Protective effects of caffeic acid phenethyl ester on rotenone-induced myocardial oxidative injury

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Abstract

Rotenone, an insecticide, causes toxicity through inhibition of mitochondrial electron transport chain at complex I and oxidative injury to the tissues. The aim of the present study was to determine in vivo effects of rotenone on myocardium and cardio-protective effects of caffeic acid phenethyl ester (CAPE), an antioxidant agent, against rotenone toxicity in rats. The rats were divided into three groups: untreated control, rotenone (2.5 mg/kg/day for 60 days, i.p.) and rotenone + CAPE groups. CAPE was administrated i.p. 10 μmol/kg/day for 62 days started two days before first dose rotenone injection. The malondialdehyde, nitric oxide levels and xanthine oxidase activity of rotenone group was significantly higher than control and rotenone + CAPE groups (p < 0.05). However, catalase activity in the rotenone group was decreased in comparison with the other groups (p < 0.05). The superoxide dismutase activity of rotenone group was insignificantly decreased compared to the others. In conclusion, rotenone caused lipid peroxidation in myocardial tissue and CAPE treatment prevented this rotenone-induced lipid peroxidation in rats. CAPE might be a cardio-protective agent against myocardial toxicities.

Keywords: Rotenone; Heart; Oxidants/antioxidants; Lipid; Peroxidation; Nitric oxide; CAPE

1. Introduction

Rotenone is the major ingredient and principal component of cube resin from a potent botanical insecticide and pesticide especially against a mite which affects colonies of the honey bees [1]. It is a
naturally found in the environment due to distribution via human being and may be a risk for human and environmental health [2].

Rotenone affects mitochondrial functions and acts as a specific inhibitor of complex I (NADH:ubiquinone oxireductase) of electron transport chain in the inner mitochondrial membrane [3]. Bashkatova et al. [4] suggested that oxidative stress associated with partial inhibition of complex I was a crucial factor for neurodegeneration occurring under rotenone administration and might be an important factor in neuronal cell death observed in neurodegenerative disorders. It was reported that rotenone produces cell injury through the inhibition of respiratory chain and induces a non-specific central nervous system and systemic toxicity [5]. The accumulation and aggregation of reactive oxygen species (ROS) has been known as a main reason of rotenone-induced toxicity [6,7]. Oxygen radicals are normally produced during metabolic process, however a harmful state, known as oxidative stress, can occur when their production is accelerated or when cellular protective mechanisms are impaired [8]. Mitochondria is one of the main production place of ROS, and the mitochondrion ROS levels are elevated in a variety of pathological conditions such as toxic chemicals resultant with oxidative cellular injuries [9,10]. The results of Bashkatova et al. provide the first direct evidence that there is no acute toxic effect of rotenone, only chronic administration of rotenone increases NO tissue level. They found that rotenone at the low dose of 1.5 mg/kg enhances NO generation in all studied brain areas after both 30 and 60 days of administration [4].

Caffeic acid phenethyl ester (CAPE) which is an active component of honeybee propolis extracts, completely blocks production of free radicals in human neutrophils and xanthine–xanthine oxidase system at a concentration of 10 μM [8,11]. CAPE was found to be a protective agent against anthra-cyclines-induced neuronal oxidant injury. [12]. Fadillioglu et al. [13] demonstrated that CAPE has antioxidant protective effects on myocardial tissue against oxidative injury induced by doxorubicin, an anthracycline anticancer drug.

To our knowledge, the mechanisms underlying oxidative damage of the myocardium of rats produced by rotenone and a possible role of NO and lipid peroxidation processes during chronic rotenone administration have not yet been studied. The objective of the present study was to investigate in vivo effects of rotenone on myocardial lipid peroxidation, antioxidant status and nitric oxide (NO) levels and protective effects of CAPE on possible rotenone-induced myocardial injury in rats.

2. Materials and methods

2.1. Animals and experimental procedure

Male Wistar Albino rats (7 weeks old, 220–240 g) were fed ad libitum with standard rat food and tap water, housed in quiet rooms with 12:12-h light–dark cycle (7 am to 7 pm), and kept in individual cages in a controlled room (at 25 °C, 75% humidity). The experiments were performed in accordance with “Guide for the Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 85–23, 1985” and approved by the Local Ethics Committee. Rats were randomly assigned to one of the three groups: control (n = 7), rotenone group (n = 7), rotenone plus CAPE group (n = 7). Rothenone (Sigma, St. Louis, MO) emulsified in sunflower oil at 2.5 mg/ml was given intraperitoneally once a day at 1 ml/kg for 60 days [4]. CAPE was administrated i.p. 10 μmol/kg b.wt./day [8,13] for 62 days started two days before single dose rotenone injection. Control group rats were given sunflower oil instead of rotenone injection. Rotenone group rats were treated only with rotenone for 60 days. Rotenone + CAPE group rats were treated with rotenone for 60 days and CAPE for 62 days (started two days before beginning of the rotenone treatment).

Rotenone-treated animals were monitored for overall health, weight loss, and activity during the course of rotenone administration. Control animals always gained weight, while rotenone-treated animals were generally characterized by weight loss. But there was no statistical difference for weight loss between the groups.

At the 60th day of rotenone treatment, rats were sacrificed by decapitation with the use of a guillotine. The hearts were removed rapidly within 25–50 s and washed two times with cold saline solution, placed into glass bottles, labeled, and
stored in a deep freeze (−30 °C) until processing (maximum 10 h). Tissues were homogenized in four volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.4) using a glass Teflon homogenizer (Ultra Turrax IKA T25 Basic, Germany) after cutting of the hearts into small pieces with a scissors (for 2 min at 5000 rpm). Analysis of malondialdehyde (MDA), nitric oxide (NO), and protein levels were carried out at this stage. The homogenate was then centrifuged at 5000 g for 60 min to remove debris. Clear supernatant fluid was taken, analysis of catalase (CAT) and xanthine oxidase (XO) activities as well as protein concentration was carried out at this stage. The supernatant solution was extracted with an equal volume of an ethanol/chloroform mixture (5/3, volume per volume). After centrifugation at 5000 g for 30 min, the clear upper layer (the ethanol phase) was taken and used in the analysis of superoxide dismutase (SOD) activity and protein assays. All preparation procedures were performed at +4 °C.

2.2. Malondialdehyde determination

The MDA level was determined by a method based on its reaction with thiobarbituric acid (TBA) at 90–100 °C [14]. In the TBA test reaction, MDA or MDA-like substances and TBA react together for production of a pink pigment having an absorption maximum at 532 nm. The reaction was performed at pH 2–3 at 90 °C for 15 min. The sample was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm (UV-GBC Cintra 10e spectrophotometer, Austria). The results were expressed as nanomole per gram protein in heart tissues according to a standard graphic, which was prepared with serial dilutions of standard 1,1,3,3-tetramethoxypropane.

2.3. Nitric oxide determination

As NO measurement is very difficult in biological specimens, tissue nitrite (NO$_2^-$) and nitrate (NO$_3^-$) were estimated as an index of NO production. The method for heart nitrite and nitrate levels was based on the Griess reaction [15]. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite + nitrate) was measured spectrophotometrically at 545 nm after conversion of nitrate to nitrite by copperized cadmium granules. A standard curve was established with a set of serial dilutions (10$^{-8}$–10$^{-3}$ mol/L) of sodium nitrite. Linear regression was done by using the peak area from the nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as micromole per gram protein in heart tissue.

2.4. Superoxide dismutase activity determination

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al. [16]. The principle of the method is based on the inhibition of nitroblue tetrazolium reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the heart homogenate. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the nitroblue tetrazolium reduction rate. SOD activity was also expressed as units per milligram tissue protein.

2.5. Xanthine oxidase activity determination

Tissue XO (EC 1.2.3.2) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbency at 293 nm, according to Prajda and Weber’s method [17]. A calibration curve was constructed by using 10–50 mU/mL concentrations of standard XO solutions (Sigma X-1875). One unit of activity was defined as 1 µmol uric acid formed per minute at 37 °C, pH 7.5, and expressed as units per gram protein in heart homogenate.

2.6. Catalase activity determination

Catalase (CAT, EC 1.11.1.6) activity was determined according to the Aebi’s method [18]. The principle of the assay is based on the determination of the rate constant (s$^{-1}$, k) or the H$_2$O$_2$
decomposition rate at 240 nm. Results were expressed as k/g tissue protein.

2.7. Protein determinations

Protein assays were made by the method of Lowry et al. [19].

2.8. Statistical analysis

Data were analyzed by using a commercially available statistics software package (SPSS for Windows v. 9.0, Chicago, USA). Distribution of the groups was analyzed with one sample Kolmogrov–Smirnov test. All groups showed normal distribution, so that parametric statistical methods were used to analyze the data. One-way ANOVA test was performed and Post Hoc multiple comparisons were done with LSD. Results were presented as means ± SEM. p values <0.05 were regarded as statistically significant.

3. Results

The MDA level in the rotenone group was significantly higher than in other groups (p < 0.05) (Fig. 1). There was no significant difference between MDA levels of control and rotenone plus CAPE groups. Xanthine oxidase activity was increased in the rotenone group in comparison with the control and rotenone plus CAPE groups (p < 0.05) (Table 1). On the other hand, CAT activity in the rotenone group were decreased in comparison with the other groups (p < 0.05) (Table 1). The SOD activity of rotenone group was insignificantly decreased compared to the others (Table 1). There was no significant difference in SOD and CAT activities between the groups. The NO level of rotenone group was higher in myocardial tissue in comparison with control and rotenone plus CAPE groups (p < 0.05). There was no significant difference in NO levels of control and rotenone plus CAPE groups (Fig. 2).

![Fig. 1: Malondialdehyde (MDA) levels of myocardial tissue after rotenone and rotenone plus CAPE treatment in rats. (a) p < 0.05 in comparison with control. (b) p < 0.05 in comparison with rotenone + CAPE.](image1)

![Fig. 2: Nitric oxide (NO) levels of myocardial tissue after rotenone and rotenone plus CAPE treatment in rats. (a) p < 0.05 in comparison with control. (b) p < 0.05 in comparison with rotenone + CAPE.](image2)

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/mg protein)</th>
<th>CAT (k/g protein)</th>
<th>XO (U/g prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-Control (n = 7)</td>
<td>1.648 ± 0.119</td>
<td>0.263 ± 0.008*</td>
<td>14.958 ± 0.940*</td>
</tr>
<tr>
<td>II-Rotenone (n = 7)</td>
<td>1.400 ± 0.080</td>
<td>0.206 ± 0.006</td>
<td>18.065 ± 1.043</td>
</tr>
<tr>
<td>III-Rotenone + CAPE (n = 7)</td>
<td>1.688 ± 0.139</td>
<td>0.256 ± 0.012*</td>
<td>15.047 ± 0.988*</td>
</tr>
</tbody>
</table>

* p < 0.05 significant in comparison with rotenone group.
4. Discussion

Heart is an important organ for mitochondrial function due to its high need of oxygen-dependent energy production. Mitochondrial dysfunction, leading to energy impairment and/or oxidative stress, is associated with a number of diseases such as hypoxia, ischemia–reperfusion injury, and neurodegenerative diseases. One of the most common ways to simulate these toxic processes experimentally is to use different mitochondrial specific inhibitors [20]. Rotenone causes tissue damage to the cellular organism via its toxic effects on mitochondrial electron transport system. Rotenone inhibits specifically mitochondrial complex I which is an important in the initiation of oxygen radicals that produce cellular injury via lipid peroxidation [21,22]. It was explained that mitochondrial complexes I and III are common sites for superoxide anion generation leading to oxidative stress [23,24]. The toxic effects of mitochondrial complex I and II inhibitors were dependent on oxidative stress and lipid peroxidation. Rotenone has been shown to block the flow of electrons from complex I to ubiquinone, thus promoting electron flow through the flavine mononucleotide–complex I site and superoxide anion production [20].

Yuki et al. [25] used rotenone to induce cellular mitochondrial impairment model in primary cultured cardiomyocytes from neonatal rats. They demonstrated that rotenone gradually decreased the pH of the culture medium with incubation time and caused slight cell injury. Our study demonstrated that rotenone caused lipid peroxidation in myocardial tissue. Also, our results made us think that mitochondrial ROS production was not the only way due to high XO activity in rotenone group. The enzyme activity of XO in rotenone group was significantly increased in comparison with control group. Xanthine oxidase catalyzes the conversion of hypoxanthine to uric acid with superoxide anion production and indirectly hydroxyl radical formation. It was shown that doxorubicin administration resulted in high myocardial XO activities in rats [26].

Rotenone induces mitochondrial superoxide formation; the possibility that calcium efflux and depolarisation were due to peroxynitrite formation from reaction of superoxide with NO was investigated. It was shown that simultaneous exposure of mitochondrial membranes to NO donors and rotenone led to peroxynitrite formation [27]. The present study indicated that rotenone administration resulted in high NO level. The antioxidant enzymes, SOD and CAT, were decreased in rat hearts after rotenone treatment. However, the decrease in SOD activity was not significant in the present study. The oxidative injury due to rotenone caused consumption of the endogenous antioxidants. The treatment with antioxidants to the regimen may prevent this loss of antioxidants and lipid peroxidation.

In this study, CAPE treatment caused decrease in lipid peroxidation as well as XO activity that is a way of superoxide production. CAPE has been shown to possess anti-inflammatory, immunomodulatory, and antioxidant properties [28]. Recent studies have shown that CAPE displays antioxidant properties against oxidant conditions that cause tissue injury [8,28–30]. It was demonstrated that CAPE treatment reduced myocardial infarct size in myocardial ischemia–reperfusion injury [31]. Okutan et al. indicated that CAPE inhibited lipid peroxidation and regulated antioxidant enzymes stress in the diabetic heart [32]. In addition, our colleagues showed that CAPE prevented bleomycin-induced lung fibrosis in rat via antioxidant mechanism [33]. CAPE prevented lipid peroxidation-induced by renal ischemia–reperfusion injury [29], brain ischemia–reperfusion injury [29], doxorubicin-induced cardiotoxicity [13], and nephrotoxicity [8]. The present study demonstrated similarly in literature that CAPE treatment caused decrease in MDA level and increase in antioxidant enzyme activities. Also, CAPE treatment prevented rotenone-induced NO increase in rat heart. Fadillioglu et al. demonstrated that doxorubicin-induced myocardial toxicity resulted in high NO production and CAPE treatment prevented this NO increase [26]. CAPE was shown to inhibit NO production and iNOS protein expression induced by lipopolysaccharide plus interferon-γ as well as iNOS mRNA expression and nuclear factor-κB binding activity in a concentration-dependent manner in RAW 264.7 macrophages [28]. CAPE exhibits its anti-inflammatory effect by inhibiting
the gene expression and the catalytic activity of NOS. The results of Hosnuter et al.’s [34] study demonstrated that the high levels of NO were reduced by CAPE in burn injury, an inflammatory situation.

In conclusion, rotenone is a toxic agent for heart tissue and causes oxidative damage to the myocardium and CAPE treatment against the rotenone toxicity prevents the lipid peroxidation via both decrease production of O$_2^{-}$ and scavenger effect against ROS. In the light of the findings of this experimental study, we suggest that CAPE may have a role on the protection of rotenone-induced oxidant injury in heart. Further, in vivo and in vitro studies are required to determine whether CAPE has an exact protective role in the rotenone-induced myocardial toxicity.

References


