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Journal of Cellular Biochemistry 108:860–866 (2009)

Journal of Cellular Biochemistry

Involvement of the JNK Activation in Terbinafine-Induced p21 Up-Regulation and DNA Synthesis Inhibition in Human Vascular Endothelial Cells

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ABSTRACT

Previously, we demonstrated that the extracellular signal-regulated kinase (ERK)-mediated pathway contributes to the terbinafine (TB)-induced increases of p21 and p53 protein level as well as decrease of DNA synthesis in human umbilical venous endothelial cells (HUVEC). The aim of this study is to examine the involvement of c-Jun NH2-terminal kinase (JNK) in the TB-induced increase of p21 protein level and DNA synthesis inhibition. Western blot analysis and kinase assay demonstrated that TB treatment increased both the protein level and the kinase activity of JNK1/2 in HUVEC. Transfection of HUVEC with JNK1 dominant negative (DN-JNK1) prevented the TB-induced increases of p21 and p53 protein level and decrease of DNA synthesis, suggesting that JNK1/2 activation is involved in the TB-induced cell cycle arrest in HUVEC. Moreover, over-expression of mitogen-activated protein kinase (MEK)-1 prevented the TB-induced increase of JNK1/2 protein levels, suggesting that MEK-1 is an upstream inhibitor of JNK. Transfection of HUVEC with DN-JNK1 prevented the TB-induced inhibition of ERK phosphorylation, suggesting that JNK1/2 might serve as a negative regulator of ERK. Taken together, our results suggest that JNK activation is involved in the TB-induced inhibition of ERK phosphorylation, p53 and p21 up-regulation and DNA synthesis inhibition in HUVEC. J. Cell. Biochem. 108: 860–866, 2009.

KEY WORDS: TERBINAFINE; p53; p21; ERK; JNK

Angiogenesis, the formation of new blood vessels from preexisting vessels, occurs extensively during embryonic development and in wound healing. A pathological imbalance in this process contributes to numerous malignant, ischemic, infectious, inflammatory and immune disorders [Carmeliet, 2005]. It is well-known that the major steps in angiogenesis include: (a) proteolytic breakdown of the basement membrane [Bellon et al., 2004], (b) migration of endothelial cells toward the angiogenic stimulus, (c) endothelial cell proliferation, and (d) lumen formation [Bussolino et al., 1997; Gupta and Qin, 2003]. A useful approach for the treatment of pathological angiogensis might exist in the further development of novel anti-angiogenic drugs.

Angiogenesis is regulated by various growth factors. Through their transmembrane receptors, growth factors activate signaling pathways leading to an array of responses. Mitogen-activated protein kinase (MEK) and ERK isoforms, which are involved in the proliferation of endothelial cells [Nakagami et al., 2000, 2001], appear to be the principal signaling pathways in growth factor signaling. It has been reported that ERK inhibition resulted in cell apoptosis, which was accompanied by increased p53 accumulation [Kim et al., 2002; Kwon et al., 2004]. Moreover, JNK and its downstream target c-jun have also been demonstrated to positively regulate angiogenesis via activation of endothelial cell proliferation, migration and proteolysis [Uchida et al., 2008]. However, it has also

Grant sponsor: National Science Council of the Republic of China; Grant numbers: NSC 94-2320-B-038-005, NSC 95-2320-B-038-019.
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Received 25 June 2009; Accepted 21 July 2009 • DOI 10.1002/jcb.22314 • © 2009 Wiley-Liss, Inc.
Published online 19 August 2009 in Wiley InterScience (www.interscience.wiley.com).

been showed that sustained activation of the JNK pathway can result in the attenuation of the mitogen-activated ERK pathway [Shen et al., 2003]. JNK can phosphorylate several transcription factors including Jun D, ATF2, ATF3, Elk-1, Elk-3, p53, RXRa, RARb, AR, NFAT4, HSF-1, and c-Myc [Johnson and Nakamura, 2007]. Thus, in the context of apoptosis, the nuclear activity of JNK may increase pro-apoptotic genes expressions and/or decrease pro-survival genes expressions [Dhanasekaran and Reddy, 2008].

Terbinafine (TB), a synthesized oral antimycotic drug, has been used as an inhibitor of fungal ergosterol biosynthesis [Petranyi et al., 1984]. It has been reported that TB has relative few drug interaction and is safe for clinical uses [Abdel-Rahman and Nahata, 1997]. Previously, we have demonstrated TB suppresses proliferation of various tumor cells in vitro and in vivo by inhibiting DNA synthesis and activating apoptosis [Lee et al., 2003]. We also demonstrated that TB could inhibit the proliferation and migration of cultured HUVEC, the formation of capillary-like tube, and sprouting of capillaries [Ho et al., 2004]. The TB-induced cell cycle arrest in HUVEC occurred when the cyclin-dependent kinase 2 (CDK2) was inhibited, just as the protein level of p21 was increased. In delineation of the molecular mechanisms underlying TB-induced DNA synthesis inhibition in HUVEC, we found that the ERK-mediated pathway contributes to the TB-induced up-regulation of p53 protein, which in turn increases the p21 protein level, and finally causes cell cycle arrest in HUVEC [Ho et al., 2008]. In the present study, we further examine the possible role of JNK in MEK/ERK suppression and DNA synthesis inhibition caused by TB. Here, we showed that TB increased the protein level and the activity of JNK1/2, which contributed to the TB-induced inhibition of ERK action and p53 as well as p21 up-regulation in human vascular endothelial cells. These experimental findings reported below highlight the molecular mechanisms of TB-induced anti-proliferation of human vascular endothelial cells.

MATERIALS AND METHODS

CELL CULTURE

HUVEC were cultured with M199 medium containing 10% fetal bovine serum (FBS), sodium heparin (5 units/ml), endothelial cell growth supplement (ECGS, 0.03 g/L, Biomedical Technologies Inc., MA) and antibiotics in gelatin-coated plates, and incubated at 37°C in 5% CO_2 in air. Cells from passages 5 to 9 were used.

WESTERN BLOT ANALYSES

To determine the protein levels in HUVEC, the total proteins were extracted and Western blot analyses were conducted as previously described. Briefly, HUVEC were cultured in 15 cm Petri dishes. After reaching subconfluence, the cells were rendered quiescent and then treated with various concentrations of TB and incubated at 37°C. At different time points, the cells were washed with phosphate buffer saline (NaCl 0.8%, Na_2HPO_4 0.144%, KH_2PO_4 0.024%, KCl 0.02%, pH 7.4), incubated with extraction buffer (Tris 0.05 M, pH 7.5, NaCl 0.15 M, PMSF mM, NP-40 1%, 0.1% SDS) on ice, and then centrifuged at 12,000*g* for 30 min. The cell extract was then boiled in a ratio of 3:1 with sample buffer (Tris–HCl 0.25 M, pH 6.8, glycerol 40%, dithiothreitol 0.4 M, SDS 8% and bromophenol blue 0.2%). Electrophoresis was carried out using 12% SDS–polyacrylamide gel (2 h, 110 V, 0.04 A, 50 μg protein per lane). Separated proteins were transferred to PVDF membranes (1 h, 0.4 A), treated with 5% fat-free milk powder (Anchor, Manurewa, NZ) to block the nonspecific IgGs, and incubated for 1 h with specific antibody for p21, p53, p-ERK, ERK or G3PDH (Jackson ImmunoResearch Laboratories, PAA). The blots were then incubated with anti-mouse, anti-rabbit, or anti-goat IgG (Jackson ImmunoResearch Laboratories) linked to HRP (1:1,000) for 1 h. Subsequently, the blots were developed using the ECL (enhanced chemiluminescence) system (Amersham Biosciences, UK). The bands were quantified by densitometry, using Image Pro-Plus 4.5 Software (Media Cybernetcis, MD).

IMMUNOPRECIPITATION AND KINASE ACTIVITY ASSAY

As previously described [Liang et al., 1999], equal amounts of total cellular lysate were immunoprecipitated with anti-JNK1 antibody (2 μg/L) and protein A/G-PLUS agarose for 15 h at 4°C. Kinase assay was carried out in 45 μl of kinase buffer (0.04 mol/L Tris–NaOH pH 7.5, 0.5 mol/L NaCl, 0.1% NP-40, 0.006 mol/L EDTA, 0.006 mol/L EGTA, 0.01 mol/L β-glycerophosphate, 0.01 mol/L NaF, 0.01 mol/L PNPP, 300 μmol/L sodium orthovanadate, 0.001 mol/L benzamidine, 0.002 mol/L PMSF, 10 g/L aprotinin, 1 g/L leupeptin, and 0.001 mol/L DTT) containing 5 μmol/L cold ATP, 5 μCi [γ-^{32}P] ATP (Amersham Biosciences), and 1 μg GST-c-Jun fusion protein (Santa Cruz Biotechnology, CA) as substrate, and incubated for 20 min at 25°C. Each sample was mixed with 8 μl of 5× Laemmli's loading buffer to stop the reaction. The samples were analyzed by 8% SDS–PAGE, and the gel was then dried and subjected to autoradiography.

[3H]THYMIDINE INCORPORATION

The [3H]thymidine incorporation was performed as previously described [Lin et al., 2002]. Briefly, HUVEC were applied to 24-well plates in growth medium (M199 plus 10% FBS and ECGS). After the cells had grown to 70–80% confluence, they were rendered quiescent by incubation for 24 h in M199 containing 2% FBS. M199 supplemented with 10% FBS and 0.05% DMSO (control) or TB was added to the cells and the cultures were allowed to incubate for 24 h. During the last 3 h of the incubation without or with TB, [3H]thymidine (Amersham Biosciences) was added at 1 μCi/ml (μCi = 37 kBq). Incorporated [3H]thymidine was extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

PREPARATION OF PLASMID CONSTRUCTS

JNK dominant negative (DN-JNK) construct. DN-JNK1 construct was a great gift from Dr. Min-Liang Kuo (Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan) [Antlsperger et al., 2003].

Constitutive active MEK-1 construct. MEK-1 cDNA was a great gift from Prof. Ruey-Hwa Chen (Institute of Biological Chemistry, Taipei, Taiwan) [Chen et al., 2005].

CELL TRANSFECTION

Transfection was performed using jetPEI-HUVEC transfection reagent (Polyplus Transfection, Bioparc, France) and following

the manufacturer's protocol. Briefly, jetPEI-HUVEC/DNA mixture was added drop-wise onto the DMEM-Glutamax medium (Invitrogen Corporation, CA) containing 2% FBS, mixed gently, and incubated in a humidified 37°C incubator for 4 h. After the growth medium was replaced with fresh growth medium containing 10% FBS for 24 h, HUVEC was rendered quiescent by incubation in M199 containing 2% FBS for 24 h. The transfected cells were then incubated in M199 supplemented with 10% FBS and TB was added to the cells and the cultures were allowed to incubate for the indicated hours. In these experiments, cells transfected with empty vector were served as a control.

STATISTICS

All data were expressed as the mean value ± SEM. Comparisons were subjected to one way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

RESULTS

TB INCREASES THE PROTEIN LEVEL AND ENZYME ACTIVITY OF JNK IN HUVEC

Previously, we demonstrated that the ERK-mediated pathway contributes to the TB-induced cell cycle arrest in HUVEC [Ho et al., 2008]. To investigate whether the JNK-mediated pathway is also involved in the TB-induced cell cycle arrest in HUVEC, we performed Western blot analysis and kinase assay to examine the changes of the protein level and enzyme activity of JNK in the TB-treated HUVEC. In response to TB treatment, the protein level (Fig. 1a) and enzyme activity (Fig. 1b) of JNK1/2 in HUVEC were increased in a concentration-dependent manner.

INVOLVEMENT OF JNK1/2 IN THE TB-INDUCED INCREASES OF p21 AND p53 EXPRESSION AND INHIBITION OF DNA SYNTHESIS IN HUVEC

Our previous study has demonstrated that TB treatment induced up-regulation of p53 and p21 in HUVEC. Since JNK1/2 has been suggested to play a role in mitogenic and growth factor signaling, we studied whether the TB-induced JNK1/2 activation is involved in TB-induced up-regulation of p53 and p21 in HUVEC. As shown in Figure 2a, TB treatment increased the levels of p21 and p53 protein in HUVEC transfected with empty vector. In contract, transfection of HUVEC with DN-JNK1 prevented the TB-induced increases of p21 and p53 protein level. To further investigate the involvement of JNK1/2 activation in cell cycle arrest caused by TB treatment, thymidine incorporation assay was conducted. As illustrated in Figure 2b, DN-JNK1 transfection completely prevented the TB-induced inhibition of DNA synthesis in HUVEC.

INVOLVEMENT OF MEK-1 SUPPRESSION IN THE TB-INDUCED UP-REGULATION OF JNK

Our previous study demonstrated that the MEK-1/ERK pathway mediates the up-regulations of p21 and p53 caused by TB [Ho et al., 2008]. To further investigate the involvement of MEK-1 in JNK1/2 up-regulation caused by TB, we over-expressed the MEK-1 in HUVEC. As shown in Figure 3, TB treatment concentration-dependently

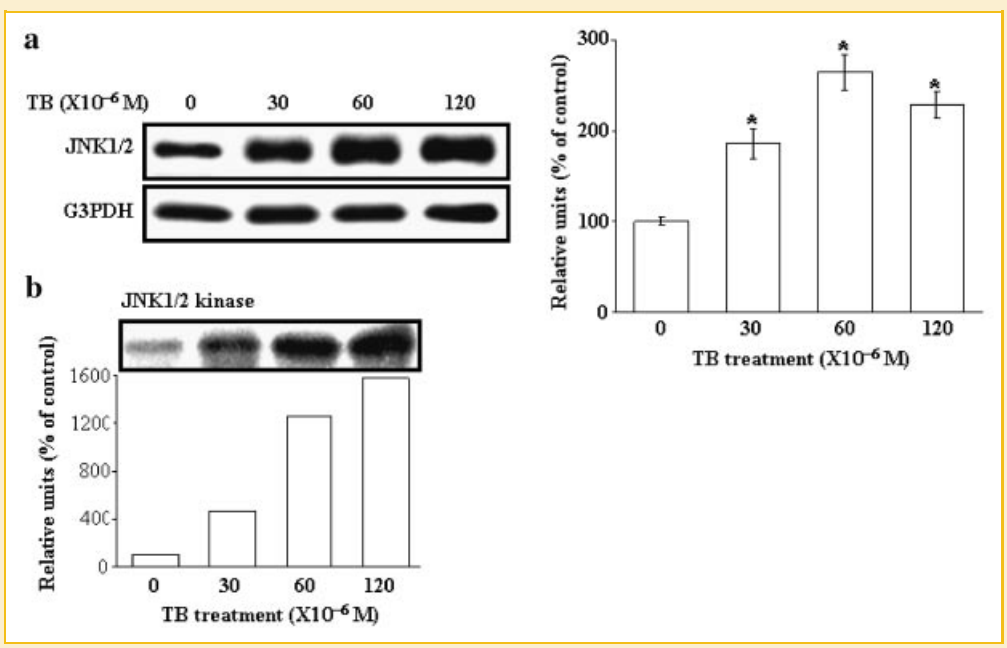

Fig. 1. The protein level and the activity of JNK1/2 in HUVEC were increased by TB treatment. After treatment with TB for 24 h, total protein was extracted for protein detection using Western blot analysis. a: TB (0–120 μM) concentration-dependently increased the levels of JNK1/2 protein (left panel). The right panel shows the quantified results of JNK1/2 protein levels, which were adjusted with corresponding G3PDH protein level and expressed as percentage of control. Four samples were analyzed in each group, and values represent the means ± SEM. *$P < 0.05$ different from control. b: TB (0–120 μM) concentration-dependently increased the activity of JNK1/2 in HUVEC.

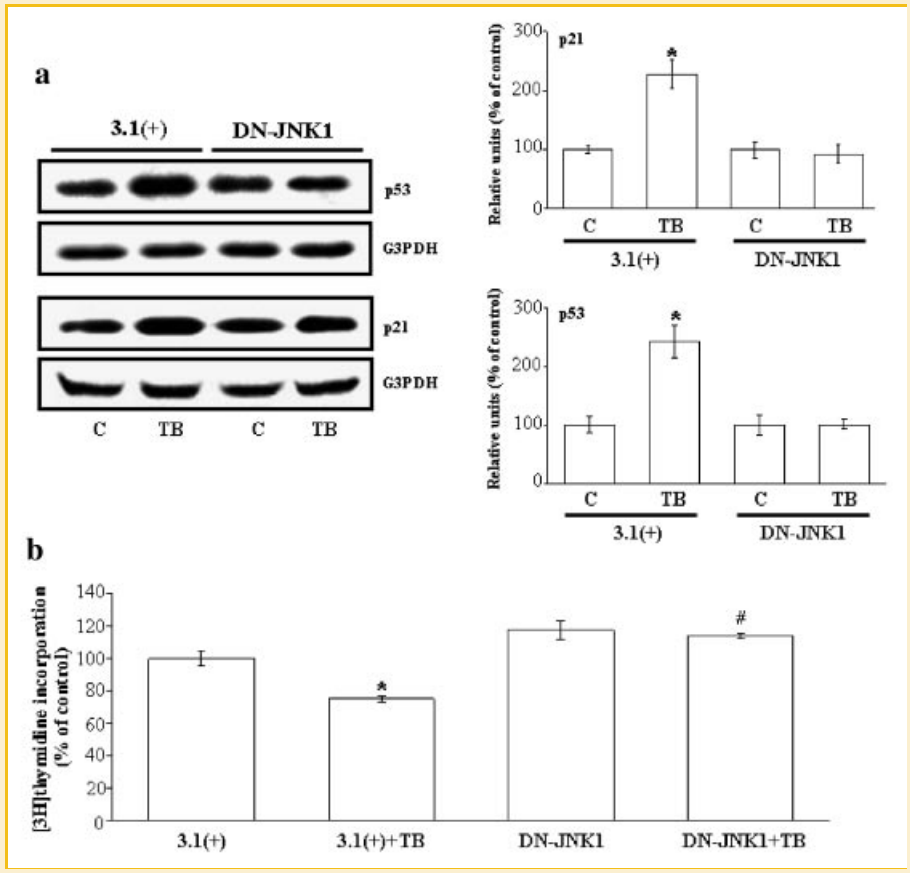

Fig. 2. Involvement of JNK in the TB-induced increases of p53 and p21 protein, and decrease of DNA synthesis. After treatment with TB for 24 h, the cells were processed for total protein extraction and Western blot analysis. a: DN-JNK1 transfection prevented the TB-induced increases of p21 and p53 protein (left panel). HUVEC were transiently transfected with control vector (pcDNA 3.1) or vector coding for DN-JNK1 followed by TB (120 μM) treatment for 24 h. Right panel shows the quantified results of p21 and p53 protein level, which were adjusted with corresponding G3PDH protein level and expressed as percentage of control. Three samples were analyzed in each group, and values represent the means ± SEM. $^{*}P < 0.05$ different from control. b: DN-JNK1 transfection prevented the TB-induced decrease of thymidine incorporation in HUVEC. HUVEC were transiently transfected with control vector (pcDNA 3.1) or vector coding for DN-JNK1 followed by TB (120 μM) treatment for 24 h. Four samples were analyzed in each group, and values represent the means ± SEM. $^{*}P < 0.05$ different from empty control vector group without TB treatment. $^{\#}P < 0.05$ different from TB treatment group without DN-JNK1 transfection.

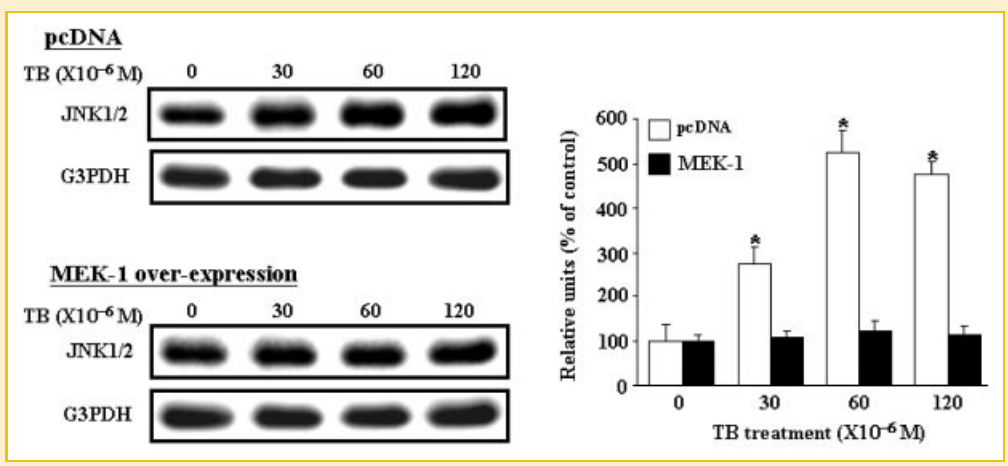

Fig. 3. Involvement of MEK-1 suppression in the TB-induced increase of JNK1/2 protein. TB (0–120 μM) treatment concentration-dependently increased the levels of JNK1/2 protein in HUVEC transfected transiently with control vector (pcDNA 3.1) (top panel), but not with vector coding for constitutive active MEK-1 (bottom panel). Right panel shows the quantified results of JNK1/2 protein level, which were adjusted with corresponding G3PDH protein level and expressed as percentage of control. Three samples were analyzed in each group, and values represent the means ± SEM. $^{*}P < 0.05$ different from empty control vector group without TB treatment.

increased the levels of JNK1/2 protein in the vector-transfected HUVEC (upper panel). In contrast, over-expression of MEK-1 prevented the TB-induced up-regulation of JNK1/2 (lower panel), suggesting that suppression of MEK-1 might be involved in the TB-induced up-regulation of JNK1/2.

JNK1/2 IS AN UPSTREAM REGULATOR OF ERK-MEDIATED PATHWAY IN THE TB-INDUCED DNA SYNTHESIS INHIBITION

Previously, we demonstrated that inhibition of the ERK action contributed to the TB-induced p21 up-regulation and DNA synthesis inhibition in HUVEC [Ho et al., 2008]. In the present study, our data suggest that MEK-1 might be an upstream inhibitor of JNK expression and activity. Accordingly, we studied the relationship between the JNK1/2 activation and ERK inactivation in the TB-treated HUVEC. As illustrated in Figure 4, DN-JNK1 transfection prevented the TB-induced reduction of ERK phosphorylation in HUVEC.

DISCUSSION

Previously, we demonstrated that treatment of HUVEC with TB caused a decrease of MEK, which in turn up-regulated p53 through the inhibition of ERK phosphorylation, and finally led to an increase of p21 expression and cell cycle retardation at the G0/G1 phase [Ho et al., 2004, 2008]. Since ERK/JNK cross-activation has been demonstrated and the JNK-mediated pathway has been proposed to be involved in the regulation of cell proliferation [Steelman et al., 2004], we further investigated whether the JNK-mediated pathway is also involved in the TB-induced up-regulation of p21 and DNA synthesis inhibition in HUVEC. The findings of the present study demonstrate that the protein levels (Fig. 1a) and kinase activity (Fig. 1b) of JNK1/2 in HUVEC were concentration-dependently increased by TB treatment. DN-JNK1 transfection prevented the TB-induced decrease of ERK activity (Fig. 4), increases of p21 and p53 proteins (Fig. 2a) and inhibition of DNA synthesis (Fig. 2b). To our knowledge, this is the first demonstration that increased JNK activity is involved in the TB-induced suppression of ERK activity, which in turn increases p21 and p53 protein levels, and finally reduced the DNA synthesis in HUVEC.

Mitogen-activated protein kinases (MAPKs) represent a family of serine-threonine kinases involved in a wide range of cellular responses including proliferation, differentiation, cell survival, and apoptosis [Hagemann and Blank, 2001; Kyosseva, 2004]. Three major mammalian MAPK subgroups including ERK, JNK, and p38, have been identified. ERK1/2 has been known to be induced by variety of growth factors and hormones, whereas JNK1/2 is strongly activated by cellular stress stimuli [Kyriakis and Avruch, 2001; Manning and Davis, 2003; Kim et al., 2006]. The JNK-mediated pathway has been indicated to be involved in many forms of stress-induced apoptosis [Davis, 2000; Lin, 2003]. It has been indicated that JNK plays a pro-apoptotic role in stress-induced neuronal cell death via transcriptional activation of c-Jun [Xia et al., 1995]. Previous study also showed that JNK-null embryonic fibroblasts are resistant to apoptosis induced by UV irradiation and other stress stimuli [Tournier et al., 2000]. JNK-mediated apoptosis has been reported to result from the phosphorylation of p53 family of proteins by JNK [Fuchs et al., 1998a,b]. JNK inhibits ubiquitin-mediated degradation of p53 by phosphorylating p53 at Ser6, which stabilizes the levels of p53 [Oleinik et al., 2007]. Phosphorylation of p53 has been suggested to be critically required for the apoptotic pathway [Oleinik et al., 2007]. As p53 is involved in the expression of several pro-apoptotic genes such as Bax, apoptotic pathways activated by JNK might play an important role in p53-mediated up-regulation of pro-apoptotic genes [Dhanasekaran and Reddy, 2008]. Although JNK1/2 has been often linked to induction of apoptosis and stress, JNK has been demonstrated to play a protective role and to support cell survival under some circumstances. It is now clear that JNK has very diverse roles in the regulation of cell proliferation and survival in some cell types [Kapur et al., 2002; Du et al., 2004; Jacobs-Helber and Sawyer, 2004]. Recently, evidence is accumulating to suggest that JNK signaling is involved in cell growth and proliferation of

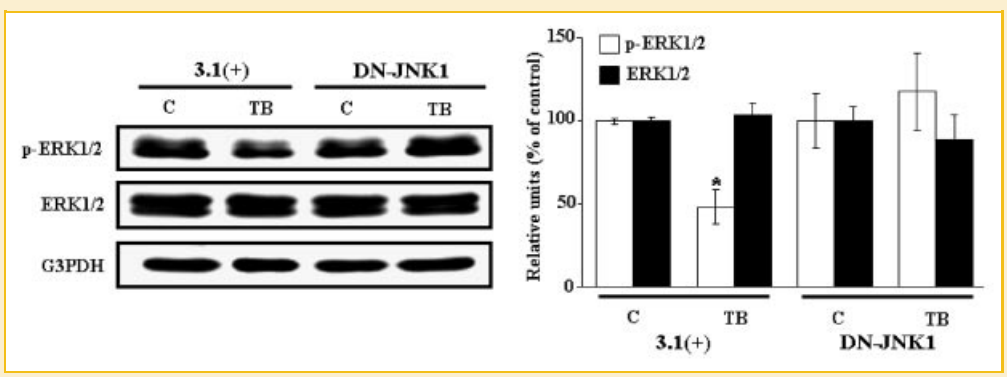

Fig. 4. JNK is an upstream regulator of ERK-mediated pathway in HUVEC. Transfection of the cells with DN-JNK1 abolished the TB-induced ERK inactivation in HUVEC. HUVEC were transiently transfected with control vector (pcDNA 3.1) or vector coding for DN-JNK1 in 10% FBS. After transfection, the cells were rendered quiescent by incubation for 24 h in M199 containing 2% FBS, and then treated with TB (120 μM) or DMSO (control) in 10% FBS for 2 h before the cells were subjected to total protein extraction and Western blot analysis. Right panel shows the quantified results of ERK and p-ERK protein level, which were adjusted with corresponding G3PDH protein level and expressed as percentage of control. Three samples were analyzed in each group, and values represent the means ± SEM. $^{*}P < 0.05$ different from empty control vector group without TB treatment.

unstressed cells. JNK1/2 has been shown to promote proliferation in response to platelet-derived growth factor and during liver regeneration [Huang et al., 2006]. Depletion of JNK with antisense oligonucleotides inhibited cell growth of human glioblastoma cells [Potapova et al., 2000]. Therefore, it is apparent that JNK1/2 also plays a role in mitogenic and growth factor signaling as well.

The various MAPK cascades (e.g., ERK1/2, JNK, p38, and ERK5) are often illustrated in the literature as linear cascades, and indications for cross-talk between the various cascades are limited [Garrington and Johnson, 1999; Gallo and Johnson, 2002]. Although JNK has been proposed to be involved in the regulation of cell proliferation [Steelman et al., 2004], and demonstrated to be the final mediator for ERK to stimulate cell proliferation with ERK/JNK cross-activation [Pedram et al., 1998], observations of persistent activation of JNK have now been frequently associated with proliferation inhibition and cell death in a variety of cells [Hu et al., 1999]. Negative cross-talk between the stress-activated MLK-SEK-JNK-Jun pathway and the ERK/MAPK pathway has been reported. The attenuation of the mitogen-activated ERK pathway by sustained activation of the JNK pathway was observed [Shen et al., 2003]. The opposite effect of ERK and JNK on p21 expression has also been demonstrated in human epidermoid carcinoma A431 cells [Huang et al., 2006].

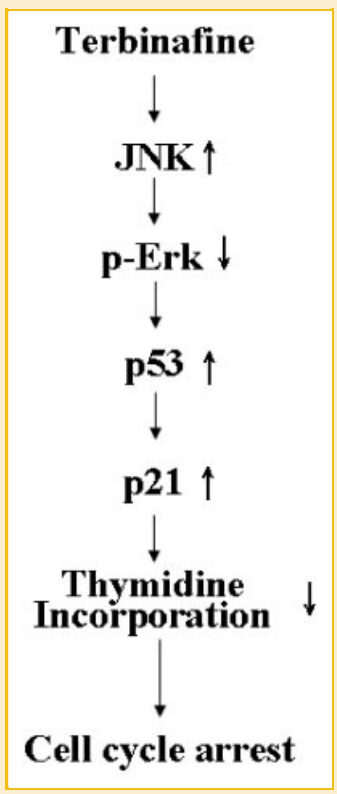

Fig. 5. Model for TB-induced p21 protein increase and DNA synthesis inhibition in HUVEC. TB increases the JNK activity and subsequently inhibits the ERK phosphorylation. Inactivation of ERK causes an increase of p53, which in turn up-regulates the p21 protein, and finally reduces the DNA synthesis of HUVEC.

In the present study, we demonstrated the involvement of JNK1/2 activation in the TB-induced DNA synthesis inhibition. Our results showed that TB treatment led to the increase in JNK1/2 activity in HUVEC. Transfection with DN-JNK1 prevented the TB-induced decrease of ERK phosphorylation (Fig. 4), suggesting that JNK1/2 is an upstream inhibitor of ERK and activation of JNK1/2 causes a decrease in phosphorylated ERK level, which in turn increases the p21 protein level, and finally decreases the thymidine incorporation into HUVEC. Surprisingly, our results indicated that increases of JNK1/2 activity caused growth inhibition instead of stimulation in HUVEC. It has been suggested that JNK operates in a manner dependent on the cell-type, stimulus, and context, with the duration of activation and the specific spectrum of substrates targeted. Our results suggested that in addition to a stimulatory effect of JNK1/2 activation on cell proliferation, JNK1/2 activation might also cause inhibition of cell proliferation.

In conclusion, this study provides the evidence that TB caused JNK activation and subsequent ERK inactivation, and eventually resulted in the inhibition of endothelial cell proliferation. Based on the results of the present study, we propose a model of the molecular mechanisms through which TB inhibits the DNA synthesis of HUVEC. A depiction of this model is shown in Figure 5.

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