

Effect of trolox C on cardiac contracture induced by hydrogen peroxide

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Abstract

Hydrogen peroxide (H₂O₂) perfused into the aorta of the isolated rat heart induces a positive inotropic effect, with cardiac arrhythmia such as extrasystolic potentiation or cardiac contractures, depending on the dose. The last effect is similar to the "stone heart" observed in reperfusion injury and may be ascribed to lipoperoxidation (LPO) of the membrane lipids, to protein damage, to reduction of the ATP level, to enzymatic alterations and to cardioactive compounds liberated by LPO. These effects may result in calcium overload of the cardiac fibers and contracture ("stone heart"). Hearts from male Wistar rats (300-350 g) were perfused at 31°C with Tyrode, 0.2 mM trolox C, 256 mM H₂O₂ or trolox C + H₂O₂. Cardiac contractures (baseline elevation of the myograms obtained) were observed when hearts were perfused with H₂O₂ (Tyrode: 5.9 ± 3.2; H₂O₂: 60.5 ± 13.9% of the initial value); perfusion with H₂O₂ increased the LPO of rat heart homogenates measured by chemiluminescence (Tyrode: 3,199 ± 259; H₂O₂: 5,304 ± 133 cps mg protein⁻¹ 60 min⁻¹), oxygen uptake (Tyrode: 0.44 ± 0.1; H₂O₂: 3.2 ± 0.8 nmol min⁻¹ mg protein⁻¹) and malonaldehyde (TBARS) formation (Tyrode: 0.12 ± 0; H₂O₂: 0.37 ± 0.1 nmol/ml). Previous perfusion with 0.2 mM trolox C reduced the LPO (chemiluminescence: 4,098 ± 531), oxygen uptake (0.51 ± 0) and TBARS (0.13 ± 0) but did not prevent the H₂O₂-induced contractures (33.3 ± 16%). ATP (Tyrode: 2.84 ± 0; H₂O₂: 0.57 ± 0) and glycogen levels (Tyrode: 0.46 ± 0; H₂O₂: 0.26 ± 0) were reduced by H₂O₂. Trolox did not prevent these effects (ATP: 0.84 ± 0 and glycogen: 0.27 ± 0). Trolox C is known to be more effective than α-tocopherol or γ-tocopherol in reducing LPO though it lacks the phytol portion of vitamin E to be fixed to the cell membranes. Trolox C, unlike vitamin A, did not prevent the glycogen reduction induced by H₂O₂. Trolox C induced a positive chronotropic effect that resulted in higher energy consumption. The reduction of energy level seemed to be more important than LPO in the mechanism of H₂O₂-induced contracture.

Key words

- Trolox C
- "Stone heart"
- Free radicals
- Hydrogen peroxide

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Introduction

Bianchini and Belló (1) demonstrated a positive inotropic effect with cardiac arrhythmia induced by hydrogen peroxide (H_2O_2) by using whole perfused rat hearts. The injection of 0.15 ml of 128 mM H_2O_2 into the aorta of the isolated rat heart (perfused at constant pressure) induces cardiac contracture ("stone heart") (2) that is partially antagonized by nifedipine (3) or more efficiently by 0.1 mM indomethacin (4).

The mechanisms that seem to be responsible for these cardiac effects may be attributed to lipoperoxidation (LPO) of membrane lipids (5), to protein damage, to reduction of the ATP level induced by H_2O_2 , to enzymatic alterations (6) and to cardioactive compounds such as prostaglandins and leukotrienes liberated by LPO (4). These mechanisms may result in cytoplasmic calcium overload inducing cardiac contracture.

During the LPO process there is singlet oxygen formation that accelerates this oxidation, thus increasing the changes in membrane permeability.

Tocopherols are considered to be singlet oxygen quenchers (7,8). Trolox C, the polar portion of vitamin E, is water soluble and is reported to be more active than α - and γ -tocopherols and may interfere with the chain reaction of lipid peroxidation by reacting directly with peroxy and alkoxy radicals (9).

The aim of the present study was to test the hypothesis that trolox C can protect the rat heart from the oxidative stress induced by hydrogen peroxide.

Material and Methods

Male Wistar rats (300-350 g) were obtained from the Central Animal House of the Universidade Federal do Rio Grande do Sul. The animals were housed in plastic cages (47 x 34 x 18 cm, three animals per cage) lined with sawdust changed every 48 h, in

air-conditioned quarters and had free access to tap water and pelleted food (Purina, Nutripal, Porto Alegre, RS, Brazil).

Animals were killed by a blow to the head and the chest was opened. The heart was carefully dissected from its connections and the aorta retroperfused with Tyrode solution of the following composition: 120 mM NaCl, 5.4 mM KCl, 1.8 mM $MgCl_2$, 1.25 mM $CaCl_2$, 27 mM $NaHCO_3$, 2.0 mM NaH_2PO_4 , 1.8 mM Na_2SO_4 and 11.1 mM glucose, pH = 7.4. This solution was maintained at 31°C, gassed with $O_2:CO_2$ (95%/5%) and used for perfusion at a constant pressure of 8.02 kPa.

Four groups (six hearts each) were studied: a) control: hearts perfused with Tyrode for 30 min; b) trolox: hearts perfused with Tyrode for 10 min and with Tyrode plus trolox C (0.2 mM; Aldrich Chemical Company, Milwaukee, WI) for another 20 min; c) H_2O_2 : hearts perfused with Tyrode for 10 min and with Tyrode plus 256 mM H_2O_2 (Perhidrol, Merck, Darmstadt, Germany) for another 20 min; d) trolox and H_2O_2 : hearts perfused with Tyrode plus trolox C (0.2 mM) for 10 min and Tyrode plus H_2O_2 (256 mM) for another 20 min.

The hydrogen peroxide dose was selected according to a previously established dose-concentration curve (2). The trolox dose employed was the same as used by Wu et al. (10).

In order to record the heart beats, the apex of the left ventricle was attached to a microdisplacement myograph transducer by means of a heart clip. Using a lifter, the load was increased until the heart worked at the L_{max} level. The coronary flow (drops/min) was recorded using a piezoelectric drop counter. The results obtained during the last minute of the experiment are reported as percent of the values obtained over a period of 1 min measured between the 9th and 10th min after the beginning of perfusion.

The contractile force (g) from each heart was determined by measuring the amplitude

of the myograms obtained at 10 min of perfusion (just before change of the perfusion fluid), and 20 min later. Contracture was considered to occur when the relaxation phase was significantly reduced and was determined by measuring the baseline elevation of the myograms and reported as percent of the value measured at 10 min of perfusion. Heart rate was measured from the myograms and the values obtained in the last minute of the experiment are reported as percent variation of the values obtained between the 9th and 10th minute of perfusion.

The transducers were attached to a recorder. All recording devices were from Narco Bio-System (Houston, Texas).

At the end of the experiments, a slice (± 200 mg) obtained from the left ventricle was used for glycogen determination according to the method of Van Handel (11). Each remaining heart was then homogenized in an ultra-Turrax blender using 1 g of tissue in 5 ml 140 mmol/l potassium chloride containing 20 mmol/l phosphate buffer, pH = 7.4. The protein of the homogenate was measured by the method of Lowry et al. (12). The suspension (approximately 1 mg of protein/ml) was added to 3 mmol/l tert-butyl hydroperoxide (Sigma Chemical Company, St. Louis, MO) and assayed for chemiluminescence (13) in an LKB Rack Beta liquid scintillation spectrometer model 1215 (LKB-Produkter AB, Bromma, Sweden). Chemiluminescence is reported as counts per second per mg protein of the homogenate.

Aliquots were used for malonaldehyde determination according to the technique of Buege and Aust (14) for thiobarbituric acid reactive substances (TBARS).

Aliquots were also used for oxygen uptake determination ($\text{nmol min}^{-1} \text{mg protein}^{-1}$) using an oxymeter with a Clark electrode purchased from FQ/IQUIFIB/MADEIC, Buenos Aires, Argentina.

In order to determine the adenosine-5'-triphosphate (ATP) level, the hearts of 16 rats (300-350 g) were perfused ($N = 4$ per

group) in the same manner as the groups previously reported with Tyrode, 0.2 mM trolox C, 256 mM H₂O₂ and trolox C followed by H₂O₂. After perfusion, the hearts were homogenized and used for enzymatic determination of ATP (Sigma Diagnostics), according to Adams (15).

Results are reported as means \pm SEM. Statistical evaluation was performed by two-way ANOVA followed by the Scheffé *t*-test or the Student *t*-test for independent samples using the SPSS-PC software (SPSS, Inc., Chicago, IL). $P < 0.05$ was taken to be significant.

Results

Figure 1 shows typical myograms obtained from hearts perfused with the different solutions. Perfusion with H₂O₂ induced cardiac contractures, i.e., a 60% decrease in the relaxation phase with respect to the myograms observed at 10 min of perfusion (60.5 ± 13.9). Previous perfusion with 0.2 mM trolox C did not significantly reduce the contracture induced by H₂O₂ (33.3 ± 16.1) ($F_{\text{cal}} = 1.64$). A small nonsignificant increase in the relaxation phase was demonstrable when hearts were perfused only with

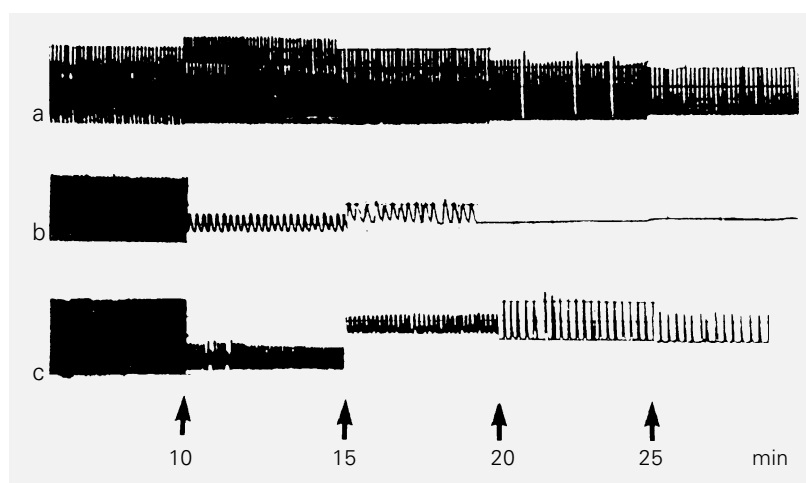


Figure 1 - Typical records obtained from hearts perfused with trolox C (0.2 mM) (a), H₂O₂ (256 mM) (b) and trolox C followed by H₂O₂ (c). The figures show myograms for the last minute of the control period followed by records obtained for 1 min after the time indicated by the arrows.

0.2 mM trolox C. The experiments were repeated with perfusion with 2 mM trolox C but the results were almost the same, i.e., trolox C did not block the cardiac contractures induced by H_2O_2 (data not shown).

Figure 2 shows the kinetic profile (77 min) of chemiluminescence initiated by tert-butyl hydroperoxide in homogenates of hearts perfused with 0.2 mM trolox C, 256 mM H_2O_2 and trolox C followed by H_2O_2 .

Table 1 shows the results obtained by various methods used to study LPO. H_2O_2 injection increased oxygen uptake, TBARS levels and chemiluminescence of heart homogenates. In the hearts previously perfused with 0.2 mM trolox C, all these phenomena were reduced, indicating LPO reduction.

Table 2 shows the contracture, heart rate and coronary flow (% of the initial values) of the different groups. It should be pointed out that trolox C increased the heart rate and coronary flow but did not prevent the contractures and the coronary constriction induced by H_2O_2 .

H_2O_2 reduced the heart glycogen concentration and trolox C did not prevent this reduction (Table 3). The ATP level was reduced by

H_2O_2 and previous perfusion with trolox C did not inhibit this effect (Table 3).

Discussion

The H_2O_2 dose used to induce cardiac contractures in the present study was high, far above the possible concentration level that would be observed in the ischemia-reperfusion phenomenon. The low temperature used ($31^\circ C$) could explain, in part, the high dose employed, but the most important fact is that perfusion was performed at constant pressure. H_2O_2 is so potent a coronary constrictor that it almost completely blocks coronary flow within the first 5 s. To avoid coronary constriction, hearts were perfused at a constant flow. In this condition, contractures similar to those obtained in this study could be induced by doses as low as 0.25 mM. This H_2O_2 concentration would occur in the ischemia-reperfusion injury (16).

H_2O_2 can react with iron ions resulting in hydroxyl radical formation. This radical can initiate the LPO of membrane lipids. The lipoperoxide production may change membrane permeability leading to the formation of hydrophilic "pores" (5).

The arachidonic acid present in cell membranes can be liberated by LPO and form various cardioactive compounds. In particular, it can be converted into prostaglandins or leukotrienes (17). Fatty acids or their oxidation products may act as ionophores. The energy loss and the presence of "pores" result in intracellular calcium overload that blocks muscle relaxation.

The overall results indicate that H_2O_2 induced LPO and cardiac contracture and that trolox C (0.2 or 2 mM) was effective in preventing the H_2O_2 -induced LPO but not the contractures.

During LPO there is singlet oxygen formation that increases this oxidative process (7). Tocopherols and trolox C are known to be singlet oxygen quenchers capable of reacting with LPO products. In our observa-

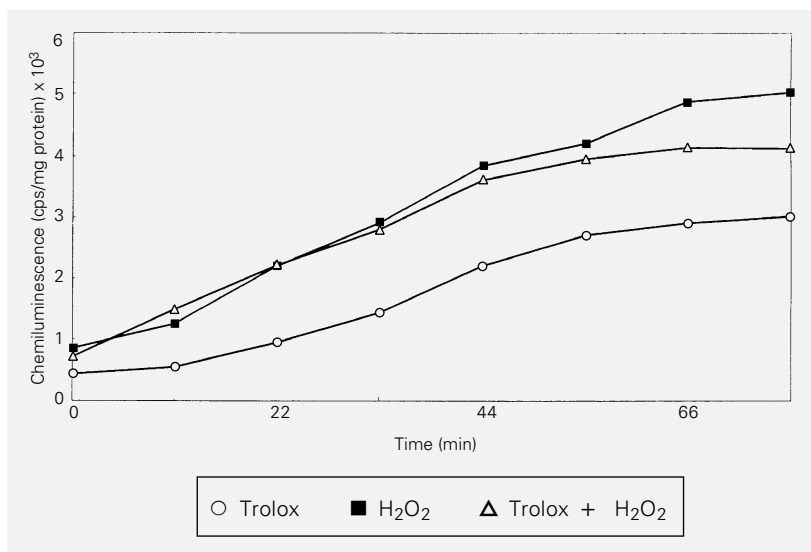


Figure 2 - Kinetic profile of chemiluminescence initiated by tert-butyl hydroperoxide (counts per second per mg protein) from homogenates of hearts perfused with trolox C (0.2 mM), with hydrogen peroxide (256 mM) or with trolox C followed by hydrogen peroxide.

Table 1 - Different methods to measure lipoperoxidation.

Data are reported as means \pm SEM for 6 hearts in each group. *P<0.05 compared to the respective control (Student *t*-test); NS, not different from the control group. +P<0.05 compared to H₂O₂ group (ANOVA, Scheffé test). The control group was perfused with Tyrode only.

	Control	H ₂ O ₂ (256 mM)	Trolox C (0.2 mM)	Trolox C + H ₂ O ₂
TBARS (nmol/ml)	0.12 \pm 0.02	0.37 \pm 0.10*	0.23 \pm 0.10 ^{NS}	0.13 \pm 0.03 ^{NS+}
Oxygen uptake (nmol min ⁻¹ mg protein ⁻¹)	0.44 \pm 0.15	3.21 \pm 0.80*	0.44 \pm 0.15 ^{NS*}	0.51 \pm 0.07 ^{NS+}
Chemiluminescence (cps mg protein ⁻¹ 60 min ⁻¹)	3,199 \pm 259	5,304 \pm 133*	3,324 \pm 358 ^{NS*}	4,098 \pm 531 ^{NS}

Table 2 - Contracture, heart rate and coronary flow (% of initial value) of the different groups.

Data are reported as means \pm SEM for 6 hearts in each group. *P<0.05 compared to the respective control; NS, not different from the H₂O₂ group. +P<0.05 compared to H₂O₂ group (ANOVA, Scheffé *t*-test).

	Control	Trolox C (0.2 mM)	H ₂ O ₂ (256 mM)	Trolox C + H ₂ O ₂
Contracture	5.9 \pm 3.2	13.1 \pm 4.5	60.5 \pm 13.9*	33.3 \pm 16.0 ^{NS}
Heart rate	53.3 \pm 7.2	88.8 \pm 2.6*	6.9 \pm 5.0*	36.1 \pm 9.5+
Coronary flow	46.2 \pm 10.2	83.7 \pm 3.8*	4.2 \pm 1.7*	23.6 \pm 12.1 ^{NS}

tion, the reductions in oxygen uptake, TBARS levels and chemiluminescence all suggest that singlet oxygen and/or products of the LPO were quenched or scavenged by trolox C. However, the present study indicates that effective protection by trolox C against H₂O₂-induced lipid peroxidation is not associated *per se* with effective protection against H₂O₂-induced contracture.

Schraufstatter et al. (6) observed that H₂O₂ can activate a nuclear enzyme, poly (ADP-ribose) polymerase, that uses NAD as substrate. The activation reduces the level of ATP necessary for the ionic pumps. H₂O₂ can also inhibit glycolysis, reducing the ATP stores necessary to maintain the fundamental ionic difference between the two cell membrane sides. Moreover, we observed that glycogen and ATP levels were reduced by H₂O₂ and trolox C was unable to prevent this reduction. On the other hand, vitamin A was more effective in protecting the heart

from the oxidative stress induced by H₂O₂, by reducing the contractures and LPO and by maintaining the glycogen level (Table 2) (18). The contracture was probably prevented by vitamin A by means of energy conservation.

H₂O₂, at the dose employed, provoked

Table 3 - Glycogen content (g%) and ATP values (μ mol/g dry tissue) in hearts perfused with different solutions.

Data are reported as means \pm SEM for 4 hearts in each group. *P<0.05 compared to the respective control (Student *t*-test).

	Glycogen	ATP
Control	0.46 \pm 0.06	2.84 \pm 0.05
Trolox C (0.2 mM)	0.18 \pm 0.01*	2.27 \pm 0.42
H ₂ O ₂ (256 mM)	0.26 \pm 0.01*	0.57 \pm 0.44*
Trolox C + H ₂ O ₂	0.27 \pm 0.03*	0.84 \pm 0.24*

depression of the contractile function (“stunning”). Trolox C, unlike vitamin A, increased the heart rate (18). This may result in greater energy consumption, reducing the energy available for the ionic pumps, leading to an elevation of intracellular calcium. The increased intracellular calcium level (that may be responsible for the contracture) can activate calcium-dependent phospholipases with subsequent release of fatty acids from membranes and activation of the arachidonic acid cascade. It is known that cardiac contractures can be induced by prostaglandins (19). The calcium overload results in cardiac stiffening known as “stone heart” or cardiac contracture.

It can also be seen in Table 2 that trolox C

increased coronary flow and heart rate, which in turn also increased energy consumption. The vasodilation induced by trolox C is a result that merits further investigation.

Trolox C does not have the phytol portion of vitamin E that fixes this vitamin to the cell membrane (20). This localization is strategic to protect the cell membrane against lesions induced by oxidative stress. But the reduction of the energy level seemed to be more important than LPO in the mechanism of cardiac H₂O₂-induced contractures.

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