Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2008;22:287-294

Accepted: June 02, 2008

Caveolin and Proteasome in Tocotrienol Mediated Myocardial Protection

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Key Words

Caveolin • Proteasome • Tocotrienol • Heart • Ischemia/Reperfusion

Abstract

The effect of different isomers of tocotrienol was tested on myocardial ischemia reperfusion injury. Although all of the tocotrienol isomers offered some degree of cardioprotection, gamma-tocotrienol was the most protective as evident from the result of myocardial apoptosis. To study the mechanism of tocotrienol mediated cardioprotection, we examined the interaction and/or translocation of different signaling components to caveolins and activity of proteasome. The results suggest that differential interaction of MAP kinases with caveolin 1/3 in conjuncture with proteasome stabilization play a unique role in tocotrienol mediated cardioprotection possibly by altering the availability of pro-survival and anti-survival proteins.

Introduction

Tocotrienols and tocopherols together compose vitamin E family. Tocotrienols exist in four different isoforms, alpha, beta, gamma and delta, based on the number of methyl groups on the chromanol ring. Although all the isomers have antioxidant property, each of them has its own biological activity. Many studies indicate that tocotrienols have anti-cancer properties. Tocotrienol-rich fraction of palm oil induces cell cycle arrest and apoptosis selectively in human prostate cancer cells [1]. Tocotrienols have been found to protect cultured cortical neurons from oxidative cell induced cell death through the activation of MAP kinase and PI-3-Kinase [2]. Tocotrienols also possess cardioprotective abilities. For example, dietary tocotrienols reduce concentration of plasma cholesterol, apolipoprotein B, thromboxane B2 and platelet factor IV in pigs with inherited hyperlipidemias [3]. Tocotrienol also reduced plasma cholesterol levels in human pilot study [4]. We showed earlier that ischemia resulted in an inhibition of 26S proteasome and a proteasome inhibitor (MG132) attenuated post ischemic

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recovery associated with increased accumulation of ubiquitinated proteins in the myocardium [5]. We have also showed protective ability of palm oil is mediated through proteasome stabilization [6]. A recent observation from our laboratory indicated that caveolin-MAP kinase interaction plays an important role in developing survival signal in the myocardium [7]. Caveolin is a cholesterol binding protein located in the lipid raft, which serves as a platform for organizing and integrating signal transduction process. Since MAP kinases are involved in cellular protection and caveolin plays a role in the generation of survival signal, we hypothesized that proteasome and caveolin might be involved in tocotrienol mediated cardioprotection. The present study was designed to study the cardioprotective abilities of the different isomers of tocotrienol (alpha, gamma, and delta) and to determine how their actions were controlled by proteasome and caveolin.

Materials and Methods

Materials

Different isomers of tocotrienols (alpha, delta, and gamma) were purchased from BASF, Florham Park, NJ. Antibodies against caveolin-1, caveolin-3, p38MAPKβ, were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. P38MAPKα, caspase-3, Cytochrome-c were purchased from Cell Signaling technology, MA. Suc-LLVY-AMC, lactacystin, and the following proteasome subunit antibodies: polyclonal β5, monoclonal α3 (MCP106) and Rpt5 (TPB1-19) were obtained from Biomol Research Lab (Plymouth Meeting, PA).

Animal

All animals used in this study received humane care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" [DHHS Publication No. (NIH) 85-23 revised 1985]. The experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (250-300 g body wt) were fed ad libitum regular rat chow and had free access to water. Rats were divided into four groups. Three groups were gavaged [forcefully injected into the stomach by using a stomach needle (1.2 mm diameter) every day] with three different isomers of tocotrienol (alpha, delta, and gamma) for 30 days while the forth group was gavaged with vehicle only. Tocotrienol isomers were dissolved in 0.01% ethanol and gavaged at a conc. of 0.3 mg/kg body weight whereas control animals were gavaged with 0.01% methanol. After 30 days, treated animals were sacrificed and the hearts were excised, perfused and subjected to 30 min ischemia and 120 min reperfusion.

Isolated rat heart preparation

Rats were anesthetized with pentobarbital sodium (80 mg/kg ip, Abbott Laboratories, North Chicago, IL), and heparin sodium (500 IU/kg iv, Elkins-Sinn, Cherry Hill, NJ) was administered for anticoagulation. After ensuing sufficient depth of anesthesia, the hearts were excised and perfused via retrograde Langendorff mode at constant perfusion pressure of 100 cm of water (10kPa) for stabilization [8]. The perfusion buffer consisted of a modified Krebs-Henseleit buffer [containing (in mM) 118 NaCl, 4.7 KCl, 1.7 $CaCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 10 glucose]. After 10 min, when cardiac function had attained a steady state, all perfused hears were subjected to 30 min global ischemia followed by 120 min reperfusion.

Estimation of Apoptosis

Caspase-3 activation/cleavage are a critical determinant of apoptosis and most common assay for examining caspase-3 activation/cleavage is immumoblotting [9]. Cytochrome-c initiates the proteolytic activation of caspase, which in turn cleave protein to produce the morphological and biological changes of apoptosis. Generally, cytochrome-C remains inside mitochondria. Stress and/or death signal increase the permeability of the inner mitochondrial membrane and swelling of mitochondrial ruptures the outer mitochondrial membrane and release pro-apoptotic proteins including cytochrome c. Cytosolic cytochrome c level is another most important determinant of apoptosis [10, 11]. In this present investigation level of apoptosis was estimated by the degree of expression of caspase-3 and cytochrome-c in cytosolic and mitochondrial fractions of different groups of heart. Heart tissues were homogenized in ice cold buffer (25 mM Tris-Hcl, 25nM NaCl, 1mM Na-orthovanadate, 10mM NaF, 10mM pyrophosphate, 10nM okadoic acid, 0.5mM EDTA, 1mM PMSF, and protease inhibitor cocktail). Cytosolic and mitochondrial fractions were prepared using standard graded centrifugation. Western blot analysis was then performed with antibodies against full length caspase-3, cleaved caspase-3 and cytochrome-c according to established protocols.

Isolation of Caveolin-rich Membrane Fractions

The hearts were homogenized in sodium bicarbonate buffer containing protease inhibitor cocktail, pH 11.0 using a Polytron homogenizer [three 10s bursts] [Brinkman Instruments, Westbury, N.Y.]. The homogenate was sonicated [three 20s bursts], and adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in MBS [25 mM Mes, pH 6.5, 0.15 M NaCl] and placed at the bottom of an ultracentrifuge tube. A 35% discontinuous sucrose gradient was formed above [4 ml of 5% sucrose/4 ml of 35% sucrose - both in MBS containing 250 mM sodium carbonate] and centrifuged at 39,000 rpm for 16-20 h in an SW41 rotor [Beckman Instruments, Palo Alto, CA]. From the top of each gradient, 1 ml gradient fractions were collected to yield a total of 12 fractions as described elsewhere [12]. Caveolin migrates mainly in fractions 5 and 6 of these sucrose density gradients [12].

Immunoprecipitation with Caveolin 1 and Caveolin 3

Caveolin rich fractions (fractions 5 and 6) were used for immunoprecipitation. Immunoprecipitation was performed with Protein-A sepharose CL-B4 (Pharmacia Biotech Inc) using a polyclonal antibody against caveolin-1 or caveolin -3 [Santa Cruz Biotechnology, Santa Cruz, CA] Incubation conditions were maintained as instructed by the supplier. Western blot analysis was then performed with antibodies against P38MAPKα, P38MAPKβ, c-Src, HO-1 and eNOS according to standard methods.

Proteasome Activity

26S Proteasome assay. The chymotryptic activity representing the β5 subunit was analyzed using the method of Reinheckel et al [13] as specifically modified for heart tissue by Powell et al [14]. For these assays, the reaction mixtures contained 30 μg protein and was conducted in the presence of ATP (0 to 56 μmol/l). Specificity of the assay was confirmed by inclusion of Lactacystin, 10 μmol/l.

26S Proteasome subunit determination

Cardiac tissue was homogenized in HEPES buffer containing (mmol/L): NaCl 137, KCl 4.6, KH_2PO_4 1.1, $MgSO_4$ 0.6, EDTA 1, Digitonin, 0.01%, plus a cocktail of protease inhibitors (leupeptin, 5 μg/ml; aprotinin, 5 μg/ml; pepstatin, 7 μg/ml; and phenylmethylsulfonyl fluoride, 40 μg/ml) at 4°C and then centrifuged at 10,000*g* to obtain the soluble fraction. Cellular proteins (50 μg) were separated on 4-20% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA) using standard SDS-Polyacrylamide gel electrophoresis with immunoblotting carried out using standard techniques and developed with an enhanced chemiluminescence (ECL) kit (Perkin Elmer Life Sciences, Boston MA). Membranes were probed with antibodies specific for the β5, α3 and Rpt5 subunits of the 26S proteasome.

Statistical Analysis

Experiments were performed with at least three animals per group. The statistical analysis was performed by analysis of variance followed by Bonferroni's correction for any differences between the mean values of all groups. Differences between data were analyzed for significance by performing a Student's *t*-test. The results were considered significant if $p < 0.05$.

Results

Cardio protection by different isomers of tocotrienol

To study the level of apoptosis in tocotrienol treated heart, caspase-3 and cytochrome C levels were examined by Western blot analysis [9-11]. We have found that the level of full length caspase-3 is high in gamma and alpha tocotrienols treated hearts compared to the I/R and delta tocotrienol treated heart. The level of cleaved caspase-3 was very high in I/R heart compared to the gamma tocotrienol treated heart. Alpha and delta tocotrienols also showed a decrease in cleaved caspase-3 level compared to I/R.

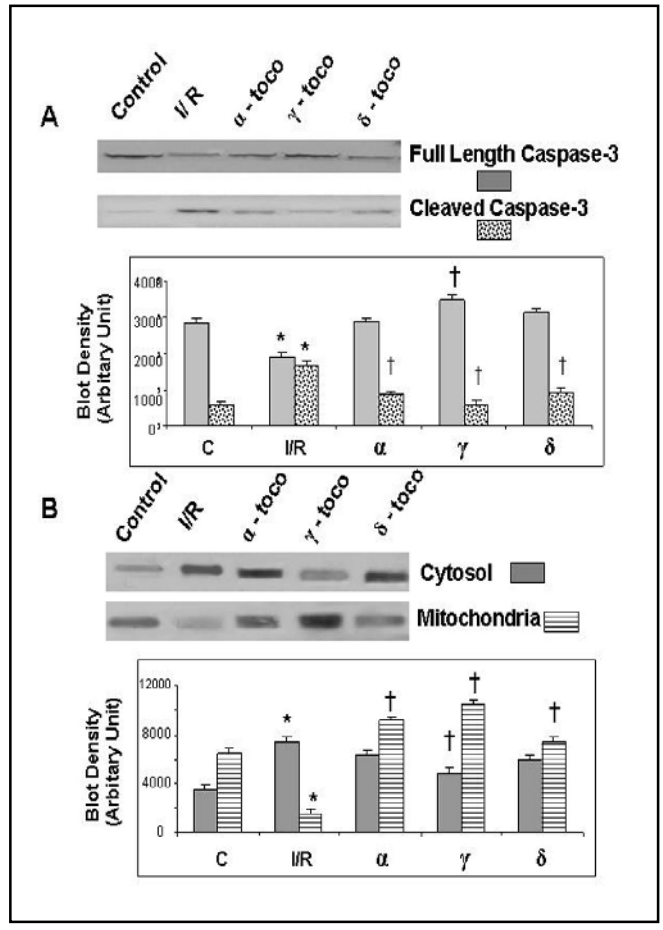

Fig. 1. Effect of different isomers of tocotrienols (alpha, gamma and delta) on the: A) caspase-3 (full length and cleaved), B) Cytochrome-c (cytosolic and mitochondrial fraction). The results are mean ± SEM. *p<0.05 vs control, †p<0.05 vs I/R [α-toco = α-tocotrienol + I/R, γ-toco = γ-tocotrienol + I/R, δ-toco = δ-tocotrienol + I/R].

In case of cytochrome-C, we tested the level of cytochrome-c in both cytosolic and mitochondrial fractions of the heart and found that the level of myocardial cytochrome c were significantly higher in cytosolic fraction compared to the mitochondrial fraction in I/R heart. In the tocotrienol treated groups, levels of myocardial cytochrome c were significantly higher in mitochondrial fraction compared to the cytosolic fraction, only exception being delta tocotrienol. Among alpha and gamma tocotrienols, gamma showed highest level of cytochrome C in the mitochondrial fraction (Fig 1).

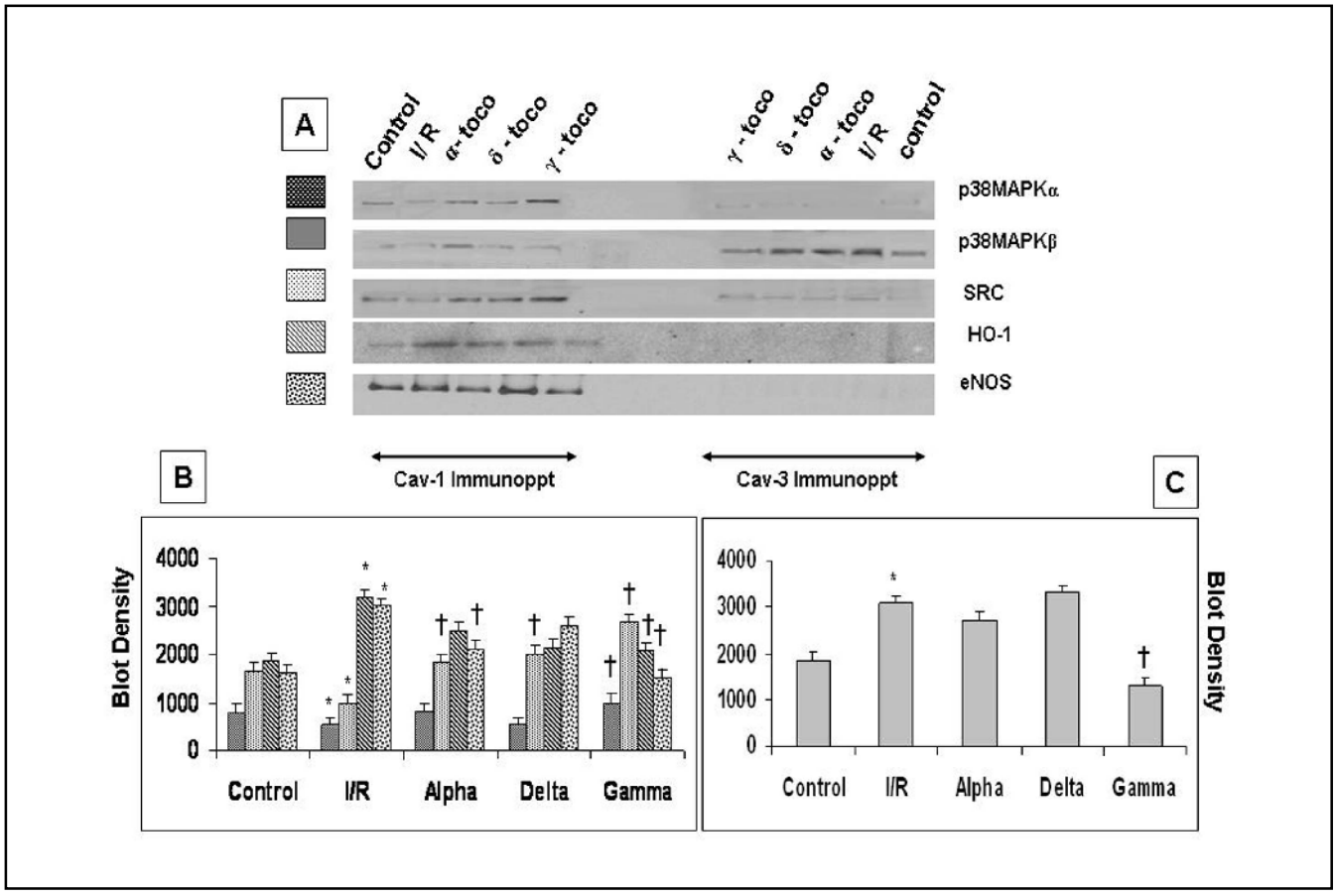

Fig. 2. A) Differential interaction of p38MAPK, heme oxygenase (HO-1), Src kinase and eNOS with caveolin-1 and caveolin-3 in tocotrienol (alpha, gamma and delta) treated hearts and non-treated hearts. B) Blot density of caveolin-1 immunoprecipitated sample. C) Blot density of caveolin-3 immunoprecipitated sample (mainly blot density of p38MAPKβ). The results are mean ± SEM. *$p<0.05$ vs control, †$p<0.05$ vs I/R [α-toco = α-tocotrienol + I/R, γ-toco = γ- tocotrienol + I/R, δ-toco = δ- tocotrienol + I/R].

Differential interaction with caveolin 1 and caveolin 3

Caveolin-rich fractions (5th and 6th fractions) of sucrose density gradient of all groups of hearts were immunoprecipitated with caveolin-1 and caveolin -3. The examination of the existence of p38MAPKα/β in the caveolin immunoprecipitated sample by Western blot revealed that high amount of p38MAPKα bound with caveolin-1 whereas interaction of caveolin-3 with p38MAPKα was very negligible. In contrast, high amount of p38MAPKβ bound with Caveolin-3 whereas its binding with caveolin-1 was very negligible. In I/R hearts, binding of p38MAPKα with caveolin -1 was much less compared to the tocotrienol treated heart. Among the different isomers of tocotrienols, binding of p38MAPKα with caveolin -1 was much higher in gamma compared to alpha and delta isomers. In between alpha and delta isomers, caveolin-1 and p38MAPKα interaction was higher in alpha than delta. However, the binding of p38MAPKβ with caveolin-3 in I/R heart was much higher than gamma tocotrienol treated heart. Alpha and delta tocotrienol treated hearts showed similar kind of p38MAPKβ and caveolin-3 interaction like I/R heart (Fig 2).

The existence of total Src Kinase in the caveolin immunoprecipitated sample by Western blot revealed that high amount Src kinase bound with Caveolin-1 whereas its binding with caveolin-3 was very negligible. In I/R hearts, binding of Src with caveolin-1 was much lower compared to tocotrienol treated hearts. Among the different isomers of tocotrienol, Src and caveolin-1

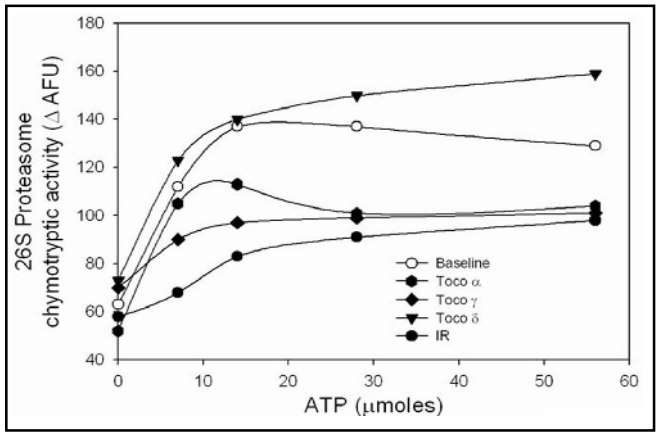

Fig. 3. Preservation of postischemic 26S-proteasome functions by different isomers of tocotrienol.

interaction was much higher in gamma compared to alpha and delta isomers. Among the alpha and delta isomers, interaction was higher in alpha than delta. (Fig 2)

Existence of heme oxygenase (HO-1) and eNOS were also tested in immunoprecipitated sample and found that HO-1 and eNOS bound with caveolin-1, whereas their binding with caveolin-3 was very negligible. In I/R heart, caveolin-1 and HO-1 interaction was higher compared to the tocotrienol treated heart. Among the different isomers of tocotrienols, caveolin-1 and HO-1 interaction was much less for gamma tocotrienol treated compared to that for alpha and delta isomers. In case of eNOS, its interaction with caveolin-1 was much higher in I/R and delta-tocotrienol treated hearts compared to the alpha and gamma tocotrienol treated hearts. (Fig 2)

The effect of tocotrienols on post ischemic 26S proteasome activity and subunit protein levels

Consistent with previous studies, ischemia/ reperfusion resulted in a 50% loss of proteasome activity (Figure 3). All of the tocotrienol isomers afforded some degree of protection against loss of proteasome activity; however a distinct pattern was apparent. The least effective was γ-tocotrienol followed by α-tocotrienol, and then δ-tocotrienol, which was the most effective, not only for preserving activity, but also for increasing activity at the higher concentrations of ATP. All three of the proteasome subunits that were analyzed tended to be increased after ischemia/reperfusion (Figure 4) with the Rpt5 and α3 subunit protein levels increased about 50%. Curiously, the isomers of tocotrienol seemed to prevent this effect with the only exception being a 60% increase in the Rpt5 subunit protein in the δ-tocotrienol treatment group.

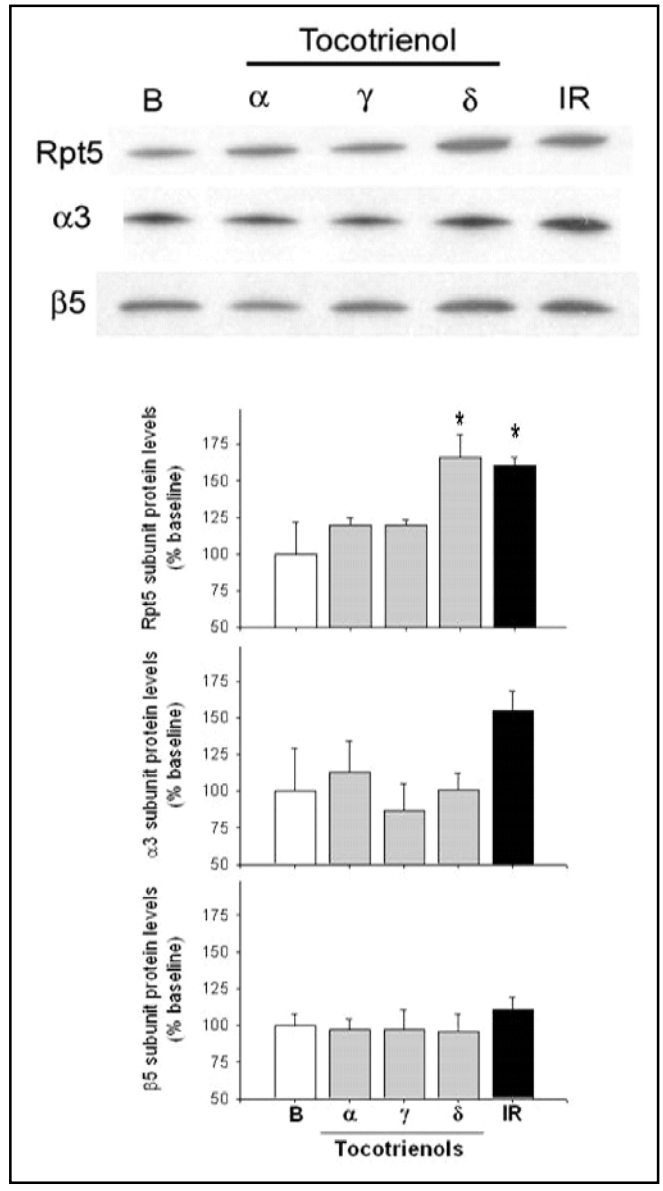

Fig. 4. Changes in proteasome subunit (Rpt5, α3, β3) protein levels in response to treatment with different isomers of tocotrienols (alpha, gamma and delta) and ischemia/ reperfusion. The results are mean ± SEM. *p<0.05 vs B [B= baseline/ control].

Discussion

The present investigation demonstrated different levels of cardioprotection by different isomers of tocotrienol and how their actions were controlled by caveolin and proteasome. A recent investigation from our laboratory showed that differential translocation and/or interaction of p38MAPKα/β with caveolin1/3, which functioned as a switch for the conversion of I/R induced death signal into preconditioning (PC) induced survival signal [7]. Earlier investigation from our laboratory also

showed that tocotrienol-rich fraction of palm oil could reverse ischemia-reperfusion mediated cardiac dysfunction by proteasome stabilization [6]. So, we reasoned that caveolin interaction might play an important role in tocotrienol-induced cardioprotection and/or tocotrienol induced survival signal. We also wished to examine whether the proteasome activity would vary with the different degree of cardioprotection by different isomers of tocotrienol.

Consistent with our earlier investigations, the present study also showed that p38MAPKα binds with caveolin -1 and p38MAPKβ binds with caveolin-3. In general, p38MAPKα is linked with death signal while p38MAPKβ is linked with survival signal [15]. In this present investigation we also tested the interaction of caveolin with Src, eNOS and HO-1. eNOS and HO-1 are pro-survival signaling components and Src is anti-survival signaling component.

The Src kinases belong to the family of nonreceptor tyrosine kinases, which mediate a wide variety of intracellular signaling, including those mediating DNA synthesis and proliferation. Myocardial ischemia-reperfusion caused an induction of c-Src protein expression [16]; inhibition of c-Src with PPI reduced the extent of cellular injury. Activation of Src kinase is associated with many degenerative diseases, including cardiovascular diseases, oncogenesis, and neurodegenerative diseases [17]. In mammalian tumors expressing the *neu* proto-oncogene, c-Src tyrosine kinase activity is elevated [17]. Markedly elevated levels of c-Src kinase activity was detected in human skin tumors [18]. Myocardial ischemia-reperfusion caused an induction of c-Src protein expression [19]; inhibition of c-Src with PPI reduced the extent of cellular injury. Here in the present investigation, we have found that increased translocation of c-Src to caveolin-1 in tocotrienol treated heat, subjecting the heart to reduced c-Src, whereas there was less translocation of c-Src in I/R heart, rendering the heart more abundance to c-Src. Among the different isomers of tocotrienol, gamma showed the highest degree of c-Src translocation to caveolin-3.

Heme oxygenase is a cyto-protective protein, commonly found in heart and has been found to display relevant cardioprotective actions by diverse mechanisms [20]. The increased activity of heme oxygenase leads to the formation of billiverdin and bilirubin, antioxidant compounds with potential protective actions [21]. In a recent study, HO-1 showed cardio protective action through CO in rat heart [22]. In rabbit model, HO-1 has been shown to play an important role in attenuating post-ischemic myocardial injury. Recently, in both rat and mice model of diabetes, HO-1 significantly decreased the infract zone in myocardium caused by ischemia reperfusion [23, 24]. In our present investigation, we have found more HO-1 translocation to caveolin-1 in I/R heart and less HO-1 translocation to caveolin-1 in tocotrienol treated heart, thereby increasing the availability of HO-1 protein to induce a survival signal. Among alpha, delta & gamma tocotrienols, gamma showed the least translocation of HO-1 and at the same time, higher cardio protective ability.

Nitric oxide (NO) derived from vascular endothelium has many physiological effects related to the protection of the ischemic-reperfused heart [25]. In cardiomyocytes, the NO-mediated regulations of cardioprotective enzymes are crucial for cell survival. It is now clear that NO, either endogenous or exogenous, represents one of the defense against myocardial ischemia-reperfusion injury [26]. Nitric oxide has also been reported to reduce calcium overload and mitochondrial permeability during ischemia reperfusion and at the same time activate mitochondrial K (ATP) channel [27]. Here in the present investigation only a negligible amount eNOS bound with caveolin-1 in tocotrienol treated heart whereas more eNOS bound with caveolin-1 in I/R heart indicating generation of a survival signal by free eNOS in the tocotrienol treated heart. Compared to alpha and gamma isomers, in delta tocotrienol treated heart caveolin-1 and eNOS interaction is much higher, which indicates that in delta tocotrienol treated heart, less eNOS is available to generate cardioprotection compared to the alpha and gamma tocotrienols. Overall observation indicates less cardio protective ability of delta tocotrienol.

In brief, in tocotrienol-treated hearts there was reduced association of p38MAPKβ, HO-1, eNOS (pro-survival signaling components) and increased association of p38MPAKα and Src kinase (anti-survival signaling components) with caveolin, indicating decreased availability of death signaling components and increased availability of pro-survival signaling components to induce cardioprotection. In I/R heart, interaction was completely reverse.

The most salient observation of the proteasome studies is a reverse relationship between efficacy of proteasome protection by tocotrienol isomers and their effect on myocardial infarct and apoptosis. In general, δ-tocotrienol produced the most proteasome protection but was the least efficacious in protecting the heart, whereas γ-tocotrienol was the least effective in preserving proteasome activity and the most effective in protecting the heart, with the α-isomer somewhere in the middle.

First, this observation does confirm our previous study [5, 6], however suggests that the overall protective effects of tocotrienols is not simply related to changes in Src kinase activation even though this effect was reproduced in the current study. Second, it is not clear why there was a differential effect of the tocotrienol isomers on proteasome activity, but could be related to differential compartmentalization or antioxidant potential with the δ-isomer reported to be the most effective [28, 29]. In addition, it appears that proteasome may respond to the stress of ischemia/reperfusion by up regulation of proteasome subunits even though these appear not to be functional. This phenomena was only observed in the δ-tocotrienol hearts, at least with respect to the Rpt5 subunit, and it is clear that any increased proteasome subunits are functional in this treatment group.

However, a more important interpretation of these results is that while preservation of proteasome activity is important for recovery of the ischemic heart, too much may be detrimental. Studies [30, 31] have suggested that proteasome subunits are subject to the same types of posttranslational regulation as any other signaling molecule and may actually be regulated by many of the same pathways that it itself regulates thus suggesting dynamic control within a certain range. It stands to reason that any movement outside of the control range may be detrimental, hence inhibition of cardiac proteasome during ischemia diminishes recovery [32, 33], and too much protection is not as protective to cardiac function overall. The cardiac hypertrophy literature provides precedence for this type of phenomena as activation of proteasome is associated with maladaptive hypertrophy secondary to pressure overload [34]. Exactly why this occurs is not clear, but it is tempting to speculate that too much proteasome activity may prevent some of the adaptive responses necessary for cardiomyocyte survival Whatever the mechanism, it does appear that differences in proteasome-mediated degradation of different proteins could affect their availability for association with different caveolin/lipid rafts and controls cardioprotection.

Acknowledgements

Funded in part by NIH HL068936 (to SRP) and HL22599, HL34360 and 56322 (to D.K.D).

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