upstream of JNK and p38 and there interrelationship with reactive oxygen species generation. (AI,AII) Time-dependent study of the effect of (AI) theaflavins (TF) and (AII) thearubigins (TR) on the upstream kinases JNK and p38. A375 cells (5×10^7 per treatment) were treated with (AI) TF or (AII) TR at 50 μg/mL and the expression patterns of phosphorylated (p)-apoptosis signal-regulating kinase (ASK) 1, ASK1, p-MAPK kinase (MKK) 3/6, MKK3, p-MKK4, MKK4, and β-actin were observed at 0, 1, 3, 6, 12, and 24 h of treatment. Protein from the total-cell lysate was subjected to SDS-PAGE and western blotting was carried out using p-ASK1, ASK1, p-MKK3/6, MKK3, p-MKK4, MKK4, and β-actin primary antibodies. Representative blots from three independent experiments gave identical results. The relative intensity of each band after normalization with the intensity of β-actin in a blot (below each western blot) was measured. (B) Dose-dependent study of the effect of TF and TR on p-ASK1 protein expression. A375 cells (5×10^7 per treatment) were treated with TF or TR at 0, 25, 50, 75, and 100 μg/mL and the expression pattern of p-ASK1 was observed at 6 h of treatment. Representative blots from three independent experiments gave identical results. The relative intensity of each band was measured after normalization with the intensity of β-actin in a blot. (C) Effect of H_2O_2 at different concentrations (0, 10, 20, and 50 μM) on the expression of p-ASK1 after 6 h of TF or TR treatment. A375 cells (5×10^7 per treatment) were treated with H_2O_2 at different concentrations for 30 min at 37°C and then treated with TF or TR at 50 μg/mL for 6 h. Protein from the total-cell lysate was subjected to SDS-PAGE and western blotting using p-ASK1 antibody. Representative blots from three independent experiments gave identical results. The relative intensity of each band was measured after normalization with the intensity of β-actin in a blot. (D) Effect of different concentrations of *N*-acetyl cystein (NAC) on the expression of phosphorylated form of ASK1 at 6 h of TF or TR treatment. A375 cells (5×10^7 per treatment) were pretreated with NAC (0, 2, 5, 10 mM) for 30 min at 37°C and then treated with TF or TR at 50 μg/mL for 6 h. Protein from the total-cell lysate was subjected to SDS-PAGE and western blotting using p-ASK1 antibody. Representative blots from three independent experiments gave identical results. The relative intensity of each band was measured after normalization with the intensity of β-actin in a blot.

Disclosure Statement

This is to certify that there is no personal or financial conflict of interest in any form whatsoever among the authors of this article.

Abbreviations

ASK	apoptosis signal-regulating kinase
BCIP	5-bromo-4-chloro-3-indolyl phospate, p-toluidine
DCF-DA	Dichlorofluorescein diacetate
DCFH-DA	2′,7′-dichlorofluorescein diacetate
EGCG	Epigallocatechins gallate
ERK	extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
MAP	mitogen-activated protein
MAPK	MAP kinase
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
NAC	*N*-acetyl cystein
NBT	nitro blue tetrazolium
NF	nuclear factor
p	phosphorylated
ROS	reactive oxygen species
TBS	Tris-buffered saline
TF	theaflavin
TR	thearubigin

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