

Characteristics of Catechin- and Theaflavin-Mediated Cardioprotection

HENRYK DREGER, MARIO LORENZ, ALEXANDRA KEHRER, GERT BAUMANN, KARL STANGL, AND VERENA STANGL¹

Medizinische Klinik mit Schwerpunkt Kardiologie und Angiologie (Campus Mitte), Charité – Universitätsmedizin Berlin, D-10117 Berlin, Germany

Catechins and theaflavins—the main polyphenolic substances of green and black tea, respectively—exert a plethora of beneficial effects on the cardiovascular system. In a model of H₂O₂-mediated oxidative stress, we investigated the effects of epigallocatechin-3-gallate (EGCG) and theaflavin-3,3'-digallate (TF3) on neonatal rat cardiomyocytes. Pretreatment with EGCG or TF3 1 hr prior to induction of oxidative stress by H₂O₂ effectively protected cardiac myocytes as determined by measuring release of lactate dehydrogenase after 24 hrs. Longer pre-incubation times resulted in significant loss of protection. To enable further mechanistic insight, we investigated expression of antioxidative enzymes and activation of prosurvival signaling cascades. Whereas mRNA levels of glutathione peroxidase 3, superoxide dismutase 1, and catalase were not influenced by both polyphenols, heme oxygenase (HO-1) was selectively upregulated by EGCG—but not by TF3. However, inhibition of HO-1 did not diminish polyphenol-mediated cardioprotection. While EGCG and TF3 activated Akt, extracellular signal-regulated kinase 1/2, and p38 mitogen-activated protein kinase, inhibition of these kinases did not attenuate polyphenol-mediated protection. Loading of cardiomyocytes with dichlorofluorescein revealed that intracellular levels of reactive oxygen species were significantly reduced after treatment with EGCG or TF3 as early as 30 mins after induction of oxidative stress. In conclusion, activation of prosurvival signaling kinases and upregulation of antioxidative enzymes do not play a major role in tea polyphenol-mediated cardioprotection. *Exp Biol Med* 233:427–433, 2008

Key words: oxidative stress; cardiac myocytes; epigallocatechin-3-gallate; theaflavin-3,3'-digallate; heme oxygenase 1

Introduction

Ranking after water, tea represents the second most frequently consumed beverage worldwide and is one of the major sources of dietary polyphenols. Vast epidemiological data indicate an inverse correlation between incidence rates of cardiovascular diseases and tea consumption (1–3). These findings have prompted numerous studies with evidence that tea polyphenols exert a plethora of antioxidant, anti-inflammatory, antiproliferative, and antithrombotic effects (4).

Oxidative stress plays a major role in the pathophysiology of various cardiovascular diseases such as atherosclerosis and ischemia-reperfusion injury. It is characterized by elevated levels of partially reduced metabolites, for example, hydrogen peroxide, superoxide anions, and hydroxyl radicals. These reactive oxygen species (ROS) severely damage proteins, DNA, and lipids. Accordingly, treatment with antioxidants (5, 6), chelators (7), and antioxidative enzymes (8–11) has been suggested to improve cardiac function, enhance myocyte survival, and reduce infarction size in models of ischemia-reperfusion injury of the heart.

Both catechins and theaflavins—the major polyphenolic ingredients of green and black tea, respectively—are excellent electron donors and efficient scavengers of free radicals such as superoxide anions, singlet oxygen, nitric oxide, and peroxynitrite (12, 13). The beneficial cardiovascular effects of tea consumption have therefore been primarily attributed to the potent ROS scavenging properties of tea polyphenols. In addition, there is some evidence that the induction of endogenous antioxidative enzymes by tea polyphenols further contributes to their antioxidative effect. Wu *et al.* observed a PI3K/Akt-dependent induction of heme oxygenase 1 (HO-1) in human endothelial cells after treatment with epigallocatechin-3-gallate (EGCG) that led to protection against oxidative stress (14).

The aim of our study was to investigate whether EGCG and theaflavin-3,3'-digallate (TF3) protect cardiac myocytes against oxidative stress and to elucidate whether additional

¹ To whom correspondence should be addressed at Medizinische Klinik m. S. Kardiologie und Angiologie (CCM), Charité – Universitätsmedizin Berlin, Charitéplatz 1, D – 10117 Berlin, Germany. E-mail: verena.stangl@charite.de

Received October 29, 2007.
Accepted December 03, 2007.

DOI: 10.3181/0710-RM-292
1535-3702/08/2334-0427\$15.00
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effects of tea polyphenols—beyond their radical scavenging properties—contribute to cardioprotection.

Materials and Methods

Primary Cardiomyocyte Cell Culture. Model of Oxidative Stress. Cardiomyocytes were prepared as described previously (15) and treated with 50 μ M EGCG (Sigma-Aldrich, Munich, Germany), 20 μ M TF3 (kindly provided by Mitsui Norin Food Research Laboratories, Japan), or solvent (water) for 1, 4, 8, or 24 hrs. For induction of oxidative stress, cells were washed with phosphate-buffered saline (PBS) and incubated with 100 μ M H₂O₂ for 30 mins in serum-free medium. For further experiments, cells were kept in serum-free M199 for up to 24 hrs (Fig. 1A).

Inhibition of RNA and Protein Synthesis, HO-1, and Signaling Cascades. Cardiomyocytes were incubated with inhibitors of HO-1 (10 μ M tin protoporphyrin IX [SnPPiX], Frontier Scientific, Logan, UT); RNA polymerase II (5 μ g/ml α -amanitin, Sigma-Aldrich, Munich, Germany); protein synthesis (1 μ g/ml cycloheximide [CHX], Sigma-Aldrich, Munich, Germany); phosphoinositide kinase-3 (PI3K) (100 nM wortmannin, Calbiochem, Darmstadt, Germany); mitogen-activated protein kinase-1 (MEK1) (50 μ M PD98059, Calbiochem, Darmstadt, Germany); p38 mitogen-activated protein kinase (p38 MAPK) (50 μ M SB203580; Calbiochem, Darmstadt, Germany); or solvent (0.1% dimethyl sulfoxide [DMSO]) 30 mins before addition of tea polyphenols to the medium. After 1 hr, cardiomyocytes were washed and treated with or without H₂O₂ for 30 mins. Following removal of H₂O₂, cells were kept in serum-free medium for 24 hrs. Fresh inhibitors were added after each wash step to ensure sufficient inhibition throughout the experiment.

Determination of Lactate Dehydrogenase Release. To provide a marker for cell damage, we quantified the release of lactate dehydrogenase (LDH) using the CytoTox-ONE Homogenous Membrane Integrity Assay (Promega, Mannheim, Germany) 24 hrs after removal of H₂O₂.

RNA Preparation and Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis. Cardiomyocytes were treated with 50 μ M EGCG, 20 μ M TF3, or solvent for 1 hr, followed by a wash step and incubation for another 4 hrs with fresh, serum-free medium. Total RNA was extracted as described elsewhere (16), and cDNA was amplified by RT-PCR using a 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR primers for HO-1, glutathione peroxidase 3 (GPx3), superoxide dismutase 1 (SOD1), catalase, and the housekeeping gene hypoxanthine phosphoribosyltransferase cDNAs were purchased from TIB MOLBIOL (Berlin, Germany). Relative expression of target genes was normalized to expression under control conditions by the comparative C_t-method ($2^{-\Delta\Delta C_t}$).

To inhibit mRNA synthesis, myocytes were treated with 5 μ g/ml α -amanitin 30 mins before addition of tea polyphenols. Fresh inhibitor was added after each wash step to ensure sufficient inhibition of RNA polymerase II throughout the experiment.

Western Blot Analysis. Total cell extracts were prepared in RIPA buffer (50 mM Tris, [pH 7.5] 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing protease complete, (Roche Applied Science, Mannheim, Germany) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate). Cellular debris was removed by centrifugation. Protein content was determined using the Bradford method (Pierce, Rockford, IL). Total protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and membranes were probed with the phospho-specific antibodies against Akt, extracellular signal regulated kinase 1/2 (ERK 1/2), and p38 MAPK (Cell Signaling, Beverly, MA). Western blots were stripped and reprobed against total α -actin (Sigma-Aldrich, Munich, Germany) as a loading control.

Measurement of Intracellular Reactive Oxygen Species Formation. Cardiomyocytes were loaded for 30 mins at 37°C in the dark with 25 μ M dichlorofluorescein diacetate (DCFDA, Invitrogen, Karlsruhe, Germany). DCFDA is cleaved by cellular esterases to nonfluorescent dichlorofluorescein, which is oxidized by intracellular ROS to the fluorescent product dichlorofluorescein. Stained cells were treated for 1 hr with 50 μ M EGCG, 20 μ M TF3, or solvent, followed by a wash step and incubation with 100 μ M H₂O₂ in PBS buffer for 30 mins. Cardiomyocytes were then trypsinized, and fluorescence was quantified in a CyAn ADP flow cytometer (DakoCytomation, Glostrup, Denmark).

Statistics. Data are expressed as mean $\pm$ SEM. We calculated significance by *t*-test or one-way ANOVA where appropriate (SPSS 11.0, Chicago, IL). An error probability of *P* < 0.05 was regarded as significant.

Results

Tea Polyphenols Protect Cardiomyocytes Against Oxidative Stress. Pretreatment of myocytes with EGCG (50 μ M) and TF3 (20 μ M) completely abolished H₂O₂-induced LDH release as a marker for cell damage when tea polyphenols were added 1 hr before H₂O₂. Longer incubation times from 4 to 24 hrs resulted in significant decrease and eventual complete loss of tea polyphenol-mediated cardioprotection (Fig. 1B).

Induction of Antioxidative Enzymes Is Not Requisite for Tea Polyphenol-Mediated Cardioprotection. To elucidate whether upregulation of antioxidative enzymes contributes to the observed protective effects, we measured mRNA levels of SOD1, GPx3, and catalase in cardiomyocytes. mRNA levels of these antioxidative enzymes were not affected by EGCG or TF3 treatment

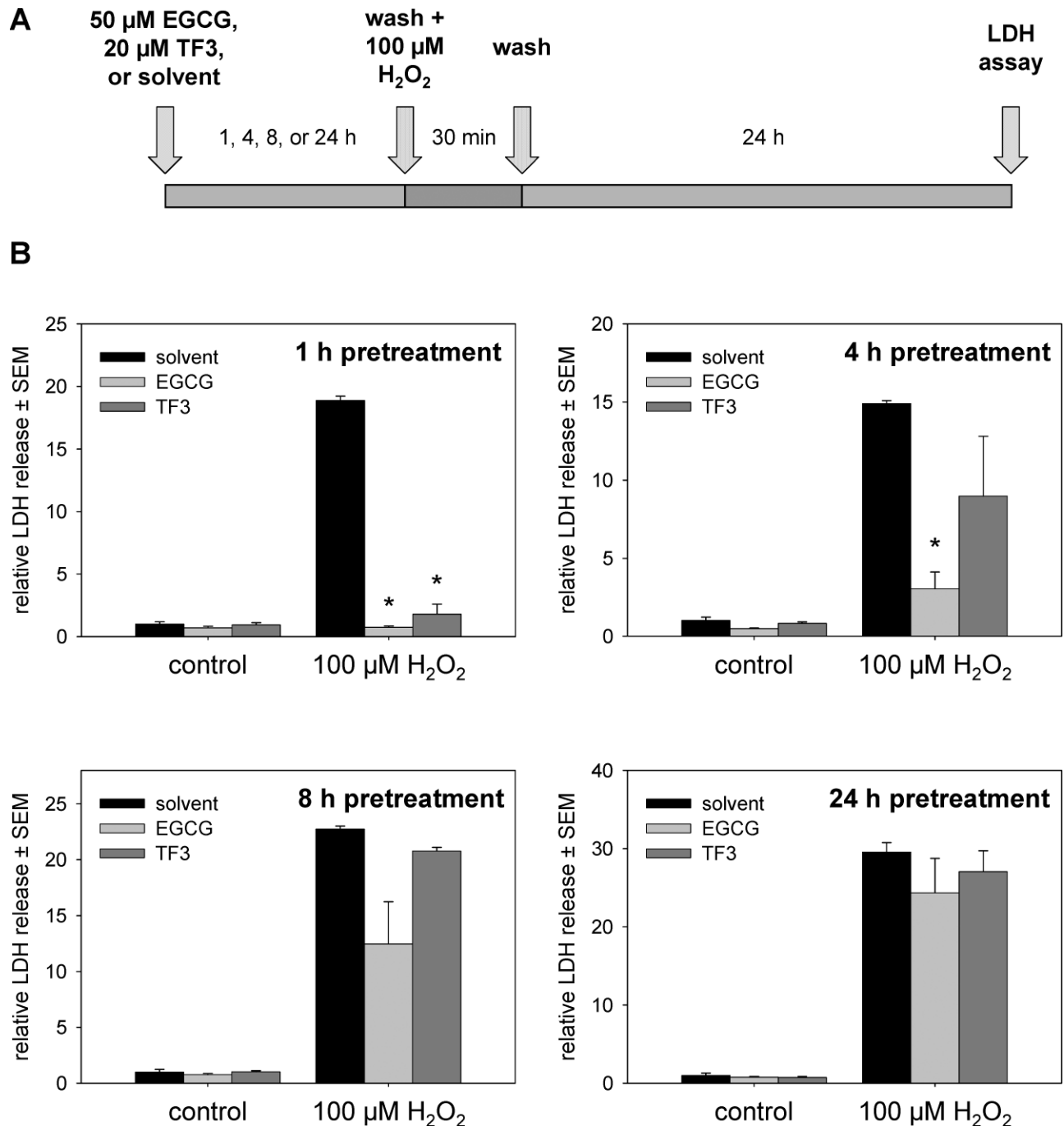


Figure 1. EGCG and TF3 protect cardiomyocytes against H_2O_2 -mediated cell damage. (A) Cells were pretreated with 50 μ M EGCG, 20 μ M TF3, or solvent for 1, 4, 8, or 24 hrs, followed by incubation with 100 μ M H_2O_2 for 30 mins. (B) LDH release as a marker for cell damage was quantified 24 hrs after removal of H_2O_2 ($n = 3$, * $P < 0.05$ versus solvent + H_2O_2).

(Fig. 2A, left panel). However, EGCG significantly induced the expression of HO-1 mRNA by 3.9-fold. This upregulation was prevented by pretreatment with the RNA polymerase II inhibitor α -amanitin, which suggests *de novo* synthesis of HO-1 mRNA. In contrast, TF3 failed to significantly increase HO-1 mRNA levels (Fig. 2A, right panel).

Next, we addressed the question of whether induction of HO-1 or *de novo* synthesis of other cellular antioxidative

enzymes contributes to tea polyphenol-mediated cardioprotection. We accordingly pretreated cardiomyocytes with the HO-1 inhibitor SnPPiX, the RNA polymerase II inhibitor α -amanitin, and the protein synthesis inhibitor cycloheximide, 30 mins before incubation of cells with EGCG or TF3. As depicted in Figure 2B, none of the above inhibitors significantly modulated polyphenol-mediated cardioprotection after challenging cells with H_2O_2 as assessed by quantification of LDH release (Fig. 2B).

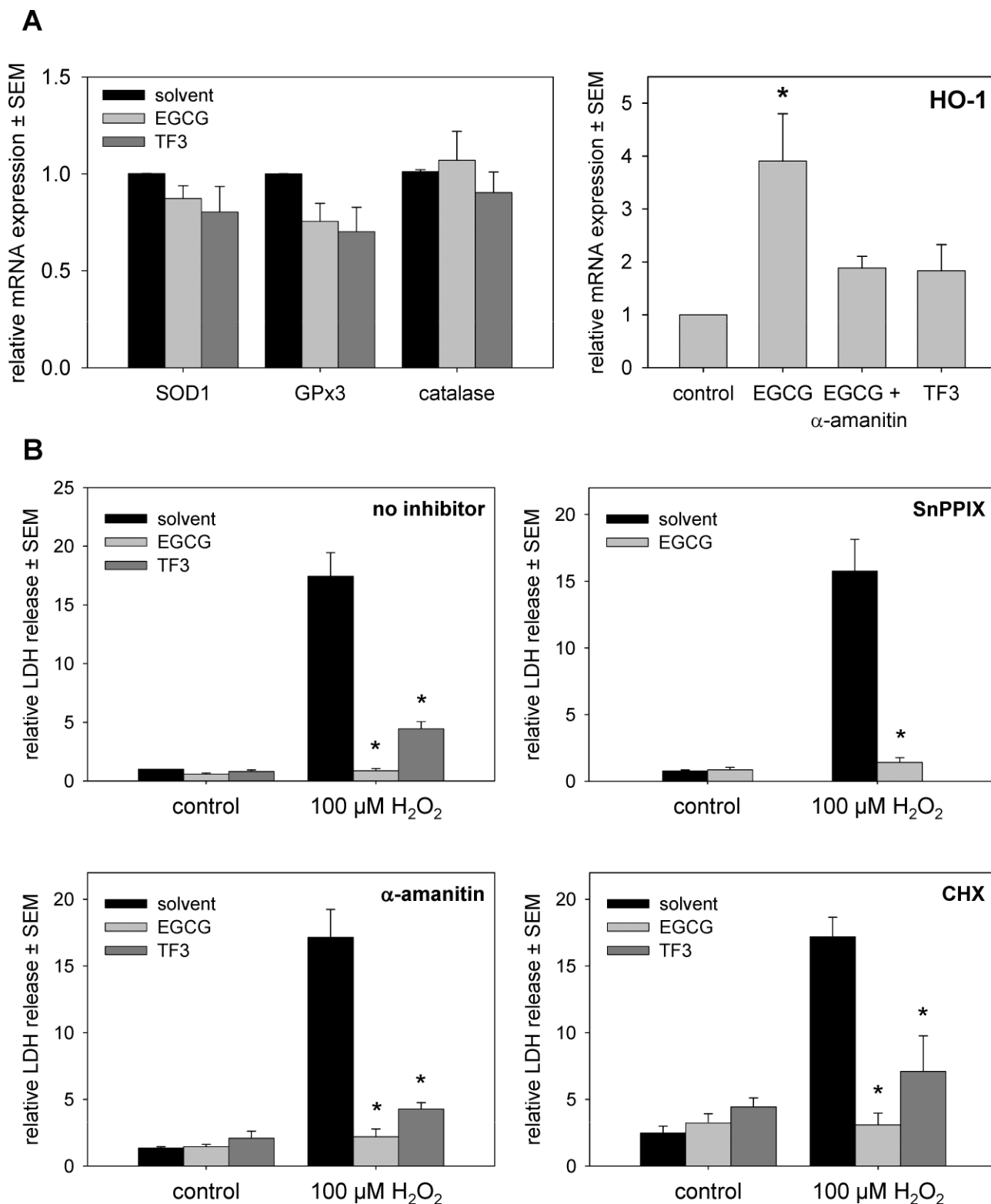


Figure 2. Tea polyphenols confer cardioprotection independently of induction of antioxidative enzymes. (A) mRNA expression of SOD1, GPx3, catalase, and HO-1, 5 hrs after treatment with 50 μ M EGCG, 20 μ M TF3, or solvent for 1 hr. Where indicated, RNA synthesis was inhibited by incubation with 5 μ g/ml α -amanitin ($n = 3$, $*P < 0.05$ versus solvent control). (B) LDH release, 24 hrs after induction of oxidative stress by incubation with H_2O_2 . Myocytes were pretreated with inhibitors of HO-1 (10 μ M tin protoporphyrin IX, SnPPIX), mRNA synthesis (5 μ g/ml α -amanitin), or protein synthesis (1 μ g/ml cycloheximide, CHX). 30 mins after treatment with inhibitors, 50 μ M EGCG or 20 μ M TF3 were added for 1 hr, followed by incubation with 100 μ M H_2O_2 for 30 mins ($n = 3$, $*P < 0.05$ versus solvent + H_2O_2). Since TF3 did not induce HO-1 expression, it was not included in experiments using SnPPIX as an HO-1 inhibitor.

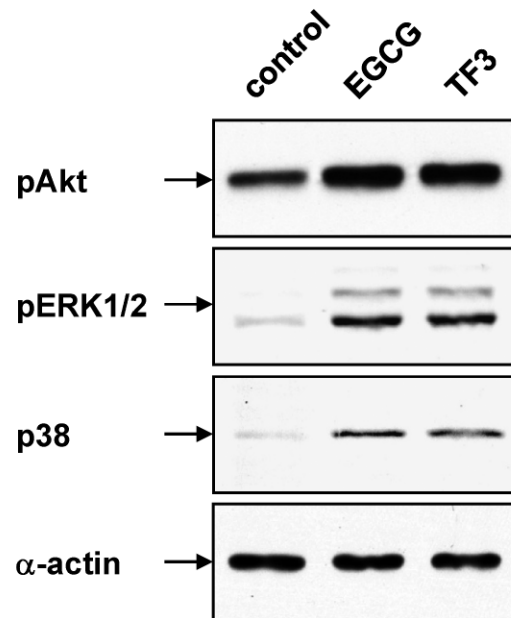
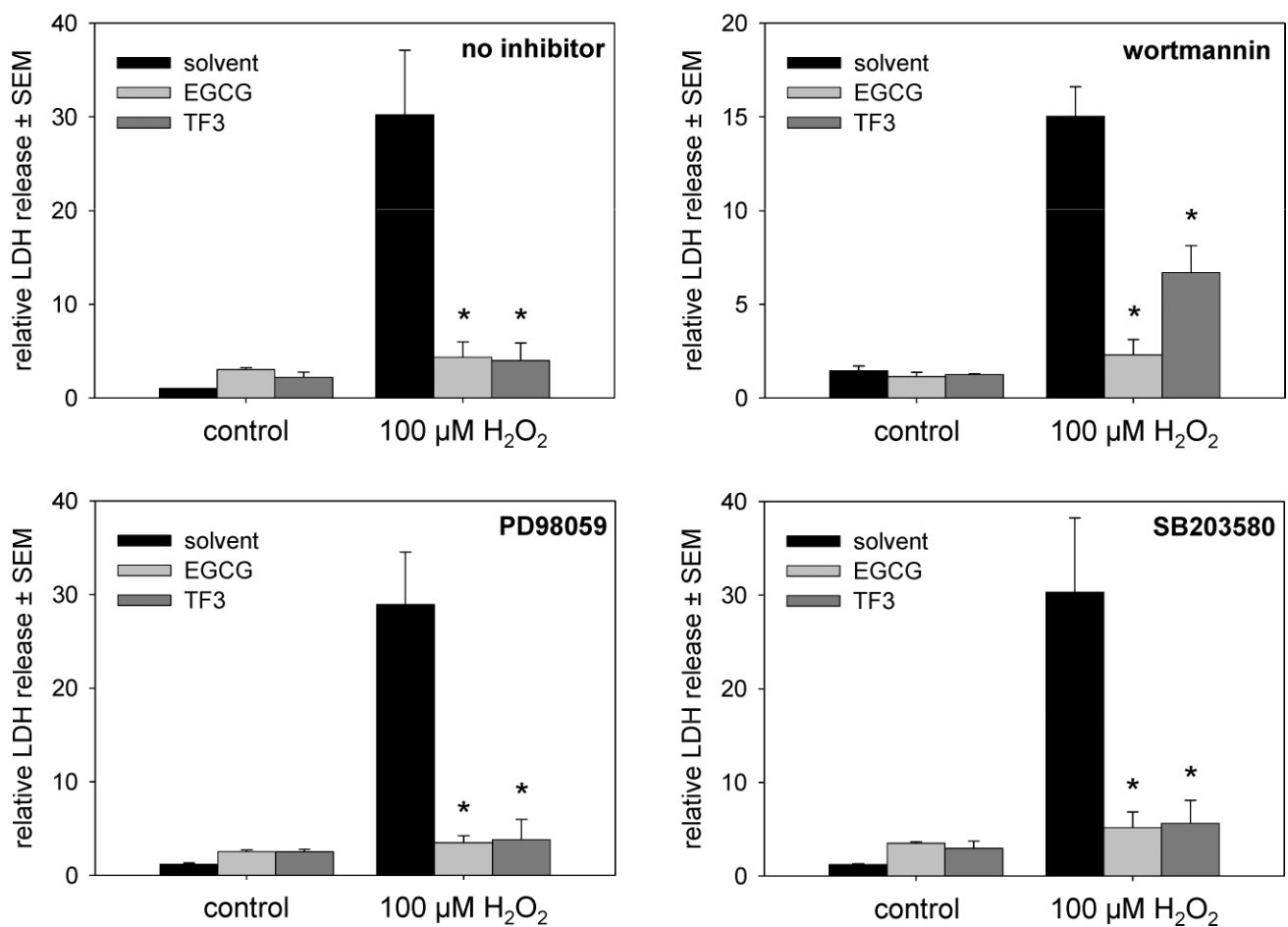
A**B**

Figure 3. Tea polyphenols confer cardioprotection independently of activation of protective signaling cascades. (A) Western blots of phospho-Akt, phospho-ERK1/2, phospho-p38 MAPK, and α-actin, 1 hr after treatment with 50 μM EGCG or 20 μM TF3. (B) LDH release, 24 hrs after induction of oxidative stress by incubation with H₂O₂. Myocytes were pretreated with inhibitors of PI3K (100 nM wortmannin), MEK1 (50 μM PD98059), or p38 MAPK (50 μM SB203580). Thirty mins after treatment with inhibitors, 50 μM EGCG or 20 μM TF3 were added for 1 hr, followed by incubation with 100 μM H₂O₂ for 30 mins (*n* = 3, **P* < 0.05 versus solvent + H₂O₂).

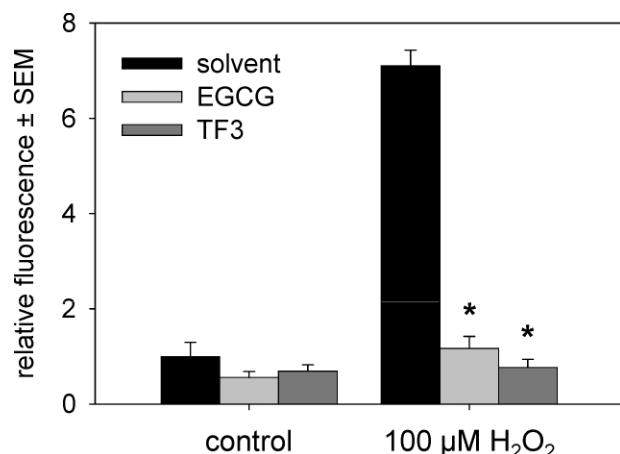


Figure 4. Tea polyphenols suppress ROS formation. Myocytes were loaded with 25 μ M DCFDA for 30 mins, and treated with 50 μ M EGCG or 20 μ M TF3 for 1 hr, followed by induction of oxidative stress with 100 μ M H₂O₂. Cells were trypsinized 30 mins after addition of H₂O₂ ($n = 3$, * $P < 0.05$ versus solvent + H₂O₂).

Tea Polyphenol-Mediated Cardioprotection Does Not Depend on Activation of Protective Signaling Cascades. To gain further insights into the mechanisms contributing to EGCG- and TF3-dependent reduction of cell damage after oxidative stress, we analyzed major signaling kinases involved in the regulation of apoptosis by phospho-specific immunoblotting. Treatment of cells with EGCG or TF3 resulted in pronounced phosphorylation, that is, activation of Akt, ERK 1/2, and p38 MAPK (Fig. 3A). To determine whether activation of these kinases is requisite for the observed tea polyphenol-mediated cardioprotection, we pretreated myocytes with inhibitors of PI3K (wortmannin), MEK1 (PD98059), and p38 MAPK (SB203580), 30 mins before incubation of cells with EGCG or TF3. As shown in Figure 3B, cardioprotection by EGCG and TF3 remained unaffected by inhibition of any of these kinases (Fig. 3B).

EGCG and TF3 Suppress ROS Formation. Since the upregulation of antioxidative enzymes and the activation of prosurvival kinases are evidently not involved in EGCG- and TF3-mediated cardioprotection, we investigated the ability of both tea polyphenols to suppress ROS levels. After loading myocytes with a fluorescent marker for intracellular ROS, treatment with EGCG or TF3 significantly reduced ROS-mediated fluorescence as early as 30 mins after induction of oxidative stress (Fig. 4). These data suggest that the beneficial effects of tea polyphenols are due to their ability to directly scavenge free radicals.

Discussion

Here we show that both EGCG and TF3—the main polyphenols in green and black tea, respectively—are capable of protecting cardiac myocytes against oxidative stress. Pretreatment with these polyphenols for 1 hr completely abolished H₂O₂-induced cellular damage,

whereas longer pre-incubation times resulted in rapid decrease of polyphenol-mediated protection. In accordance with the half-life of the polyphenols in cell culture, the beneficial effects of EGCG and TF3 were completely lost after pre-incubation of 24 and 8 hrs, respectively (17, 18). Since upregulation of endogenous antioxidants would be expected to result in more delayed and sustained cardio-protection, our observations argue for direct protective properties of these polyphenols.

In concurrence with our findings, polyphenol-mediated cytoprotection has primarily been attributed to the ability of polyphenols to effectively scavenge free radicals (19). However, a number of studies in different cell types suggest additional effects of polyphenols. Townsend and coworkers demonstrated inhibition of apoptosis by EGCG-mediated activation of signal transducer and activator of transcription-1 (STAT-1) in cardiac myocytes during postischemic reperfusion (20). In an *in vivo* model of myocardial infarction, Aneja et al. observed ameliorated myocardial damage after treatment with EGCG that correlated with reduced degradation of nuclear factor κ B inhibitor α (I κ B α) and nuclear factor κ B activity (21). In addition, Negishi et al. reported upregulation of catalase in aortas of spontaneously hypertensive rats after oral consumption of green tea polyphenols (22). Whereas the expression of SOD1, GPx3, and catalase remained unaffected by both polyphenols in cardiac myocytes, we observed selective upregulation of HO-1 by EGCG. In a recent study, induction of HO-1 in cardiac myocytes by resveratrol—a polyphenolic phytoalexin found in red grapes—was shown to reduce infarction size in an *in vivo* model of myocardial ischemia reperfusion injury (23). Upregulation of HO-1 by EGCG, therefore, may in fact contribute to its protective effects on cardiac myocytes. Indeed, Wu and coworkers recently demonstrated that induction of HO-1 by EGCG in endothelial cells correlated with protection against oxidative stress (14). In our study, however, direct inhibition of HO-1 as well as inhibition of RNA and protein synthesis did not diminish EGCG-mediated protection of cardiac myocytes which indicates that upregulation of HO-1 plays no major role in cardioprotection by EGCG.

To obtain further insights into the underlying mechanisms involved in antioxidative protection, we assessed the effects of both polyphenols on prosurvival pathways in cardiomyocytes. Both EGCG and TF3 activated Akt, ERK 1/2, and p38 MAPK that are known to exert anti-apoptotic effects (24–26). Surprisingly, inhibition of these pathways did not significantly influence the protective effects of tea polyphenols—which indicates that activation of these signaling cascades is not required for EGCG- and TF3-mediated cardioprotection.

While a recent publication suggests that EGCG itself exerts oxidant activities (27), we did not observe an increase of ROS levels after treatment with EGCG alone. On the contrary, further experiments revealed a suppression of ROS

levels by polyphenol treatment as early as 30 mins after induction of oxidative stress.

The polyphenol concentrations used in our model are well within the range of doses employed by most previous *in vitro* studies (14, 20). However, due to the low bioavailability of catechins and theaflavins, tea consumption results in polyphenol plasma levels that are considerably lower (28). Therefore, further *in vivo* studies are needed to confirm our data.

Taken together, EGCG and TF3 render protection in cardiomyocytes against oxidative stress independently of upregulation of antioxidative enzymes or activation of prosurvival kinases.

We thank Minoo Moobed and Wanda Michaelis for their excellent technical assistance.

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