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Resveratrol Mediated Activation of CREB Through Adenosine A_3 Receptor by Akt -Dependent and –Independent Pathways

Samarjit Das, Arpad Tosaki, Debasis Bagchi, Nilanjana Maulik, Dipak K Das.

Cardiovascular Research Center, University of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT, 06030-1110 (S.D., N.M., D.K.D.), Department of Pharmacology, University of Debrecen, Debrecen, Hungary (A.T.), Pharmacy Sciences, Creighton Univ Med Ctr, , Omaha, Nebraska, 68178 (D.B.)

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Correspondence: Dipak K. Das, Ph.D.
Cardiovascular Research Center
University of Connecticut
School of Medicine
Farmington, CT 06030-1110

Tel: (860) 679-3687
Fax: (860) 679-4606
Email: DDAS@NEURON.UCHC.EDU

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LIST OF NON-STANDARD ABBREVIATIONS

CREB: Cyclic AMP responsive element binding protein; PKA: Protein kinase A; MAPK: Mitogen-activated protein kinases; NO: Nitric oxide; NOS: Nitric oxide synthase; KHB: Krebs Henseleit bicarbonate; PC: Preconditioning; CREB: cAMP response element binding protein; MEK: Mitogen-activated extracellular signal-regulated protein kinase

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ABSTRACT

A recent study documented a role of Adenosine A_3-Akt-CREB survival signaling in resveratrol preconditioning of the heart. In this study, we demonstrate that resveratrol mediated CREB activation can also occur through an Akt-independent pathway. Isolated rat hearts were perfused for 15 min with KHB (Krebs-Henseleit bicarbonate) buffer containing resveratrol in the absence or presence of an adenosine A_3 receptor blocker, MRS 1191, PI-3-kinase inhibitor LY 294002, a MEK inhibitor, PD 098059, or a combination of LY 294002 and PD 098059. All hearts were subsequently subjected to 30 min ischemia followed by 2 h reperfusion. Cardioprotection was examined by determining infarct size, cardiomyocyte apoptosis and ventricular recovery. Resveratrol phosphorylated both Akt and CREB that was blocked by MRS 1191, which also abolished cardioprotective abilities of resveratrol. LY 294002 completely inhibited Akt phosphorylation, but partially blocked the phosphorylation of CREB. Inhibition of PI-3- kinase also partially blocked resveratrol's ability to precondition the heart. PD 098059 partially blocked the phosphorylation of CREB and resveratrol-mediated cardioprotection. Pre-perfusing the hearts with LY294002 and PD 098059 together completely abolished the phosphorylation of CREB simultaneously inhibiting resveratrol-mediated cardioprotection. The results indicate that resveratrol preconditions the hearts through adenosine A_3 receptor signaling that triggers the phosphorylation of CREB through both Akt-dependent and –independent pathways leading to cardioprotection.

INTRODUCTION

Resveratrol, a naturally occurring phytoalexin abundant in grapes and red wines, has been found to provide cardioprotection through a mechanism involving pharmacological preconditioning (Hattori et al, 2002; Imamura et al, 2002; Das et al, 2005; Bradamante et al, 2000). The cardioprotective effects of red wine have been attributed to the resveratrol present in red wine (Hung et al, 2004) through diverse mechanisms including its ability to inhibit low density lipoprotein (LDL) (Chen and Pace-Asciak, 1996), to block platelet aggregation (Bertelli et al, 1996) and induce NO production (Ma et al, 1993). Nitric oxide (NO) appears to be involved in resveratrol mediated cardioprotection (Hattori et al, 2002; Imamura et al, 2002; Bradamante et al, 2003; Hung et al, 2004; Chen and Pace-Asciak, 1996), which also plays a crucial role in ischemic preconditioning of the heart (Tosaki et al, 1998; Guo et al, 1999). Several reports exist in the literature to indicate a role of adenosine in resveratrol mediated preconditioning. Similar to NO, Adenosine is also involved in resveratrol preconditioning (Bradamante et al, 2003; Das et al, 2005).

A recent study showed that resveratrol provides cardioprotection through a cyclic AMP responsive element binding protein (CREB) –dependent Bcl-2 survival signaling triggered by adenosine A_3-receptor activation (Das et al, 2005). This study provides evidence that resveratrol preconditions the heart through the activation of adenosine A_1 and A_3 receptors, the former transmitting a survival signal through PI-3-kinase-Akt-Bcl_2 signaling pathway, while the latter protects the heart through a CREB-dependent Bcl_2 pathway in addition to Akt-Bcl_2 pathway.

Adenosine receptor occupancy can lead to the activation of the cAMP-PKA system as well as of p38 MAPK and p42/44 MAPK, all of which can activate the CREB transcription factor system (Chio et al, 2004). Interestingly p38MAPK, p42/44 MAPK are also involved in ischemic

preconditioning (Sato et al, 2000; Ping et al, 1999). The MAPK (mitogen-activated protein kinase) pathway is one of the main signal transduction cascades that links extracellular stimuli to proliferation and survival. The upstream signaling component of MAPK, also known as MEK (mitogen-activated extracellular signal-regulated protein kinase), has been found to play a role in the preconditioning process (da Silva et al, 2004). In order to gain further insight of the adenosine A3 receptor and CREB regulation of resveratrol preconditioning of the heart, we examined the relative contribution of MEK and Akt signaling triggered by adenosine receptor activation. The results of our study revealed that resveratrol potentates an adenosine A3 receptor mediated CREB dependent survival signal, which may or may not involve PI-3-kinase-Akt pathway.

MATERIALS AND METHODS

Resveratrol. Resveratrol (trans-3, 4', 5-trihydroxystilbene), a natural phytoalexin, was obtained from Sigma Chemical Co. (St. Louis, MO, USA). A highly specific blocker of the adenosine A_3 receptor MRS 1191 (3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6–phenyl-1,4-(±)–dihydropyridine-3,5-dicar-boxylate) and PD 098,059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, a MEK inhibitor, were also purchased from the same company. LY 294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), a PI-3-kinase inhibitor, was obtained from Calbiochem, CA. The drugs were dissolved in 0.1% DMSO, and the aliquots were frozen at 4°C. Control experiments contained the vehicle (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 gms. 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): pre-perfused the isolated hearts for 15 min with KHB i) containing 0.1% DMSO as a control group; ii) KHB containing 10 µM resveratrol; iii) 10 µM resveratrol + 1 µM MRS 1191; iv) 10 µM resveratrol+3 µM LY 294002; v) 10 µM resveratrol+ 20 µM PD098059 or 10 µM resveratrol + 20 µM 098059+3 µM LY 294002. All hearts were then subjected to 30 min ischemia followed by 2 h reperfusion. Control experiments were performed with vehicle (DMSO) only, MRS 1191 only, LY 294002 only or PD 098059 only.

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 the hearts were perfused for 15 min with KHB in the absence or presence of vehicle

(control) or various combinations of different drugs as shown in Figure 1. 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) (Engelman et al, 1995). 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, a 10 % (w/v) solution of triphenyl tetrazolium in phosphate buffer was infused into aortic cannula for 20 min at 37 °C (Das et al, 2005). The hearts were excised and stored at –70 °C. Sections (0.8 mm) of frozen heart were fixed in 2% Para formaldehyde, placed between two cover slips and digitally imaged using a Microtek ScanMaker 600z. To quantitative the areas of interest in pixels, a NIH image 5.1 (a

public-domain software package) were used. The infarct size was quantified and expressed in pixels.

TUNEL Assay for assessment of Apoptotic Cell Death. Immunohistochemical detection of apoptotic cells was carried out using TUNEL (Maulik et al, 2000) using APOP TAG® kit (Oncor, Gaaithesburg, MD). The heart tissues were immediately put in 10% formalin and fixed in an automatic tissue-fixing machine. The tissues were carefully embedded in the molten paraffin in metallic blocks, covered with flexible plastic moulds and kept under freezing plates to allow the paraffin to solidify. The metallic containers were removed and tissues became embedded in paraffin on the plastic moulds. Prior to analyzing tissues for apoptosis, tissue sections were deparaffinized with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, 70%). Then tissues 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 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 NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA and 1 mM PMSF (Ray et al, 1999). 100 µg of protein of each heart homogenate was incubated with 1 µg of antibody against the phospho-Akt, CREB, and p-CREB. (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 then stripped and reblotted with specific antibodies against G-6-P dehydrogenase, which served as loading control. The resulting blots were digitized, subjected to densitometric scanning using a standard NIH image program, and normalized against loading control.

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 followed by Bonferoni's correction 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. There were no differences in baseline function between the nine groups. In general, there were no significant differences between resveratrol vs. control and also between (Resveratrol + MRS) or (Res + PD) od (Res + LY) or (Res + PD + LY) vs. resveratrol on heart rates and coronary flow (Table I). In accordance with our previous study, upon reperfusion, the absolute values of all functional parameters were decreased in all the groups as compared with the respective baseline values. Group II (Resveratrol) displayed significant recovery of post ischemic myocardial function. The cardioprotective effects of resveratrol were evidenced by significant differences in the LVDP from R-30 onwards (Table I), the difference is especially apparent at R-60 (110.26±1.19 mmHg vs. 88.01±9.57 mmHg) and at R-120 (87.8±1.74 mm Hg vs. 42.5±7.62 mm Hg) and also in the LVdp/dt at R-120 (1391.8±104.7 mm Hg/sec vs. 899.83±86.75 mmHg/sec). Aortic flow was

markedly higher in the Res group from R-30 onwards at the all rest three points. R-30 (66.1±3.62 ml/min vs. 36.03±12.7 ml/min), R-60 (43.53±5.33 ml/min vs. 19.24±6.48 ml/min) and R-120 (14.88±2.36 ml/min vs. 4.29±1.43 ml/min). With the use of adenosine A_3 receptor inhibitor (MRS 1191), resveratrol lost its cardioprotective effects which were evidenced by significant differences in the post ischemic period of LVDP from R-30 onwards, the decrease is prominent at R-60 (97.86±4.16 mmHg and 94.56±4.14 mm Hg, respectively, vs. 110.26±1.19 mmHg) and R-120 (75.73±3.69 mmHg and 72.07±3.27 mm Hg, respectively, vs. 87.8±1.74 mmHg) and also from the significant decrease of LVdp/dt at R-120 (980.16±62.5 mmHg/sec and 904±74 mm Hg/sec, respectively, vs. 1391.8±104.7 mmHg/sec). This is also confirmed from the Aortic flow value; which is markedly lower throughout the whole reperfusion period (Table I).

LY294002 or PD 098059 when used with Resveratrol, they each partially abolished the **cardioprotective** effect of Resveratrol, which were evidenced from LVDP from R-60 onwards; the decrease is more prominent at R-120 (69.97 $\pm$ 11.96 mmHg or 61.35 $\pm$ 10.05% vs. 87.8±1.74 mmHg, respectively). The same result also evidenced from LVdp/dt R- 30 onwards; the decrease is more prominent at R- 60 (1908.67 $\pm$ 249.55 mmHg/sec and 2114.7 $\pm$ 119.29 mmHg/sec vs. 2843 $\pm$ 79.48 mmHG/sec, respectively,) and also at R – 120 (932.33 $\pm$ 207.45 mm Hg/sec and 877.7 $\pm$ 187.27 mmHg/sec vs. 1391.8 ± 104.7 mmHg/sec, respectively). The cardioprotective effects were completely abolished when both PD 098059 and LY 294002 were used together (Table 1).

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. 33.79±2.74%). When resveratrol was used along with MRS 1191 or PD

098,059 or in combination of PD + LY, they prevented the reduction in infarct size observed with resveratrol alone Thus, the infarct size was significantly higher in resveratrol + MRS1191 groups as compared to the resveratrol group is 26.33±2.45%, as shown in the Figure 2 (top). Inhibition of PI-3-kinase with LY 294002 or MEK with PD 098059 also increased the infarct size (24.9 ± 2.3% and 28.2 ± 1.96%, respectively) compared to resveratrol group. The myocardial infarct size was further increased when both PD 098059 and LY 294002 were used together (30.05 ± 4.9%).

Effects of Resveratrol on Cardiomyocyte Apoptosis. The percent of apoptotic cardiomyocytes was significantly reduced in resveratrol group as compared to the control (3.7±1.2% vs. 22.7±1.5%). Similar to infarct size, when resveratrol was used along with MRS 1191 or PD 098,059 or in combination of PD + LY, they prevented the reduction in apoptotic cardiomyocytes observed with resveratrol alone. Thus, the apoptosis was significantly higher in the resveratrol + MRS1191 group as compared to the resveratrol group (20.9 ± 1.7% vs. 3.7 ± 1.2%); as shown in the Figure 1 (bottom). Inhibition of PI-3-kinase with LY 294002 or MEK with PD 098059 also increased the number of apoptotic cardiomyocytes to (17.4 ± 1.2% and 18.9±1.4 %, respectively) compare to resveratrol group. The cardiomyocyte apoptosis was further increased when both PD 098059 and LY 294002 were used together (24.3 ± 1.1%).

Effects of Resveratrol and Adenosine receptors Antagonists on the Expression of Akt and CREB. Resveratrol significantly enhanced the phosphorylation of Akt and CREB, **supported our previous study.** As shown in Figure 3 and Figure 4, phosphorylation of Akt was increased by 10-12 fold and CREB by 6-7 fold. The resveratrol-mediated induction of Akt and

its subsequent phosphorylation were reduced significantly by MRS 1191 and LY 294002, but not with PD 098059. In contrast, any one of the three blockers inhibited the phosphorylation of CREB. As shown in Figure 4, LY 294002 and PD 098059 partially, but MRS 1191 and LY294002 plus PD 098059 almost completely abolished resveratrol mediated CREB phosphorylation.

DISCUSSION

The results of the present study demonstrated that resveratrol phosphorylated both Akt and CREB that was blocked by MRS 1191, which also abolished cardioprotective abilities of resveratrol. LY 294002 completely inhibited Akt phosphorylation, but partially blocked the phosphorylation of CREB. Inhibition of PI-3- kinase also partially blocked resveratrol's ability to precondition the heart. PD 098059 partially blocked the phosphorylation of CREB and resveratrol-mediated cardioprotection. Pre-perfusing the hearts with LY294002 and PD 098059 together completely abolished the phosphorylation of CREB simultaneously inhibiting resveratrol-mediated cardioprotection. The results indicate that resveratrol preconditions the hearts through adenosine A_3 receptor signaling that triggers the phosphorylation of CREB through both Akt-dependent and –independent pathways leading to cardioprotection.

A number of studies including our own have indicated the ability of resveratrol to pharmacologically precondition the heart (Das et al, 2005; Hung et al, 2000; Giovannini et al, 2001). Ischemic preconditioning (PC) refers to the paradoxical mechanism by which cyclic episodes of brief reversible ischemia, each followed by another brief periods of reperfusion render the heart tolerant to subsequent ischemic reperfusion injury (Sato et al, 2000; Engelman et al, 1995; Flack et al, 1991). PC is a complex phenomenon, which occurs through multiple

interrelated cascades of events. A variety of neurohumoral factors are released during the onset of PC that include among many intracellular mediators, adenosine (De Jonge et al, 2002; Maddock et al, 2002; Asimakis et al, 1993). Both adenosine A_1 and A_3 receptors have been implicated in PC-mediated cardioprotection (De Jonge et al, 2002; Klinger et al, 2002; Schulte and Fredholm, 2002; Schulte et al, 2004). The same adenosine has recently been implicated in resveratrol preconditioning (Bradamante et al, 2003). In addition, nitric oxide (NO), which is a powerful mediator of PC (De Jonge et al, 2002), is also involved in resveratrol PC (Das et al, 2005; Bradamante et al, 2003). Resveratrol activates both iNOS and eNOS, which presumably contributed towards the ability of resveratrol to provide cardioprotection. In a recent study, resveratrol failed to precondition mouse hearts devoid of any copies of iNOS gene suggesting a crucial role of iNOS in resveratrol PC (Imamura et al, 2002). A study from our laboratory have indicated the role of that both A_1 and A_3 receptors are involved in resveratrol preconditioning, and both use PI-3-kinase-Akt signaling pathway (Das et al, 2005). The resveratrol-mediated increased Akt phosphorylation occurred at serine 478 site. The increased Akt phosphorylation was blocked by MRS and CPT, suggesting the involvement of both A_1 and A_3 receptor in Akt signaling. Interestingly, LY 294002 abolished the cardioprotective effects of resveratrol indicating PI-3-kinase as the upstream signaling molecule for resveratrol PC.

Akt is a critical regulator of PI-3-kinase-mediated cell survival (Kuwahara et al, 2000). A large number of studies have demonstrated in various cell types that constitutive activation of Akt is sufficient to block cell death induced by a variety of apoptotic stimuli (Zhu et al, 2001). Akt is activated by PC as a result of activation of PI-3-kinase leading to the activation of PKC and endothelial NO synthase (eNOS) (Gliki et al, 2002). Several recent studies showed phosphorylation of Akt as a result of adenosine A_3 receptor activation. For example, A_3

adenosine receptor activation triggered phosphorylation of PKB/Akt and protected rat basophilic leukemia 2H3 mast cells from apoptosis (Gao et al, 2001). Low concentrations of ethanol activate cell survival promoting PI-3-kinase/Akt pathway in endothelial cells by an adenosine receptor-dependent mechanism (Liu et al, 2002). In another study, adenosine receptor was found to regulate insulin-induced activation of PI-3-kinase/PKB in rat adipocytes (Takasuga et al, 1999).

Protein kinase B or Akt has been recognized as a survival factor by its ability to inhibit apoptosis (Downward, 1998). Akt, a member of serine/threonine kinase family, is a major target of PI-3-kinase, which enhances the level of lipid second messenger, PI-3,4,5-triphosphate upon stimulation leading to its binding to PH domain of Akt (Falasca et al, 1998). This results in the translocation of Akt from cytosol to plasma membrane, where it becomes activated by phosphorylation on Thr^{308} and Ser^{473}. Finally, Akt is detached from the membrane, and goes to to both cytosol and nucleus, where it regulates gene expression by the stimulation of transcription factors including NFkB and CREB (Brunet et al, 2001). Several down stream targets of Akt have been recognized to be apoptosis regulatory molecules including bcl-2-family member BAD (Yang et al, 2003), and cAMP response element-binding protein (CREB) (Shaywitz and Greenberg, 1999). We recently showed that resveratrol could induce the expression of Bcl-2, which was inhibited by A_1 and A_3 receptor antagonism. Additionally, the downstream target molecules of Bcl_2, BAD and CREB were phosphorylated with resveratrol. CSC and MRS 1191 significantly inhibited the phosphorylation of BAD, indicating that resveratrol-mediated Akt-Bad survival signal was regulated by both A_1 and A_3 adenosine receptors PI-3-kinase and Akt signaling pathways were also found to play a critical role in the prevention of apoptotic cell death by adenosine A_3 receptor activation (Das et al, 2005).

CREB, a major nuclear transcription factor that transduces cAMP activation of gene transcription, is another regulatory downstream target molecule of Akt (Shaywitz and Greenberg, 1999). CREB has been recognized as an important nuclear factor for cell survival. Overexpresson of a dominant negative CREB transgene induced apoptosis in T cells (Barton et al, 1996). A recent study showed that CREB contributed to cell survival in response to growth factor stimulation (Du and Montminy, 1998). Our results showed simultaneous induction of CREB and Bcl_2 in response to resveratrol treatment. The promoter region of Bcl_2 contains a cAMP-response element (CRE) site and the transcription factor CREB has been recognized as a positive regulator of Bcl_2 expression. Like NFκB, CREB is also a target for several signaling pathways mediated by a variety of stimulation. For example, IGF-1-stimulated CREB phosphorylation was decreased by Wortmannin, an inhibitor of PI-3-kinase, suggesting a role of Akt in CREB activation.

An alternative survival pathway via CREB that may bypass PI-3-kinase-Akt signaling has recently been described (Frodin and Gammeltoft, 1999). In this report, CREB phosphorylation was found to occur through the activation of the MAP kinase pathway via activation of p90rsk. In another study, relaxin activated CREB through a Akt-independent signaling pathway (Zhang et al, 2002), In this case, CREB may be phosphorylated via MEK/MAPK/p90rsk/CREB or cAMP-PKA signaling pathway, or both (Telgmann et al, 1997). In another related study, dopamine induced PI-3-kinase independent activation of Akt in striatal neurons indicating a new route to CREB phosphorylation (Brami-Cherrier, 2002).

In summary, the results of this study demonstrated that resveratrol preconditioning is mediated by adenosine A_3 receptors that trigger CREB phosphorylation via both PI-3-kinase-Akt and via MEK-CREB pathways. Resveratrol-mediated phosphorylation of Akt and CREB was

blocked by MRS 1191, which also abolished cardioprotective abilities of resveratrol, indicating a crucial role of adenosine A3 receptor for resveratrol preconditioning. The fact that LY 294002 completely inhibited Akt phosphorylation, but partially blocked the phosphorylation of CREB resulting in partial inhibition of resveratrol's ability to precondition the heart, suggests that PI-3-kinase-Akt-CREB signaling pathway is at least partially responsible for the cardioprotection achieved by resveratrol. Partially blockage of CREB phosphorylation and resveratrol-mediated cardioprotection by PD 098059 indicates negative role of PI-3-kinase/Akt signaling in CREB activation. This receives further support from the finding that LY294002 and PD 098059 together completely abolished the phosphorylation of CREB simultaneously inhibiting resveratrol-mediated cardioprotection. The results indicate that resveratrol preconditions the hearts through adenosine A_3 receptor signaling that triggers the phosphorylation of CREB through both Akt-dependent and –independent pathways leading to cardioprotection.

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FIGURE LEGENDS

FIGURE 1. Experimental protocol. Isolated rat hearts were perfused for 15 min with KHB buffer in the absence or presence of vehicle only or with various combinations of drugs. The arrows represent the time points at which various parameters were measured. First level of five arrows represents the five different points where the ventricular functions were recorded. Second level arrow represents the point where infarct size and apoptosis were measured and the third level arrow shows the point where the tissue was taken for the Western blot analysis for Akt and CREB.

KHB, Krebs-Henseleit bicarbonate buffer; PD 098,059, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; LY-294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; MRS-1191, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate. CREB, cAMP response element-binding protein. Akt, Protein kinase B**.**

FIGURE 2. Effects of Resveratrol, PD 098,059, LY 294002 and MRS 1191 on myocardial infarct size and cardiomyocyte apoptosis. The isolated hearts from control (n=6), Resveratrol pre-perfused in absence or presence of either PD 098,059 or LY 294002 or MRS 1191 (n=6) rats were subjected to 30 min global ischemia followed by 2 h of reperfusion in working mode. Infarct size was measured by TTC dye method while cardiomyocyte apoptosis was evaluated by Tunnel method in conjunction with antibody against α-myosin heavy chain. Results are expressed as Means ± SEM. *$p < 0.05$ vs. control; **p,0.05 vs. ischemia/reperfusion; †$p<0.05$ vs. resveratrol; §$p<0.05$ vs. Resveratrol+PD098059 or resveratrol+LY294002

FIGURE 3. Western blot analysis of Akt and p-Akt proteins. The results of phosphorylated Akt (p-Akt) are shown in bar graphs as means ± SEM of 6 different experiments per group. Representative Western blots are shown at the top of the bar graphs. The density of the Akt blots did not change, which also served as control.*$p < 0.05$ vs. baseline or ischemia/reperfusion. †$P < 0.05$ vs. resveratrol alone.

FIGURE 4. Western blot analysis of CREB and p-CREB proteins. The results of phosphorylated CREB (p-CREB) are shown in bar graphs as means ± SEM of 6 different experiments per group. Representative Western blots are shown at the top of the bar graphs. The density of the CREB blots did not change, which also served as control. *$p < 0.05$ vs. baseline or ischemia/reperfusion. †$P < 0.05$ vs. resveratrol alone; §$p<0.05$ vs. resveratrol+LY294002 or resveratrol+PD098059.

TABLE 1

Effects of Resveratrol and the inhibitors of Adenosine A_3 receptors, MEK (1/2) and PI-3- Kinase on ventricular functions

	Group	Baseline	10 min R	30 min R	60 min R	120 min R
Heart Rate	**Control**	345 ± 31	316 ± 26	365 ± 17	344 ± 35	414 ± 24
(beats/min)	**Resveratrol**	352 ± 15	368 ± 20	382 ± 21	394 ± 16	411 ± 13
	PD 098,059	383 ± 17	371 ± 17	392 ± 6.5	405 ± 5.7	439 ± 5.9
	LY 294002	380 ± 21	358 ± 19	388 ± 23	411 ± 24	441 ± 38
	MRS 1191	390 ± 20	372 ± 20	388 ± 18	408 ± 8.7	422 ± 6.2
	Res + MRS	364 ± 23	368 ± 17	384 ± 13	413 ± 8.8	433 ± 7.7
	Res + PD	415 ± 26	372 ± 20	386 ± 14	406 ± 9.8	426 ± 10.2
	Res + LY	377 ± 28	395 ± 5.7	391 ± 18	420 ± 3.4	458 ± 8.2
	Res + PD + LY	385 ± 14	355 ± 12	393 ± 6.9	416 ± 4.1	438 ± 3.4
LVDP	**Control**	127 ± 3.2	107 ± 5.4	104 ± 7.1	88 ± 9.6	53 ± 4.6
(mm Hg)	**Resveratrol**	126 ± 3	116 ± 2.4	122 ± 2.6 *	110 ± 1.2 *	88 ± 1.7 *
	PD 098,059	127 ± 3.2	109 ± 4	111 ± 4.4	92 ± 3	57 ± 5.1
	LY 294002	131 ± 1.6	123 ± 6.7	127 ± 7.1	100 ± 8.9	52 ± 6.4
	MRS 1191	119 ± 4.2	106 ± 3.7	97 ± 3.1	80 ± 4	40 ± 2.7
	Res + MRS	123 ± 2.8	102 ± 3.7	109 ± 3.1	98 ± 4.2 #	76 ± 3.7 #
	Res + PD	129 ± 2.5	116 ± 4	116 ± 3.4	107 ± 3.4	61 ± 4 #
	Res + LY	127 ± 5.3	109 ± 2.1	109 ± 5.4	92 ± 9.9	70 ± 12
	Res + PD + LY	128 ± 1.2	102 ± 4	103 ± 2.8 #	84 ± 2.3 #	59 ± 4.7 #
LVdp/dt	**Control**	3319 ± 115	2412 ± 250	2472 ± 235	1881 ± 403	900 ± 87
(mm Hg/sec)	**Resveratrol**	3324 ± 95	2720 ± 131	3013 ± 64 *	2843 ± 79 *	1425 ± 110 *
	PD 098,059	3365 ± 82	2470 ± 68	2600 ± 159	1853 ± 100	843 ± 78
	LY 294002	3044 ± 115	2536 ± 76	2266 ± 78	1705 ± 129	996 ± 98
	MRS 1191	2985 ± 152	2493 ± 230	2371 ± 151	1572 ± 231	898 ± 78
	Res + MRS	2932 ± 212	2164 ± 270	2326 ± 238	2077 ± 286 #	980 ± 63 #
	Res + PD	3578 ± 115	2998 ± 127	2902 ± 113	2115 ± 119 #	878 ± 187 #
	Res + LY	3116 ± 21	2261 ± 85	2347 ± 136	1908 ± 250	932 ± 207
	Res + PD + LY	3218 ± 52	2524 ± 86	2623 ± 27 #	1716 ± 92 #	707 ± 71 #

	Group	Baseline	10 min R	30 min R	60 min R	120 min R
Aortic Flow	**Control**	72 ± 5.2	43 ± 12.9	36 ± 12.7	19 ± 6.5	4.3 ± 1.4
(ml/min)	**Resveratrol**	72 ± 3.5	61 ± 4.3	66 ± 3.6 *	44 ± 5.3 *	14.9 ± 2.4 *
	PD 098,059	71 ± 3.4	46 ± 3.8	41 ± 4.8	20 ± 3	3.65 ± 1
	LY 294002	71 ± 3.5	40 ± 2.6	27 ± 2.7	8.7 ± 1.2	6.6 ± 1
	MRS 1191	60 ± 3.2	44 ± 3.8	32 ± 4.6	14 ± 2.2	2.8 ± 1
	Res + MRS	63 ± 3.9	36 ± 5.3	39 ± 5 #	29 ± 5.7 #	7 ± 1 #
	Res + PD	79 ± 2.2	49 ± 8	44 ± 7.4 #	19 ± 5.7 #	3 ± 1 #
	Res + LY	75 ± 4.4	62 ± 4.3	58 ± 7.4	34 ± 11.6	11 ± 7.5
	Res + PD + LY	64 ± 7	35 ± 9.8	31 ± 6.9 #	10 ± 3.4 #	1.9 ± 1 #
Coronary Flow	**Control**	24 ± 1.4	22 ± 1	23 ± 1.9	23 ± 1.3	22 ± 1.2
(ml/min)	**Resveratrol**	24 ± 1.3	23 ± 1.5	26 ± 1.1	25 ± 1.6	23 ± 1.5
	PD 098,059	21 ± 1.4	20 ± 1.2	21 ± 1	21 ± 1.5	20 ± 1.8
	LY 294002	24 ± 1.2	22 ± 1.1	24 ± 1.1	25 ± 1.5	25 ± 1.5
	MRS 1191	28 ± 1	25 ± 1.2	23 ± 1	22 ± 1	20 ± 1
	Res + MRS	28 ± 1	28 ± 1	28 ± 1.4	27 ± 1.5	25 ± 1.5
	Res + PD	20 ± 1.5	23 ± 2	22 ± 1.8	22 ± 1.1	21 ± 1
	Res + LY	22 ± 1.3	21 ± 1.5	21 ± 0.2	23 ± 2.3	23 ± 2.3
	Res + PD + LY	21 ± 1	19 ± 1.1	20 ± 1	20 ± 1.4	19 ± 1.9

LVDP: Left ventricular developed pressure; LVdp/dt: Maximum first derivatives of developed pressure; R: Reperfusion. Results are expressed as mean ± SEM of 6 animals as group * $p<0.05$ Res vs. Control and # $p<0.05$ (Res + MRS) or (Res + PD) or (Res + LY) or (Res + PD + LY) vs. Res

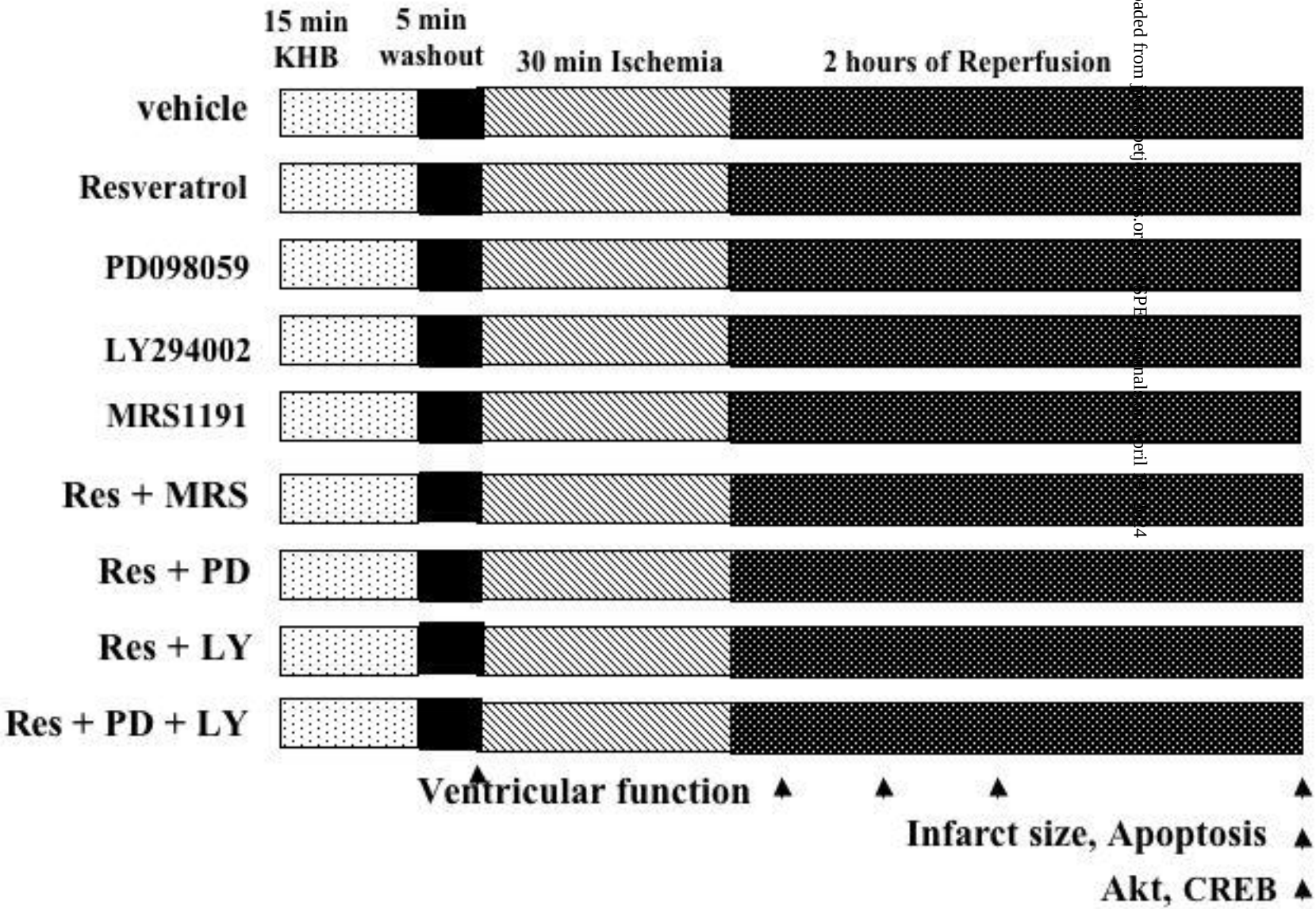

Figure 1

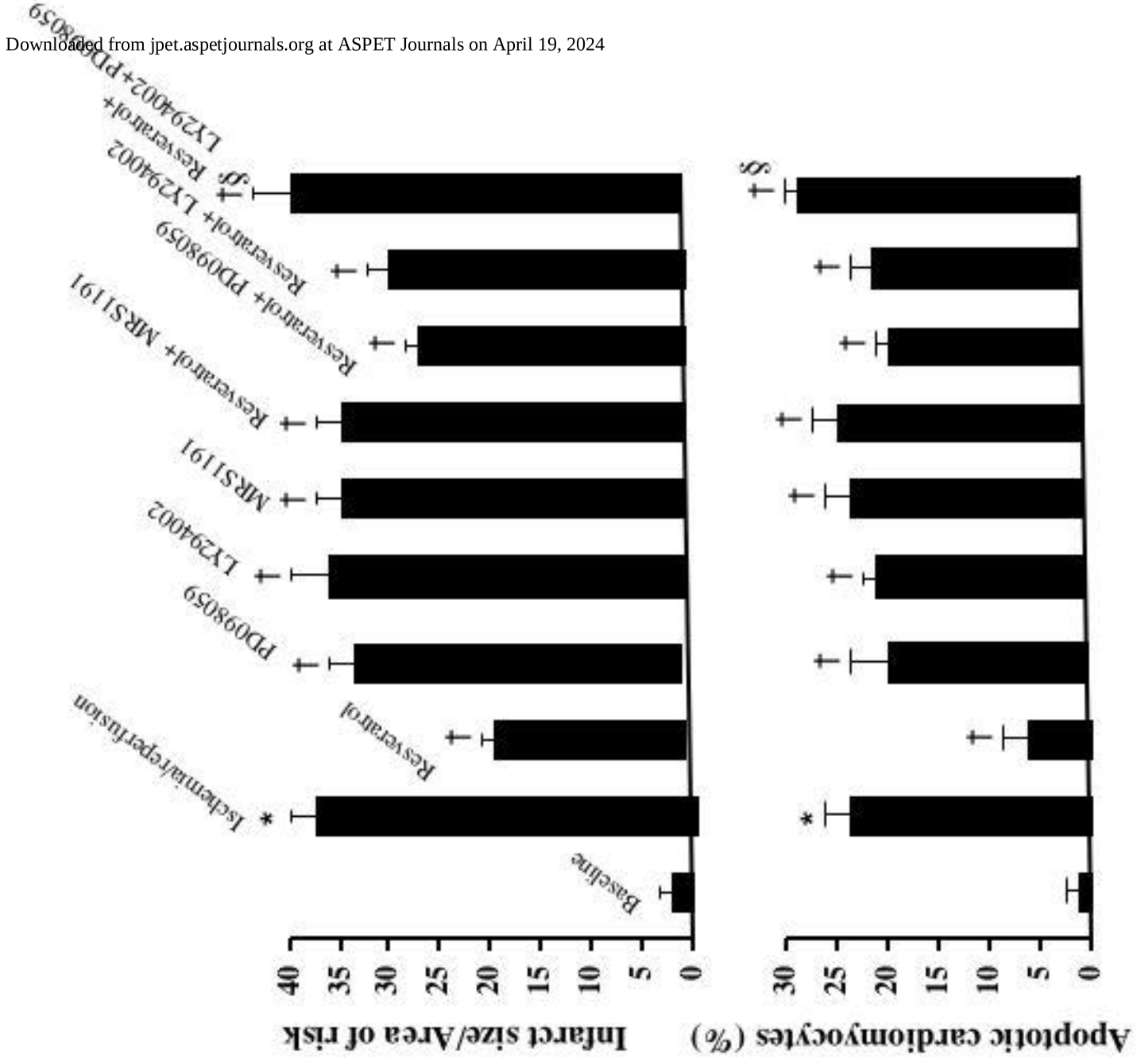

Figure 3

Figure 4