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Oleuropein prevents oxidative myocardial injury induced by ischemia and reperfusion

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Abstract

The potential protective effects of oleuropein, a dietary antioxidant of olive oil, has been investigated in the isolated rat heart. The organs were subjected to 30 minutes of no-flow global ischemia and then reperfused. At different time intervals, the coronary effluent was collected and assayed for creatine kinase activity as well as for reduced and oxidized glutathione. In addition, the extent of lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substance concentration in cardiac muscle. Pretreatment with 20 μg/g oleuropein before ischemia resulted in a significant decrease in creatine kinase and reduced glutathione release in the perfusate. The protective effect of oleuropein against the post-ischemic oxidative burst was investigated by measuring the release, in the coronary effluent, of oxidized glutathione, a sensitive marker of heart's exposure to oxidative stress. Reflow in ischemic hearts was accompanied by a prompt release of oxidized glutathione; in ischemic hearts pretreated with oleuropein, this release was significantly reduced. Membrane lipid peroxidation was also prevented by oleuropein. The reported data provide the first experimental evidence of a direct cardioprotective effect of oleuropein in the acute events that follow coronary occlusion, likely because of its antioxidant properties. This finding strengthens the hypothesis that the nutritional benefit of olive oil in the prevention of coronary heart disease can be also related to the high content of oleuropein and its derivatives. Moreover, our data, together with the well documented antithrombotic and antiatherogenic activity of olive oil polyphenols, indicate these antioxidants as possible therapeutic tools for the pharmacological treatment of coronary heart disease as well as in the case of cardiac surgery, including transplantation.

Keywords: Heart; Ischemia; Oleuropein; Olive oil; Antioxidant; Polyphenol

1. Introduction

Oleuropein is a phenolic antioxidant that is present in elevated concentration in olives and olive oil, influencing their sensory organoleptic properties and being responsible for their typically bitter and pungent aroma [1,2]. This complex phenol can be hydrolyzed either to hydroxytyrosol and elenolic acid glucoside or to oleuropein aglycone and glucose (Fig. 1).

Oleuropein and its derivatives have a variety of biochemical roles [3–5], including anti-inflammatory and antithrombotic activities [6]. These polyphenols are able to prevent low-density lipoprotein oxidation [7,8] and platelet aggregation [9] and to inhibit lipoxygenases and eicosanoid production [9,10]. Furthermore, as we have directly demonstrated, hydroxytyrosol is able to counteract reactive oxygen species (ROS)–mediated cytotoxicity in human cell systems, including Caco-2 cells [11] and erythrocytes [12]. Finally, its metabolism and transport have been amply explored [4,13–15].

However, despite the well established data supporting the hypothesis that phenolic components significantly contribute to the health beneficial effect of olive oil intake, a direct cardioprotective effect of these molecules has not yet been explored. Therefore, to elucidate further the contribution of olive oil antioxidant in the prevention of coronary

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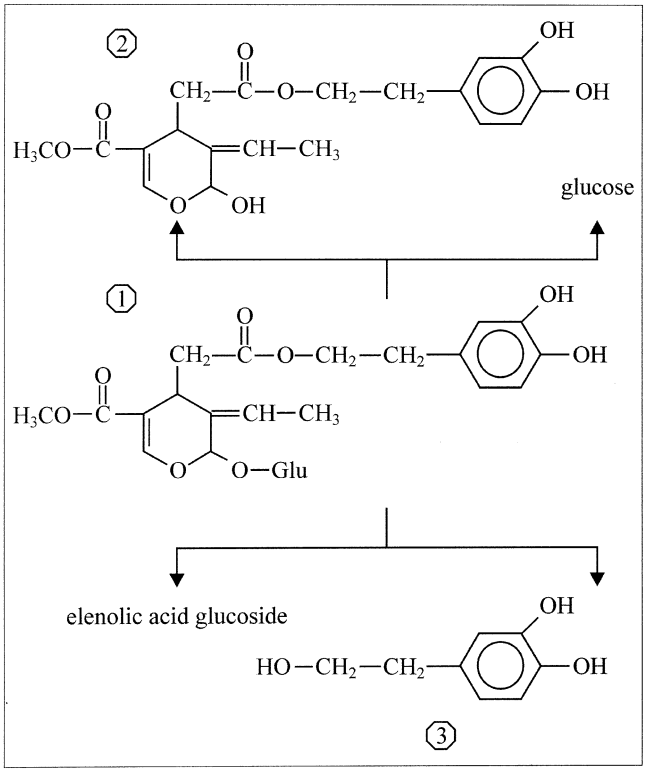

Fig. 1. Chemical structure of oleuropein and its derivatives. 1 = oleuropein; 2 = oleuropein aglycone; 3 = hydroxytyrosol.

heart disease (CHD), we have investigated the effect of oleuropein against oxidative myocardial injury induced by ischemia and reperfusion.

Considerable evidence indicates that both ROS and reactive nitrogen species (RNS) are involved in the cellular responses to hypoxia and subsequent oxidative injury during reoxygenation, in different organs [16,17]. Data supporting the relevant role of ROS and RNS in ischemia/reperfusion (I/R) injury include detection of oxidated and nitrated biomolecules [18,19] as well as lipoperoxidation end-products [20,21]. Moreover, increased free-radical production has been directly evidenced by the paramagnetic resonance and spin-trapping technique [22]. Extended periods of anoxia or severe ipoxia may eventually lead to cell death, by either necrosis or apoptosis or by both [23,24].

From a clinical point of view, I/R-induced biochemical alterations play a key role in the pathogenesis of several diseases including myocardial infarction [16,17], one of the most common causes of mortality in western countries. Therefore, elucidation of the key role played by oxidative stress in I/R-induced myocardial damages could lead to new nutritional strategies to reduce tissue injury, either by preventing formation of both oxygen and nitrogen reactive species or by scavenging them through dietary antioxidants [25].

In the present study, we examined the possible protective effect of oleuropein in preventing I/R-induced oxidative injuries using isolated rat heart subjected to global ischemia and then reperfused.

2. Methods and materials

2.1. Chemicals

Oleuropein was purchased from Extra Synthese (Geney, France). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Perfusion buffer

The perfusion buffer consisted of the following: 117 mmol/L NaCl, 6.0 mmol/L KCl, 3.0 mmol/L$CaCl_2$, 1.0 mmol/L $MgSO_4$, 0.5 EDTA, 16.7 mmol/L glucose, and 24 mmol/L $NaHCO_3$, pH 7.4. High-purity–grade reagents from Carlo Erba (Milan, Italy) were dissolved in twice-distilled water and the buffer was equilibrated at 37° C with a gas mixture of 95% O_2–5% CO_2.

2.3. Isolated heart preparation

Experiments were conduced in conformity with the "Guiding Principles for Research Involving Animals (Guide for the Care and Use of Laboratory Animals, DHEW publication No. (NIH) 80-23. Revised 1978, reprinted 1980, Office of Science and Health Reports, DRR/NIH, Bethesda, MD)." Isolated hearts were prepared as previously reported [26]. Male Sprague-Dawley (250–300 g) rats were heparinized and anesthetized with thiopental by intraperitoneal injection. The hearts were excised and perfused in the retrograde Langendorff mode under constant flow. The flow rate of each heart was initially adjusted to achieve a perfusion pressure of 70–80 mm Hg. The right ventricle was paced at 360 beats/min through a stimulation catheter connected to a Harvard Research stimulator (Harvard Apparatus, South Natick, MA).

After a 20-minute equilibration period, hearts were subjected to 30 minutes of global ischemia by cross-clamping the perfusion line. During ischemia hearts were kept at 37°C and the pacer turned off. After this period, hearts were reperfused for 1 hour and the coronary effluent samples were collected at different time intervals for creatine kinase (CK) and glutathione assays of both the oxidized and reduced forms. At the end of the experiment, hearts were removed from the perfusion apparatus, weighed, and processed to evaluate lipoperoxidation.

2.4. CK assay

Myocardial damage was evaluated by CK activity (expressed as U/min/g wet weight), measured in the coronary

effluent using a commercial kit from Abbott Clinical Chemistry (Abbott Park, IL).

2.5. Reduced glutathione and oxidized glutathione assays

Glutathione levels in the coronary effluent was measured by the glutathione reductase/5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) recirculating assay [27]. To measure total glutathione (*i.e.*, oxidized glutathione [GSSG] + reduced glutathione [GSH]), aliquots of coronary effluent were incubated at 25°C for 10 minutes, with potassium phosphate buffer 6 mmol/L, NADPH 149 μmol/L, EDTA 1 mmol/L, and DTNB 150 μmol/L in the presence of 1 U/mL GSSG reductase; the absorbance was then read at 412 nm.

For selective measurement of GSSG release, aliquots of coronary effluent were immediately added to 1 mmol/L EDTA and 5 mmol/L N-ethylmaleimide (NEM) to prevent artifactual oxidation of GSH. The samples were allowed to stand for 10 minutes at 0°C and then extracted seven times with water-satured dithyl ether to remove excess NEM.

Glutathione concentrations were expressed as nanomoles of GSH equivalents released per minute per gram of wet weight. GSH release was calculated from the difference between total and oxidized glutathione.

2.6. Evaluation of lipid peroxidation in the cardiac tissue

Both atria and the right ventricle were removed, and the left ventricle was homogenized in NaCl 1.15% containing SDS 5%, 1:3 (w/v). The homogenate was centrifuged at 10,000 × *g* for 1 hour at 4°C and the supernatant used to measure lipoperoxidation end products according to a modified thiobarbituric acid (TBA) method [20,28]. Briefly, 0.4 mL of the sample was treated with 1 mL of 20% acetic acid and 1 mL of 1% TBA and incubated for 15 minutes at 90°C in a water bath. After cooling, the samples were extracted with 2 mL of butanol/pyridine (15:1) and the absorbance was read at 532 nm. Thiobarbituric acid–reactive substance (TBARS) concentration, expressed as nmol/g wet weight, was obtained using the value of 153,000 $(\text{mol/L})^{-1} \cdot \text{cm}^{-1}$ as the extinction coefficient of TBA adducts.

2.7. Effect of olive oil oleuropein

To study the potential cardioprotective effects of oleuropein, hearts were perfused with the phenolic compound at a concentration of 50 μmol/L for 15 minutes (corresponding to 20 μg/g of wet weight), before induction of ischemia.

2.8. Statistical analysis

Results are reported as means ± SD ($n = 6$). The area under the curve (AUC) test was used to evaluate data on the time course of post-ischemic release of CK, GSH, and GSSG (Figs. 2 and 3). AUC values, calculated from data of all OE-treated *versus* nontreated samples, were compared by means of the Student *t* test ($P < 0.05$). Differences between data on the cardiac TBARS levels (Fig. 4) were analyzed for significance by performing a Student *t* test ($P < 0.05$).

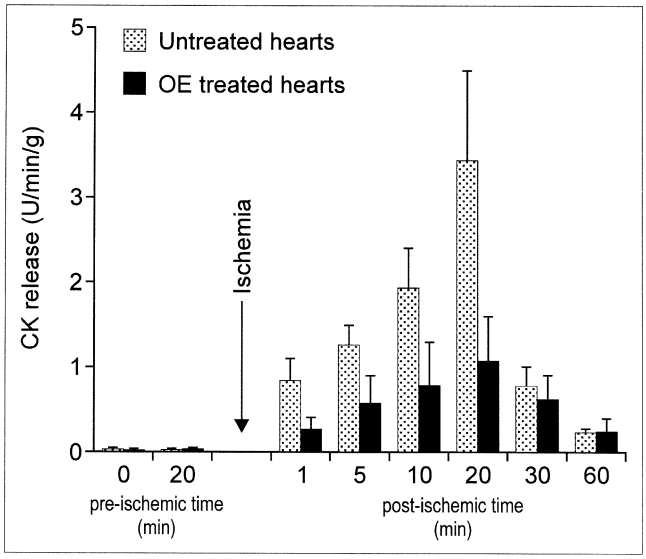

Fig. 2. Effect of oleuropein (OE) on creatine kinase (CK) release in the coronary effluent after ischemia/reperfusion of isolated rat hearts. Isolated hearts were subjected to 30 minutes of global ischemia and then reperfused. At different time intervals the coronary effluent was collected and CK activity measured as described in the Methods and materials section. Data (mean ± SD; $n = 6$) were analyzed by the area under the curve (AUC) test. AUC values, calculated from data of all OE-treated *versus* nontreated samples, were compared by the Student *t* test ($P < 0.05$).

3. Results

3.1. Effect of oleuropein on I/R-induced release of CK

In clinical practice, the severity of cardiac I/R injury is related to increased serum concentration of cardiac enzymes, including CK. Therefore, we selected this biochemical marker of cellular damage to test the possible protective effect of oleuropein against I/R-induced lysis of cardiac cells. Isolated hearts were subjected to 30 minutes of global ischemia and were then reperfused; at different time intervals, the coronary effluent was collected and assayed for CK activity.

As shown in Fig. 2, ischemic treatment results in an extensive, time-dependent release of the cardiac enzymes in the coronary effluent after reperfusion; the post-ischemic release of CK starts rapidly after reflow and reaches its maximum after 20 minutes. When hearts are treated with oleuropein (20 μg/g tissue), a significant decrease in CK release can be observed.

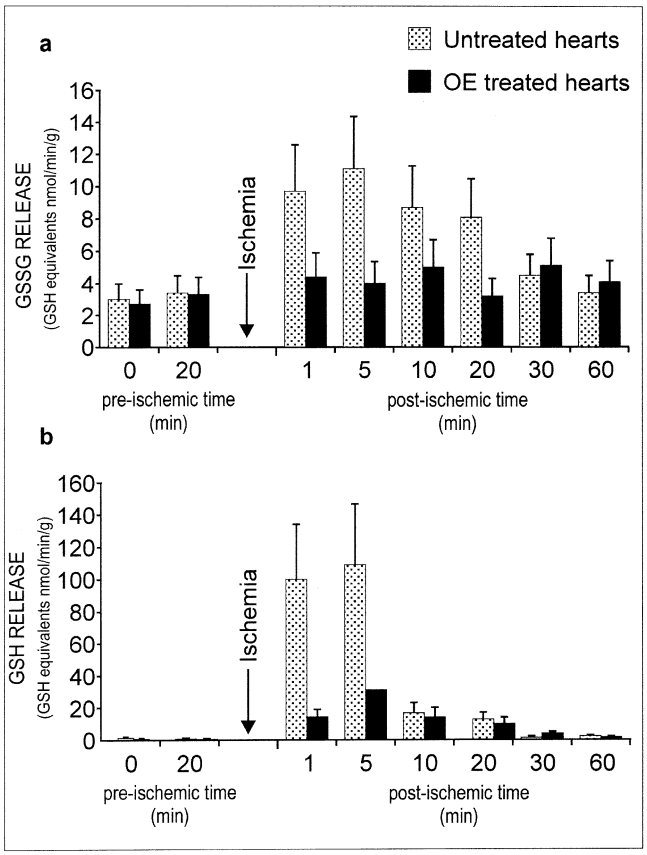

Fig. 3. Effect of oleuropein (OE) on glutathione release in the coronary effluent after ischemia/reperfusion of isolated rat hearts. Isolated hearts were subjected to 30 minutes of global ischemia and then reperfused. At different time intervals the coronary effluent was collected and glutathione levels measured as described in the Methods and materials section. Data (mean ± SD; $n = 6$) were analyzed by the area under the curve (AUC) test; AUC values, calculated from data of all OE-treated *versus* untreated samples, were compared by the Student t test ($P < 0.05$). Panel a shows the time course of the efflux of oxidized glutathione; panel b shows the time course of the efflux of reduced glutathione.

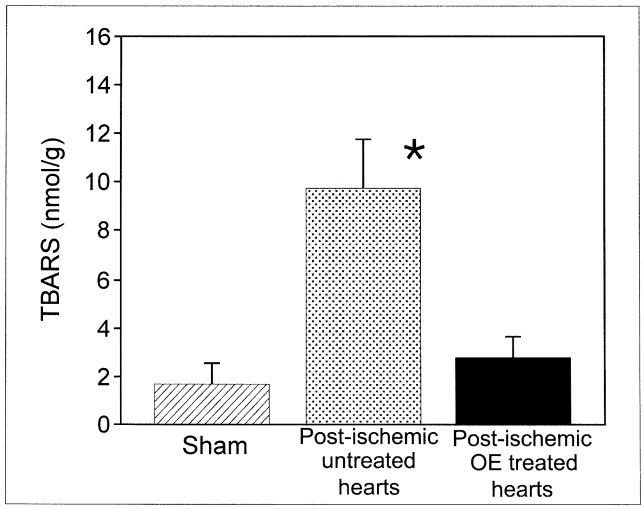

Fig. 4. Effect of oleuropein (OE) on thiobarbituric acid–reactive substance (TBARS) formation in the cardiac tissue after ischemia/reperfusion of isolated rat hearts. Isolated hearts were subjected to 30 minutes of global ischemia and then reperfused. After 1 hour of reperfusion, hearts were removed from the perfusion apparatus and TBARS measured as described in the Methods and materials section. Data (mean ± SD; $n = 6$) were analyzed by the Student t test. *$P < 0.05$ compared to sham samples.

3.2. Effect of oleuropein on I/R-induced glutathione release

The protective effect of oleuropein against the post-ischemic oxidative burst was investigated by measuring the release, in the coronary effluent, of the oxidized glutathione, a sensitive marker of heart's exposure to oxidative stress [29]. It has been previously demonstrated that a significant increase in GSSG intracellular levels, as well as a stimulation of its cardiac energy–dependent efflux, occur in post-ischemic hearts [26,29].

As shown in Fig. 3a, a modest release of GSSG also occurred before ischemic perfusion. Reflow of ischemic hearts was accompanied by a prompt release of GSSG; the GSSG efflux peaked 5 minutes after reperfusion and remained significantly higher than baseline values up to 20 minutes. In ischemic hearts pretreated with oleuropein, GSSG release was significantly prevented.

In addition, levels of the reduced form of glutathione, washed out from injured myocytes, were also measured in the same coronary effluent samples (Fig. 3b).

Release of GSH before ischemic perfusion was negligible in all the samples examined. After reperfusion, a dramatic increase in GSH release was observable that was 10 times higher than GSSG efflux; this basically reflected the different intracellular concentrations of the oxidized and reduced forms of the sulfur compound. Also, in this case, oleuropein was also shown to be dramatically protective, as it limits the release of GSH in the antioxidant-treated hearts (Fig. 3b).

3.3. Effect of oleuropein on I/R-induced lipoperoxidation

To investigate the specific molecular oxidative alterations that ultimately result in cardiac tissue injury, the levels of TBARS were measured in both I/R- and oleuropein-treated hearts and compared with those in control samples. It should be stressed, in this respect, that lipid peroxidation is thought to be a major mechanism involved in I/R-induced impairment of cardiac function [20,21].

As shown in Fig. 4, TBARS baseline value in normally perfused control hearts averaged 2.27 ± 0.46 nmol/g wet weight. In post-ischemic hearts, after 1 hour of reperfusion, a 5-fold increase in TBARS concentration was observable, indicating a severe oxidative alteration of the cardiac membrane phospholipids. Oleuropein appeared to be completely protective in that no significant increase in TBARS was observable in the antioxidant-treated hearts compared to sham samples.

4. Discussion

The results reported in this paper provide the first experimental evidence of a direct cardioprotective effect of oleuropein in the acute events that follow coronary occlusion.

During the last decade, a number of studies have focused attention on the crucial role of nonvitamin dietary antioxidants such as polyphenols. Data have been collected indicating that the elevated phenolic antioxidant content of the components of the Mediterranean diet, together with antioxidant vitamins, greatly contributes to the health-beneficial effects of this diet. In this respect, our findings and those in the literature confirm that the nutritional benefit of olive oil in preventing CHD should be ascribed not only to the elevated oleic acid content but also to the antioxidant properties of oleuropein and its derivatives. Moreover, recent data from an animal study by Coni et al. indicate that the dietary intake of olive oil polyphenols could contribute to modulate the antioxidant balance *in vivo*. These investigators demonstrated that rabbits fed with an oleuropein-rich diet show a higher serum antioxidant capacity and an increased resistance to lipoperoxidation compared to animals receiving a standard diet [30].

It is noteworthy that in our experimental system, a significant protection against I/R-induced oxidative stress was observed in rat hearts pretreated with as little as 20 mg/kg of tissue wet weight, a dose comparable to the average daily intake of biophenols from olive oil in the Mediterranean diet [30]. Therefore, daily intake of extra-virgin olive oil containing high levels of phenolic compounds could be useful to maximize the protective properties of antioxidants, thereby contributing to the prevention of pathologic conditions with etiologies or progression related to free-radical–mediated citotoxicity.

Finally, our data, together with the that on the well documented antithrombotic and antiatherogenic activity of olive oil polyphenols [3–6], indicate that these antioxidants are possible therapeutic tools for pharmacological treatment of CHD as well as being useful in relation to cardiac surgery and transplantation [4,13–15].

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