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## Accepted Manuscript

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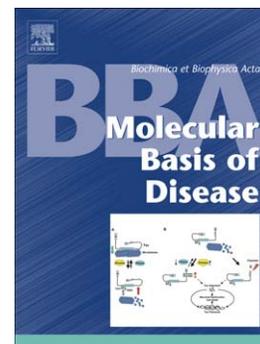
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**CARDIOPROTECTION WITH  $\alpha$ -TOCOPHERYL PHOSPHATE:  
AMELIORATION OF MYOCARDIAL ISCHEMIA REPERFUSION  
INJURY IS LINKED WITH ITS ABILITY TO GENERATE A  
SURVIVAL SIGNAL THROUGH AKT ACTIVATION**

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**Running Title:** *Cardioprotection with  $\alpha$ -tocoperyl phosphate*

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**ABSTRACT.**

The emerging potential of  $\alpha$ -tocopheryl phosphate, a phosphoric acid ester of  $\alpha$ -tocopherol, in health benefits was tested gavaging this compound (5 mg/kg body wt) to a group of rats for a period of thirty days while the control rats were given water only. After thirty days, the rats were sacrificed, the hearts excised, and the isolated hearts were perfused by working mode. Both control and experimental hearts were subjected to 30 min global ischemia followed by 2 h of reperfusion. The tocopheryl phosphate fed rats exhibited significant cardioprotection as evidenced by improved ventricular performance and reduced myocardial infarct size and cardiomyocyte apoptosis. Supplementation with  $\alpha$ -Tocopheryl phosphate converted MAP kinase-induced death signal into a survival signal by enhancing anti-apoptotic p42/44 ERK kinase and p38 MAPK $\beta$  and reducing pro-apoptotic proteins p38 MAPK $\alpha$  and JNK. In concert, the phosphorylation of pro-apoptotic c-Src was also reduced. Tocopheryl phosphate increased the DNA binding of the redox-sensitive transcription factor NF $\kappa$ B and potentiated the activation of anti-death protein Bcl-2 and survival signaling protein Akt. The results of this study demonstrated for the first time that tocopheryl phosphate could ameliorate myocardial ischemic reperfusion injury by converting ischemia/reperfusion-mediated death signal into a survival signal by modulating MAP kinase signaling.

**KEY WORDS**

Tocopheryl phosphate, Cardioprotection,  $\alpha$ -tocotrienol, Ischemia reperfusion injury, c-Src, Akt, MAP kinase

**ABBREVIATIONS**

LVDP: Left ventricular developed pressure

LVdp/dt: Maximum first derivative of developed pressure

AF: Aortic flow

TTC: Triphenyl tetrazolium chloride

KHB: Krebs-Henseleit bicarbonate buffer

I/R: Ischemia reperfusion

WHO: World Health Organization

NF $\kappa$ B: Nuclear factor kappa B

MAP kinase: Mitogen-activated protein kinase

## INTRODUCTION

Epidemiologic evidence exists in the literature to support the notion that vitamin E, especially  $\alpha$ -tocopherol can reduce risk of coronary heart disease [1, 2]. Experimental studies using laboratory animals have supported cardioprotective abilities of  $\alpha$ -tocopherol [3-5]; but randomized clinical trials have demonstrated very little, if any, benefit from vitamin E supplementation. For example, many large prospective studies including MONICA [Monitoring Trends in Cardiovascular Disease] trial organized by WHO [World Health Organization], NHS [Nurses' Health Study], and HPPS [health Professionals' Follow-up Study] showed only a little evidence in favor of cardioprotective effectiveness of vitamin E [6-8].

Recently, an ester derivative of phosphate with the hydroxyl group of tocopherol,  $\alpha$ -tocopheryl phosphate ester, has been described as a novel natural form of tocopherol [9, 10]. Similar to  $\alpha$ -tocopherol,  $\alpha$ -tocopheryl phosphate is ubiquitously present in biological tissues as well as plant kingdom [11]. One of the major differences between  $\alpha$ -tocopherol and  $\alpha$ -tocopheryl phosphate is that the former is readily oxidized by oxygen while the later is quite stable against oxidation.  $\alpha$ -tocopherol is practically insoluble in water whereas  $\alpha$ -tocopheryl phosphate is water soluble [12]. Very little information is available about biological and/or physiological effectiveness of  $\alpha$ -tocopheryl phosphate. A recent study has shown atherosclerotic-preventing effect of  $\alpha$ -tocopheryl phosphate, and such effect was more pronounced than that of the acetate derivative [13]. It has been also shown that  $\alpha$ -tocopheryl phosphate is highly resistant to hydrolysis, both chemical and enzymatic indicating that its effect is not related to the hydrolytic liberation of free tocopherol [12, 13, 18]. In a few studies it was shown that  $\alpha$ -tocopheryl phosphate showed more potency than the non esterified molecule possibly behaving as an activated form of tocopherol. [12, 18]

We reasoned that because of these properties,  $\alpha$ -tocopheryl phosphate could serve as a superior cardioprotective compound. To test this, a group of rats were fed  $\alpha$ -tocopheryl phosphate [5 mg/kg body wt] for a month. After 30 days, the rats were sacrificed, the hearts were excised and isolated hearts were subjected to 30 min ischemia followed by 2 h reperfusion. The results showed significant cardioprotection with  $\alpha$ -tocopheryl phosphate as evidenced by improved ventricular function and reduced myocardial infarction and cardiomyocyte apoptosis when compared with a control group.  $\alpha$ -Tocopheryl phosphate also generated a survival signal against ischemia reperfusion injury by inducing the activation of Akt and Bcl-2.

#### **MATERIALS AND METHODS.**

**Chemicals.**  $\alpha$ -Tocopheryl phosphate was obtained from Phosphagenics Ltd, Australia. All other chemicals were of analytical grade and were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise specified.

**Animals.** All animals used in this study received humane care in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Sprague Dawley male rats weighing between 250-300 gm were used for the experiment. The rats were randomly assigned to two groups: control and treated. The rats were fed *ad libitum* regular rat chow with free access to water. The treated rats were gavaged with  $\alpha$ -tocopheryl phosphate (5 mg/kg body wt/day) for thirty days while the control group received water only.

The rats were anesthetized with sodium pentobarbital (80 mg/kg, bw i.p.), (Abbott Laboratories, North Chicago, IL, USA) and anticoagulant with heparin sodium (500 IU/kg., bw

i.v.) (Elkins-Sinn Inc., Cherry Hill, NJ, USA) injection. 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 [14]. 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. At the end of 10 min, after the attainment of steady state cardiac function, baseline functional parameters were recorded. The hearts were then subjected to 30 min of global ischemia followed by 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 30-, 60- and 120- min reperfusion.

***Assessment of Cardiac function.*** 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) [14]. Heart Rate (HR), 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 (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 1% (w/v) solution of triphenyl tetrazolium chloride in phosphate buffer was infused into aortic cannula for 20 min at 37°C [15]. The hearts were excised and stored at -70°C. Sections (0.8 mm) of frozen heart were fixed in 2% paraformaldehyde, placed between two cover slips and digitally imaged using a Microtek Scan Maker 600z. To quantitate 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 the Assessment of Apoptotic Cell Death.** Immunohistochemical detection of apoptotic cells was carried out using TUNEL [15] using Dead-End TM Fluorometric TUNEL System (Promega, Madison, WI). 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%, 80%, 70%). Then the TUNEL experiment was done according to the manufacturer's instructions. The fluorescence staining was viewed with a fluorescence microscope (AXIOPLAN2 IMAGING) (Carl Zeiss Microimaging, Inc. NY). The number of apoptotic cells was counted and expressed as a percent of total myocyte population.

**Preparation of Cytosolic, nuclear and mitochondrial extracts.** Tissues (frozen in liquid nitrogen and stored at -80°C) were homogenized in 1 ml buffer A (25 mM Tris-HCl, pH 8, 25 mM NaCl, 1mM Na-Orthovanadate, 10mM NaF, 10mM Na-Pyrophosphate, 10nM Okadaic acid, 0.5mM EDTA, 1mM PMSF, and 1x Protease inhibitor cocktail) in a Polytron-homogenizer.

Homogenates were centrifuged at 2000 rpm at 4<sup>0</sup>C for 10 min and the nuclear pellet was resuspended in 500 µl of Buffer A with 0.1% Triton X-100 and 500mM NaCl. Supernatant from the above centrifugation was further centrifuged at 10,000 rpm at 4<sup>0</sup>C for 20 min, and the resultant supernatant was used as cytosolic extract. The mitochondrial pellet was resuspended in 200-300 µl of Buffer A with 0.1% Triton X-100. The nuclei pellet and mitochondrial pellet were lysed by incubation for one hour on ice with intermittent tapping. Homogenates were then centrifuged at 14,000 rpm at 4<sup>0</sup>C for 10 min, and the supernatant was used as nuclear lysate and mitochondrial lysate respectively. Cytosolic, nuclear and mitochondrial extracts were aliquoted, snap frozen and stored at -80<sup>0</sup>C till use. Total protein concentration in cytosolic, nuclear and mitochondrial extract was determined using BCA Protein Assay Kit (Pierce, Rockford, IL).

**Western blot Analysis.** Proteins were separated in SDS-PAGE and transferred to nitrocellulose filters [16]. Filters were blocked in 5% nonfat dry milk, and probed with primary antibody for overnight at 4<sup>0</sup>C. Primary antibodies such as Bcl-2, Akt, phospho-Akt(Ser 473), P38 β and Glyceraldehyde-6-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA; whereas Src, phospho-Src (Ser 416), JNK, phospho-JNK (Thr183/Tyr185), P38 alpha, ERK, phospho- ERK (Thr180/Tyr182) were obtained from Cell Signaling Technology, Beverly, MA. All primary antibodies were used at the dilution of 1:1000. Protein bands were identified with horseradish peroxidase conjugated secondary antibody (1:2000 dilution) and Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA). GAPDH was used as loading control. The resulting blots were digitized, subjected to densitometric scanning using a standard NIH image program, and normalized against loading control.

**EMSA for DNA Binding Activity of NFκB.** To determine NFκB binding activity, nuclear proteins were isolated from the heart according to the method described previously [17]. In short, about 150 mg of left ventricle from heart tissue was homogenized with ice-cold buffer A (20mM HEPES, pH7.9, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2mM EDTA, 1mM DTT, 0.1% Triton X-100 and protease inhibitors {PMSF, leupeptin, aprotinin and pepstatin}) in homogenizer. Homogenates were centrifuged at 2000 rpm at 4<sup>0</sup>C for 15 min and the nuclear pellet was resuspended in five volumes of buffer B that was essentially buffer A except containing 500 mM NaCl. The nuclei were lysed by incubation for one hour on ice with intermittent tapping. Homogenates were then centrifuged at 10,000 rpm at 4<sup>0</sup>C for 15 min supernatants were aliquoted and snap frozen at -80<sup>0</sup>C until used. Protein concentration was estimated using the Pierce Protein Assay kit (Pierce Chemical Co., Rockford, IL).

NFκB oligonucleotide (5'-AGTTGAGGGGACTTCCAGG-3') (2μl [5 ng/μl]) was labeled using 5μl 10x T4 polynucleotide kinase as previously described [17]. DNA- protein binding reaction was carried out in 40 μl binding buffer (20 mM HEPES, pH7.9, 3% glycerol, 60mM NaCl, 1.5mM MgCl<sub>2</sub>, 1.0mM EDTA and 1mM DTT) containing 30 μg nuclear extract, 1μg poly (dI-dC), and 32P-labeled probe (40,000 CPM) on ice for 40 min. Protein-DNA complex were fractionated on 8% acrylamide gel in 0.5X TBE at 200 V, 4<sup>0</sup>C until the dye hit the bottom. After electrophoresis, gels were dried and exposed to Kodak X-ray film at -80<sup>0</sup>C. Autoradiographic results were evaluated quantitatively by an image analyzer. The binding signal from each sample was measured in the same sized area. Competition reaction was carried out using 100 fold molar excess unlabeled competitor DNA. Therefore, the specific binding was calculated by subtracting non-specific binding from total binding.

**Statistical Analysis.** The values for myocardial functional parameters, total and infarct volumes and infarct sizes and cardiomyocyte apoptosis are all expressed as the mean  $\pm$  standard error of mean (SEM). Analysis of variance test followed by Bonferroni'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  $\alpha$ -Tocopheryl Phosphate on Left Ventricular Function.** Fig. 1 shows the cardiac function obtained in rats pretreated with 5 mg/kg body wt. of  $\alpha$ -tocopheryl phosphate or vehicle only. The hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion. There were no significant different in the baseline parameters between the groups. In hearts treated with 5 mg/kg body wt.  $\alpha$ -tocopheryl phosphate a significant post-ischemic recovery in aortic flow (AF), left ventricular developed pressure (LVDP), and first derivative of maximum left ventricle developed pressure (LVDP max dp/dt) were observed after 30-, 60-, and 120 min of reperfusion in comparison with the vehicle treated control group. Similarly to the AF, LVDP, and LVDP max dp/dt significant change was found in coronary flow (CF), but only after 2h of reperfusion. The heart rate did not show significant different between the two groups.

**Effects of  $\alpha$ -Tocopheryl Phosphate on Myocardial Infarct.** After 30 min ischemia followed by 120 min of reperfusion myocardial infarct size was determined by TTC method. As fig. 2 shows  $\alpha$ -tocopheryl phosphate treatment significantly reduced the infarct size compared to the vehicle treatment. In case of vehicle treatment the infarct size was  $36.5 \pm 2.5\%$ , in the  $\alpha$ -tocopheryl phosphate treated group the infarct size was only  $21.8 \pm 2.1\%$ .

***Effects of  $\alpha$ -Tocopheryl Phosphate on Cardiomyocyte Apoptosis.*** As shown in Fig. 3, in case of ischemic control group rat (I/R) cardiomyocyte apoptosis determined by Tunel method was about  $30\pm 2\%$  at the end of reperfusion. There were very less amount of apoptotic cells  $5\pm 0.5\%$  in the hearts perfused with the KHB buffer without subjecting to ischemia and reperfusion (control). Again,  $\alpha$ -tocopheryl phosphate significantly reduced the number of apoptotic cardiomyocytes to  $11\pm 1.5\%$ .

***Effects of  $\alpha$ -Tocopheryl Phosphate on MAP Kinase Signaling.***

We examined the effect of  $\alpha$ -tocopheryl phosphate on MAP kinase signaling. Fig. 4 shows that ischemia can reduce the activity of anti apoptotic ERK 44/42 and p38 MAPK $\beta$  but treatment with  $\alpha$ -tocopheryl phosphate can prevent this reduction of these anti apoptotic members. Fig. 4 also shows that ischemia reperfusion can increase the activity of pro-apoptotic p38MAPK $\alpha$  and JNK but these activations were reduced after treatment with  $\alpha$ -tocopheryl phosphate.

***Effects of  $\alpha$ -Tocopheryl Phosphate on the DNA binding of NF $\kappa$ B.***

To check whether  $\alpha$ -tocopheryl phosphate induces NF $\kappa$ B activities, we performed electrophoretic mobility shift assay with a double stranded radiolabeled oligo nucleotide harboring the canonical NF $\kappa$ B binding sequence (5'-AGTTGAGGGGACTTCCCAGG-3'). As shown in Fig. 5, NF $\kappa$ B binding activity diminished upon ischemia as compared to non-ischemic control while it substantially increased for the  $\alpha$ -tocopheryl phosphate treated hearts. Out of four NF $\kappa$ B binding activities such induced, at least three (C1-3) had p65 as a constituent as evident from super-shift assay.

*Effects of  $\alpha$ -Tocopheryl Phosphate in the generation of Survival Signal.*

We examined the effects of  $\alpha$ -tocopheryl phosphate on the members of the survival signal pathway. Figure 6 shows the results. The phosphorylation of Akt was reduced after ischemia/reperfusion, but significantly upregulated with  $\alpha$ -tocopheryl phosphate treatment. The phosphorylation of c-Src was increased after ischemia reperfusion but in case of  $\alpha$ -tocopheryl phosphate treated group the activation of pro-apoptotic factor c-Src was significantly reduced. Bcl-2 was downregulated after ischemia/reperfusion, but tocopheryl phosphate prevented the loss of Bcl-2 protein due to ischemia/reperfusion.

**DISCUSSION.**

The present study demonstrates that nutritionally supplemented  $\alpha$ -tocopheryl phosphate can reduce myocardial ischemia reperfusion injury as evidenced by its ability to improve ventricular function and reduce infarct size and cardiomyocyte apoptosis. Supplementation with tocopheryl phosphate appears to potentiate a survival signal through the activation of Akt and Bcl-2 and by switching MAP kinase-induced death signal into a survival signal.

$\alpha$ -Tocopheryl phosphate is a water soluble phosphoric acid ester of  $\alpha$ -tocopherol that exists ubiquitously in both animal and plant kingdom. Nutritionally supplemented tocopheryl phosphate results in a minor increase of the non esterified form [18] therefore the effects of  $\alpha$ -tocopheryl-phosphate appeared not to be related to the release of free tocopherol. As mentioned earlier,  $\alpha$ -tocopheryl phosphate is resistant to hydrolysis and oxidation, and it can liberate  $\alpha$ -tocopherol only by strong alkaline hydrolysis or very slowly by alkaline phosphatase [12].

Only recently, health benefits of  $\alpha$ -tocopheryl-phosphate have become apparent. For example,  $\alpha$ -tocopherol phosphate has recently been found to protect against ultraviolet B-

induced damage in cultured mouse skin [19]. A mixture of  $\alpha$ -tocopheryl phosphate and di- $\alpha$ -tocopheryl phosphate inhibited cell proliferation and CD36 mRNA and protein expression leading to the inhibition of oxLDL uptake [13]. Another recent study showed that the atherosclerotic-preventing effect and CD36 inhibitory effect of the phosphate derivative was more pronounced than the acetate derivative of  $\alpha$ -tocopherol [20]. Data on the tissue distribution of  $\alpha$ -Tocopheryl phosphate after supplementation are not available yet. Consequently, a distinction between a pharmacological and a nutritional intervention is not possible at the present time. However, the present study demonstrates the ability of  $\alpha$ -tocopheryl phosphate to reduce myocardial ischemia reperfusion injury by enhancing ventricular performance and reducing myocardial infarct size and cardiomyocyte apoptosis.

Existing evidence supports the role of  $\alpha$ -tocopheryl phosphate as a signaling molecule. However, direct evidence of performing intracellular signaling was never shown previously. To the best of our knowledge, the present study reports for the first time that tocopheryl phosphate can ameliorate myocardial ischemia reperfusion injury by activating survival signal through the phosphorylation of Akt and enhancing the expression of anti-death protein Bcl-2. Our study clearly demonstrated a reduction of Akt phosphorylation in the ischemic reperfused myocardium. Not only did  $\alpha$ -tocopheryl phosphate prevent such a reduction of Akt phosphorylation, it actually increased the phosphorylation over the baseline. Similarly, Bcl-2 was almost negligible in the ischemic reperfused heart, but increased several-fold with  $\alpha$ -tocopheryl phosphate. These findings are consistent with the previous reports, where Akt acts at the level of mitochondria to release cytochrome c via Bcl-2 in adult cardiomyocytes [21]. PI-3-kinase-Akt signaling pathway appears to play a critical role in the prevention of ischemia/reperfusion-induced cell death [22].

The pro-apoptotic factor c-Src was also significantly lowered as a result of  $\alpha$ -tocopheryl phosphate treatment. The Src kinase belongs to the family of nonreceptor tyrosine kinases, which modulate a wide variety of intracellular signaling. Including those mediating DNA synthesis and proliferation. Activation of Src kinase is associated with many degenerative diseases including ischemic heart disease [23]. Myocardial ischemia reperfusion causes an induction of c-Src protein expression, and inhibition of c-Src with PPI can restore the extent of cellular injury [24]. The present results also support these previous reports.

Interestingly,  $\alpha$ -tocopheryl phosphate could modulate the MAP kinase signaling by converting MAP kinase-induced death signal into a survival signal. Ischemia reperfusion generated a death signal as a result of reduced activity of anti-apoptotic ERK 42/44 and p38MAPK $\beta$  in concert with an activation of pro-apoptotic p38MAPK $\alpha$  and JNK.  $\alpha$ -Tocopheryl phosphate completely reversed the MAP kinase signaling by inducing the activation of ERK 42/44 and p38MAPK $\beta$  and the reduction of JNK and p38MAPK $\alpha$ . A significant number of reports exist in the literature demonstrating a crucial role of MAP kinase signaling in modulating the death signal induced by ischemia/reperfusion [25]. Many cardioprotective agents function by changing such death signal into survival signal. For example, ischemic preconditioning protects the ischemic myocardium by activating p42/44 ERK and p38MAPK $\beta$  and reducing p38MAPK $\alpha$  and JNK phosphorylation [26]. P38 MAPK signaling appears to be of particular interest, as one of the isomers p38MAPK $\alpha$  potentiates a death signal while another isomer p38MAPK $\beta$  potentiates a survival signal [27]. It is interesting to note that tocopheryl phosphate can differentially regulate p38MAPK signaling.

A number of recent studies have determined an essential role of NF $\kappa$ B in myocardial survival following ischemia and reperfusion. For example, myocardial protection through

ischemic preconditioning cannot be achieved unless there is an increased DNA binding of NF $\kappa$ B [28]. The results of the present study support the previous findings of reduced NF $\kappa$ B in the ischemic reperfused myocardium, and further demonstrate a significant increase the DNA binding activity of NF $\kappa$ B after  $\alpha$ -tocopheryl phosphate treatment.

In summary, the results of the present study demonstrate the cardioprotective ability of  $\alpha$ -tocopheryl phosphate. Cardioprotection was achieved by changing ischemia reperfusion-mediated death signal into a survival signal by modulating MAP kinase signaling and inhibition of c-Src activity. The anti-death signal was realized through Akt-Bcl-2 survival pathway indicating the ability of  $\alpha$ -tocopheryl phosphate to generate a survival signal.

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**FIGURE LEGENDS.**

**Figure 1.** The effect of  $\alpha$ -tocopheryl phosphate on heart rate, coronary flow, aortic flow, left ventricular developed pressure, and first derivative of left ventricular developed pressure (dp/dt). n=6 in each group. Mean  $\pm$  SEM, comparisons were made to the values of drug-free control group. \*p<0.05. RE: reperfusion.

**Figure 2.** The effect of  $\alpha$ -tocopheryl phosphate on myocardial infarct size was determined by TTC method. n=3 in each group. Mean  $\pm$  SEM, comparisons were made to the values of drug-free control group. \*p<0.05.

**Figure 3.** Effects of  $\alpha$ -tocopheryl phosphate on cardiomyocyte apoptosis. Isolated heart from control (non ischemic) rats were perfused with the KHB buffer without subjecting to ischemia and reperfusion. Isolated hearts from ischemic control rats [I/R] (n=6) and  $\alpha$ -tocopheryl phosphate treated rats (n=6) were subjected to 30 min of global ischemia followed by 2h reperfusion. Cardiomyocyte apoptosis was measured by Tdt-mediated dUTP nick end labeling with Promega kit. Panel A is total number of cells (red channel). Panel B is total number of apoptotic cells (green channels). Panel C is merged imaged of panel A and panel B (yellow dots in merged channel are the apoptotic cells). Values are Mean  $\pm$  SEM \*p <0.05 vs. control. †p<0.05 vs. I/R. Representative photomicrographs are shown below the bar graphs.

**Figure 4.** Western blot analysis to check the effects of  $\alpha$ -tocopheryl phosphate on the phosphorylation of p44/42 MAP Kinase, JNK and the expression of p38 $\beta$  and p38 $\alpha$ . The isolated rat hearts were perfused for 15 min with KHB buffer. The hearts were subjected to 30 min global ischemia followed by 2 h of reperfusion in the working mode. At the end of

the experiments, the hearts were frozen at liquid nitrogen. The phosphorylated proteins are shown on the top of non phosphorylated proteins, which also served as the controls. GAPDH was used as a loading control for p38 $\beta$  and p38 $\alpha$ . Figures are representative images of three different groups, and each experiment was repeated at least thrice. Values are Mean  $\pm$  SEM. \*p <0.05 vs. control, †p <0.05 vs. I/R.

**Figure 5.** Induction of NF- $\kappa$ B binding activity by  $\alpha$ -tocopheryl phosphate in rat heart. Nuclear extracts were prepared from hearts isolated from rats fed with  $\alpha$ -tocopheryl phosphate or the vehicle followed by ischemia-reperfusion. Thirty micrograms of extracts were tested for NF $\kappa$ B binding activities by electrophoretic mobility shift assay using radiolabeled cognate binding site (5'-AGTTGAGGGGACTTTCCCAGG-3') as probes. Lane A: control; lane B: ischemia/reperfusion; lane C:  $\alpha$ -tocopheryl phosphate treatment followed by ischemia-reperfusion; lane D: extract in lane C plus p65 antibody (1:5 dil); Lane E: extract in lane C plus p65 antibody (undiluted); lane F: extract in lane C plus 100 fold molar excess unlabeled NF $\kappa$ B binding sequence as competitor. The NF $\kappa$ B specific complexes are marked as C1-4, of which C1-3 is super-shifted by the p65 antibody and thus contains p65 as a constituent.

**Figure 6,** Western blot analysis of p-Akt, Akt, p-Src, Src, and Bcl-2 proteins in heart tissue obtained from control (non ischemic), I/R (ischemic control) and  $\alpha$ -tocopheryl phosphate treated group. The isolated rat hearts were perfused for 15 min with KHB buffer. The hearts were then subjected to 30 min global ischemia followed by 2 h of reperfusion in the working mode. At the end of the experiments, the hearts were frozen at liquid nitrogen temperature. The phosphorylated proteins are shown on the top of non phosphorylated proteins, which

also served as the controls. GAPDH was used as a loading control for Bcl-2 protein. Figures are representative images of three different groups, and each experiment was repeated at least thrice. Values are Mean  $\pm$  SEM. \* $p < 0.05$  vs. control. † $p < 0.05$  vs. I/R.

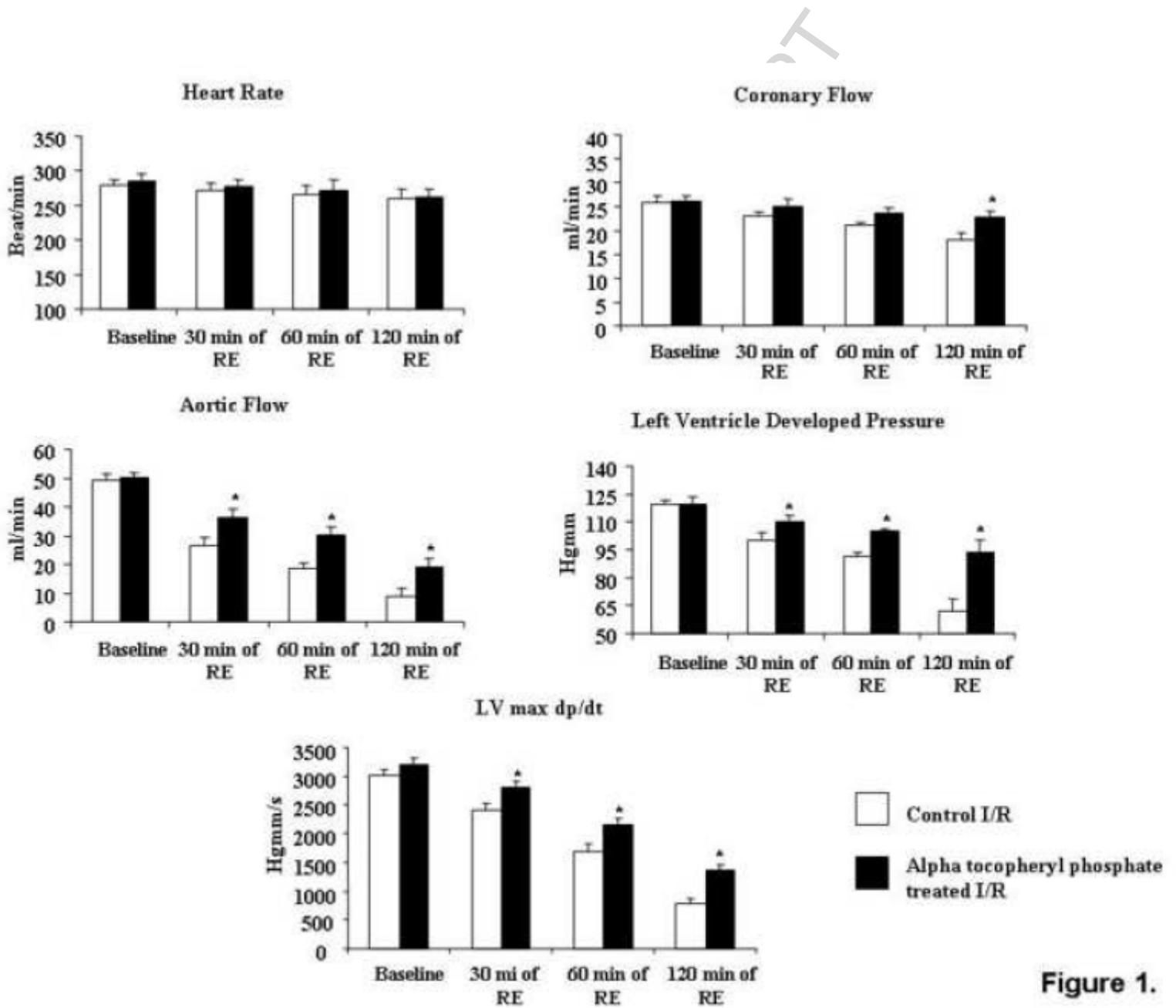


Figure 1.

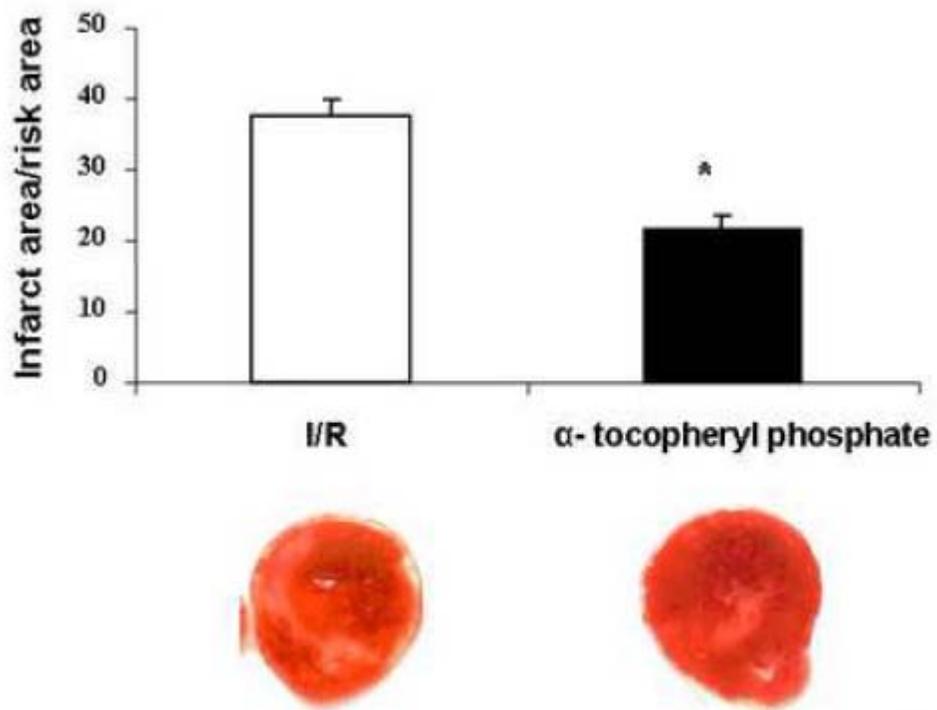


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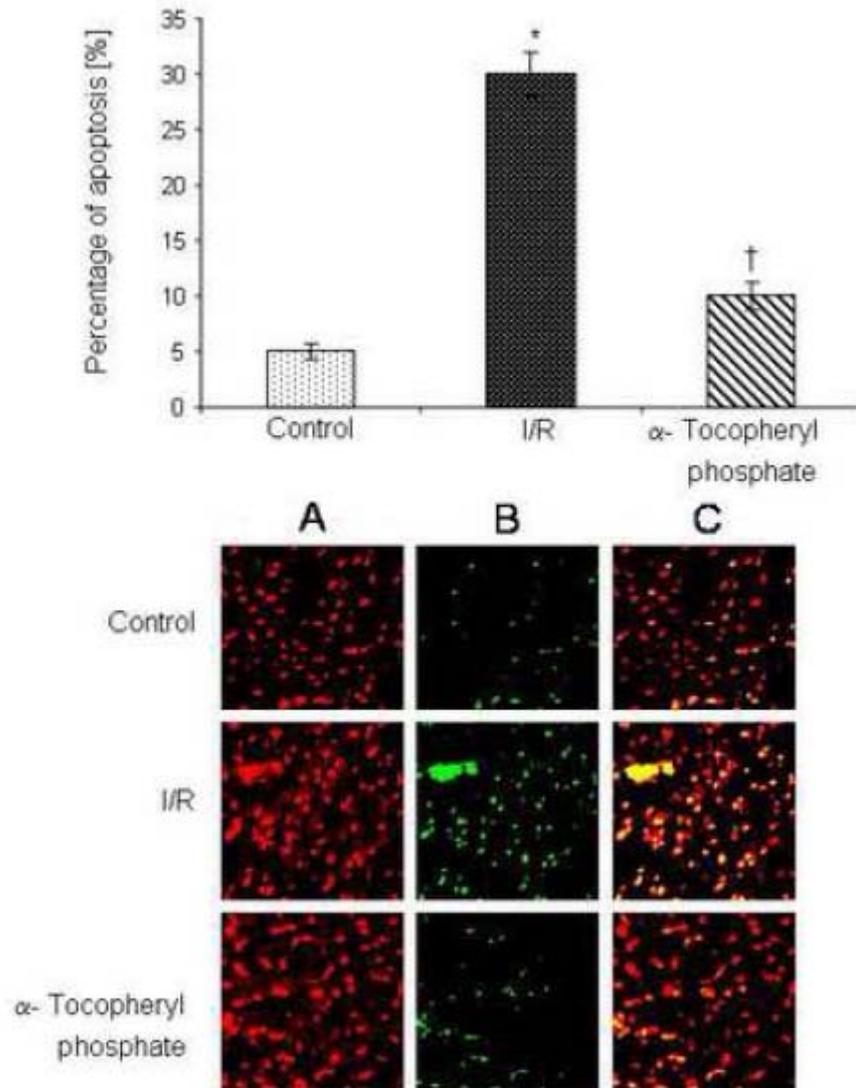


Figure 3.

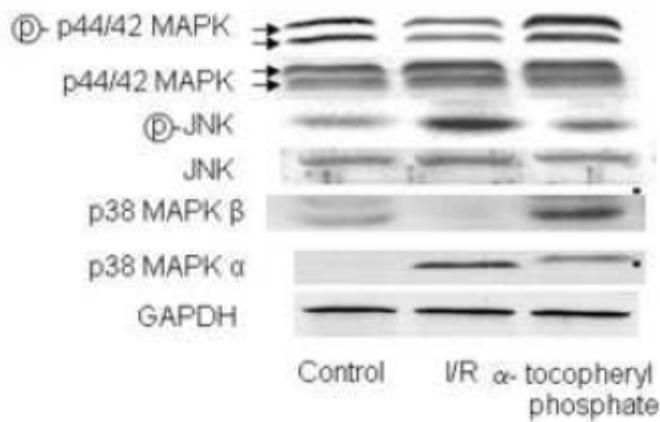
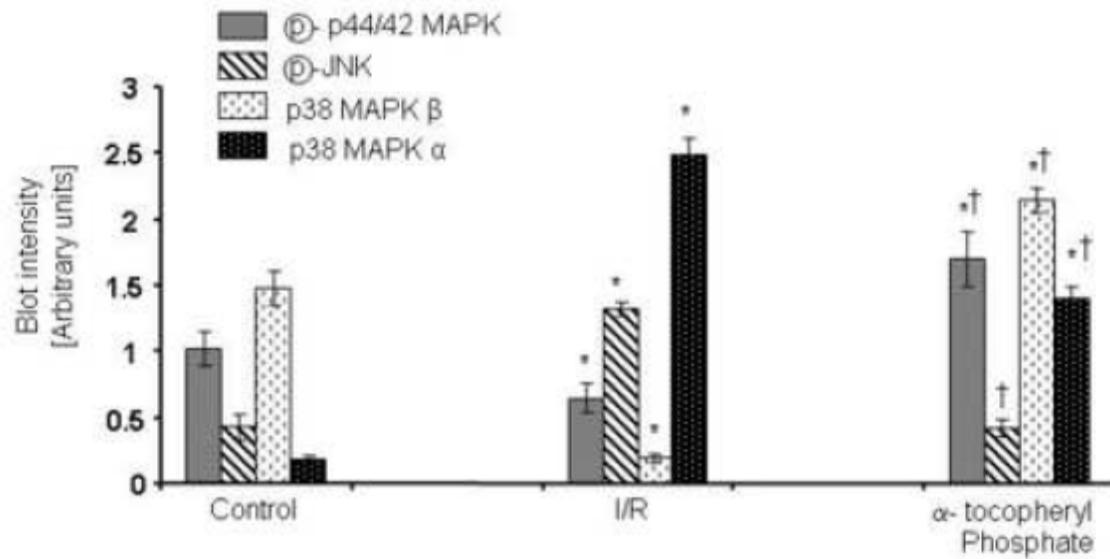


Figure 4.

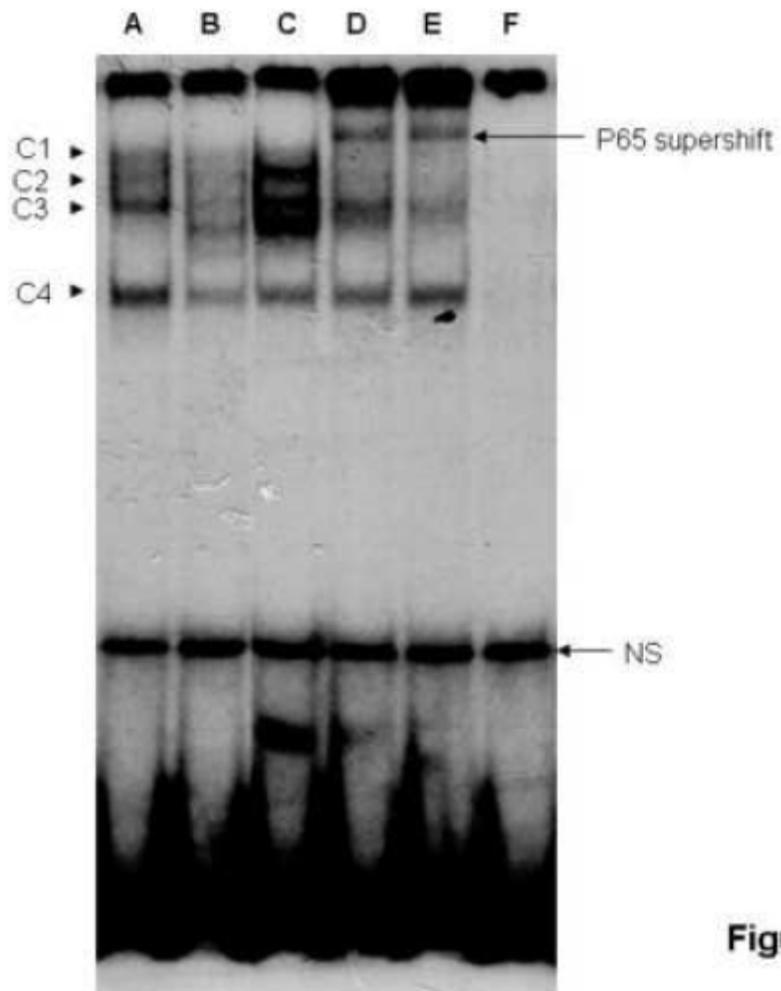


Figure 5.

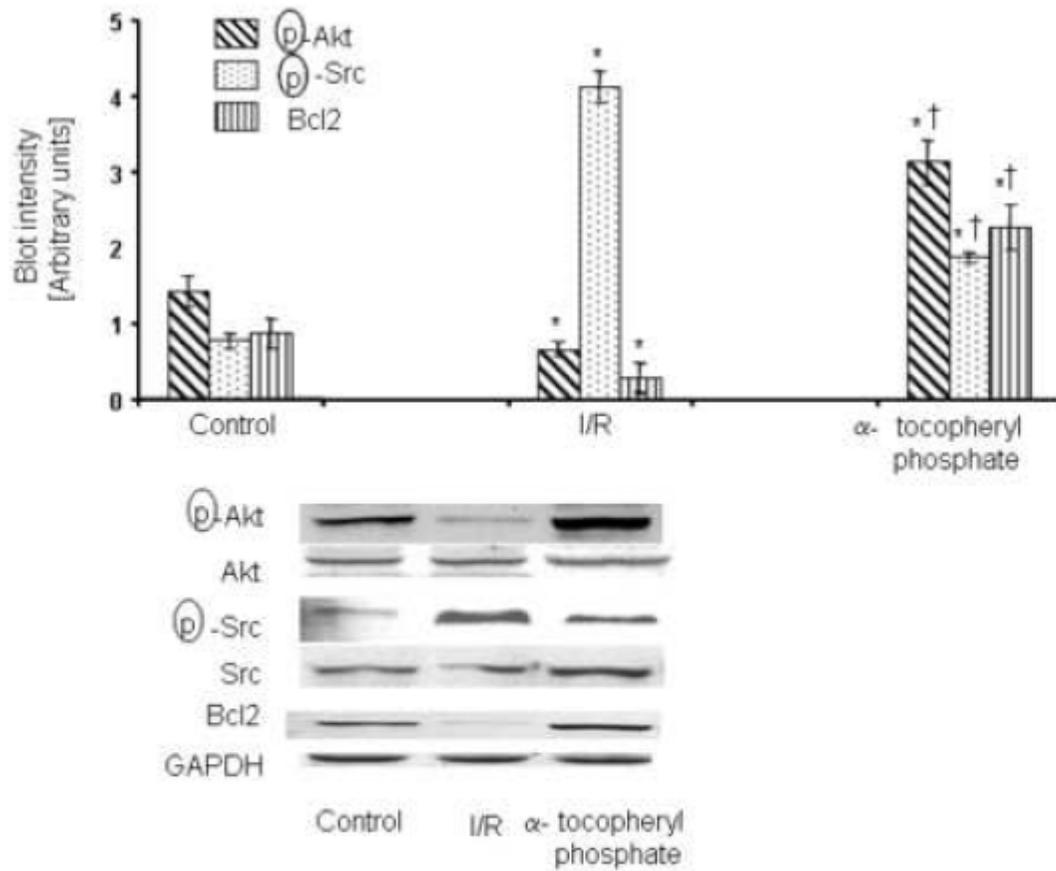


Figure 6