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POTENTIATION OF A SURVIVAL SIGNAL IN THE ISCHEMIC HEART BY RESVERATROL THROUGH p38MAPK-MSK-1-CREB SIGNALING

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ABBREVIATIONS

CREB: cAMP response-element binding protein; MAPK, mitogen-activated protein kinase; DMSO, dimethyl sulfoxide; KHB, Krebs-Henseleit bicarbonate; LVDP, left ventricular developed pressure; LVdp/dt, maximum first derivative of developed pressure; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; p-CREB, phosphorylated cAMP response-element binding protein; R, reperfusion; PC, preconditioning; ERK, Extracellular regulated kinase; NO, nitric oxide; iNOS, inducible nitric oxide synthase

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 CREB-dependent PI-3-kinase-Akt-Bcl2 pathway. The present study was designed to determine if similar to ischemic preconditioning, resveratrol uses MAP kinases as upstream signaling targets. The isolated rat hearts were pre-perfused for 15 min with KHB buffer in the absence (control) or presence of ERK1/2 inhibitor PD098059, p38MAPK inhibitor SB202190, MSK1 inhibitor H-89, PKA inhibitor KT5720, resveratrol only, resveratrol plus PD098059, resveratrol plus SB202190, resveratrol plus H-89 or resveratrol plus KT5720. In consistent with previous reports, resveratrol provided cardioprotection as evidenced by its ability to improve post-ischemic ventricular function, reduction of myocardial infarct size and cardiomyocyte apoptosis. The cardioprotection afforded by resveratrol was partially abolished with PD098059 or SB202190 suggesting that both ERK1/2 and p38MAPK play roles in resveratrol-mediated preconditioning. A MSK-1 inhibitor, H-89 abolished resveratrol mediated preconditioning indicating MSK-1 to be the downstream target molecule for both ERK1/2 and p38MAPK. *KT5720 had no effect on resveratrol mediated cardioprotection.* Corroborating with these results, Western blot analysis revealed phosphorylation of ERK1/2, p38MAPK, MAPKAP kinase 2 and MSK1 with resveratrol, and inhibition of phosphorylation with corresponding inhibitors. These results showed for the first time that resveratrol triggers a MAP kinase signaling pathway involving ERK1/2 and p38MAP kinase, the former using MSK1 as the downstream target while the later using both MAPKAP kinase 2 and MSK1 as downstream targets.

INTRODUCTION

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; Das et al, 2005; Sato et al, 2000, Hattori et al, 2002). The cardioprotective mechanisms of resveratrol include antioxidant (Das et al, 2005; Ray et al, 1999), anti-inflammatory (Sato et al, 2000; Das et al, 2005), and anti-platelet (Bertelli et al, 1996) activities, all of which involve nitric oxide signaling (Das et al, 2005; Hattori et al, 2002; Imamura et al, 2002; Chen et al, 1996). 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; Das et al, 2005; Hattori et al, 2002), it has been postulated that resveratrol protects the hearts through preconditioning.

Recently, resveratrol was found to protect the ischemic heart through the upregulation of adenosine A1 and A3 receptors (Das et al, 2005), a property shared by ischemic preconditioning (Bradamante et al., 2000; Hattori et al., 2002; Imamura et al., 2002; Das et al., 2005). In this study, resveratrol induced the expression of Bcl2 and caused its phosphorylation along with the phosphorylation of CREB, Akt and Bad. A PI-3-kinase inhibitor LY294002 partially blocked the cardioprotectyive abilities of resveratrol suggesting that resveratrol transmits a survival signal through a CREB-dependent PI-3-kinase-Akt-Bcl2 signaling pathway. Subsequent studies determined that such survival signal through the activation of CREB could also occur through an Akt-independent pathway (Das et al, 2005).

Ischemic preconditioning, the state-of-the-art techniques of cardioprotection involves MAP kinases as upstream signaling molecules (Baines et al, 2002; Fryer et al, 2001). Whether resveratrol also transmits survival signals through a signaling cascade involving MAP kinases is not known. A recent study demonstrated that in mouse epidermal cells, resveratrol activated ERKs, JNKs 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 SB202190 or stable expression of a dominant negative mutant of ERK2 or p38kinase impaired resveratrol-induced p53-dependent transcriptional activity and apoptosis suggesting that both ERKs and p38MAPK mediate resveratrol-induced p53 phosphorylation. The present study was designed to investigate if similar to ischemic preconditioning, resveratrol preconditioning also involve MAP kinase signaling.

MATERIALS AND METHODS

Resveratrol. Resveratrol (trans-3, 4', 5-trihydroxystilbene), a natural phytoalexin, ERK1/2 inhibitor, PD098059 and p38MAPK blocker, SB202190 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The MSK-1 blocker, H-89, and PKA inhibitor KT5720 were purchased from Calbiochem Corp. (San Diego, CA, USA). The drugs were dissolved in DMSO, and the aliquots were kept at 4°C. Control experiments used the vehicle (0.01% DMSO) only.

Animals. All animals used in this study received humane care in compliance with the principles of the laboratory animal care formulated by the National Society for Medical Research and 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 Number NIH 85-23, revised 1985). Sprague Dawley male rats weighing between 250-300 gm 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 (Figure 1): perfused for 15 min with KHB with i) vehicle (DMSO) only; ii) PD 098,059 only; iii) SB 202190 only; iv) H-89 only; v) KT5720 only; vi) KHB containing 10 µM resveratrol; vii) 10 µM resveratrol + 20 µM PD 098,059; viii) 10 µM resveratrol + 10 µM SB 202190; ix) 10 µM resveratrol + 1 µM H-89 or x) 10 µM resveratrol+10 µM KT5720. All hearts were then subjected to 30 min ischemia followed by 2 h reperfusion.

Prior to performing our experiment with 10 µM resveratrol, we determined the optimal dose of resveratrol as 10 µM by studying four different doses [3.7 µM, 7.4 µM, 10 µM and 25 µM]. Resveratrol at 3.7 µM and 7.4 µM had no cardioprotective effects while at 25 µM the effects were slightly, but not significantly, lower 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, USA) and anticoagulant with heparin sodium (500 IU/kg., i.v.) (Elkins-Sinn Inc., Cherry Hill, NJ, USA). After ensuring sufficient depth of anesthesia thoracotomy was performed, 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 Krebs-Henseleit bicarbonate buffer (KHB) (in mM: sodium chloride 118, potassium chloride 4.7, calcium chloride 1.7, sodium bicarbonate 25, potassium biphosphate 0.36, magnesium sulfate 1.2, and glucose 10). The Langendorff preparation was switched to the working mode following the washout period as previously described (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 either KHB with vehicle (DMSO) or any of the blockers (Control), Resveratrol at a concentration of 10 μM or a combination of Resveratrol and the any of blockers for a duration of 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 then 2 h of reperfusion. The first 10 min of reperfusion was in the retrograde mode to allow for post ischemic stabilization and there after, 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, USA) connected to a side arm of the aortic cannula, the signal was amplified using a Gould 6600 series signal conditioner and monitored on a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA, USA) (11). Heart Rate (HR), Left Ventricular develops 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 (AF) was measured using a calibrated flow-meter (Gilmont Instrument Inc., Barrington, IL, USA) and coronary flow (CF) 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 [Na2HPO4 88 mM, NaH2PO4 1.8 mM] for 20 min at 37oC. 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 cm3 of each slice were then calculated on the basis of slice weight to remove the introduction of any errors due to non-uniformity 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.

TUNEL Assay for assessment of Apoptotic Cell Death. Immunohistochemical detection of apoptotic cells was carried out using TUNEL (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 percent of total myocytes 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 NaF, 10 mM pyrophosphate, 10 µM okadaic acid, 0.5 mM EDTA and 1 mM PMSF (Sato et al, 2000). 100 µg of protein of each heart homogenate was incubated with 1 µg of antibody against the phospho- CREB, p38MAPK, MSK-1, MAPKAP kinase2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at 4^{o}C. The immune complexes were precipitated with protein A Sepharose, immunoprecipitates separated by SDS-PAGE 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, p38MAPK, MSK-1, MAPKAP

kinase2. 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 ± standard error of mean (SEM). Analysis of variance test was first carried out to test for any differences between the mean values of all groups. If differences between 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 μM and 7.4 μM resveratrol [Figure 2 and Figure 3] supporting our previous results (Ray et al, 1999). There was no effect on myocardial infarct size in any of these doses of resveratrol (Figure 3). In consistent with previously published papers, the maximum beneficial effect was noticed at 10 μM resveratrol. At 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 concentrations including 10 μM concentration, there were no differences in baseline function between all the ten groups. In general, there were no significant differences between resveratrol vs. control, PD98059, SB202190 , H-89, and KT5720 (not shown) as well as vs. Resveratrol + PD98059, Resveratrol + SB202190, Resveratrol + H-89 or resveratrol + KT5720 vs. Resveratrol on aortic flow and coronary flow **(**Figure 4, bottom**).** As was expected, upon

reperfusion, the absolute values of all functional parameters were decreased in all the groups as compared with the respective baseline values. Resveratrol group displayed significant recovery of post ischemic myocardial function. Aortic flow (Figure 4, top) was markedly higher in the resveratrol group from R-30 onwards at the all rest three points. R-30 (66.1 ± 3.62 ml/min vs. 36.02 ± 12.7 ml/min), R-60 (43.5 ± 5.34 ml/min vs. 19.24 ± 6.48 ml/min) and R-120 (14.9 ± 2.36 ml/min vs. 4.28 ± 1.43 ml/min). The cardio protective effects of resveratrol were evidenced by significant differences in the LVDP from R-30 onwards at the all rest three points **(**Figure 5, top**),** the difference was especially apparent at R-30 (121.7 ± 2.64 mm Hg vs. 103.57 ± 7.1 mm Hg), R-60 (110.27 ± 1.2 mmHg vs. 88.02 ± 9.57 mmHg) and at R-120 (87.8 ± 1.74 mm Hg vs. 52.5 ± 4.56 mm Hg). For LVdp/dt (Figure 5, bottom), resveratrol mediated increased recovery was apparent at R-60 (2843 ± 79.48 mmHg/sec vs. 1880.5 ± 403.3 mm Hg/sec) and at R-120 (1391.8±104.7 mm Hg/sec vs. 899.83±86.75 mmHg/sec). With the use of SB202190 and PD098059 resveratrol partially lost it cardio-protective effect, but with H-89 resveratrol significantly lost its cardio protective effects, which were evidenced by significant differences in the post ischemic period of LVDP from R-30 onwards at all of the three time points. PD 098,059 or SB 202190 did not decrease the LVDP at R-30 or at R-60 level but with H-89 the decrease was prominent both at R-30 (105.5 ± 5.78 mm Hg vs. 121.7 ± 2.64 mmHg) and R-60 (83.9 ± 4.75 mm Hg vs. 110.27 ± 1.2 mmHg). At R-120 the decrease is significant with all the inhibitors [R-120 (61.35 ± 4.05 mmHg, 68.62 ± 3.59 mmHg, and 60.63 ± 6.27 mmHg, respectively, vs. 87.8 ± 1.74 mmHg)]. The same effect of PD 098,059, SB 202190 and H-89 on resveratrol preconditioning also reflects from the significant decrease of LVdp/dt at R-60 (2114.7 ± 119.29 mmHg/sec, 1760.5 ± 158.44 mmHg/sec and 1633.17 ± 225.59 mmHg/sec, respectively, vs. 2843 ± 79.48 mmHg/sec) and at R-120 (877.7 ± 187.27 mmHg/sec, 866.7 ± 96.61 mmHg/sec and 862.3 ± 126.32 mm Hg/sec, respectively, vs. 1425.2 ± 109.72 mmHg/sec). But with

H-89 the significant decrease also observed at R-30 (2566.5 ± 143.17 mmHg/sec vs. 3012.7 ± 64.02 mmHg/sec) apart from the other time points. This was also confirmed from the Aortic flow value; which is markedly lower at R-30 onwards at all the rest three time points with all the inhibitors. KT5720 had no effect on resveratrol mediated ventricular recovery.

Effects of Resveratrol on Myocardial Infarct size. Infarct size (percent of infarct vs. total area at risk) was noticeably reduced in resveratrol group as compared to the control (18.17 ± 2.08% vs. 34.7 ± 2.74%) (Figure 6, top). This infarct zone was increased significantly when resveratrol were used along with SB202190 and PD98, 059 (30.4 ± 2.44 % and 29.8 ± 1.98 %, respectively, vs. 18.17 ± 2.08%). When resveratrol was used along with H-89 the infarct zone was further increased compared to the other two inhibitors (33.6 ± 2.62%) as shown in the Figure 6, top. KT5720 did not have any effect on infarct size lowering ability of resveratrol.

Effects of Resveratrol on Cardiomyocyte Apoptosis. The percent of apoptotic cardiomyocytes was significantly reduced in resveratrol group as compared to the control (35.1±1.2% vs. 21.7±1.5%) (Figure 6, bottom). This apoptotic cell death was increased significantly when resveratrol was used along with PD98059, SB202190 or H-89., Thus, the apoptosis was significantly higher in resveratrol+PD98059 (12.5±2.0%), resveratrol+SB202190 (11.8±1.6%) and resveratrol + H-89 (20.8±2.5) groups as compared to the resveratrol group (5.1±1.2%) (Figure 4, bottom)). Significant differences also existed between resveratrol+H-89 and resveratrol+PD98059 or SB202190 groups. In contrast, there was no difference between resveratrol and resveratrol + KT5720 groups.

Effects of Resveratrol on the phosphorylation of p38MAPK, MAPKAP kinase2, MSK-1 and CREB. The dose-response curve for the activation and phosphorylation of ERK1/2, p38MAPK, and Akt is shown in Figure 7. There was no activation of any of the kinases (shown in black bars); however, at both 10 μM and 25 μM doses increased phosphorylation of ERK1/2, p38MAPK and Akt occurred (shown in 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 MAP kinases. As shown in Figure 8, phosphorylation of ERK1/2, p38 MAP kinase, and MAPKAP kinase 2 was increased significantly as compared to control. Resveratrol-mediated increased phosphorylation of ERK1/2 (Figure5, top) was reduced by PD98059, but not with SB202190, increased phosphorylation of p38MAP kinase (Figure 8, middle) and MAPKAP kinase Figure 8, bottom) was reduced by SB202190, but not with PD98059 (Figure 8, middle and bottom).

The phosphorylation pattern of MSK-1 and CREB is shown in Figure 6. Resveratrol increased the phosphorylation of both MSK-1 (Figure 9, top) and CREB (Figure 9, bottom. Increased phosphorylation of MSK-1 and CREB was reduced significantly by either PD98059 or SB202190. Resveratrol-mediated increased phosphorylation of MSK-1 and CREB was almost abolished by H-89, but not with KT5720**.**

DISCUSSION

The most important finding of this study is that resveratrol increased the phosphorylation of by ERK1/2 and p38 MAPK, which in turn phosphorylated MSK1 leading to the activation of CREB, suggesting that phosphorylation of MSK1 and subsequent activation of CREB occurred via

both p38MAPK ERK1/2. Resveratrol also increased the phosphorylation of MAPKAP kinase 2, the downstream target of p38 MAP kinase. In consistent with these results, cardioprotective abilities of resveratrol were partially abolished either with an ERK1/2 inhibitor, PD 098059 or with a p38 inhibitor, SB202190 and almost completely with a MSK-1 blocker, H-89. 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 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, anti-platelet, and vasorelaxant activities (Sato et al, 2000; Bertelli et al, 1996; Orallo et al, 2002). Striking similarities of the cardioprotective properties between resveratrol and NO prompted the researcher to determine the role of NO in resveratrol-mediated cardioprotection. A direct role of NO was shown from a study, which found resveratrol-mediated increase in NOS activity in cultured pulmonary artery endothelial cells, suggesting that resveratrol could afford cardioprotection by affecting the expression of NOS (Hsieh et al, 1999). In consistent with these results, resveratrol was found to protect isolated working rat hearts through the upregulation of iNOS (Das et al, 2005; Hattori et al, 2002). Resveratrol failed to provide cardioprotection in iNOS knockout mice devoid of any copy of iNOS gene further supporting the role of NO (Imamura et al, 2002). In a more cent study, resveratrol reduced myocardial ischemia/reperfusion injury through both iNOS-dependent and iNOS independent manner (Hung et al, 2004). Similar to NO, resveratrol significantly reduced the amount of proadhesive molecules including sICAM-1, sVCAM-1 and E-Selectin in the ischemic reperfused myocardium (Das et al, 2005).

Resveratrol has been known to modulate MAP kinase signaling. Among the three MAP kinases, ERK1/2 is involved in cell proliferation, while p38MAPK 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, JNKs and p38MAPK in the mouse epidermal cells, which subsequently enhanced serine-15 phosphorylation of p53 (She et al, 2001). Dominant negative mutant of ERK2 or p38MAPK 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, relatively higher amount of resveratrol (1-10 mM) induced activation and nuclear translocation of ERK1/2 (Shih et al, 2002). Interestingly, at higher concentration (even at 50-100 μM) resveratrol appears 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 et al, 1999). Another related study showed that resveratrol activated JNKs at the same dose that inhibited tumor promoter-induced cell transformation (She et al, 2002). Thus, it appears that resveratrol can cause activation of MAPK in some cells, while 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 p38MAPK and ERK1/2. In concert, inhibition of p38MAPK with SB 202190 or ERK1/2 with PD098059 partially abolished the effect of preconditioning. MSK-1, a mitogen- and stress-activated protein kinase-1, is situated downstream of ERK1/2 and p38MAPK. MSK1 that belongs to AGC family of kinases and is related in structure to ribosomal p70 S6 subfamily

can be activated by both ERK1/2 and p38MAPK (Figure 7). MSK-1 as well as MSK-2 can be directly activated both in vitro and in vivo by p42/44 ERK and p38MAPK in cultured cells (Deak et al, 1998). In another study, MSK-1 and MSK-2 activities were increased 400-500% and 200-300%, 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 (Yu et al, 2001). The activity of MAPKAP kinase 2, the downstream target of p38 MAPK, increased 3.1-fold while MSK-1, downstream of both ERK1/2 and p38MAPK increased 2.4-fold at the same time. In the present study, resveratrol-mediated increase in MSK-1 appears to be the result of the activation of both p38MAPK and ERK1/2, because inhibition of either p38MAPK or ERK1/2 resulted in partial downregulation of MSK-1.

MSK-1 is required for cyclic AMP response element (CRE)-binding protein (CREB) and the closely related activating transcription factor (ATF1) activation after mutagenic or stress stimuli. Upon phosphorylation, they recruit the co-activator 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, 2005). Another related study demonstrated activation of CREB by resveratrol though Akt- dependent as well as Akt-independent pathways (Das et al, 2005). 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 MAP kinase signaling pathways including classical ERK pathway and stress activated p38MAPK pathway (Shaywitz and Greenberg, 1999). Recent studies determined that MSKs are the major growth factor-regulated CREB kinase (Wiggin et al, 2002). In the present study, resveratrol-mediated CREB activation

appears to occur through the phosphorylation of MSK1 since the inhibition of MSK1 abolished the phosphorylation of CREB. Previous studies demonstrated the involvement of CREB in transmitting resveratrol-mediated survival signal through the activation of Bcl2 (Das et al, 2005). Thus, it appears that resveratrol activates CREB through the phosphorylation of MSK1.

In this study, 1 μM H-89 was used to block MSK activation. However, this compound can also block PKA. A recent study showed 2 μM H-89 enhanced post-ischemic 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 H-89, which abolished resveratrol mediated cardioprotection, KT5720 did not alter resveratrol mediated ventricular recovery, nor it had 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 p38MAPK. 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 p38MAPK leading to the phosphorylation of MAPKAP kinase 2, which in turn upregulates heat shock protein 27 (HSP 27) (Chevalier et al, 2000). In this study, resveratrol could phosphorylate MAPKAP kinase 2 via the activation of p38MAP kinase as 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 MAP kinase signaling pathway. Thus, resveratrol activates both ERK1/2 and p38MAPK both of which contributes towards the

phosphorylation of MSK1. There appears to be two downstream targets for p38MAPK, MSK1 and MAPKAP kinase 2. MSK1 in turn activates CREB, which was previously shown to transmit survival signal by activating Bcl 2.

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Footnotes

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FIGURE LEGENDS

Figure 1. Experimental protocol

Figure 2. Dose-response curve of the effects of resveratrol on myocardial performance. The isolated rat hearts were perfused for 15 min with KHB buffer in the absence or presence of four different doses [3.7 µM, 7.4 µM, 10 µM and 25 µM] of resveratrol. The hearts was made globally ischemic for 30 min followed by 2 h of reperfusion in the working mode. The cardiac function were determined at the indicated times Results are expressed as Means ± SEM of four to six hearts per group. *p<0.05 vs. control; †p<0.05 vs. resveratrol.

Figure 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 µM, 7.4 µM, 10 µM and 25 µM] of resveratrol. The hearts was 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 Means ± SEM of four to six hearts per group. *p<0.05 vs. control; †p<0.05 vs. resveratrol.

Figure 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 was 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 Means ± SEM of six hearts per group. *p<0.05 vs. control.

Figure 5. 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 was made globally ischemic for 30 min followed by 2 h of reperfusion in the working mode. The developed pressure (top) and the maximum first derivative of developed pressure (bottom) were determined at the indicated times Results are expressed as Means ± SEM of six hearts per group. *p<0.05 vs. control; †p<0.05 vs. resveratrol.

Figure 6. 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 was 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 end of the experiments Results are expressed as Means ± SEM of six hearts per group. *p<0.05 vs. control; †p<0.05 vs. resveratrol.

Figure 7. Dose-response curve of the effects of resveratrol on the phosphorylation of ERK1/2, p38MAPK and Akt. The isolated rat hearts were perfused for 15 min with KHB buffer in the presence of four different concentrations of resveratrol. The hearts was 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 in black bars while the non-phosphorylated proteins are shown in white bars . The average of three experiments (Means±SEM) is shown as bar graphs.

Figure 8 Effects of resveratrol and various inhibitors used to block the effects of resveratrol preconditioning on the phosphorylation of ERK1/2 (top), p38MAPK (middle) and MAPKAP kinase2 (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 was 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 non-phosphorylated proteins, which also served as the controls. The average of four experiments (Means±SEM) is shown as bar graphs on the top of the representative Western blots.

■ Nonphosphorylated ⊡ Phosphorylated

Figure 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 was 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 non-phosphorylated proteins, which also served as the controls. The average of four experiments (Means±SEM) is shown as bar graphs on the top of the representative Western blots.

Figure 10. MSK-1 and CREB are the downstream targets of ERK and p38 MAPKs.

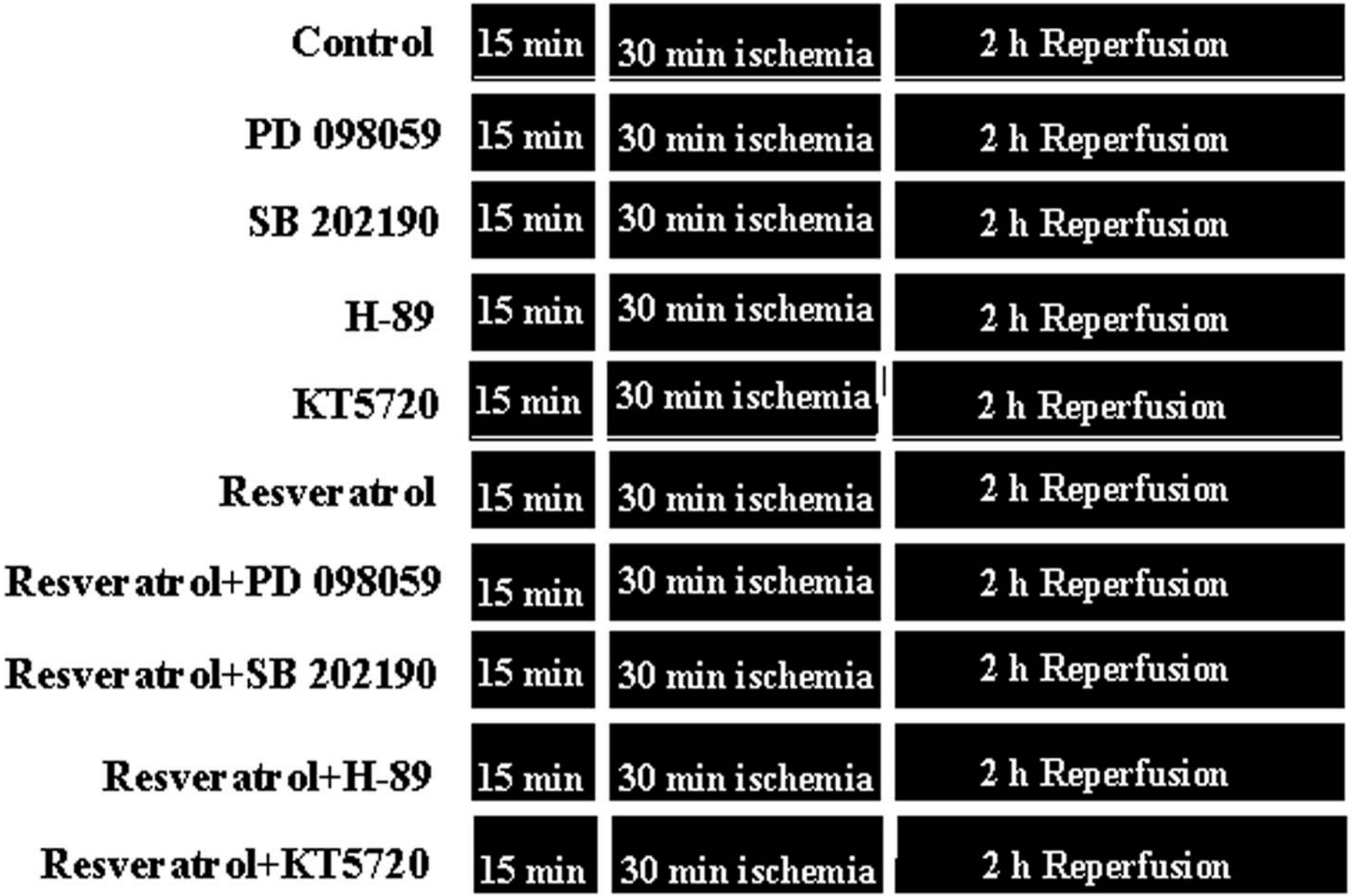

Figure 1. Experimental protocol

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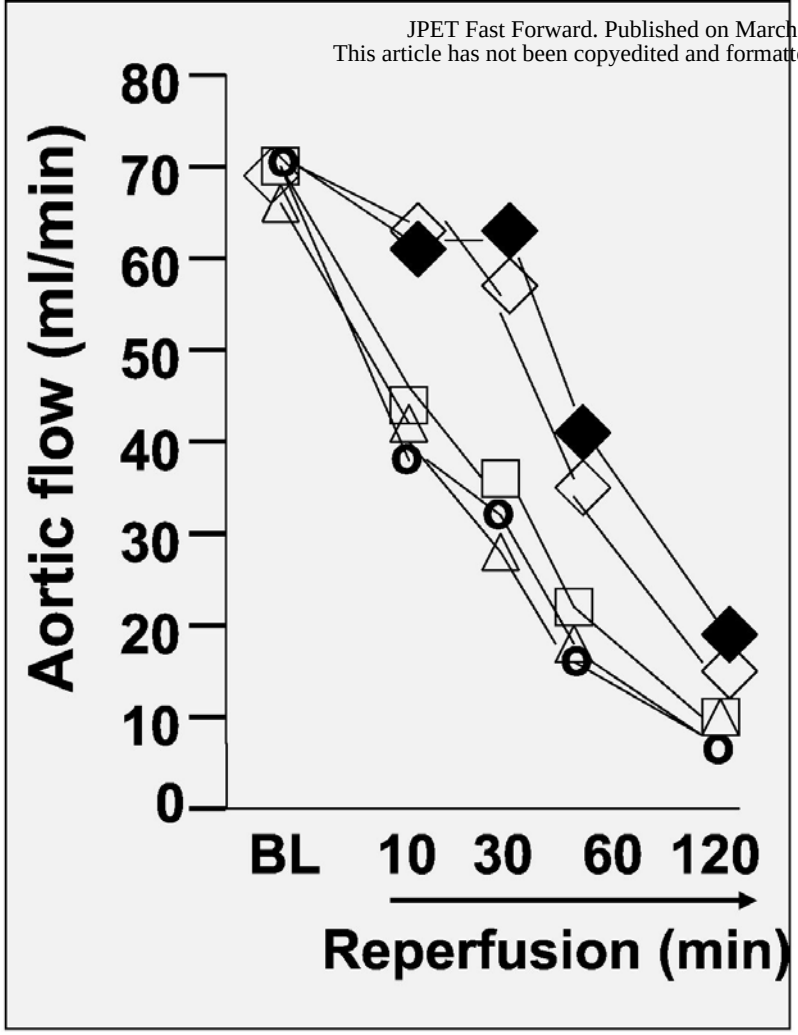

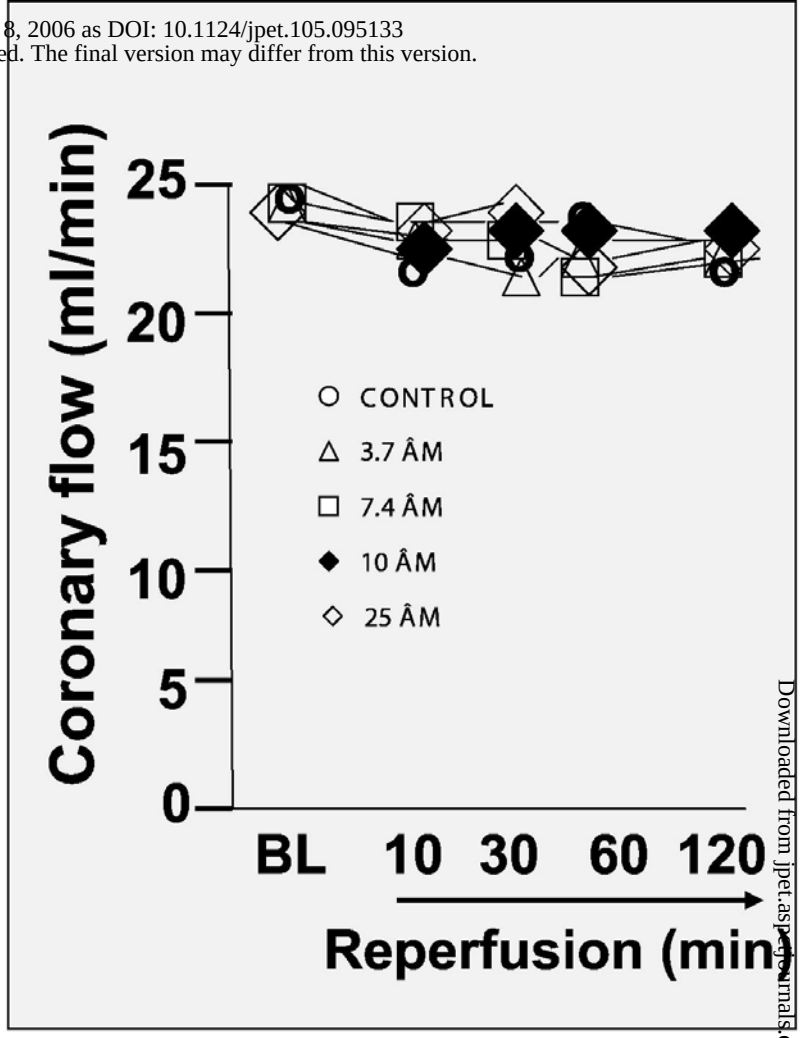

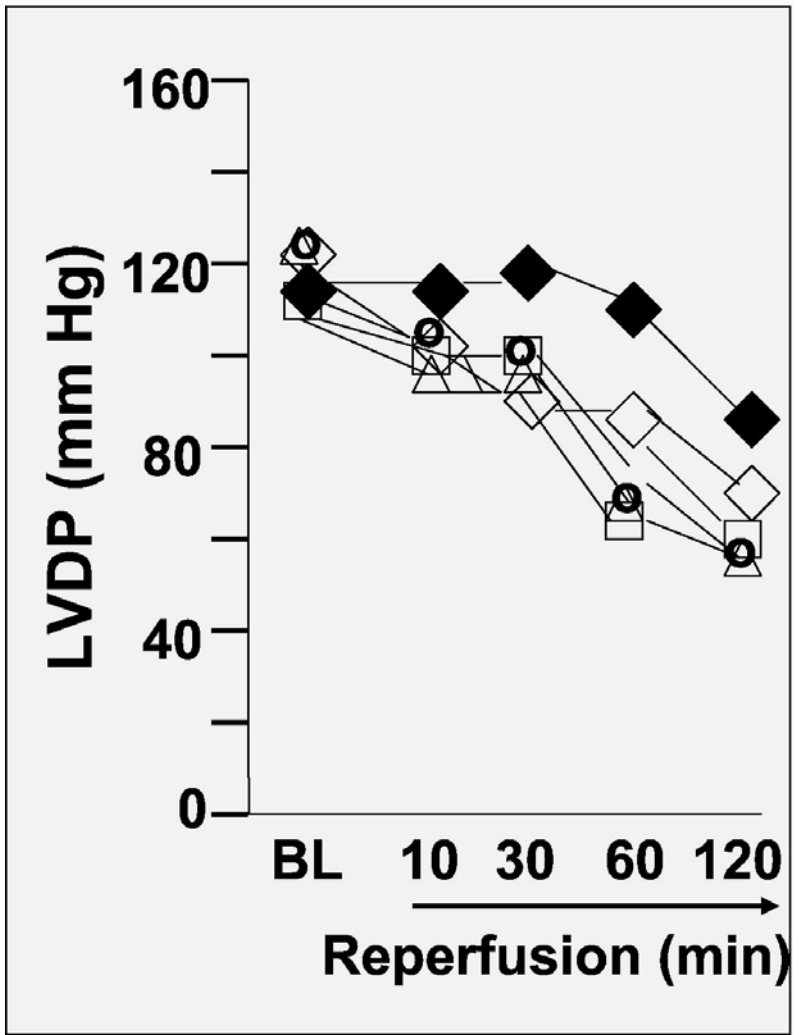

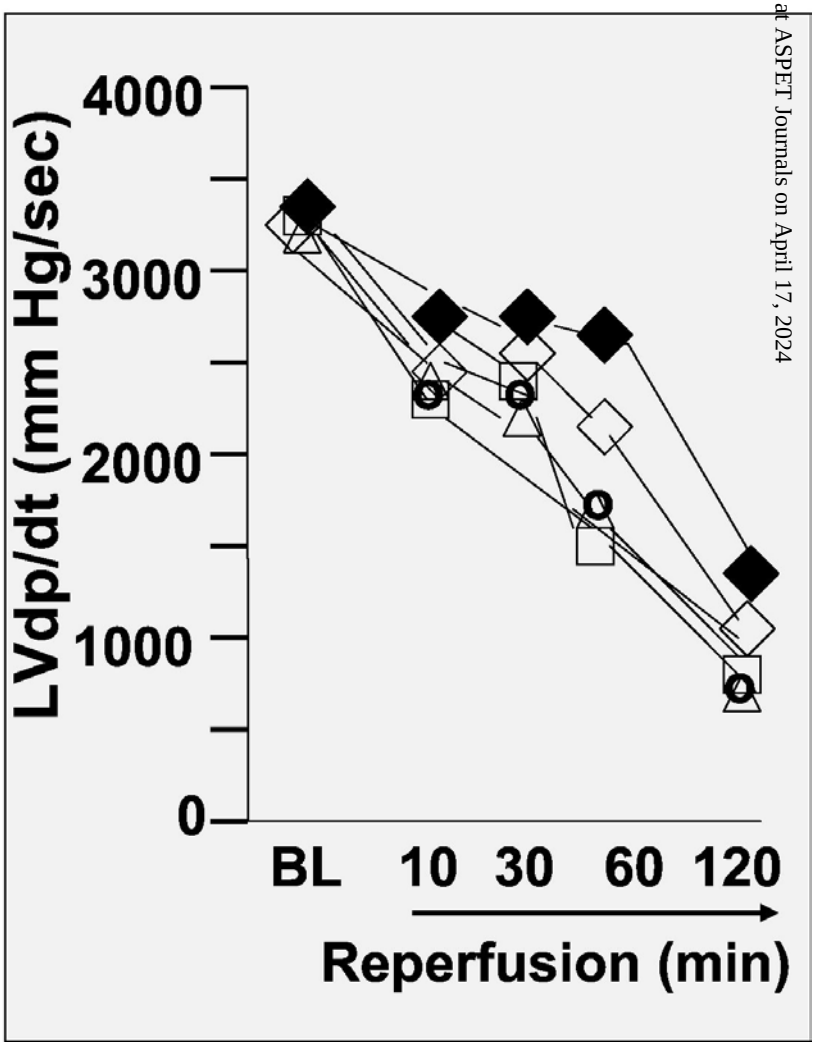

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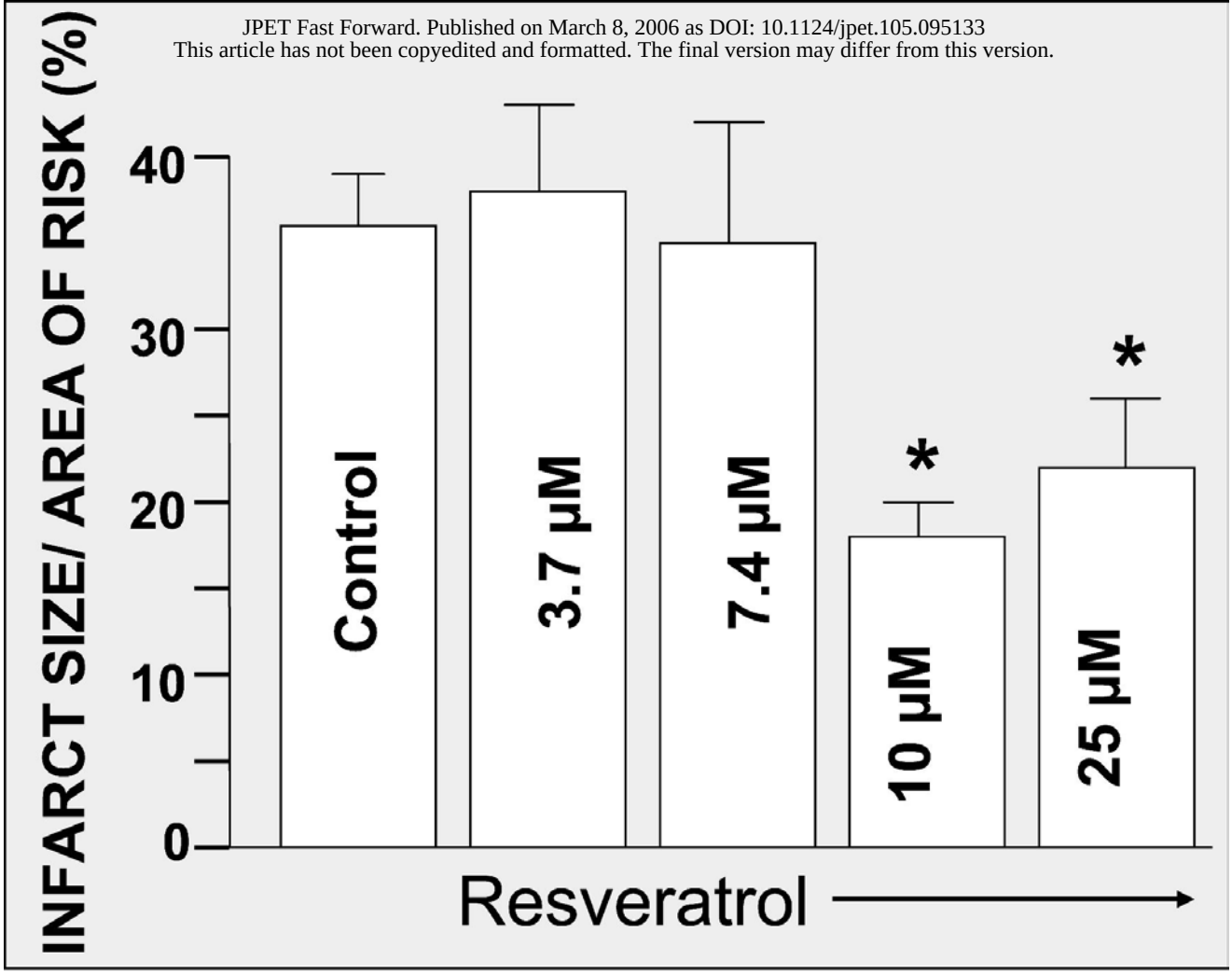

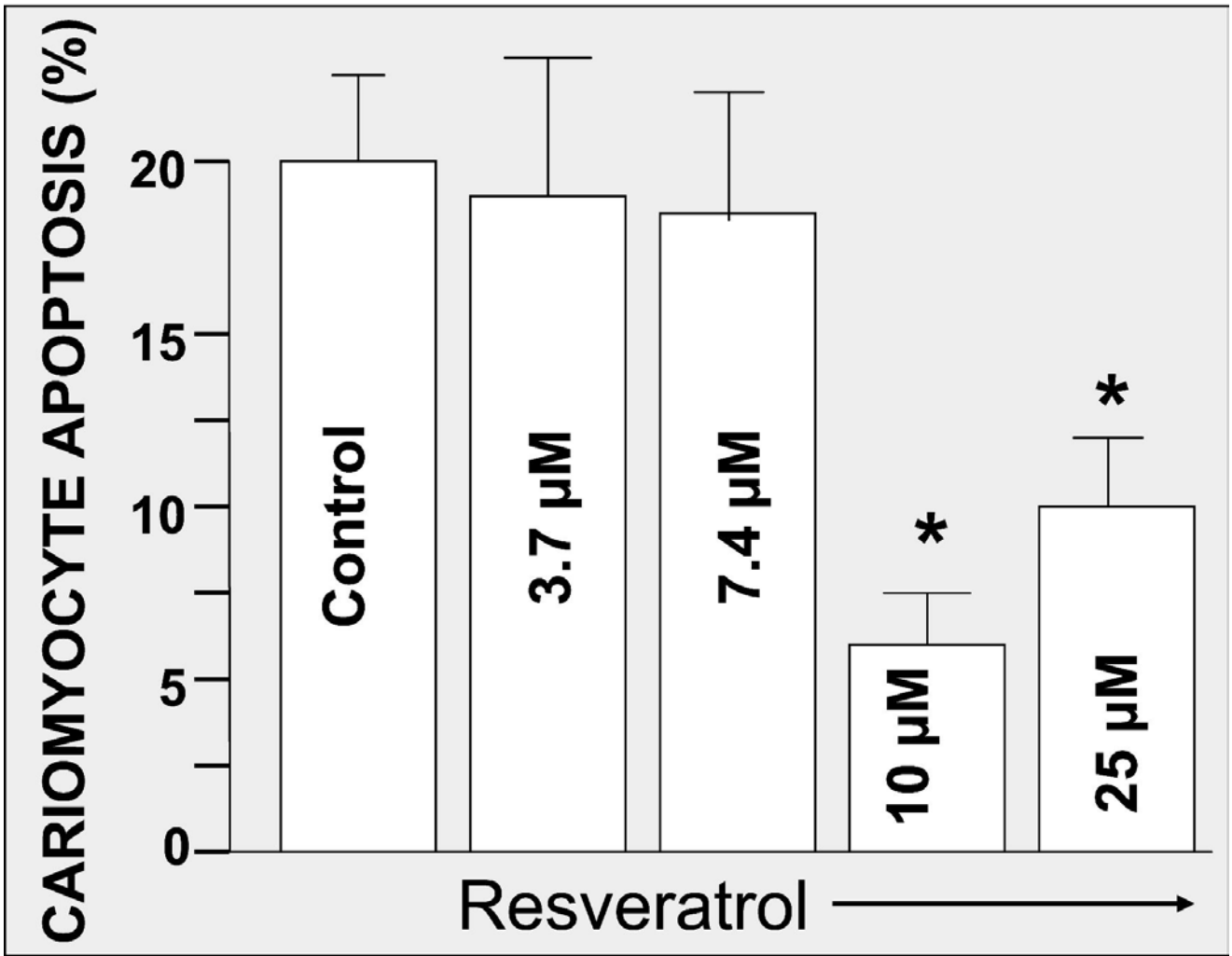

Figure 4

Figure 5

Figure 6

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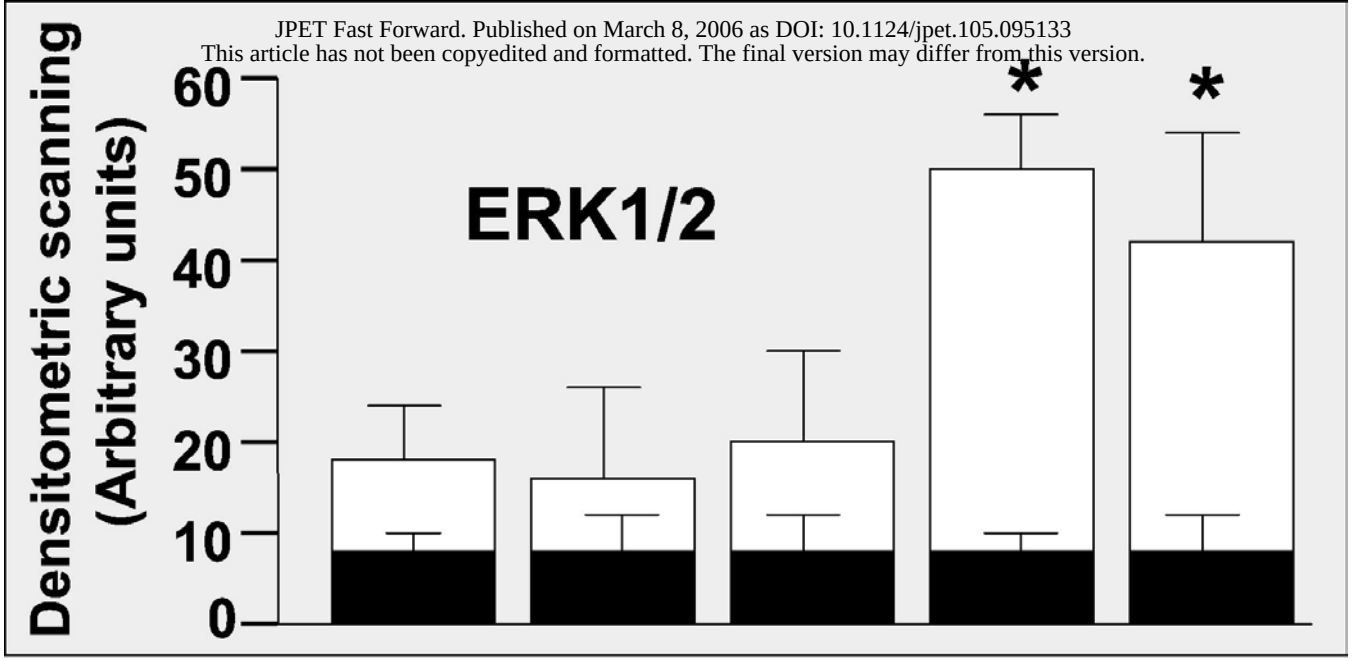

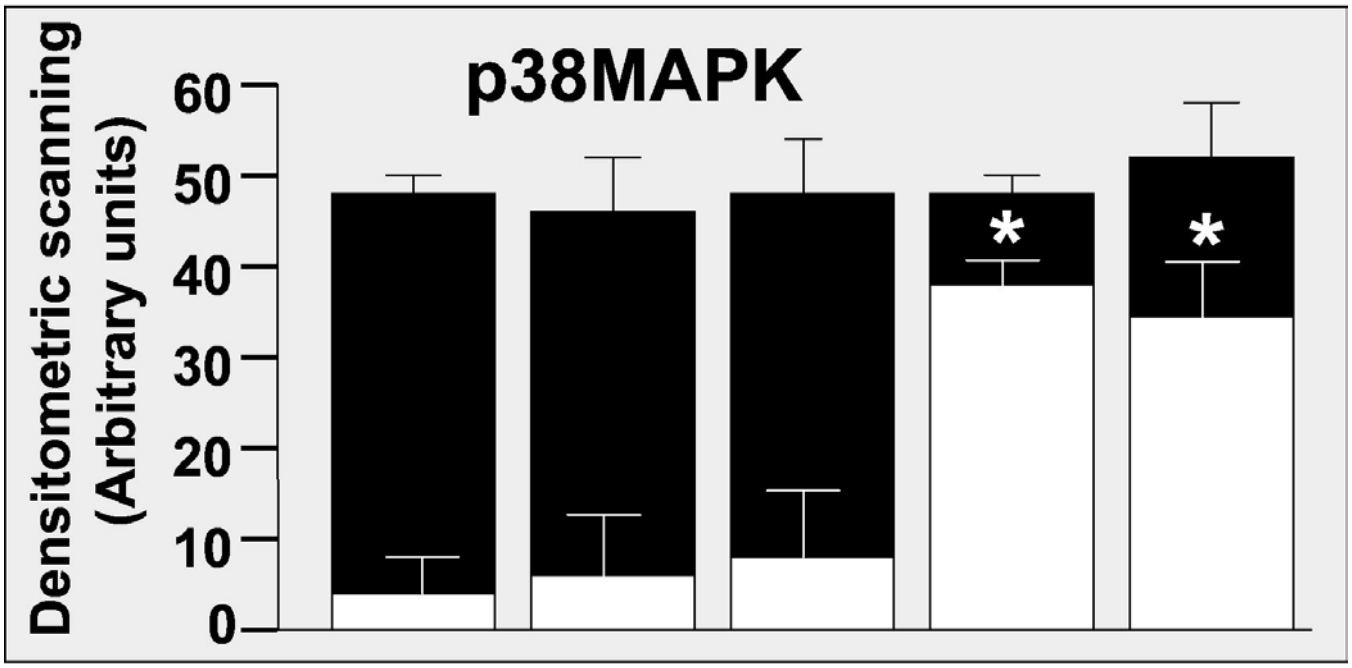

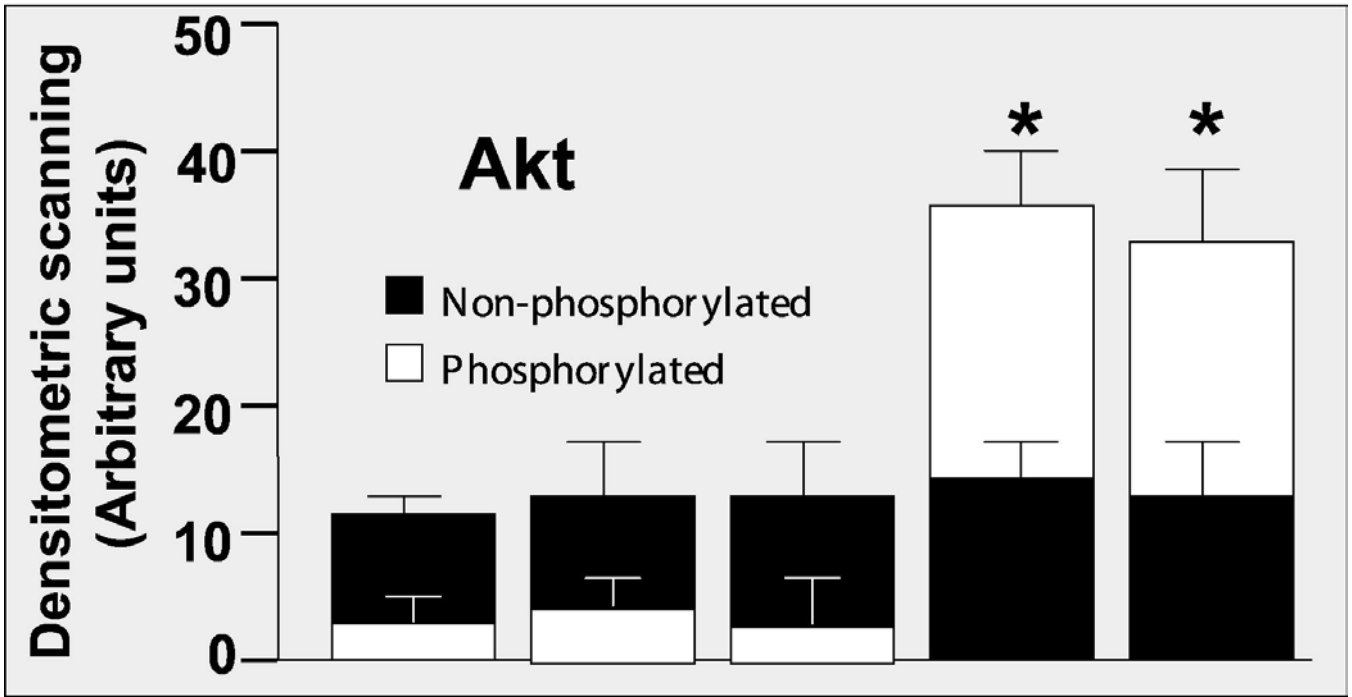

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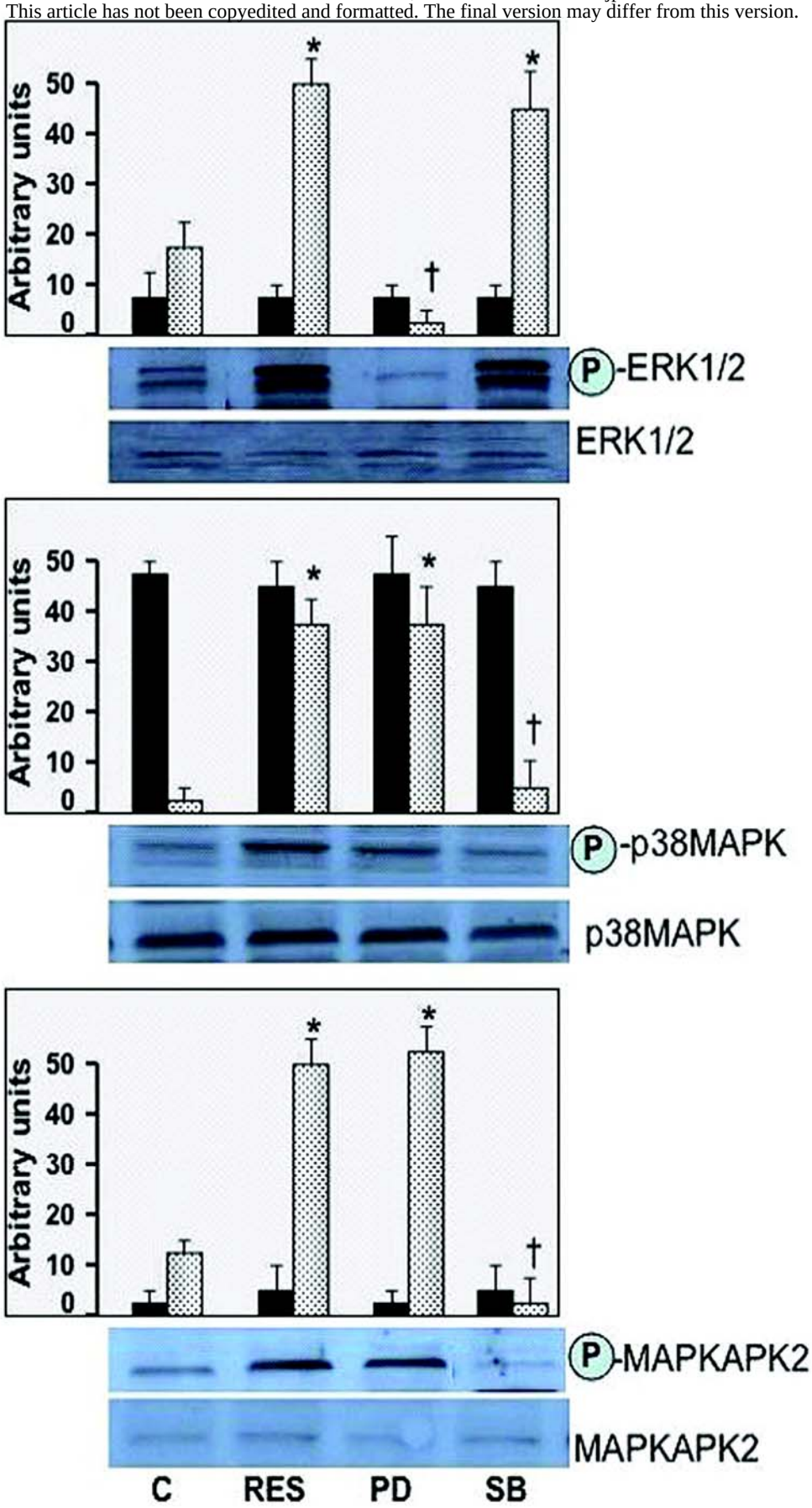

Figure 8

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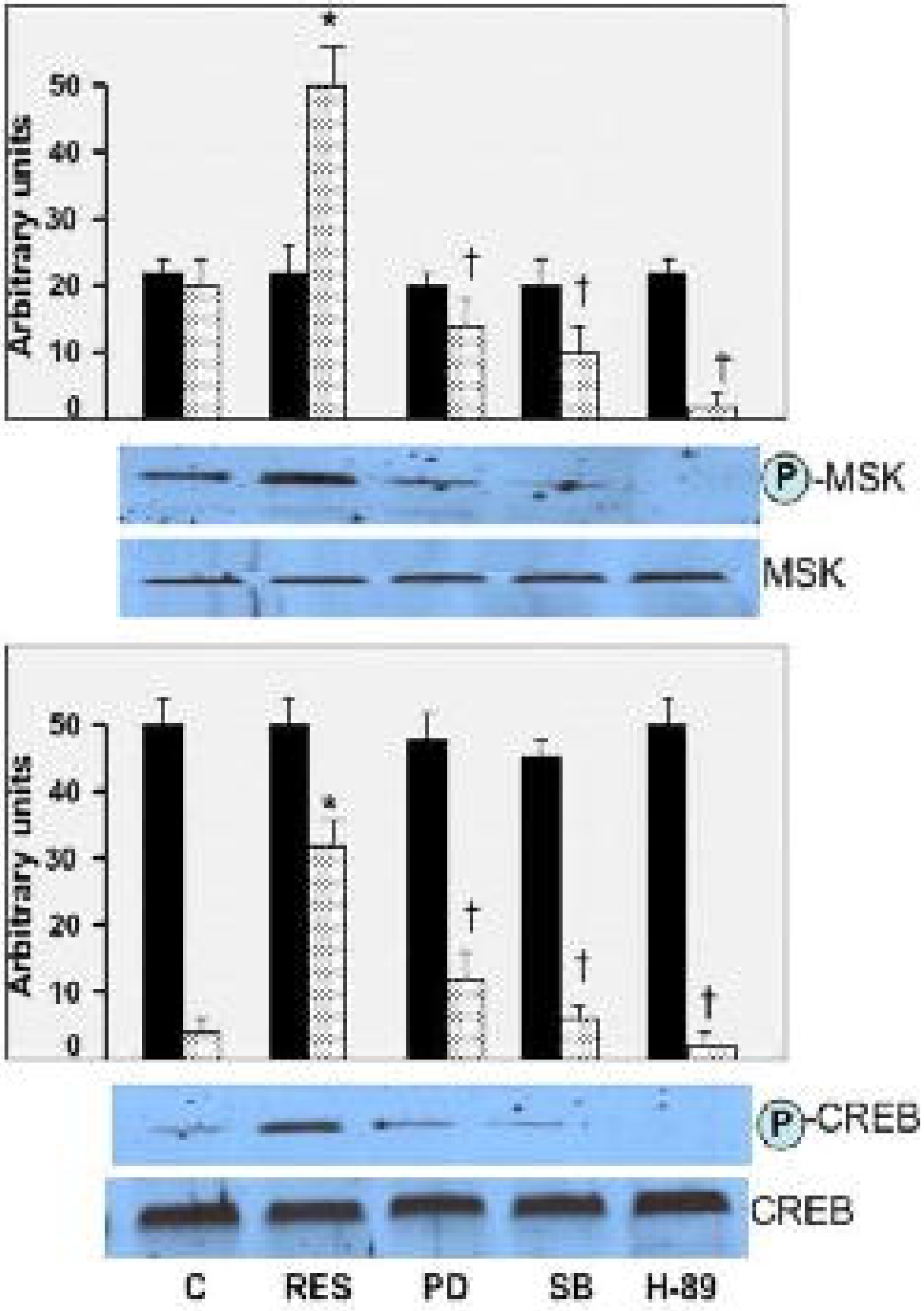

Figure 9

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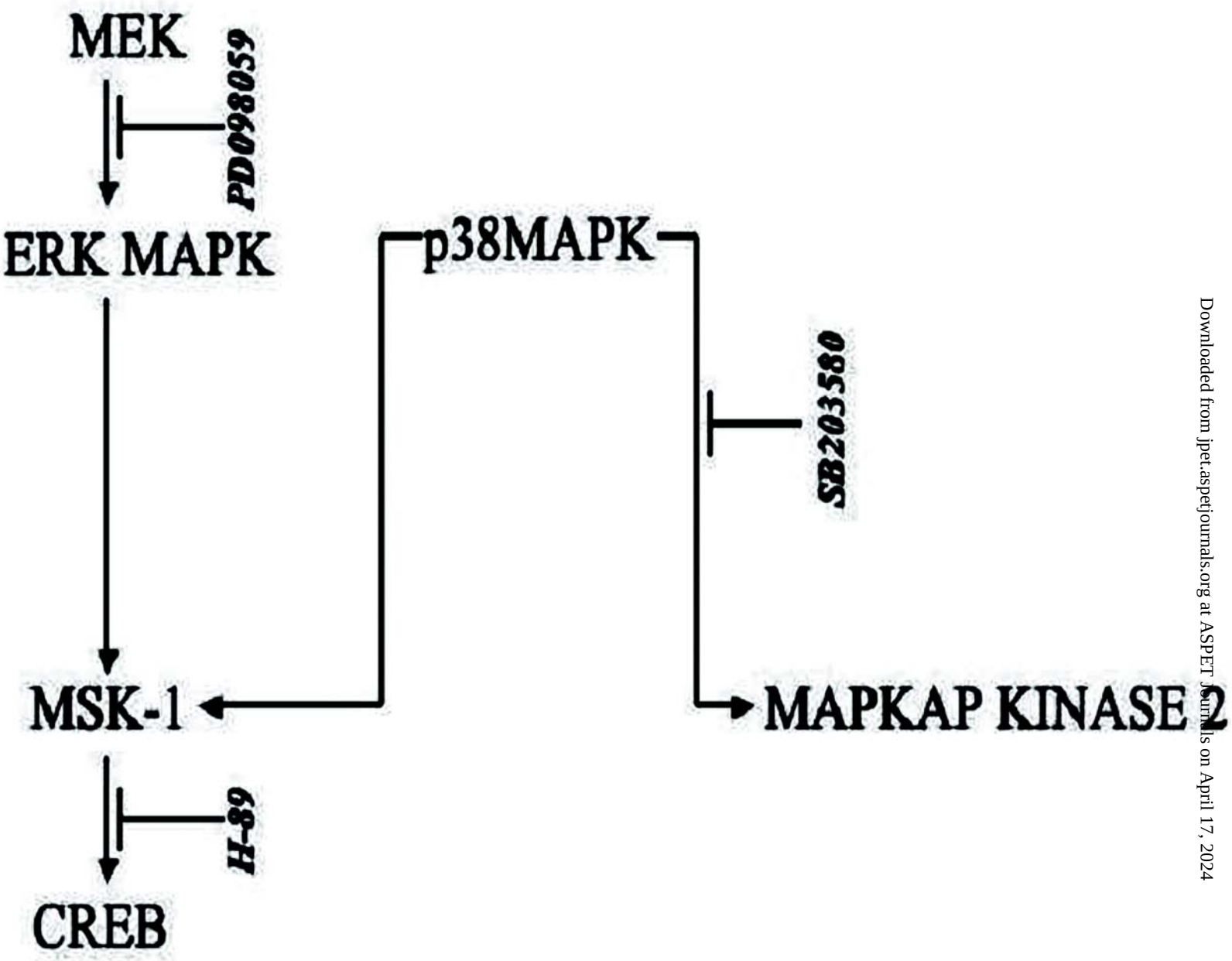

Figure 10