Potentiation of a Survival Signal in the Ischemic Heart by Resveratrol through p38 Mitogen-Activated Protein Kinase/ Mitogen- and Stress-Activated Protein Kinase 1/cAMP Response Element-Binding Protein Signaling

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ABSTRACT

Resveratrol (3,4′,5-trihydroxy-trans-stilbene), a naturally occurring polyphenolic compound found abundantly in grape skins and red wines, has been found to pharmacologically precondition the heart against ischemia reperfusion injury through the potentiation of a survival signal involving cAMP response element-binding protein-dependent phosphatidylinositol 3-kinase-Akt-BclII pathway. The present study was designed to determine whether, similar to ischemic preconditioning, resveratrol uses mitogen-activated protein kinases (MAPks) as upstream signaling targets. The isolated rat hearts were preperfused for 15 min with Krebs-Henseleit bicarbonate buffer in the absence (control) or presence of extracellular signal-regulated kinase (ERK) 1/2 inhibitor 2′-amino-3′-methoxyflavone (PD98059), p38 MAPK inhibitor 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB-202190), mitogen- and stress-activated protein kinase 1 (MSK-1) inhibitor N-[2-((4-bromocinnamylamino)ethyl]-5-isoquinoline (H89), protein kinase A inhibitor (S,S,10S,12R)-2,3,9,10,11,12-hexahydro-10hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3fg:2′,3′,2″,3″-kl]-pyrrolo[3,4-][1,6]benzodiazocine-10-carboxylic acid hexyl ester (KT5720), resveratrol only, resveratrol plus PD98059, resveratrol plus SB-202190, resveratrol plus H89, or resveratrol plus KT5720. Consistent with previous reports, resveratrol provided cardioprotection as evidenced by its ability to improve postischemic ventricular function, reduction of myocardial infarct size, and cardiomyocyte apoptosis. The cardioprotection afforded by resveratrol was partially abolished with PD98059 or SB-202190, suggesting that ERK1/2 and p38 MAPK play roles in resveratrol-mediated preconditioning. An MSK-1 inhibitor, H89, abolished resveratrol-mediated preconditioning, indicating MSK-1 to be the downstream target molecule for both ERK1/2 and p38 MAPK. KT5720 had no effect on resveratrol-mediated cardioprotection. Corroborating these results, Western blot analysis revealed phosphorylation of ERK1/2, p38 MAPK, MAPK-activated protein (MAPKAP) kinase 2, and MSK-1 with resveratrol and inhibition of phosphorylation with corresponding inhibitors. These results showed for the first time that resveratrol triggers an MAPK signaling pathway involving ERK1/2 and p38 MAPK, the former using MSK-1 as the downstream target and the latter, using both MAPKAP kinase 2 and MSK-1 as downstream targets.

Resveratrol (3,4′,5-trihydroxy-trans-stilbene), a naturally occurring polyphenolic compound found abundantly in grape skins and red wines, has been found to pharmacologically precondition the heart against ischemia reperfusion injury (Hung et al., 2000; Sato et al., 2000b; Hattori et al., 2002; Das et al., 2005b). The cardioprotective mechanisms of resveratrol include antioxidant (Ray et al., 1999; Das et al., 2005c), anti-inflammatory (Sato et al., 2000b; Das et al., 2006), and antiplatelet (Bertelli et al., 1996) activities, all of which in-
volve nitric oxide signaling (Chen and Pace-Aciak, 1996; Hattori et al., 2002; Imamura et al., 2002; Das et al., 2005a). Because of close resemblance of the mechanisms of action between ischemic preconditioning (Tosaki et al., 1998; Guo et al., 1999) and resveratrol-mediated cardioprotection (Ray et al., 1999; Hattori et al., 2002; Das et al., 2005b), it has been postulated that resveratrol protects the heart through preconditioning.

Recently, resveratrol was found to protect the ischemic heart through the up-regulation of adenosine A1 and A3 receptors (Das et al., 2005b), a property shared by ischemic preconditioning (Bradamante et al., 2000; Hattori et al., 2002; Imamura et al., 2002; Das et al., 2005b). In this study, resveratrol induced the expression of BclII and caused its phosphorylation along with the phosphorylation of cAMP response element-binding protein (CREB), Akt, and Bad. Phosphatidylinositol 3 kinase inhibitor LY294002 partially blocked the cardioprotective abilities of resveratrol, suggesting that resveratrol transmits a survival signal through a CREB-dependent phosphatidylinositol 3 kinase-Akt-BclII signaling pathway. Subsequent studies determined that such a survival signal through the activation of CREB could also occur through an Akt-independent pathway (Das et al., 2005c).

Ischemic preconditioning, the state-of-the-art techniques of cardioprotection, involves mitogen-activated protein kinase (MAPK) as upstream signaling molecules (Fryer et al., 2001; Baines et al., 2002). Whether resveratrol also transmits survival signals through a signaling cascade involving MAPK is not known. A recent study showed that in mouse epidermal cells, resveratrol activated extracellular signal-regulated kinases (ERK), c-Jun NH2-terminal kinases (JNK), and p38 MAPK, leading to the serine 15 phosphorylation of p53 (She et al., 2001). In this study, pretreatment of the cells with PD98059 or SB-202190 or stable expression of a dominant-negative mutant of ERK2 or p38 kinase impaired resveratrol-induced p53-dependent transcriptional activity and apoptosis, suggesting that both ERK and p38 MAPK mediate resveratrol-induced p53 phosphorylation. The present study was designed to investigate whether, similar to ischemic preconditioning, resveratrol preconditioning also involved MAPK signaling.

Materials and Methods

Resveratrol

Resveratrol (a natural phytoalexin), ERK1/2 inhibitor PD98059, and p38 MAPK blocker SB-202190 were obtained from Sigma Chemical Co. (St. Louis, MO). The mitogen- and stress-activated protein kinase 1 (MSK-1) blocker H89 and protein kinase A (PKA) inhibitor KT5720 were purchased from Calbiochem Corp. (San Diego, CA). The drugs were dissolved in dimethyl sulfoxide (DMSO), and the aliquots were kept at 4°C. Control experiments used the vehicle (0.01% DMSO) only.

Animals

All of the animals used in this study received humane care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (publication no. NIH 85-23, revised 1985). Sprague-Dawley male rats weighing between 250 and 300 g were fed ad libitum regular rat chow with free access to water until the start of the experimental procedure. The rats were randomly assigned to one of the following groups (Fig. 1), perfused for 15 min with Krebs-Henseleit bicarbonate (KHB); 1) vehicle (DMSO) only, 2) PD98059 only, 3) SB-202190 only, 4) H89 only, 5) KT5720 only, 6) KHB containing 10 μM resveratrol, 7) 10 μM resveratrol + 20 μM PD98059, 8) 10 μM resveratrol + 10 μM SB-202190, 9) 10 μM resveratrol + 1 μM H89, or 10) 10 μM resveratrol + 10 μM KT5720. All of the hearts were then subjected to 30 min of ischemia, followed by 2 h of reperfusion.

Before performing our experiment with 10 μM resveratrol, we determined the optimal dose of resveratrol as 10 μM by studying four different doses (3.7, 7.4, 10, and 25 μM). Resveratrol at 3.7 and 7.4 μM had no cardioprotective effects, whereas the effects were slightly, but not significantly, lower at 25 μM than that observed for 10 μM concentration.

Isolated Working Heart Preparation

Rats were anesthetized with sodium pentobarbital (80 mg/kg i.p.) (Abbott Laboratories, North Chicago, IL) and an anticoagulant with heparin sodium (500 IU/kg i.v.) (Elkins-Sinn Inc., Cherry Hill, NJ). After ensuring sufficient depth of anesthesia, thoracotomy was performed, and hearts were perfused in the retrograde Langendorff mode at 37°C at a constant perfusion pressure of 100 cm of water (10 kPa) for a 5-min washout period. The perfusion buffer used in this study consisted of a modified KHB (118 mM sodium chloride, 4.7 mM potassium chloride, 1.7 mM calcium chloride, 25 mM sodium bicarbonate, 0.36 mM potassium biphosphate, 1.2 mM magnesium sulfate, and 10 mM glucose). The Langendorff preparation was switched to the working mode after the washout period as described previously (Engelman et al., 1995).

At the end of 10 min, after the attainment of steady-state cardiac function, baseline functional parameters were recorded. The circuit was then switched back to the retrograde mode, and hearts were perfused with either KHB with vehicle (DMSO) or any of the blockers (control), resveratrol at a concentration of 10 μM, or a combination of resveratrol and any of the blockers for 15 min. This was followed by a 5-min washout with KHB buffer, and then the hearts were subjected to global ischemia for 30 min and 2 h of reperfusion. The first 10 min of reperfusion was in the retrograde mode to allow for postischemic stabilization and thereafter in the antegrade working mode to allow for assessment of functional parameters, which were recorded at 10-, 30-, 60-, and 120-min reperfusion.

Cardiac Function Assessment

Aortic pressure was measured using a Gould P23XL pressure transducer (Gould Instrument Systems Inc., Valley View, OH) connected to a side arm of the aortic cannula, and the signal was amplified using a Gould 6600 series signal conditioner and moni-
toired on a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA). Heart rate, left ventricular developed pressure (LVDP) (defined as the difference of the maximum systolic and diastolic aortic pressures), and the first derivative of developed pressure (dP/dT) were all derived or calculated from the continuously obtained pressure signal. Aortic flow was measured using a calibrated flowmeter (Gilmore Instrument Inc., Barrington, IL), and coronary flow was measured by timed collection of the coronary effluent dripping from the heart.

**Infarct Size Estimation**

At the end of reperfusion, the left ventricle was cut into transverse slices. The slices were incubated in 1% triphenyl tetrazolium solution in phosphate buffer (88 mM Na₂HPO₄ and 1.8 mM NaH₂PO₄) for 20 min at 37°C. This procedure distinguishes necrotic tissue from viable myocardium. The slices were stored for 48 h in 10% buffered formalin. The heart slices were photographed, and the weights of the slices were monitored. Digital images of the slices were magnified, and the area of necrosis in each slice was quantified by computerized planimetry. The risk and infarct volumes in cubic centimeters of each slice were then calculated based on the slice weight to remove the introduction of any errors caused by nonuniformity of heart slice thickness. The risk volumes and infarct volumes of each slice were summed to obtain the risk and infarct volumes for the whole heart. Infarct size was taken to be the percent infarct volume of risk volume for any one heart.

**Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay for Assessment of Apoptotic Cell Death**

Immunohistochemical detection of apoptotic cells was carried out using terminal deoxynucleotidyl transferase dUTP nick-end labeling (Maulik et al., 2000). The sections were incubated again with mouse monoclonal antibody recognizing cardiac myosin heavy chain to specifically recognize apoptotic cardiomyocytes. The fluorescence staining was viewed with a confocal laser microscope. The number of apoptotic cells was counted and expressed as a percentage of total myocyte population.

**Western Blot Analysis**

Left ventricles from the hearts were homogenized in a buffer containing 25 mM Tris-HCl, 25 mM NaCl, 1 mM orthovanadate, 10 mM MgF, 10 mM pyrophosphate, 10 μM okadaic acid, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (Sato et al., 2000a). One hundred micrograms protein of each heart homogenate was incubated with 1 μg of antibody against the phospho-CREB, p38 MAPK, MSK-1, and MAPK-activated protein (MAPKAP) kinase 2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at 4°C. The immune complexes were precipitated with protein A-Sepharose, and the immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and immobilized on polyvinylidene difluoride membrane. The membrane was immunoblotted with PY20 to evaluate the phosphorylation of the compounds. The membrane was stripped and reblotted with specific antibodies against CREB, p38 MAPK, MSK-1, and MAPKAP kinase 2. The resulting blots were digitized and subjected to densitometric scanning using a standard NIH image program.

**Statistical Analysis**

The values for myocardial functional parameters, total and infarct volumes and infarct sizes, and cardiomyocyte apoptosis are all expressed as the mean ± S.E.M. Analysis of variance test was first carried out to test for any differences between the mean values of all the groups. If differences were established, the values of the treated groups were compared with those of the control group by a modified t test. The results were considered significant if p < 0.05.

**Results**

**Effects of Resveratrol on Myocardial Function.** Initially, we performed a dose-response study to determine optimal dose of resveratrol. There were no effects on ventricular function at 3.7 and 7.4 μM resveratrol (Figs. 2 and 3), supporting our previous results (Ray et al., 1999). There was no effect on myocardial infarct size in any of these doses of resveratrol (Fig. 3). Consistent with previously published articles, the maximum beneficial effect was noticed at 10 μM resveratrol. At a higher dose (25 μM), resveratrol still exerts cardioprotective effects, but the effects tend to be slightly depressed. Subsequent studies were performed with 10 μM resveratrol.

At all of the concentrations, including the 10 μM concentration, there were no differences in baseline function among all 10 groups. In general, there were no significant differences between resveratrol versus control, PD98059, SB-202190, H89, and KT5720 (not shown) and versus resveratrol + PD98059, resveratrol + SB-202190, resveratrol + H89 and resveratrol + KT5720 versus resveratrol on aortic flow and coronary flow (Fig. 4, bottom). As was expected, on reperfusion, the absolute values of all of the functional parameters were decreased in all of the groups compared with the respective baseline values. The resveratrol group displayed significant recovery of postischemic myocardial function. Aortic flow (Fig. 4, top) was markedly higher in the resveratrol group from reperfusion (R)-30 onward at all three time points: R-30 (66.1 ± 3.62 versus 36.02 ± 12.7 ml/min), R-60 (43.5 ± 5.34 versus 19.24 ± 6.48 ml/min), and R-120 (14.9 ± 2.36 versus 4.28 ± 1.43 ml/min). The cardioprotective effects of resveratrol were evidenced by significant differ-
ences in the LVDP from R-30 onward at all three time points (Fig. 5, top); the difference was especially apparent at R-30 (121.7 ± 2.64 versus 103.57 ± 7.1 mm Hg), R-60 (110.27 ± 1.2 versus 88.02 ± 9.57 mm Hg), and R-120 (87.8 ± 1.74 versus 52.5 ± 4.56 mm Hg). For LVdp/dt (Fig. 5, bottom), resveratrol-mediated increased recovery was apparent at R-60 (2843 ± 79.48 versus 1880.5 ± 403.3 mm Hg/s) and R-120 (1391.8 ± 104.7 versus 899.83 ± 86.75 mm Hg/s). With the use of SB-202190 and PD98059, resveratrol partially lost its cardioprotective effect; however, with H89, resveratrol significantly lost its cardioprotective effects, which was evidenced by significant differences in the postischemic period of LVDP from R-30 onward at all three time points. PD98059 or SB-202190 did not decrease the LVDP at R-30 or R-60 levels, but with H89, the decrease was prominent both at R-30 (105.5 ± 5.78 versus 121.7 ± 2.64 mm Hg) and R-60 (83.9 ± 4.75 versus 110.27 ± 1.2 mm Hg). At R-120, the decrease is significant with all of the inhibitors (R-120, 61.35 ± 4.05, 68.62 ± 3.59, and 60.63 ± 6.27 mm Hg, respectively, versus 87.8 ± 1.74 mm Hg). The same effect of PD98059, SB-202190, and H89 on resveratrol preconditioning also reflects the significant decrease of LVdp/dt at R-60 (2114.7 ± 119.29, 1760.5 ± 158.44, and 1633.17 ± 225.59 mm Hg/s, respectively, versus 2843 ± 79.48 mm Hg/s) and R-120 (877.7 ± 187.27, 866.7 ± 96.61, and 862.3 ± 126.32 mm Hg/s, respectively, versus 1425.2 ± 109.72 mm Hg/s). But with H89, the significant decrease also was observed at R-30 (2566.5 ± 143.17 versus 3012.7 ± 64.02 mm Hg/s) apart from the other time points. This was also confirmed from the aortic flow value, which is markedly lower at R-30 onward at all

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**Fig. 3.** Dose-response curve of the effects of resveratrol on myocardial infarction and cardiomyocyte apoptosis. The isolated rat hearts were perfused for 15 min with KHB buffer in the absence or presence of four different doses (3.7, 7.4, 10, and 25 μM) of resveratrol. The hearts were made globally ischemic for 30 min followed by 2 h of reperfusion in the working mode. Myocardial infarct size (top) and cardiomyocyte apoptosis (bottom) were determined at the indicated times. Results are expressed as mean ± S.E.M. of four to six hearts per group. *, p < 0.05 versus control; †, p < 0.05 versus resveratrol.

**Fig. 4.** Effects of resveratrol and various inhibitors used to block the effects of resveratrol preconditioning on the ischemic reperfused heart. The isolated rat hearts were perfused for 15 min with KHB buffer in the absence or presence of resveratrol without or with the inhibitors. The hearts were made globally ischemic for 30 min followed by 2 h of reperfusion in the working mode. The aortic flow (top) and the coronary flow (bottom) were determined at the indicated times. Results are expressed as mean ± S.E.M. of six hearts per group. *, p < 0.05 versus control.
three time points with all of the inhibitors. KT5720 had no effect on resveratrol-mediated ventricular recovery.

**Effects of Resveratrol on Myocardial Infarct Size.** Infarct size (percentage of infarct versus total area at risk) was noticeably reduced in the resveratrol group compared with the control group (18.17 ± 2.08 versus 34.7 ± 2.74%) (Fig. 6, top). This infarct zone was increased significantly when resveratrol was used along with SB-202190 and PD98059 (30.4 ± 2.44 and 29.8 ± 1.98%, respectively, versus 18.17 ± 2.08%). When resveratrol was used along with H89, the infarct zone was further increased compared with the other two inhibitors (33.6 ± 2.62%) as shown in Fig. 6, top. KT5720 did not have any effect on infarct size-lowering ability of resveratrol.

**Effects of Resveratrol on Cardiomyocyte Apoptosis.** The percentage of apoptotic cardiomyocytes was significantly reduced in the resveratrol group compared with the control group (35.1 ± 1.2 versus 21.7 ± 1.5%) (Fig. 6, bottom). This apoptotic cell death was increased significantly when resveratrol was used along with SB-202190 and PD98059 (12.5 ± 2.0%, resveratrol + SB-202190 (11.8 ± 1.6%), and resveratrol + H89 (20.8 ± 2.5) groups compared with the resveratrol group (5.1 ± 1.2%) (Fig. 4, bottom). Significant differences also existed between resveratrol + H89 and resveratrol + PD98059 or SB-202190 groups. In contrast, there was no difference between resveratrol and resveratrol + KT5720 groups.

**Effects of Resveratrol on the Phosphorylation of p38 MAPK, MAPKAP Kinase 2, MSK-1, and CREB.** The dose-response curve for the activation and phosphorylation of ERK1/2, p38 MAPK, and Akt is shown in Fig. 7. There was no activation of any of the kinases (Fig. 7, black bars); however, at both 10 and 25 μM, increased phosphorylation of ERK1/2, p38 MAPK, and Akt occurred (Fig. 7, white bars). Resveratrol at 3.7 and 7.4 μM concentrations could not induce phosphorylation of any of these kinases. Our subsequent studies were performed with 10 μM resveratrol concentration.

At 10 μM, resveratrol significantly enhanced the phosphorylation of MAPK. As shown in Fig. 8, phosphorylation of ERK1/2, p38 MAPK, and MAPKAP kinase 2 was increased significantly compared with control. Resveratrol-mediated increased phosphorylation of ERK1/2 (Fig. 5, top) was reduced by PD98059, but not with SB-202190; increased phosphorylation of p38 MAPK (Fig. 8, middle) and MAPKAP kinase (Fig. 8, bottom) was reduced by SB-202190, but not with PD98059 (Fig. 8, middle and bottom).

The phosphorylation pattern of MSK-1 and CREB is shown in Fig. 6. Resveratrol increased the phosphorylation of both MSK-1 (Fig. 9, top) and CREB (Fig. 9, bottom). Increased phosphorylation of MSK-1 and CREB was reduced significantly by either PD98059 or SB-202190. Resveratrol-mediated increased phosphorylation of MSK-1 and CREB was almost abolished by H89, but not with KT5720.

**Discussion**

The most important finding of this study is that resveratrol increased the phosphorylation of ERK1/2 and p38 MAPK, which in turn phosphorylated MSK-1, leading to the activa-
tion of CREB, suggesting that phosphorylation of MSK-1 and subsequent activation of CREB occurred via both p38 MAPK and ERK1/2. Resveratrol also increased the phosphorylation of MAPKAP kinase 2, the downstream target of p38 MAPK. Consistent with these results, cardioprotective abilities of resveratrol were partially abolished either with an ERK1/2 inhibitor, PD98059, or with a p38 inhibitor, SB-202190, and almost completely with an MSK-1 blocker, H89. A specific blocker of PKA, KT5720, had no effect on resveratrol-mediated cardioprotection.

Inverse relationship between the consumption of red wine and incidence of cardiovascular disease has been popularly known as the French paradox (Kopp, 1998). The cardioprotective abilities of red wine have been attributed to resveratrol (Kopp, 1998; Hung et al., 2000), which possesses diverse properties, including anti-inflammatory, antiplatelet, and vasorelaxant activities (Bertelli et al., 1996; Sato et al., 2000b; Orallo et al., 2002). Striking similarities of the cardioprotective properties between resveratrol and nitric oxide (NO) prompted the researcher to determine the role of NO in resveratrol-mediated cardioprotection.

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Resveratrol has been known to modulate MAPK signaling. Among the three MAPK, ERK1/2 is involved in cell proliferation, whereas p38 MAPK and JNK are activated in response to environmental stress. In undifferentiated cells, a small amount (1 μM) of resveratrol can induce phosphorylation of ERK1/2 (Miloso et al., 1999). In retinoic acid-differentiated cells, the same amount of resveratrol induced an increase in ERK1/2 phosphorylation. Another study showed increased phosphorylation of ERK1/2, JNK, and p38 MAPK in the mouse epidermal cells, which subsequently enhanced serine 15 phosphorylation of p53 (She et al., 2001). Dominant-negative mutant of ERK2 or p38 MAPK depressed phosphorylation of p53 at serine 15. In this study, overexpression of dominant-negative mutant of JNK1 had no effect on this phosphorylation. In papillary and follicular thyroid carcinoma cell lines, a relatively higher amount of resveratrol (1–10 mM) induced activation and nuclear translocation of ERK1/2 (Shih et al., 2002). Interestingly, at higher concentrations (even at 50–100 μM), resveratrol seems to inhibit
phosphorylation of MAPK. At 37 mM concentration, resveratrol depressed MAPK activity and reduced phosphorylation of ERK1/2, JNK1, and p38 MAPK at active sites (El-Mowafy and White, 1999). Another related study showed that resveratrol activated JNK at the same dose that inhibited tumor promoter-induced cell transformation (She et al., 2002). Thus, it seems that resveratrol can cause activation of MAPK in some cells, whereas it inhibits MAPK in others. Moreover, activation/inhibition seems to be concentration-dependent; in general, it is stimulatory at lower concentration and inhibitory at higher concentration. In the present study, resveratrol at 10 μM concentrations enhanced the phosphorylation of p38 MAPK and ERK1/2. In concert, inhibition of p38 MAPK with SB-202190 or ERK1/2 with PD98059 partially abolished the effect of preconditioning. MSK-1 is situated downstream of ERK1/2 and p38 MAPK (Fig. 10). MSK-1, which belongs to the AGC family of kinases and is related in structure to the ribosomal p70 S6 subfamily, can be activated by both ERK1/2 and p38 MAPK (Fig. 7). MSK-1, as well as MSK-2, can be directly activated both in vitro and in vivo by p42/44 ERK and p38 MAPK (Deak et al., 1998). In another study, MSK-1 and MSK-2 activities were increased 400 to 500% and 200 to 300%, respectively, in exercised muscle along with an increase in MAPKAP kinase 2 (Krook et al., 2000). In a related study, ERK1/2 phosphorylation increased 7.8-fold and p38 MAPK phosphorylation increased 4.4-fold after the exercise. The activity of MAPKAP kinase 2, the downstream target of p38 MAPK, increased 3.1-fold, whereas MSK-1, downstream of both ERK1/2 and p38 MAPK, increased 2.4-fold at the same time. In the present study, resveratrol-mediated increase in MSK-1 seems to be the result of the activation of both p38 MAPK and
ERK1/2 because inhibition of either p38 MAPK or ERK1/2 resulted in partial down-regulation of MSK-1.

MSK-1 is required for CREB and the closely related activating transcription factor activation after mutagenic or stress stimuli. On phosphorylation, they recruit the coactivator CREB-binding protein, thereby effecting phosphorylation. Recently, resveratrol was found to phosphorylate CREB via adenosine A1 and A3 receptors through the activation of Akt survival pathway (Das et al., 2005b). Another related study showed activation of CREB by resveratrol through Akt-dependent and Akt-independent pathways (Das et al., 2005c). Several distinct pathways can induce CREB, which is an important nuclear factor for cell survival. For example, growth factors and stress can induce CREB phosphorylation through the activation of downstream targets of MAPK signaling pathways, including classical ERK pathway and stress-activated p38 MAPK pathway (Shaywitz and Greenberg, 1999). Recent studies determined that MSK are the major growth factor-regulated CREB kinase (Wiggin et al., 2002). In the present study, resveratrol-mediated CREB activation seems to occur through the phosphorylation of MSK-1 because the inhibition of MSK-1 abolished the phosphorylation of CREB. Previous studies showed the involvement of CREB in transmitting resveratrol-mediated survival signal through the activation of BelII (Das et al., 2005b). Thus, it seems that resveratrol activates CREB through the phosphorylation of MSK-1.

In this study, 1 μM H89 was used to block MSK activation. However, this compound can also block PKA. A recent study showed that 2 μM H89 enhanced postischemic cardiac contractile recovery and reduced infarct size (Makaula et al., 2005), presumably by reducing PKA activity. To confirm the role of MSK signaling in resveratrol preconditioning, the hearts were also treated with a specific PKA blocker, KT5720, in conjunction with resveratrol. Unlike H89, which abolished resveratrol-mediated cardioprotection, KT5720 did not alter resveratrol-mediated ventricular recovery, nor did it have any effect on infarct size-lowering ability of resveratrol. Western blot analysis revealed that KT5720 did not affect the phosphorylation of MSK or CREB induced by resveratrol. These results confirmed that MSK signaling was involved in resveratrol preconditioning.

MAPKAP kinase 2 is the downstream target for p38 MAPK. A large number of reports exist in the literature indicating that MAPKAP kinase 2 plays a crucial role in preconditioning (Maulik et al., 1999). Preconditioning potentiates the phosphorylation of p38 MAPK, leading to the phosphorylation of MAPKAP kinase 2, which in turn up-regulates heat shock protein 27 (Chevalier and Allen, 2000). In this study, resveratrol could phosphorylate MAPKAP kinase 2 via the activation of p38 MAPK because the MAPKAP kinase 2 phosphorylation was partially blocked with SB-202190.

In summary, the results of the present study showed for the first time that resveratrol triggers a preconditioning-like survival signaling by activating MAPK signaling pathway. Thus, resveratrol activates both ERK1/2 and p38 MAPK, both of which contribute toward the phosphorylation of MSK-1. There seems to be two downstream targets for p38 MAPK, MSK-1 and MAPKAP kinase 2. MSK-1 in turn activates CREB, which was previously shown to transmit survival signal by activating BelII.

**Fig. 9.** Effects of resveratrol and various inhibitors used to block the effects of resveratrol preconditioning on the phosphorylation of MSK-1 (top) and CREB (bottom). The isolated rat hearts were perfused for 15 min with KHB buffer in the absence or presence of resveratrol without or with the inhibitors. The hearts were made globally ischemic for 30 min followed by 2 h of reperfusion in the working mode. At the end of the experiments, the hearts were frozen at liquid nitrogen temperature for subsequent determination of the protein phosphorylation by Western blot analysis. The phosphorylated proteins are shown on the top of nonphosphorylated proteins, which also served as the controls. The average of four experiments (mean ± S.E.M.) is shown as bar graphs on the top of the representative Western blots. *p < 0.05 versus control; †p < 0.05 versus resveratrol.

**Fig. 10.** MSK-1 and CREB are the downstream targets of ERK and p38 MAPK. MEK, mitogen-activated protein kinase kinase.

**References**


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