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Green tea protection of hypoxia/reoxygenation injury in cultured cardiac cells

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Abstract

Antioxidant-rich diets exert a protective effect in diseases involving oxidative damage. Among dietary components, green tea is an excellent source of antioxidants. In this study, cultured neonatal rat cardiomyocytes were used to clarify the protective effect of a green tea extract on cell damage and lipid peroxidation induced by different periods of hypoxia followed by reoxigenation. Cultures of neonatal rat cardiomyocytes were exposed to 2–8 hr hypoxia, eventually followed by reoxygenation, in the absence or presence of α-tocopherol or green tea. LDH release and the production of conjugated diene lipids were measured, and appeared linearly related to the duration of hypoxia. During hypoxia, both LDH release and conjugated diene production were reduced by α-tocopherol and, in a dose dependent manner, by green tea, the 50 μg/ml being the most effective dose. Reoxygenation caused no further increase in LDH leakage, while it caused a significant increase in conjugate dienes, which absolute value was lower in antioxidant supplemented cells. Anyway, the ratio between conjugated diene production after hypoxia and after reoxygenation was similar in all groups, indicating that the severity of free radical-induced reoxygenation injury is proportional to the severity of previous hypoxic injury. Since hypoxic damage is reduced by α-tocopherol and green tea, our data suggest that any nutritional intervention to attenuate reoxygenation injury must be directed toward the attenuation of the hypoxic injury. Therefore, recommendations about a high dietary intake of antioxidants may be useful not only in the prevention, but also in the reduction of cardiac injury following ischemia. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Antioxidants; Green tea; Cultured cardiomyocytes; Hypoxia; Reoxygenation; Free radicals

1. Introduction

The pathogenetic mechanism of myocardial ischemic damage is still not completely understood, but the role of oxygen-derived free radicals in myocardial ischemia has been established, although not completely characterized. A major source of pathological free radical production due to ischemia or hypoxia may result from reflow or reoxygenation. A variety of mechanisms and mediators are involved in the pathophysiology of reperfusion injury [1]; a key event is the reintroduction of molecular oxygen and subsequent formation of reactive oxygen species (ROS), important mediators of myocardial ischemia/reperfusion injury [2–6]. The major cytotoxic effect of free radicals in the heart is supposed to be the peroxidation of lipid components of cellular and subcellular membranes. The resulting loss of cellular integrity could lead to irreversible cell injury.

Ischemia/reperfusion damage has important implications, and particular attention has been focused to the consequences of reperfusion, due to the diffusion of current techniques to restore blood flow to ischemic heart [7]. Ischemia followed by reperfusion constitutes a series of events in which the production of ROS might overwhelm the capacity of the antioxidant system [8]; thus, an additional mechanism to increase the survival of tissues exposed to free radicals produced during ischemia/reperfusion may be achieved by providing free radical scavengers, which possess the ability to protect cells against lipid peroxidation. A large variety of antioxidants have been tested for their possible protective effect in ischemia/reperfusion injury of the heart; however, the results are controversial.

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In the present investigation, using cultured rat cardiomyocytes as a model system, we have verified the possibility to counteract cell damage and lipid peroxidation induced by different periods of hypoxia, followed by reoxygenation, by the supplementation of antioxidants from a dietetic source, green tea (GT). GT is an excellent source of polyphenol antioxidants, particularly of a group of compounds, known as green tea catechins (GTCs) [9]. GT refers to a non fermented product in which GTCs are more preserved than in partially fermented (oolong or pouchong tea), or fully fermented tea (black or pu-erh tea). Results obtained with GT were analyzed in the light of those obtained with α-tocopherol (αTC) supplementation. Vitamin E, which is a chain-breaking antioxidant and inhibitor of lipid peroxidation, is the most important endogenous antioxidant [10]; pretreatment with vitamin E has been demonstrated to attenuate ischemia-reperfusion injury in isolated hearts [11, 12]. The 20μM dose used in this study has been reported to increase cellular αTC content approximately 2–3 fold, to reduce membrane lipid alteration, to enhance the recovery of contractile function and to reduce the accumulation of calcium in isolated rat hearts exposed to global ischemia and reperfusion [13,14]. The type and concentration of GT used in this study has been previously demonstrated to be highly effective in protecting from oxidation both a lipid model system of refined peanut oil and cultured cardiomyocytes exposed to a free radical generating system [15]. Nevertheless, different concentrations of GT have been used in this study in order to provide dose response data in cardiomyocytes exposed to hypoxia.

2. Methods and materials

Green tea extract (GTE) was a kind gift of Indena (Milano, Italy), and it was defined by the producer as having a polyphenol content of 75 ± 5% (w/w), and a caffeine content lower than 8% (w/w). Its HPLC analysis [15] revealed the following composition: gallic acid 5.3 ± 0.1 mg/g GTE, (-)-epigallocatechin 38.9 ± 2.5 mg/g GTE, (+)-catechin 47.9 ± 3.7 mg/g GTE, (-)-epigallocatechin gallate 270.6 ± 3.8 mg/g GTE, (-)-epicatechin 51.8 ± 3.9 mg/g GTE, (-)-gallocatechin gallate 150.6 ± 1.2 mg/g GTE, (-)-epicatechin gallate 137.5 ± 1.8 mg/g GTE, caffeine 65.4 ± 1.5 mg/g GTE. GTE was dissolved in warm double distilled water at the concentration of 1 mg/ml and kept at 4°C until use. Horse serum (HS), fetal calf serum (FCS), Ham F10 culture medium, αTC and other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). αTC was dissolved in ethanol at the concentration of 5 mM and kept at −20°C until use. Acetonitrile was from Prolabo (Paris, France), and all the other chemicals and solvents were of the highest analytical grade.

Neonatal hearts were collected from rats between 2 and 4 days of age as previously described [16]. The myocytes were plated on 100 mm plastic culture dishes at a density of approximately 2×10^6 cells/ml. The cells were grown in Ham F10 medium supplemented with 10% (v/v) HS and 10% (v/v) FCS and kept in this medium for 5 days at 37°C and 5% CO_2 under aerobic conditions in a carbon dioxide incubator (Queue). During this period the cells were beating spontaneously at a frequency of 20−40 beats/min, and reached complete confluence in a monolayer. Each culture dish contained 2.31 ± 0.26 mg protein (n = 10). The supplemented F10 medium was changed every 48 hr.

In some experiments, 20μM αTC or 5–100 μg/ml GTE were added to the culture medium at day 5 of culturing, 24 hr before the beginning of the hypoxic period.

Before starting the experiments, at day 6 of culturing, the medium was removed, cells were washed with PBS, and changed to 6 ml F10 medium without serum. In order to achieve near anoxic conditions, this medium was pregassed with bubbling 95% nitrogen-5% CO_2. In cells previously supplemented with antioxidants, the same concentrations of the antioxidants were maintained in this hypoxia medium. The culture plates were then transferred to specially designed, air tight, thermostated chambers. The experiments lasted in hypoxia for 2–8 h. Some experiments were followed by 1 h reoxygenation. At the beginning of the reoxygenation period, the hypoxia medium was changed to Ham F10 medium supplemented with 10% HS and 10% FCS and, in cardiomyocytes pretreated with antioxidants, with the same concentrations of antioxidants themselves. The hypoxic procedure reduced oxygen from 20 to 5% after 3 min, and to 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, UK) [17,18]. Reoxygenation increased oxygen to 20% within 5 min.

At the end of each experiment, cells were counted by a hemocytometer, and cell viability was determined by staining with Trypan blu (0.2%). Cell damage was assessed by measurement of the release of lactate dehydrogenase (LDH) into the medium. Aliquots of medium were collected from each dish, and analyzed spectrophotometrically for LDH activity by measuring NADH levels at 340 nm [19]. Cells were scraped off in ice-cold methanol, and the appearance of conjugated diene-containing lipids was evaluated as an index of lipid peroxidation using the method of Burton *et* al. [20] Briefly, cells, scraped from the culture plates, were extracted in chloroform:methanol:water (2:1:1 v/v). The chloroform layers from two extractions were combined and then dried under nitrogen. Samples were resuspended in a known volume of acetonitrile and absorbance determined at 235 nm.

Data are means ± S.D. of 5 different cultures. Statistical differences were evaluated using the Student's t test.

3. Results

During hypoxia, culture medium was serum deprived, so we first evaluated LDH release and conjugated diene production in cardiomyocytes maintained in serum deprived

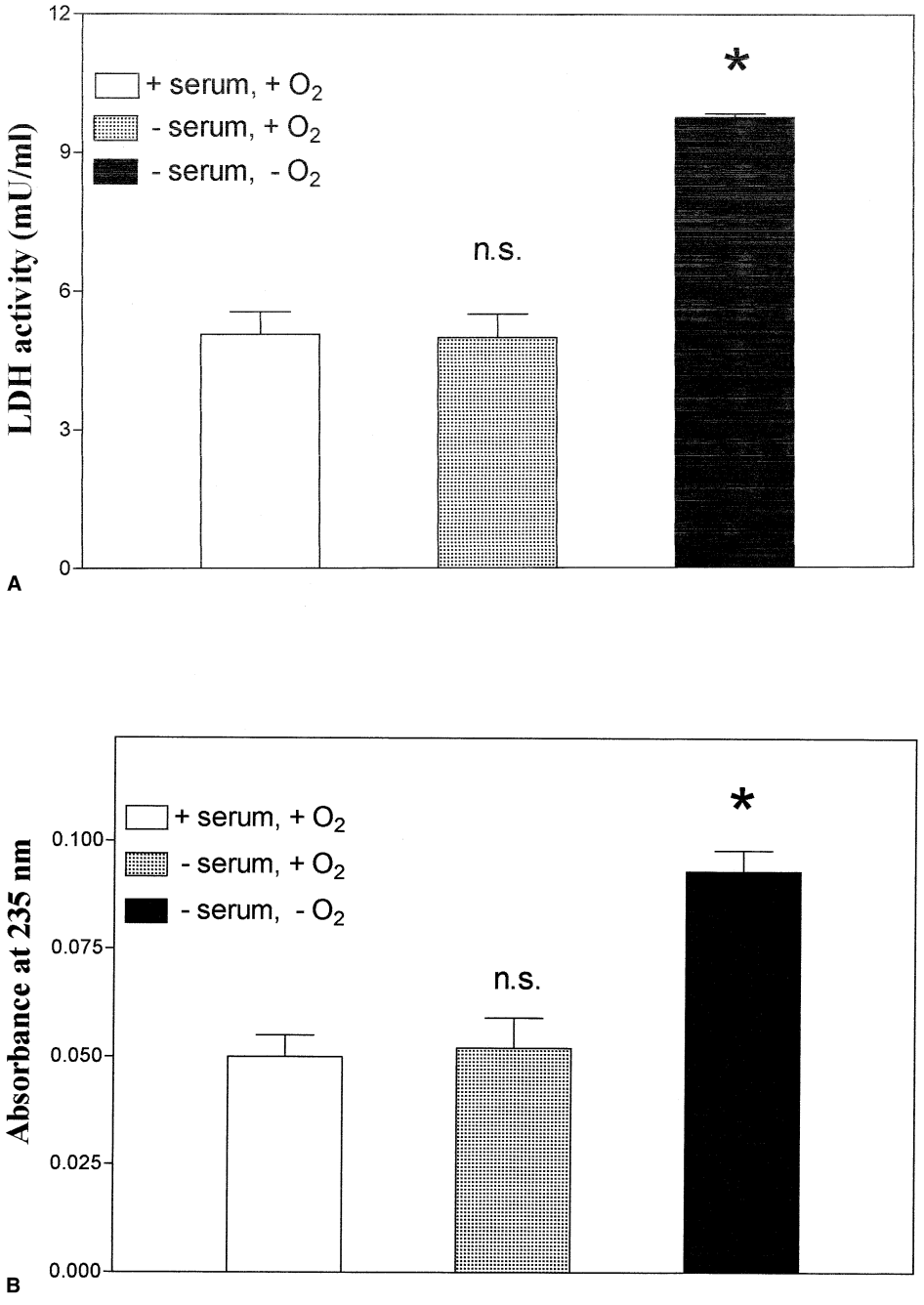

Fig. 1. LDH release and conjugated diene production in cardiomyocytes after 8 hr of serum deprivation in condition of normoxia or hypoxia. Cardiomyocytes were maintained in serum deprived medium for 8 hr, in normoxic or hypoxic conditions. **Panel A**: LDH activity was measured on aliquots of the culture media as reported under Methods. **Panel B**: Conjugated diene production was measured as 235 nm absorbance as reported under Methods. Data are means ± S.D. of five different cell cultures. Statistical analysis was performed by the Student's t test comparing control cardiomyocytes to serum deprived cardiomyocytes in condition of normoxia (n.s. = not significant) and hypoxia (* $p < 0.001$).

medium but in normoxic conditions, in order to discriminate the effect of serum deprivation from the effect of oxygen deprivation on these parameters. Serum deprivation was maintained for 8 hr, in order to reproduce the duration of the maximum hypoxic period tested. LDH release in the culture medium of cardiomyocytes after 8 hr of serum deprivation, in normoxic or hypoxic conditions, is reported in Fig. 1A and compared to control cardiomyocytes. Serum deprivation had no effects on LDH release, which appeared similar to controls and significantly lower than in serum deprived-hypoxic cells. Similarly, conjugated diene production (Fig. 1B) was similar in serum deprived–normoxic cells than in control cells, while it was significantly higher in serum deprived-hypoxic cardiomyocytes.

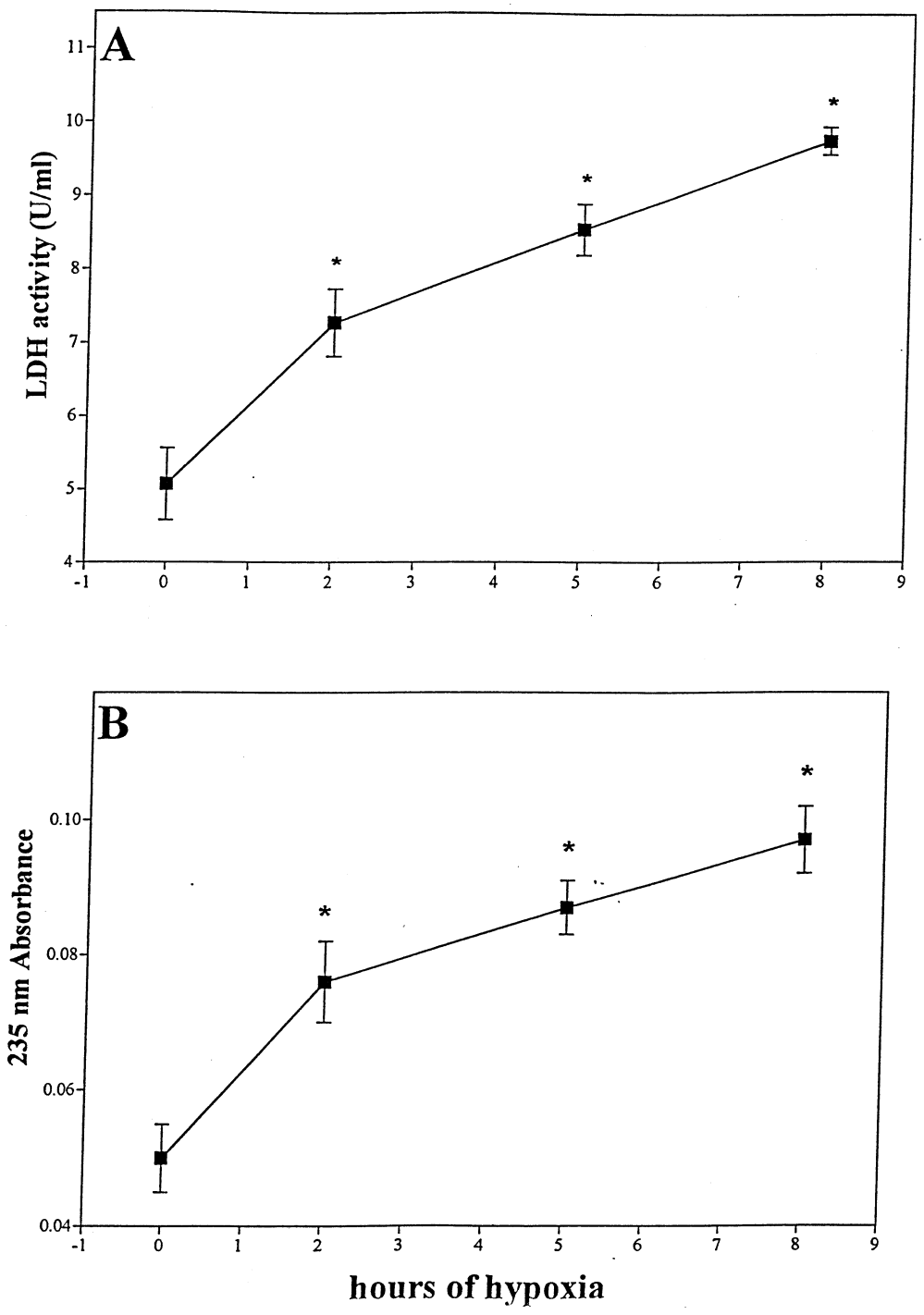

Fig. 2. LDH release and conjugated diene production in cardiomyocytes after different periods of hypoxia. Cardiomyocytes were subjected to hypoxia for different times (2–8 hr). **Panel A**: LDH activity was measured on aliquots of culture media as reported under Methods. **Panel B:** Conjugated diene production was measured as 235nm absorbance as reported under Methods. Data are means ± SD of five different cell cultures. Statistical analysis was performed by the Student's t test comparing cardiomyocytes not subjected to hypoxia (controls) to cardiomyocytes subjected to hypoxia: * $p < 0.001$.

The time-course of LDH release in the culture medium, in the absence of antioxidants, was monitored during the hypoxic periods, and is reported in Fig. 2A. Enzyme activity significantly increased after 2 hr hypoxia in comparison to normoxic cells; LDH activity was linearly related to the duration of the hypoxic period ($r = 0.971$; $p < 0.05$). The time-course of the appearance of conjugated diene containing lipids in the absence of antioxidant supplementation during the hypoxic period was used as an index of lipid peroxidation, and is reported in Fig. 2B. Similarly to data obtained measuring LDH release, exposure of cells to 2 hr hypoxia caused a significant increase in conjugated diene

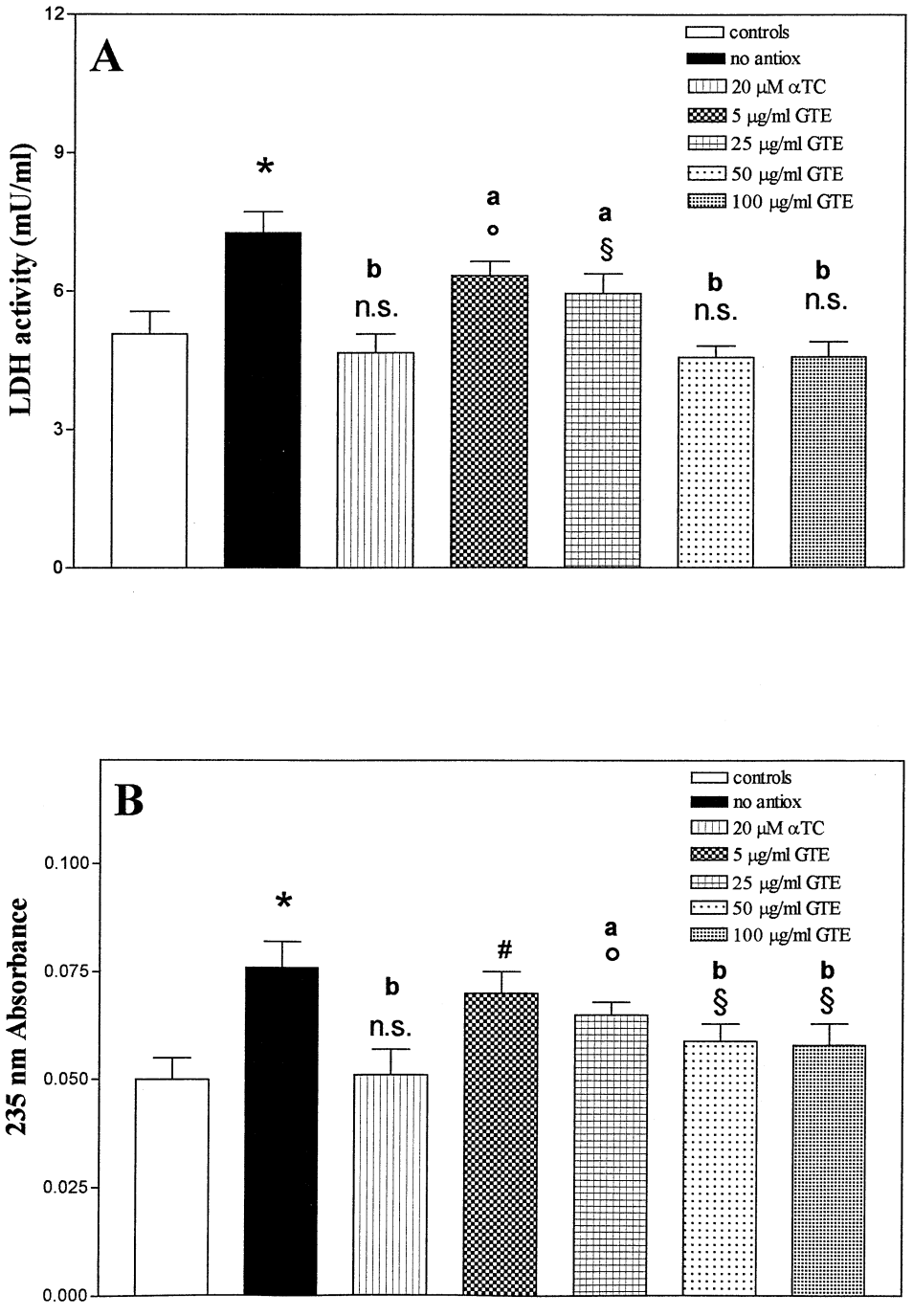

Fig. 3. LDH release and conjugated diene production in cardiomyocytes after 2 hr of hypoxia in the absence or presence of antioxidants. Some cardiomyocytes were supplemented with antioxidants 24 hr prior to the experiment, then subjected to hypoxia in the presence of the same antioxidants. **Panel A:** LDH activity was measured on aliquots of culture media as reported under Methods. **Panel B:** Conjugated diene production was measured as 235 nm absorbance as reported under Methods. Data are means ± S.D. of at least three different cell cultures. Statistical analysis was performed by the Student's t test comparing: 1) control (normoxic) cardiomyocytes to cardiomyocytes subjected to hypoxia in the absence or presence of antioxidants (n.s.= not significant; § $p < 0.05$; ° $p < 0.01$, # $p < 0.002$; * $p < 0.001$); 2) cardiomyocytes subjected to hypoxia in the absence of antioxidants vs cardiomyocytes subjected to hypoxia in the presence of antioxidants ($^a p < 0.01$, $^b p < 0.001$).

production, which was linearly related to the duration of the hypoxic period ($r = 0.943$, $p < 0.05$).

Although during hypoxia both LDH release and conjugate diene production appeared to be time-dependent, the major increase was during the first 2 hr, therefore in the following experiments, concerning the supplementation of cardiomyocytes with antioxidants, hypoxia was lasted for 2 hr.

As reported in Fig. 3A, supplementation with both 20 μM αTC or 5–100 μg/ml GTE prevented the release of LDH in the culture medium after 2 hr hypoxia, although to

different extent, while the enzyme activity was significantly increased in the medium of not supplemented cells. LDH leakage decreased proportionally with the increase of GTE concentration up to 50 μg/ml; the 100μg/ml supplementation had similar effect as the 50μg/ml one (N.S.). The correlation between GTE concentration up to 50 μg/ml and the entity of LDH release was statistically significant ($r = -0.963$, $p < 0.05$). Similarly (Fig. 3B), supplementation of cardiomyocytes with αTC completely prevented the appearance of conjugated diene lipids; a partial protection was achieved supplementing cells with GTE, and conjugated diene production was lower than in non supplemented cells. Even in conjugated diene lipid production, a correlation between GTE concentration up to 50 μg/ml and 235 nm absorbance was detected ($r = -0.963$, $p < 0.05$). Since the 50 μg/ml concentration appeared to be the lowest dose causing the maximum protective effect, the following experiments in hypoxia/reoxygenation have been performed supplementing cells with this concentration.

In Fig. 4A LDH release in the medium of cardiomyocytes not supplemented or supplemented with antioxidants after 2 hr of hypoxia followed by a 1 hr normoxic recovery period is reported. In cardiomyocytes not supplemented with antioxidants hypoxia caused an increase in LDH leakage with respect to control conditions, and no further increase was detected after 1 hr reoxygenation. Antioxidant supplementation completely prevented LDH release into the medium after both hypoxia and reoxygenation. Conjugated diene production after 2 hr of hypoxia followed by a 1 hr reoxygenation in cardiomyocytes not supplemented or supplemented with antioxidants is reported in Fig. 4B. In both unsupplemented and supplemented cells the restoration of the normoxic condition caused a significant increase in conjugate diene production, which levels appeared higher than in both control conditions and after 2 hr hypoxia. Conjugated diene production after reoxygenation appeared anyway higher in unsupplemented than in GTE supplemented or in αTC supplemented cardiomyocytes.

GTE or αTC supplementations did not influence either cell number or cell viability in normoxic conditions. Two hours hypoxia did not influence cell number, independent of the presence of αTC or GTE, but, when cells were not supplemented with antioxidants, cell viability was significantly reduced (Fig. 5). On the contrary, this parameter was not modified when cells were submitted to hypoxia in the presence of antioxidants. One hour reoxigenation did not affect cell counting, but influenced cell viability to a greater extent in cells not supplemented with antioxidants, which partially protect cardiomyocytes (Fig. 5).

4. Discussion

Lack of blood supply or ischemia underlies many of the most important diseases faced by physicians in their daily practice; many evidences suggest that reperfusion of ischemic areas may contribute to further tissue damage.

During ischemia/reperfusion, a great quantity of free radicals are generated in the heart; the reactions caused by exceeding radicals lead to lipid peroxidation of membranes [21–23], particularly in the heart which has low antioxidant defenses [24]. Although ischemia/reperfusion injury is now an accepted phenomenon, there are still many difficulties in differentiating between pre-existing damage resulting from a period of ischemia and any subsequent damage that can be attributed to the reperfusion process.

In order to clarify this point, in this study, using primary cultures of rat ventricular myocytes as a model, we have investigated about the damage induced by different periods of hypoxia and subsequent reoxygenation. The possibility to counteract ischemia/reperfusion injury by antioxidants has been investigated by supplementing cell cultures with a well known antioxidant as αTC and with GTE, rich in catechins which antioxidant properties has been extensively studied [15,25].

Since hypoxia was performed in a serum-deprived medium, we first evaluated the effect of serum starvation on LDH release and conjugated diene production. Serum deprivation does not participate in the onset of cell damage due to hypoxia, so data obtained in hypoxic cells are to ascribe to oxygen deprivation only.

Hypoxia caused a significant increase in both LDH release into cell medium and conjugated diene production, evidencing both an aspecific cell damage and an increased cell lipid peroxidation. Both LDH release and conjugated diene production were positively related to the length of hypoxia. Increased leakage of LDH during ischemia or hypoxia has been previously demonstrated [26,27]. Our data clearly show that free radical production occurs even during hypoxia, and not only during reoxygenation, since conjugated diene production increases in a time-dependent manner during oxygen deprivation, in agreement with data obtained in a perfused rabbit heart model [28,29]. It is difficult to establish what is the mechanism of ROS generation during hypoxia; possibly, in this condition the residual oxygen is sufficient to generate ROS. Data by Williams et al. indicate that iron is not implicated in ROS generation during hypoxia [29], and our data exclude the involvement of activated neutrophils, since we used virtually “pure” cultures of cardiomyocytes. A similar conclusion was drawn by Kloner et al. using rabbit hearts isolated and perfused with a neutrophyl-deprived solution [30].

Antioxidants supplemented to cardiomyocytes demonstrated a striking protective effect; in particular GTE showed a dose related protective effect on both LDH release and conjugated diene production, with the maximum effect at the 50 μg/ml concentration.

Reoxigenation for 1 hr did not cause a further increase in LDH leakage, in agreement with previous findings by Ek et al. [31]. On the contrary, oxygen readmission caused a significant increase in conjugated diene production. Al-

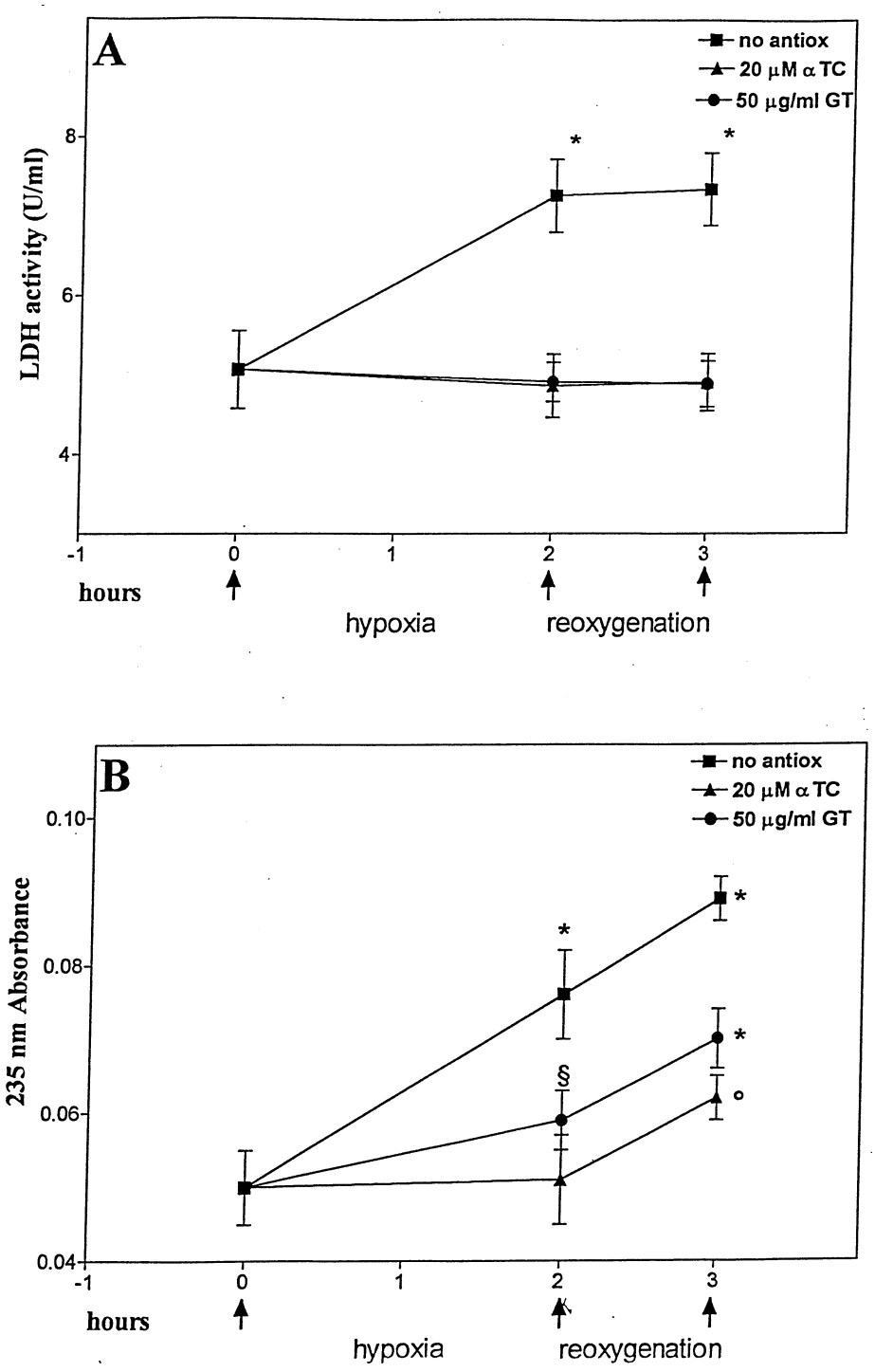

Fig. 4. LDH release and conjugated diene production in cardiomyocytes after 2 hr hypoxia followed by a 1 hr reoxigenation in the absence or presence of antioxidants. Some cardiomyocytes were supplemented with antioxidants 24 hr prior to experiments, then subjected to hypoxia for 2 hr followed by reoxygenation for 1 hr in the presence of the same antioxidants. **Panel A:** LDH activity was measured on aliquots of culture media as reported under Methods. **Panel B:** Conjugated diene production was measured as 235 nm absorbance as reported under Methods. Data are means ± S.D. of five cell cultures. Statistical analysis was performed by the Student's t test comparing control (normoxic) cardiomyocytes to cardiomyocytes after hypoxia and reoxygenation in the absence or presence of antioxidants (§ = $p < 0.05$; ° = $p < 0.01$; * = $p < 0.001$).

though the absolute value of conjugated diene level was lower in antioxidant supplemented cells than in not supplemented cells, the ratio between conjugated diene production after hypoxia and after reoxygenation was similar in not supplemented (0.85) and supplemented cells (0.82 and 0.84 for αTC and GTE supplemented, respectively), indicating that the entity of lipid peroxidation after the first hour of reoxigenation largely depends on the extent of peroxidation

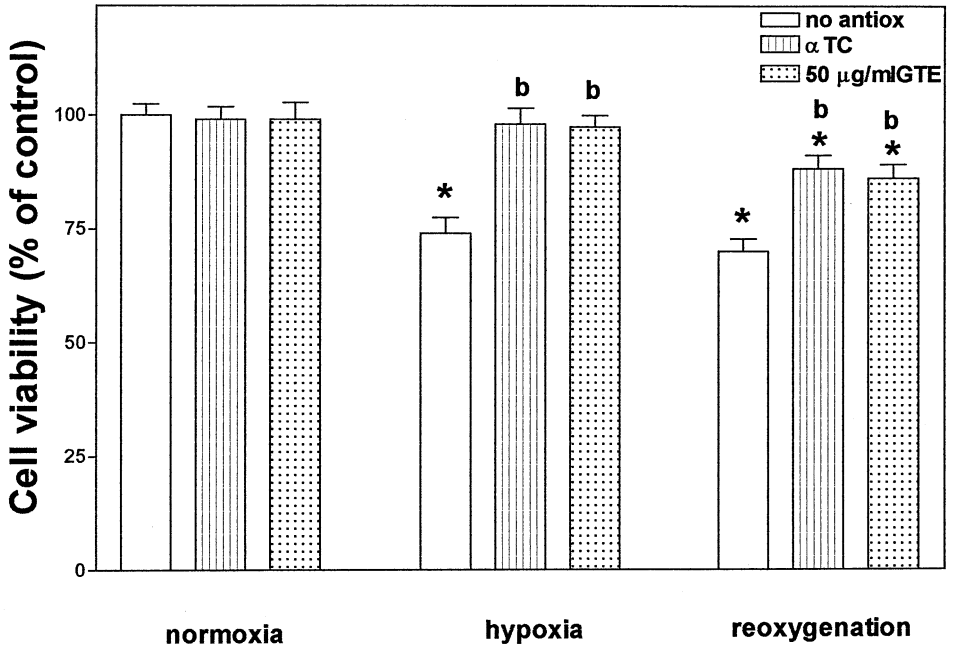

Fig. 5. Viability of cardiomyocytes in the absence or presence of antioxidants in conditions of normoxia, hypoxia and reoxygenation. Cell viability was determined as reported under Methods and expressed as % of controls (unsupplemented, normoxic cardiomyocytes). Data are means ± S.D. of five different cell cultures. Statistical analysis was performed by the Student's t test: *$p < 0.001$ vs normoxic cardiomyocytes. In hypoxia and reoxygenation, the comparison between unsupplemented cells and cells supplemented with antioxidants was statistically significant ($^b p < 0.001$).

during previous hypoxia. The invariance of this ratio is not to ascribe to a consumption of antioxidant molecules by the cells during the hypoxic period, since we refilled cell medium with fresh solutions of antioxidants at the beginning of reoxygenation.

Hypoxia injury and reoxygenation injury are not two independent entities. The term "reoxygenation injury" is a misnomer, because it is hypoxia that "sets the stage" for the development of reoxygenation injury. Many studies directly measuring free radicals in experimental models of ischemia have found that the magnitude of the free radical generation after reperfusion is proportional to the magnitude of the flow deficit during the antecedent coronary occlusion [32]. These facts support the important concept that the severity of the reoxygenation injury component is proportional to the severity of the hypoxic injury component. Accordingly, any intervention that attenuates the severity of the hypoxic injury will also, indirectly, attenuate the severity of the subsequent reoxygenation injury. Our data clearly support these statements and the hypothesis that administration of antioxidants prior to the onset of ischemia may reduce tissue damage, and suggest the importance of future researches in this direction. In this light, green tea consumption could be of particular importance, since green tea is one of the most popular drink in the world, and is an important source of polyphenols involved in the antioxidative action. The antioxidative capacity of green tea has to be ascribed to its catechin content and composition. Catechins show a mechanism of radical scavenging action due to the presence of hydroxy groups on their B ring. The antioxidative mechanisms of catechins has been fully characterized demonstrating that each molecule can scavenge four radicals per mole (for a review see [33]).

The possibility that an antioxidant-rich diet, and consequently a high plasma total antioxidant capacity, could represent a useful tool not only in the prevention of myocardial ischemia, but also in counteracting extensive tissue damage in the luckless case of ischemia onset, is now emerging [34–36]. Future researches could allow to set up a protocol of antioxidant administration and/or dietary recommendations in order to reduce the severity of cardiac injury following myocardial ischemia.

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