JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Green Tea Protects Cytoskeleton from Oxidative Injury in Cardiomyocytes

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Cardiac ischemia/reperfusion injury results in oxidative stress and poor physiological recovery. Episodes of hypoxia/reoxygenation (H/R) cause some subtle functional and structural alterations in sarcolemma, mithocondria, sarcoplasmic reticulum, nucleus, as well as cytoskeleton. In this report, by using cultured rat cardiomyocytes and laser confocal microscopy we have verified the possibility to counteract cytoskeleton alterations induced by H/R with the supplementation of an antioxidant agent, a green tea extract (GTE), and compared its effects to those of α-tocopherol. Moreover the effects of GTE on cell viability and cytosolic antioxidant activity have been evaluated. H/R induced myocardial damage occurs as histological alterations such as degeneration and disorganization of the cytoskeleton and loss of structural integrity of the nucleus. GTE supplementation increases cytosolic antioxidant activity and shows protective effects on cardiomyocyte cytoarchitecture and viability.

KEYWORDS: Green tea; cardiomyocytes; cytoskeleton; hypoxia/reoxygenation

INTRODUCTION

A large body of experimental literature supports the notion that reactive oxygen species (ROS) play an important role in cardiac ischemia/reperfusion by producing lethal cell injury (*1*−*6*). The mechanisms of the enhanced ROS generation and of ROS attack on cellular and subcellular targets are not well established, although mitochondria are considered an important locus of ROS production (*7*, *8*).

Ischemia followed by reperfusion triggers a series of events in which the production of ROS may overwhelm the defensive capacity of the antioxidant system (*9*); thus, an additional mechanism to increase the survival of tissues exposed to free radicals produced during hypoxia/reoxygenation (H/R) may be achieved by providing free radical scavengers, which possess the ability to protect cells against oxidative stress. A large variety of antioxidants has been tested for their possible protective effect against H/R injury of the heart; however, the results are controversial (*10*, *11*). Green tea (GT) is an excellent source of polyphenol antioxidants, particularly of a group of compounds known as green tea catechins (GTCs) (*12*). GT refers to a nonfermented product in which GTCs are more preserved than in partially fermented (oolong or pouchong tea) or fully fermented tea (black or pu-erh tea). GT consumption has been demonstrated to protect against chronic illnesses such as cardiovascular diseases and cancer (*13*). Moreover, GTE has been previously demonstrated to be able to influence the expression and activity of the enzyme nitric oxide synthase (iNOS) in H/R (*14*), and it has been recently demonstrated that the inducible isoform of iNOS colocalizes with the cytoskeleton, including actin microfilaments, microtubules, and intermediate filaments.

The components of the cytoskeleton are involved in a variety of cellular processes such as cell growth and division, cell movement, vesicles transportation, cellular organelle location and function, localization and distribution of membrane receptors, and cell−cell communications. The cytoskeleton in cardiac myocytes is believed to play an important role in the transduction of mechanical signals, based upon the unique distribution of the extensive cytoskeletal network involved in the maintenance of the juxtaposition of ion channels and the nuclear envelope through a delicate network, comprising actin filaments (*15*, *16*).

Actin filaments (F-actin) exist in a highly regulated and dynamic equilibrium with the monomeric form (G-actin). Since the state of actin filaments is dependent upon the amount of available ATP, and since ATP levels decrease during hypoxia (*17*), ATP depletion would result in a net depolymerization of F-actin (*18*).

The aims of the present investigation were to evaluate in cultured rat cardiomyocytes the effects of H/R on cytoskeletal alterations by confocal microscopy, on cell viability, and on cytosolic antioxidant activity and to verify the possibility to counteract H/R induced alterations by the supplementation of a Chinese green tea extract (GTE), whose content and composition in catechins is reported in (*19*), in comparison with

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10.1021/jf062859r CCC: $33.50
Published on Web 12/01/2006

α-tocopherol (α-TC), a well-known protective agent against H/R injury in isolated hearts (*20*).

MATERIALS AND METHODS

Chemicals. Horse serum (HS), fetal calf serum (FCS), Ham F10 culture medium, α-tocopherol (α-TC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 2,2′-azinobis-(3-ethylbenzothiazoline-6 sulfonic acid) (ABTS•$^+$), and FITC conjugated secondary antibody were purchased from Sigma Chemical Co. (St. Louis, MO). α-TC was dissolved in ethanol at the concentration of 5 mM and kept at −20 °C until use. All the chemicals and solvents were of the highest analytical grade. The mouse monoclonal antibody against α-tubulin, 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI), and Texas Red-X phalloidin were from Molecular Probes-Invitrogen Corporation (Carlsbad, CA). Mowiol was from Hoechst (Frankfurt, Germany).

Green Tea Extract. GTE was a kind gift from Indena (Milan, Italy) and defined by the producer as an aqueous spray-dried extract of a Chinese green tea, having a polyphenol content of 75 ± 5% (w/w). GTC content and composition of this GTE were previously determined by HPLC analysis (*19*) revealing the following composition (mg/g GTE): gallic acid 5.3 ± 0.1, (−)-epigallocatechin 38.9 ± 2.5, (+)-catechin 47.9 3 ± 7, (−)-epigallocatechin gallate 270.6 ± 3.8, (−)-epicatechin 51.8 ± 3.9, (−)-gallocatechin gallate 150.6 ± 1.2, (−)-epicatechin gallate 137.5 ± 1.8. GTE was dissolved in warm, double distilled water at the concentration of 1 mg/mL, sterilized using a 0.22 μm filter, and kept at 4 °C until use.

Cell Culture and Treatments. Primary heart cell cultures were obtained by isolation of cardiomyocytes from the ventricles of 2−4 day old Wistar rats, as previously reported (*21*). Cells were seeded at a density of 2×10^6 cells/mL and were grown in Ham F10 nutrient mixture supplemented with 10% v/v FCS and 10% v/v HS (complete medium), and they were kept in this medium at 37 °C, 5% CO_2, and 95% humidity (aerobic condition) until complete confluence. In some dishes, 50 μg/mL GTE or 20 μM α-TC was added to the culture medium 24 h before the induction of hypoxia. Simulated ischemia was achieved by culturing the cells in serum-deficient medium, but containing the previous concentrations of antioxidants, in an anaerobic workstation (BugBox, Jouan, France), saturated with 5% CO_2/95% N_2, at 37 °C. The hypoxic procedure reduced oxygen from 20 to 5% after 3 min, and to less than 1% after 10 min. The O_2 content of the atmosphere inside the chamber was <1% for the duration of the experiment, as measured by an on-line meter (Griffin and George, Fife, U.K.). In some experiments 2 h of hypoxia were followed by 1 h reoxygenation, during which oxygen increased to 20% within 5 min. In order to strictly mimic a reperfusion, the serum free-medium was replaced with complete medium containing the previous concentrations of GTE or α-TC.

Cell Viability. Cells were plated in 24-well plates (1×10^6 cells/well), and cellular damage elicited by H/R treatment was evaluated by measuring MTT reduction. MTT was added to the medium (final concentration 0.5 mg/mL) and incubated for 1 h at 37 °C. After incubation, MTT solution was removed, DMSO was added, and the absorbance was measured using a microplate spectrophotometer VICTOR3 V Multilabel Counter (Perkin-Elmer - Wellesley, MA) at a wavelength of 595 nm.

Cytosolic Antioxidant Activity (CAA). In both normoxic condition and H/R, cytosolic antioxidant activity was determined in cardiomyocytes using the method of Re et al. (*22*), based on the ability of the antioxidant molecules in the sample to reduce the radical cation of ABTS, and measured as quenching of the absorbance at 740 nm, as previously reported (*23*). Briefly, cardiomyocytes were washed 3 times with cold phosphate buffer solution (PBS). Cells were subsequently collected in 1 mL of PBS and centrifuged for 10 min at 500*g* at 4 °C, after which the supernatant was removed and the cells washed two times with 1 mL of PBS. The pellet was finally reconstituted in 0.05% Triton X-100 in PBS. Cells were than homogenized and allowed to stand at 4 °C for 30 min. Cytosolic fractions were subsequently separated by centrifugation at 10000*g* for 15 min at 4 °C. Values obtained for each sample were compared to the concentration−response curve of a standard Trolox solution and expressed as μmol of Trolox equivalent antioxidant activity per milliliter (TEAA μmol/mL).

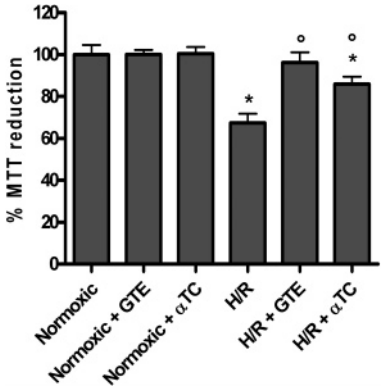

Figure 1. Protection against H/R-induced cell damage by GTE. Cardiomyocytes were supplemented with GTE or α-TC 24 h before cells were subjected to 2 h hypoxia followed by 1 h reoxygenation. Cell viability was measured by the MTT assay as reported in the Materials and Methods section. Data are expressed as percent of unsupplemented normoxic cells (=100%). Statistical analysis was performed on the values of absorbance at 595 nm by the Student's *t* test: *H/R vs normoxia. °Supplemented vs unsupplemented in the same conditions.

Confocal Microscopy. Cells were seeded on glass cover-slips and treated as described in the Cell Culture and Treatments section, then washed twice with PBS, fixed with 3% paraformaldehyde, washed with 0.1 M glycine in PBS, and permeabilized in 70% ice cold ethanol. After fixing, the cells were incubated with anti α-tubulin overnight at 4 °C. Subsequently the samples were washed with 1% BSA in PBS and incubated with FITC conjugated secondary antibody for 1 h at room temperature. Texas Red-X phalloidin was used for labeling F-actin and DAPI for labeling nuclei. Preparations were embedded in Mowiol and the multiple-exposure images were acquired by using a laser scanning confocal microscope Bio-Rad MRC 1024 (Bio-Rad, Hemel Hempstead, U.K.) equipped with a Nikon Eclipse microscope (Nikon Instech Co., Tokyo, Japan). Analysis were performed in both hypoxic and H/R conditions. To avoid reoxygenation of hypoxic cells, culture plates containing coverslips were fixed directly inside the hypoxic chamber.

Data Presentation and Statistics. Each experiment was performed at least three times, and all values were represented as means ± SD. Analysis was performed by the Student's *t* test (Prism 4, GraphPad Software Inc., San Diego, CA). Values of $p < 0.05$ were considered as statistically significant.

RESULTS

Cell Viability. To evaluate the ability of GTE to protect cardiomyoyctes from H/R induced damage, cells were supplemented with 50 μg/mL GTE or 20 μM α-TC for 24 h before H/R and the cell viability was detected by the MTT assay, as reported in the Materials and Methods section. The values obtained in unsupplemented normoxic cells were considered equal to 100%, and neither GTE nor α-TC resulted cytotoxic for cardiomyocytes. The ability of cardiomyocytes to reduce MTT was drastically reduced after H/R (67.32 ± 4.52%, $p < 0.05$) (**Figure 1**). GTE supplementation completely maintained cell viability at values similar to that of normoxic cells. α-TC supplementation showed a significant increase in cell survival following H/R, but the values were still lower than in normoxic cells.

Cytosolic Antioxidant Activity. Cytosolic TEAA of cardiomyocytes unsupplemented or supplemented with 50 μg/mL GTE or 20 μM α-TC is reported in **Figure 2**. Both GTE and α-TC significantly increased TEAA in control cells, but to a different extent, GTE being more effective than α-TC. H/R

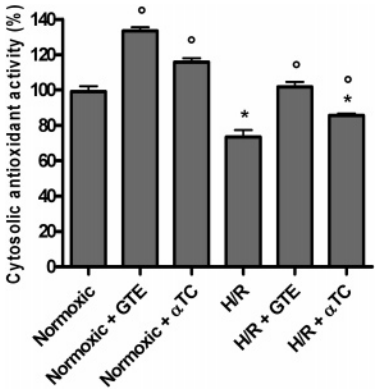

Figure 2. Cytosolic antioxidant activity in hypoxic/reoxygenated cardiomyocytes supplemented with GTE. Cardiomyocytes were supplemented with 50 μg/mL GTE or 20 μM α-TC 24 h before cells were subjected to 2 h hypoxia followed by 1 h reoxygenation. Cytosolic antioxidant activity was measured as reported in the Materials and Methods section and expressed as percent of normoxic cardiomyocytes (TEAA = 91.27 ± 7.81 μmol/mL = 100%). Statistical analysis was performed on the TEAA values using the Student's *t* test: *H/R vs normoxia. °Supplemented vs unsupplemented in the same conditions.

caused a significant decrease in TEAA levels with respect to normoxic cardiomyocytes. GTE was able to completely restore the TEAA values at levels significantly different from H/R cardiomyocytes and comparable to normoxic cells. α-TC supplementation was only able to partially restore TEAA levels after H/R, but the values were significantly lower than in normoxic cells.

Effects of GTE on H/R Induced Cytoskeletal Alterations. The effects of hypoxia on the microtubule network (MT) and on the nuclear morphology were investigated by fluorescence and immunofluorescence methods. MT was stained, and its integrity, in the absence or presence of GTE or α-TC, was observed by confocal microscopy. In normoxic conditions, in neonatal cardiac myocytes, the microtubule density was very low (**Figure 3a**), and MT (arrows) was evident only as a mitotic spindle component in the cells supplemented with GTE or α-TC (**Figure 3d,g**). Moreover, the supplementation with GTE or α-TC altered neither MT nor actin microfilaments organization, nor nuclear morphology (**Figure 3d,g**). After 2 h hypoxia, a disorganization of the MT and a significant modification of the actin organization were observed in unsupplemented cells (**Figure 3b**). In particular, an alteration of the MT patterns was observed as a loss of the perimembraneous MT, which progressively shranked at the vicinity of the nuclei. A similar cytoskeleton disorganization was observed in α-TC supplemented hypoxic cells (**Figure 3e**), while GTE supplementation partially preserved the integrity of actin microfilaments during simulated ischemia (**Figure 3h**).

After 1 h reoxygenation, a disruption of the actin network and a drastic alteration of nuclear morphology were observed in unsupplemented cells (**Figure 3c**). α-TC supplemented cells showed a similar cytoskeletal damage as reoxygenated unsupplemented cells. Furthermore, the cells showed typical apoptotic features, such as nuclear condensation and nuclear fragmentation upon DAPI staining (arrows, **Figure 3f**). On the contrary, GTE supplementation led to significant protective effects on the integrity of actin cytoskeleton and nuclear

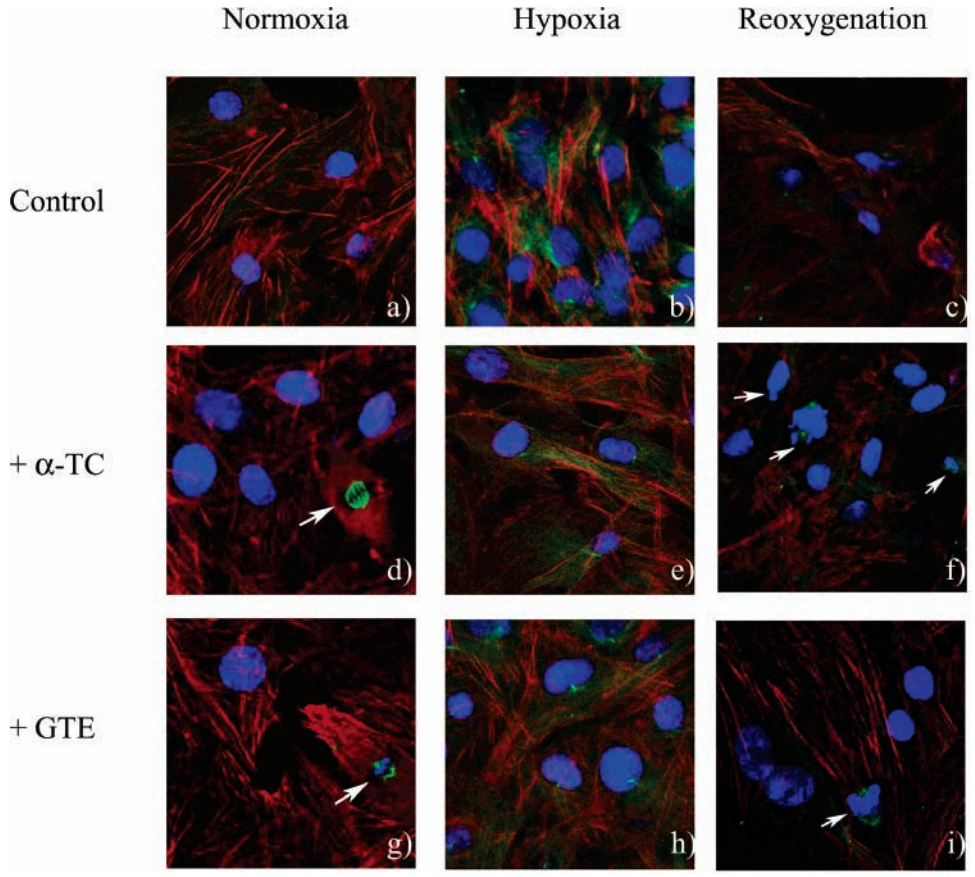

Figure 3. Representative confocal micrographs of H/R cardiomyocytes in the absence or the presence of GTE or α-TC. Cardiomyocytes were supplemented with GTE or α-TC 24 h before cells were subjected to 2 h hypoxia followed by 1 h reoxygenation. Images were acquired with a ×60 objective. Normoxic (left), hypoxic (middle), and reoxygenated (right) cells were stained for actin (red), tubulin (green), and nuclei (blue). Arrows in panels **d**, **g**, and **h** indicate mitotic spindles, and arrows in **f** evidence apoptotic nuclei.

morphology. In particular, the organization of the actin fibers and the nuclear morphology appeared similar to unsupplemented normoxic cells (**Figure 3i**). Furthermore, microtubule density appeared very low, and MT was evident only as a mitotic spindle component (arrows) as in normoxic conditions.

DISCUSSION

The implication of ROS in H/R damage is well documented, but although many factors have been proposed to play a role in the protection by antioxidant supplementation, the detailed mechanisms remain unknown (*11*, *24*, *25*). To better understand these aspects, we focused our attention on the effects of H/R on the cytoskeleton of cultured cardiomyocytes and on the possibility to counteract H/R induced alterations by GTE supplementation. We compared the protective effects of GTE to that of α-TC, which is the most important endogenous antioxidant in cardiac cells (*26*). We used α-TC at a concentration of 20 μM, since this concentration has been reported to increase cellular α-TC content by approximately 2−3-fold, to reduce membrane lipid alteration, to enhance the recovery of the contractile function, and to reduce the accumulation of calcium in isolated rat hearts exposed to global ischemia and reperfusion (*27*).

Hypoxia and reoxygenation injuries are not two independent entities. The term reoxygenation injury is a misnomer because it is hypoxia that sets the stage for the development of the reoxygenation injury (*28*). For this reason, we chose to study cell viability and cytosolic antioxidant activity in conditions resembling ischemia and reperfusion injury, and not ischemia alone. In fact the restoration of blood flow is a prerequisite for preserving a viable myocardium following an acute episode of ischemia. In our experiments hypoxia lasted 2 h, and reoxygenation 1 h, since it is known that the deleterious effects of oxygen readmission occur very rapidly (*29*, *30*).

Our results demonstrated that GTE supplementation was able to maintain cell viability in H/R cardiomyocytes at degrees comparable to normoxic cells. On the other hand, in α-TC supplemented cells, cell viability was significantly higher than in unsupplemented H/R cardiomyocytes, but was significantly lower than in normoxic cells. These findings were strictly in agreement with the data obtained for the cytosolic antioxidant activities. Only GTE evoked the ability to maintain the cytosolic antioxidant status at the same level of that of normoxic cells.

Furthermore, as shown by confocal microscopy, GTE supplementation protected the cells from the damages to nuclear morphology and cytoskeleton caused by oxygen readmission. In particular, in H/R cardiomyocytes GTE fully restored the organization of actin fibers and MT observed in normoxic conditions. On the other hand, α-TC showed only a partial protection of the cytoskeleton architecture. In addition several nuclei showing typical morphologic features of apoptotic death were observed. Indeed prolonged periods of myocardial ischemia are related to an increase in the rate of necrosis, whereas, paradoxically, reperfusion leads to an enhancement of apoptosis (*31*−*35*). Reperfusion also restores the energy required for the completion of apoptosis and can accelerate the apoptotic process (*32*−*34*). A recent study demonstrated that green tea catechins were able to prevent apoptotic cell death in cardiac myocytes following ischemia/reperfusion injury (*36*).

In this study only GTE was demonstrated to be highly effective in protecting myocardial cytoskeleton from H/R induced alterations. Why this different effectiveness of GTE versus α-TC? A possible answer lies in the ability of green tea catechins to act as amphiphilic antioxidants, both in the lipid bilayer and intracellularly, since they can cross cell membranes (*36*). On the other hand, α-TC is a lipophilic antioxidant, mainly present in the lipid bilayer, and acts indirectly on the cytosolic antioxidant status through the regeneration of cytosolic antioxidants at the membrane-cytosol interface. There are actually other possibilities to answer this question: for example, concentrations of phytochemicals in GTE, bioactive compounds other than GTCs in GTE, or non-antioxidant mechanisms might be involved.

In this study, for the first time, attention has been focused on the effects of natural antioxidants in maintaining cardiac cytoarchitecture following H/R, highlighting the unique role of amphiphilic polyphenolic molecules. The possibility to control and regulate the delicate system of interactions of cytoskeletal components by GTE consumption could be essential to ensure proper cardiac function, and a more detailed insight in these processes might provide new tools to improve the contractile efficiency of the cardiomyocytes. Future research could allow the establishment of dietary recommendations in order to reduce the severity of cardiac injury following myocardial ischemia.

ACKNOWLEDGMENT

We thank C.I.R.B. for the use of confocal microscopy.

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Received for review October 5, 2006. Accepted October 13, 2006. This work was supported by grants from MURST ex 60% 2005 S.H., L.M.

JF062859R