Cardioprotection with palm tocotrienol: antioxidant activity of tocotrienol is linked with its ability to stabilize proteasomes

Samarjit Das,1,4 Saul R. Powell,2 Ping Wang,2 Andras Divald,2 Kalanithi Nesaretnam,3 Arpad Tosaki,4 Gerald A. Cordis,1 Nilanjana Maulik,1 and Dipak K. Das1

1Cardiovascular Research Center, University of Connecticut School of Medicine, Farmington, Connecticut; 2Long Island Jewish Medical Center Campus of the Albert Einstein College of Medicine, New Hyde Park, New York; 3Malaysian Palm Oil Board, Kuala Lumpur, Malaysia; and 4University of Debrecen, Debrecen, Hungary

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TOCOTRIENOLS ARE THE ISOFORMS of vitamin E that are abundant in cereal grains, including soybeans, barley, oats, rice bran, and palm oil. They differ from corresponding tocopherols only in their alphatic tail. The isoforms of tocotrienols differ in their methyl substitution in the chromanol ring and a 16-carbon hydrocarbon tail; the α-form contains three methyl groups, the β- and γ-forms have two methyl groups, and the δ-form has only one methyl group (41). Recent studies have demonstrated many health benefits of tocotrienols, including their anticancer and tumor-suppressive activities and their ability to lower cholesterol (11, 31).

Dietary tocotrienol derived from plant sources, especially palm oil, has been found to be beneficial against a variety of degenerative diseases. For example, supplementation with dietary tocotrienols from a tocotrienol-rich fraction of palm oil (TRF) reduced the concentration of plasma cholesterol and apolipoprotein B, thromboxane B2, and platelet factor 4, indicating its ability to protect against endothelial dysfunction and platelet aggregation (32). Dietary tocotrienols reduce concentrations of plasma cholesterol, apolipoprotein B, thromboxane B2, and platelet factor 4 in pigs with inherited hyperlipidemias (34). Supplementation with TRF reduced plasma cholesterol levels in a human pilot study (33). Tocotrienols from TRF inhibited the proliferation of human breast cancer cell lines (25). Tocotrienols also suppressed the growth of murine B16 melanomas in vitro and in vivo (18). In neutural cells, tocotrienols inhibited glutamate-induced pp60src kinase activation of HT4 neuronal cells (39). Many of the signaling pathways involved in cell cycling and proliferation are regulated by the ubiquitin-proteasome system (10, 12, 27). Recent studies (4, 29) have shown that the proteasome may be involved in cell death. In view of protective effects of tocotrienols in a variety of degenerative diseases, we hypothesized that tocotrienol could protect the hearts from ischemia-reperfusion injury.

We recently showed that ischemia of the isolated rat heart results in inhibition of the 26S proteasome and have hypothesized that the degree of inhibition may be related to the extent of recovery during the postischemic period (29). In these same studies, preischemic perfusion of the isolated rat heart with the proteasome inhibitor MG132 resulted in dose-dependent decrements in postischemic recovery associated with increased accumulation of ubiquitinated proteins in the myocardium, thus supporting this hypothesis. Because many in vitro studies in a variety of cell lines (for review see Refs. 12 and 27), including cardiomyocytes (3), and in at least one study in the isolated rat heart (28) show that proteasome inhibition of sufficient degree and duration can lead to apoptosis, we examined the possibility that TRF may preserve the proteasome activity in the postischemic heart. Isolated rat hearts were perfused with TRF (0.035%) for 15 min before they were subjected to 30 min of ischemia and 2 h of reperfusion. The results indicated that TRF was able to improve postischemic ventricular dysfunction and reduce myocardial infarct size and incidence of ventricular arrhythmias. TRF also reduced the ischemia-reperfusion-induced increase in e-Src phosphorylation and stabilized 20S and 26S proteasome activities.

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MATERIALS AND METHODS

Materials. TRF was supplied by the Malaysia Palm Oil Board. All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise mentioned.

Animals. 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 until the start of the experimental procedure. The rats were randomly assigned to one of three groups: the control group comprised isolated hearts perfused with Krebs-Henseleit bicarbonate buffer (KHB) for 15 min, and hearts of the experimental groups were perfused with TRF (0.035%) or 5 μM 4-amino-5-(4-methylphenyl)-7-(t-butyl)-pyrazolo-3,4-d-pyrimidine (PPI) under identical conditions. All hearts were then subjected to 30 min of ischemia followed by 2 h of reperfusion.

Isolated working 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 sufficient depth of anesthesia was ensured, a thoracotomy was performed and hearts were perfused in the retrograde Langendorff mode at 37°C at a constant perfusion pressure of 100 cmH2O (10 kPa) for a 5-min washout period (8). The perfusion buffer consisted of a modified KHB (in mM: 118 NaCl, 4.7 KCl, 1.7 CaCl2, 1.4 NaHCO3, 1.2 KH2PO4, 1.2 MgSO4, and 10 glucose). The Langendorff preparation was switched to the working mode after the washout period, as previously described (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O (4).
RESULTS

Effects of TRF on ventricular function. There were no differences in baseline function among the six groups. In general, there were no significant differences between TRF and control in heart rate and coronary flow (Table 1). As expected, on reperfusion, the absolute values of all functional parameters were decreased in all groups compared with the respective baseline values. TRF displayed significant recovery of postischemic myocardial function. The cardioprotective effects of TRF were evidenced by significant differences in the left ventricular dP/dt from 30 min of reperfusion onward, the difference is especially apparent at 60 and 120 min of reperfusion (Table 1). Similar to TRF, PPI also improved postischemic myocardial function. The cardioprotective effects of TRF on ventricular function.

Infarct size, the apoptotic cardiomyocytes were also further reduced when a combination of TRF and PPI was used: 4.8 ± 2.2% vs. 33.4 ± 2.44% (Fig. 1, bottom). The infarct size was further reduced when a combination of TRF and PPI was used (20.5 ± 2.44% vs. 33.4 ± 2.44% (Fig. 1, top)).

Effects of TRF on myocardial infarct size. Infarct size (percentage of infarct vs. total area at risk) was significantly higher in the hearts subjected to 30 min of ischemia and 2 h of reperfusion than in the hearts that were not subjected to the ischemia-reperfusion protocol (almost at the baseline level, data not shown). The values were noticeably reduced in TRF and PPI groups compared with the group subjected to ischemia-reperfusion: 25.1 ± 2.45 and 25.6 ± 2.33%, respectively, vs. 33.4 ± 2.44% (Fig. 1, top). The infarct size was further reduced when a combination of TRF and PPI was used (20.5 ± 2.2% vs. 33.4 ± 2.44% (Fig. 1, top).

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>10 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
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<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>345.92 ± 22.45</td>
<td>332.74 ± 25.22</td>
<td>339.37 ± 15.4</td>
<td>343.4 ± 35.57</td>
<td>414.28 ± 24.38</td>
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Values are means ± SE of 6 animals per group. Isolated rat hearts were preperfused with Krebs-Henseleit bicarbonate buffer in the absence or presence of 0.035% tocotrienol-rich fraction of palm oil (TRF) and without or with 4-amino-5-(4-methylphenyl)-7-(t-buty1)-pyrazolo-3,4-d-pyrimidine (PPI) before 30 min of ischemia followed by 2 h of reperfusion. LVDP, left ventricular developed pressure; LV dP/dt, maximum 1st derivative of developed pressure. *P < 0.05 vs. control.

Effects of TRF on cardiomyocyte apoptosis. Ischemia-reperfusion caused the cells to undergo apoptosis, as expected. The percentage of apoptotic cardiomyocytes was significantly reduced in the TRF and PPI groups compared with the control group: 5.7 ± 1.3 and 6.6 ± 2.2%, respectively, vs. 22.0 ± 1.7% (Fig. 1, bottom). As observed for the infarct size, the apoptotic cardiomyocytes were also further reduced when a combination of TRF and PPI was used: 4.8 ± 0.8% vs. 22.0 ± 1.7% (Fig. 1, bottom).
TRF not only prevented this decrease, but it actually appeared to activate both proteasomes to levels significantly (P < 0.05) higher than the ischemic values. After 120 min of reperfusion, 20S proteasome activity recovered to a level not different from baseline, and TRF had no effect. However, 26S proteasome activity was still significantly (P < 0.05) depressed by 58%, which was prevented in the TRF-treated hearts (Fig. 4). Treatment of hearts with PPI had no protective effects on postischemic proteasome activities (data not shown).

**DISCUSSION**

There are several salient features of the present study. 1) Palm tocotrienol was found to provide cardioprotection, as evidenced by reduction of the ischemia-reperfusion-mediated increase in ventricular dysfunction, ventricular arrhythmias, and myocardial infarct size. 2) Palm tocotrienol reduced ischemia-reperfusion-induced activation of c-Src activities. 3) Tocotrienol stabilized proteasomes by preventing the ischemia-reperfusion-mediated reduction of 26S and 20S proteasomes. The results of the study thus showed, for the first time, that beneficial effects of tocotrienol are due to its ability to reduce c-Src activation, which is linked with the stabilization of proteasomes. Tocotrienols have extremely short half-lives; after oral ingestion, they are not recognized by α-tocotrienol transport protein, which also accounts for their low bioavailability. For this reason, TRF was used in an acute experiment to determine its immediate effects on the ischemic-reperfused myocardium. The results indicate that tocotrienol readily blocks the ischemia-reperfusion-mediated increase in Src kinase activation and proteasome inactivation, thereby providing cardioprotection.

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. Activation of Src kinase is associated with many degenerative diseases, including cardiovascular diseases, oncogene-
thereby, becomes a target for the 26S proteasome. For exam-
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the Src family of kinases (14, 15, 26), undergo suicide regu-
larases, such as protein kinase C (22) and several members of
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The Src kinases are also involved in progression of the G2-
tivity is elevated (24). Markedly elevated levels of c-Src kinase
activity were detected in human skin tumors (1). Myocardial
ischemia-reperfusion caused an induction of c-Src protein
expression (17); inhibition of c-Src with PPI reduces the extent
of cellular injury.
Signaling pathways of Src kinase involve its activation through
the activated cell surface receptors. For example, binding of ligand to platelet-derived growth factor receptors
causes ligand to be associated with and to activate the Src
family kinases (35), which trigger a cascade of events leading
to entry into the S phase and subsequent DNA replication (2).
The Src kinases are also involved in progression of the G2-
to-M transition of the cell cycle (37). In addition, Src kinases
can also transduce signals in response to cell-cell or cell-matrix
adhesion (19).
The present studies confirm previous studies (4, 29) that
presented evidence of inactivation of 20S and 26S proteasomes
during myocardial ischemia-reperfusion. When proteasome is
inhibited, cell cycle regulatory proteins and proapoptotic fac-
tors, normally inactivated by this complex, can accumulate.
Several studies have shown that many activated protein ki-
nases, such as protein kinase C (22) and several members of
the Src family of kinases (14, 15, 26), undergo suicide regu-
lation, whereby the activated form is rapidly ubiquitinated and,
thereby, becomes a target for the 26S proteasome. For exam-
ple, Blk, an Src family member, is recognized by the E3
ubiquitin-protein ligase E6AP, which promotes its ubiquitina-
tion and subsequent degradation by the 26S proteasome (26).
Other studies (27, 36) indicate that Src itself is degraded in a
ubiquitin-dependent manner and that the active form is specif-
ically targeted for degradation, thus indicating a negative
regulatory function for the proteasome. It is conceivable that
decreased proteasome activity in postischemic hearts accounts
for part of the observed increased Src and phosphorylated Src,
which normally signals ubiquitination (36). This interpretation
is strongly supported by the observation that preserving pro-
teasome activity and, in particular, 26S proteasome activity,
might mitigate this increase. On the other hand, PPI has no protec-
tive effects on postischemic proteasome activities but, rather,
directly inhibits c-Src, thus exerting an overall cytoprotective
effect that is not mediated through the proteasome. In combi-
nation, these results support the conclusion that c-Src activa-
tion has a large role in postischemic cardiac injury and dys-
function.
The notion that preserving proteasome function in the postis-
chemic heart can be protective may appear to be at odds with
two studies (5, 30) that suggest protective effects on the
ischemic myocardium of the proteasome inhibitor PS-519
(Millenium Pharmaceuticals). These studies (5, 30) used the
inhibitor to limit the inflammatory response by decreasing
leukocyte adhesion to endothelial cells. One of these studies
(5) demonstrated positive effects in the leukocyte-supple-
mented crystalloid-perfused heart preparation but failed to
observe any effect of the inhibitor, positive or negative, in the
absence of the leukocytes. Although both of these studies (5,
30) determined peripheral leukocyte 20S proteasome activity,
neither measured myocardial 20S or 26S proteasome activity,
nor did they measure levels of ubiquitin-conjugated proteins,
and it is not clear whether the beneficial effect was related to
myocardial proteasomes. The ability of proteasome inhibitors
to decrease the inflammatory response has been well docu-
mented (7) and, besides effects on leukocyte adhesion, has
been attributed to decreased ubiquitin-mediated degradation of
NF-κB nuclear translocation (6). Whether a proteasome inhib-
itor has a beneficial (anti-inflammatory) or negative (proapop-
totic) effect is notoriously dose related (21) and will be some-
what dependent on degrees of proteasome activity in the
different tissues (i.e., leukocyte vs. heart). When little or no
proteasome inhibition is present, such as after brief ischemia,
the decrease in leukocyte-mediated inflammation may be benefi-
cial. However, in the presence of decreased proteasome activity,
an inhibitor that adds to ischemia-mediated proteasome inhibition
may tip the cell toward death, but an agent, such as TRF, which protects the proteasome, may be beneficial, as
shown in this study.
The mechanism by which TRF preserves proteasome activity
is not completely understood from these studies. It is
tempting to speculate that TRF acts as an antioxidant,
preventing oxidative inactivation during ischemia. The 20S
and 26S proteasomes have been shown to be vulnerable to
oxidative inactivation, with 26S proteasome significantly
more vulnerable (36). Indeed, Bulteau et al. (4) showed that
subunits of the 20S proteasome are oxidatively modified
during myocardial ischemia. However, a recent study (20)
suggests that certain antioxidants isolated from cruciferous
vegetables are capable of upregulating expression of several

subunits of the proteasome, an effect observed 24 h after treatment. In light of the rather short treatment and post-treatment intervals used in the present experiments, it is unlikely that proteasome upregulation could account for the increased proteasome activity. Nonetheless, proteasome activity was increased to levels greater than baseline, indicating activation. This suggests mechanisms that include other than simple antioxidation, possibly redox effects, as has been suggested for the 20S proteasome (9).

In summary, ischemia-reperfusion caused ventricular dysfunction, electrical rhythm disturbances, and increased myocardial infarct size. PPI or TRF could reverse the ischemia-reperfusion-infarct size. PPI or TRF could reverse the ischemia-reperfusion-electrical rhythm disturbances, and increased myocardial infarct size. PPI or TRF could reverse the ischemia-reperfusion-association of increased ubiquinated proteins with cardiac apoptosis. 

REFERENCES


