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INHIBITORY EFFECT OF THE ANTIOXIDANT ETHOXYQUIN ON ELECTRON TRANSPORT IN THE MITOCHONDRIAL RESPIRATORY CHAIN

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Abstract—Ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline, EQ) is an antioxidant used as a preservative in animal and human foods. In a previous work, we demonstrated that EQ induces an inhibition of renal secretory mechanisms that are dependent on metabolic energy; EQ inhibits renal ATPases. In the present study, we analyzed the effects of EQ on the metabolic pathways of renal and hepatic rat cells, as well as on mitochondria and submitochondrial particles isolated from bovine heart and kidney. EQ induced a mild inhibition of oxygen uptake when it was added to whole homogenates of rat renal cortex in the presence of glucose. In contrast, a strong concentration-dependent inhibition was produced when EQ was added to preparations of intact liver mitochondria or to submitochondrial particles isolated from renal cortex. In the presence of NADH, 90% inhibition was attained at a final concentration of 1 mM EQ. The direct inhibitory effect of EQ on NADH dehydrogenase was a most relevant finding, since no inhibitor for the partial reaction of NADH-ferricyanide on this complex has been reported previously.

Key words: kidney; liver; heart; cell respiration; ATPase; polarography

Antioxidants commonly used as food preservatives have been reported to have some deleterious effects on kidney function, and renal morphological abnormalities have been noted [1]. EQ† is used as an antioxidant in industrialized animal foods and for the control of superficial scalding in apples and pears for human consumption. EQ is eliminated mainly through the kidney, and it has been reported that EQ is accumulated in the renal cortex for several days after a single dose [2, 3]. Renal lesions induced by the chronic administration of a diet containing EQ are: irregular zones of fibrosis, tubular atrophy, focal tubular dilation, lymphocytic infiltration, deposits of calcified material in the medulla, and zones of necrosis. Manson *et al.* [4] described some ultrastructural renal abnormalities related to EQ, such as chronic glomerulonephrosis, hyperplastic tubules, and epithelial and basal membrane thickening, which may block the tubular lumen. Degeneration and thickening of the mitochondrial crests have also been described [5].

Oxygen consumption is highest in the kidney of an organism, in terms of the weight of this organ, [6], and it has been suggested that it is related to the high energy requirements for transport processes, since most of these transport processes occur against electrochemical gradients. Thus, a high and continuous supply of energy is required, and it must come mainly from phosphorylation coupled to respiration. The renal cortex shows high oxygen consumption in contrast with the medulla and papilla, which obtain their energy from glycolysis [7, 8].

In a previous paper, we demonstrated that EQ inhibits the specific uptake of *p*-aminohippurate and tetraethylammonium in renal cortical slices [9]. These two compounds are considered markers of organic anion and cation tubular secretion, respectively. These secretory mechanisms are active and require energy derived from ATP hydrolysis. EQ also inhibits renal Na^+,K^+-ATPase activity [9]. In this paper, we explore the effect of EQ on the most important source of ATP in aerobic tissues, namely oxidative phosphorylation. We measured oxygen consumption in renal cortex whole homogenates, in rat liver intact mitochondria, and in submitochondrial particles isolated from renal cortex and bovine heart. EQ inhibited the respiratory function of mitochondria and of submitochondrial particles. NADH oxidation, using ferricyanide as an electron acceptor, was also inhibited by EQ. This is a most relevant finding since, to our knowledge, no inhibitors for this partial reaction of complex I have been reported previously.

MATERIALS AND METHODS

Materials. Ethoxyquin (EQ) with a purity above

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† Abbreviations: EQ, ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline); Na^+,K^+-ATPase, sodium-potassium adenosine triphosphatase; and SMP, submitochondrial particles.

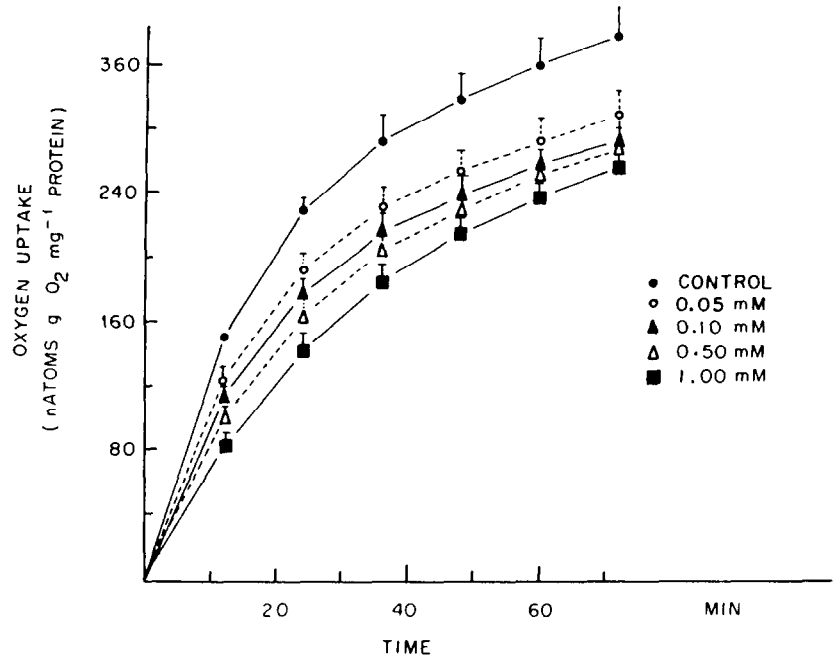

Fig. 1. Time course of the effect of EQ on the oxygen uptake of renal cortex homogenates. Kidney cortex was homogenized in Ringer's solution, and the oxygen consumption was measured in a Warburg's manometer with glucose as substrate, in the presence of increasing concentrations of EQ. Values are means ± SEM, N = 6.

94%, as assessed by gas chromatography and ultraviolet spectrometry, was donated by Insumos Químicos S.A. (México). The spectrum of this compound was similar to those measured in a standard solution obtained from the U.S.A. Environmental Protection Agency. Ethanol was used as a vehicle for EQ (0.1 M) and for antimycin (1 mg/mL), at final concentrations of <0.6%; the ethanol did not affect the measurements performed. Glucose, succinate, NADH and antimycin A were purchased from Sigma. All other chemicals were of the highest grade available and obtained from local dealers.

Experiments were carried out in adult male Wistar rats weighing 240–260 g, grown in our animal facility and maintained on standard Purina chow diet (Purina, Alief, TX) and water *ad lib.*, with temperature between 20 and 22°.

Preparation of rat kidney cortex. The rats were lightly anesthetized with ether and beheaded. Kidneys were rapidly removed and immersed in Ringer's solution with the following composition: 110 mM NaCl; 5 mM KCl; 1.2 mM $MgSO_4$; 1 mM $CaCl_2$; 1.2 mM NaH_2PO_4; 25 mM $NaHCO_3$; 7 mM glucose; and 10 mM sodium acetate, pH 7.4, at 4°. Kidney capsules were removed and slices of renal cortex were obtained with a manual microtome.

Preparation of rat liver mitochondrial fraction. Rat liver mitochondria were prepared from male rats (250 ± 10 g body weight) according to the method described by Johnson and Lardy [10].

Isolation of mitochondria from rat kidney cortex. One gram of renal cortex was homogenized in 3 mL of Ringer's solution in a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 800 *g* for 10 min. The pellet was discarded, and the supernatant was centrifuged at 15,000 *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in Tris–HCl buffer (10 mM, pH 7.4) with the sodium salt of EDTA (1 mM) and sucrose (0.25 M). All steps were carried out at 4°. Protein content was determined by the method of Lowry *et al.* [11].

Preparation of SMP. Submitochondrial particles from rat renal cortex were prepared by a modification of the method of Horstman and Racker [12]. Mitochondria from 8 g of renal cortex were resuspended in 50 mL of sucrose-EDTA buffer (1 mM sodium EDTA, 0.25 M sucrose and 10 mM Tris–HCl, pH 7.4) and were sonicated in an ice bath for 1 min at the maximum output of a Branson sonicator with a microtip. After sonication, the suspension was centrifuged at 31,000 *g* for 10 min. The supernatant fraction was decanted and centrifuged at 144,000 *g* for 1 hr. The resulting pellet was finally suspended in 500 μL of sucrose (0.25 M), and a protein recovery of 19.42 mg/mL was usually obtained. Beef heart submitochondrial particles were prepared according to the method of Loyter *et al.* [13] and stored at −70° until used.

Oxygen uptake in homogenates, intact mitochondria and isolated submitochondrial particles. Oxygen uptake in the homogenates was measured by the conventional Warburg technique, in Ringer solution containing 30 mM glucose as substrate. Measurement of oxygen uptake was started after an equilibration

Table 1. Enzyme/cytochrome content in submitochondrial particles of murine or bovine origin

Enzyme/cytochrome	pmol/mg membrane protein	
	Rat renal cortex	Beef heart*
Succinate : ubiquinone Oxidoreductase (EC 1.3.99.1) Complex II	72–123†	128
Ubiquinol : cytochrome *c* oxido reductase (EC 1.10.2.2) bc_1 Complex	216‡	195
Ferrocytochrome *c*: oxygen oxido reductase (EC 1.9.3.1) complex IV	456§	452
Cytochrome *c*	491‖	580

* Data from Hatefi and Galante [17] as cited by Hackenbrock *et al.* [18], assuming that 21% of the total mitochondrial protein is located in the inner membrane.

† Taken from the difference in absorption between dithionite-reduced minus succinate-reduced in the presence of antimycin A.

‡ Taken from the absorbance in the presence of antimycin A and assuming 2 molecules of heme of cytochrome *b* per complex.

§ Assuming 2 molecules of heme *a* per complex.

‖ Total concentration of cytochromes *c* ($c + c_1$) minus moles of bc_1 complex.

period of 12 min, at 25°. Respiration of intact mitochondria and submitochondrial particles was measured polarographically with a Clark electrode (Yellow Spring Instrument Co.). The composition of the incubation buffer for intact mitochondria was as follows: 300 mM sucrose; 5 mM potassium phosphate buffer (pH 7.4), 10 mM L-glutamate and 10 mM L-malate or, alternatively, 10 mM succinate. After recording respiration under basal conditions, ADP (0.8 mM) or gramicidin (3.33 μg) was added to stimulate respiration. Mitochondrial protein content was 3.3 mg assay, and the final volume was 3 mL. The composition of the incubation medium for submitochondrial particles was as follows: 0.01 M phosphates; 0.015 M KCl and 0.25 M sucrose, adjusted to pH 7.4. Succinate (15 mM) or NADH (0.5 mM) was used as a substrate at 25°. EQ was added at 0.1, 0.5, 1.0 and 1.5 mM, using ethanol as a vehicle (0.6%).

Spectroscopic studies on submitochondrial particles. Differential spectra of renal cortex submitochondrial particles were made in an Aminco DW 2, UV/vis spectrophotometer. To determine the concentration of cytochromes $c + c_1$, we used the absorbance difference 552–540 nm with an absorption coefficient of 18.25 mM^{-1} cm^{-1}, which is an average of the absorption coefficients of cytochrome *c*, 19 mM^{-1} cm^{-1} at 550–540 nm [14], and cytochrome c_1, 17.5 mM^{-1} cm^{-1} at 553–541 nm [15]. Cytochrome *b* was measured at 562–575 nm using an absorption coefficient of 25 mM^{-1} cm^{-1} [15]. Cytochrome oxidase was measured at 603–625 nm using an absorption coefficient of 16.6 mM^{-1} cm^{-1} [16].

NADH oxidation by potassium ferricyanide in bovine heart SMP was measured. Absorption changes at 340 nm were used to monitor NADH oxidation in SMP in an Aminco DW 2. UV/vis spectrophotometer, in the split mode. Incubation

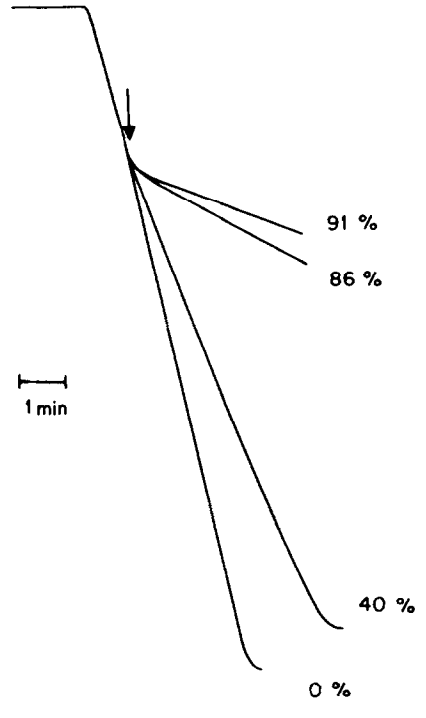

Fig. 2. Polarographic recordings of the oxygen uptake in isolated submitochondrial particles of rat kidney in the presence of NADH (0.5 mM). Arrow indicates the addition of either vehicle or EQ. Numbers show the percent of inhibition induced by the following concentrations of EQ (mM): 0.0 (0%); 0.1 (40%); 0.5 (86%) and 1.0 (91%).

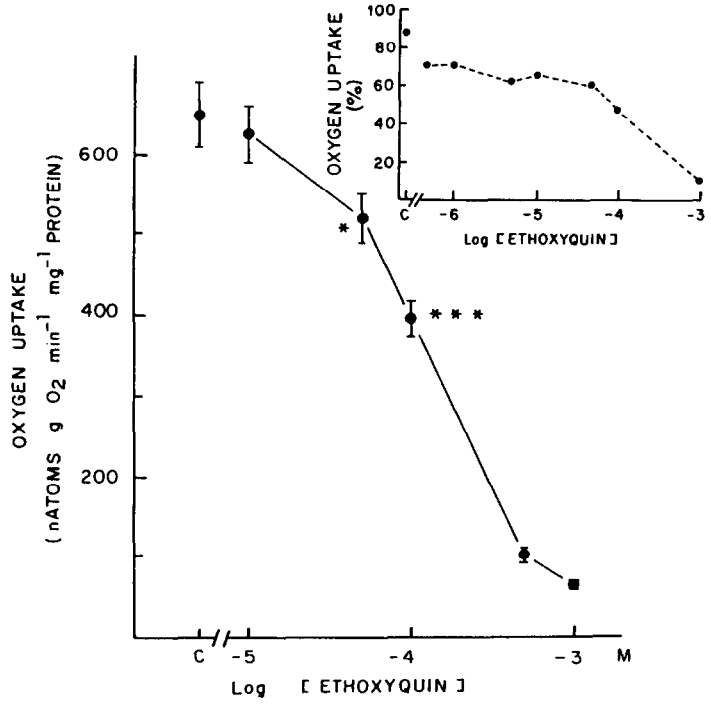

Fig. 3. Effect of ethoxyquin on oxygen uptake of renal cortex submitochondrial particles, with NADH (0.5 mM) as substrate. Values are means ± SEM, N = 6. Key: (*)$P < 0.05$; and (***)$P < 0.001$. Inset shows the effect of ethoxyquin on the oxygen uptake of submitochondrial particles isolated from bovine heart, with the same substrate, in a typical experiment.

medium composition was as follows: 250 mM sucrose; 10 mM buffer potassium phosphate; 0.2 mM NADH; 1.0 mM potassium ferricyanide, pH 7.0.

For statistical analysis, Student's *t*-test was used, and a value of $P < 0.05$ was considered to be significant.

RESULTS

Effect of EQ on oxygen uptake in renal cortex whole homogenates. Inhibition of O_2 consumption by EQ was detected when glucose was used as substrate, and this inhibition was found to be concentration dependent (Fig. 1). When the substrate was changed to succinate, no effect of EQ on O_2 consumption was detected (data not shown).

Renal cortex submitochondrial particles. Quantification of cytochromes was assessed in isolated SMP of rat renal cortex by differential spectrophotometry (reduced − oxidized spectra) under different conditions. Addition of either succinate or NADH elicited a similar response. Addition of EQ showed no significant effect, except that a longer time was required to reach anaerobiosis. The quantification obtained for cytochromes was: cytochromes $c + c_1$, 682 pmol/mg membrane protein; cytochrome *b*, 382 pmol/mg protein; and cytochromes $a + a_3$, 913 pmol/mg membrane protein. Addition of antimycin to the sample cuvette was used to cause an increase in the reduction of cytochrome *b*, up to 433 pmol/mg protein. Further reduction of the sample was attained with dithionite, resulting in an increment of the cytochrome *b* associated with succinate dehydrogenase, with a consequent increase of cytochromes *b* to 505 pmol/g protein and without modification of either cytochromes $c + c_1$ or cytochrome *a*. The comparative contents of cytochromes between renal cortex and bovine heart SMP are shown in Table 1. No significant difference between the two membrane preparations was observed.

When succinate was used with SMP, EQ (1 mM) lowered O_2 consumption from 253 ± 2 to 203 ± 6 natoms g $O_2 \cdot min^{-1} \cdot (mg\ protein)^{-1}$ (vehicle and EQ, respectively, $P < 0.05$). In contrast, when NADH was used as substrate, the polarographic recordings of the O_2 consumption showed a marked decrement (Fig. 2). EQ inhibited O_2 consumption up to 91%, at a 1.0 mM concentration. Inhibition of O_2 consumption in renal SMP showed a concentration-dependent pattern (Fig. 3) with an IC_{50} of 120 μM. The effect was not specific for the renal cortex preparation, since a similar effect of EQ was observed on bovine heart SMP (Fig. 3, inset). This suggests that the effect of EQ is not restricted to the kidney but is present in other tissues. These experiments predicted that the inhibitory effect would also be present in preparations of other organs, such as the liver. To search for additional effects of EQ on mitochondrial metabolism, we prepared intact rat liver mitochondria. The addition of EQ to mitochondria oxidizing glutamate plus

Table 2. Percent of inhibition of oxygen consumption induced by different concentrations of EQ in rat liver mitochondria

Ethoxyquin (mM)	Inhibition of oxygen consumption in coupled mitochondria (%)	Inhibition of oxygen consumption in uncoupled mitochondria (%)
0.5	31	71
1.0	71	82
1.5	81	89

L-Glutamic acid (10 mM) and L-malic acid (10 mM) were added as substrates. Rat liver intact mitochondria were used at a final concentration of protein of 3.33 mg. Coupled mitochondria: ADP (0.4 mM, final concentration) was added prior to the EQ and the rate of respiration was increased by 2.2-fold. Uncoupled mitochondria: gramicidin (3.3 μg/mL, final concentration) was added prior to the EQ, resulting in an increment in the rate of respiration of 3.2-fold.

Table 3. Percent of inhibition of NADH oxidation induced by different concentrations of EQ, with potassium ferricyanide as an electron acceptor in SMP of bovine heart

$[EQ] \times 10^{-4}$ M	Inhibition (%)
3.3	15
6.6	31
10.0	70
13.0	82

In each assay, 0.75 mg of total protein of SMP from bovine heart was used. NADH and potassium ferricyanide were added at final concentrations of 200 μM and 1 mM, respectively.

malate resulted in an inhibition of the rate of oxygen consumption. The coupled mitochondria were stimulated previously by the addition of ADP or gramicidin, as shown in Table 2. A marked inhibition was evident in the gramicidin uncoupled preparation.

To gain further insight into the mechanism of inhibition of EQ on the respiratory chain, a study of the effect of the antioxidant on NADH oxidation by ferricyanide in SMP from bovine heart was undertaken. This reaction showed a clear concentration-dependent inhibition by EQ (Table 3).

DISCUSSION

The conclusions that emerged from our observations were: (i) EQ inhibited oxygen consumption in a concentration-dependent manner, when glucose was used as a metabolic substrate in kidney homogenates; (ii) the inhibition of oxygen consumption caused by EQ in intact rat liver mitochondria, using glutamate plus malate as oxidizable substrates, was observed in coupled (after ADP addition) and uncoupled (after gramicidin) conditions; (iii) the inhibition of electron transport by EQ was also observed in SMP from beef heart and rat kidney cortex when NADH was the substrate; and (iv) the oxidation of NADH in the presence of ferricyanide (an artificial electron acceptor) was impaired by EQ in bovine heart SMP.

These observations have not been described previously for this antioxidant and point to an inhibitory effect that is not restricted to one organ or to one species. They also provide additional evidence of a deleterious effect of EQ on the kidney, liver and heart, and suggest that since the antioxidant is affecting a most important cellular function, this effect may also be present in other tissues. The inhibition was found to be concentration dependent, and since this antioxidant is retained in the renal cortex for longer periods of time than in other tissues, chronic exposure may result in its accumulation at this location. The consequences of this accumulation on the transport processes that occur in the cortex have been only partially explored [9]. The weaker inhibition of oxygen consumption by EQ, observed for oxygen consumption in whole homogenates and for glutamate plus malate oxidation by coupled rat liver mitochondria, indicates that the activity of the EQ-sensitive site is in large excess of the capacity of the respiratory chain under ADP control, at least at the concentration of ADP that was used in this study. Through the same reasoning, the EQ-sensitive site is needed at nearly full capacity during maximal respiratory activity in the presence of gramicidin in mitochondria or in SMP using NADH as a substrate.

From the data obtained in this work, we suggest that EQ acts on site I of the mitochondrial respiratory chain, but that the locus of its action is different from that of rotenone or piericidin [19, 20]. This suggestion is based on the studies of Galante and Hatefi [21], who have shown that oxidation of NADH by ferricyanide is insensitive to rotenone and piericidin. In mammals, NADH: ubiquinone oxidoreductase (Complex I) contains 41 different subunits, which give a total molecular mass of 907 kDa [22]. Electron microscopy analysis of *Neurospora crassa* complex I revealed an unusual L-shaped structure, with two distinguishable arms. The longer arm is in the interior of the membrane and the shorter arm is in the peripheral part [23]. The membrane-bound segment contains at least one iron sulfur center. A rotenone binding site was identified in a membrane-bound subunit by photoaffinity labeling [24]. The peripheral part protrudes into the matrix space and contains the NADH-binding site,

the FMN, and at least three iron–sulfur centers. This peripheral part can be fractionated by chaotropic agents, and this gives rise to a flavoprotein fraction (FP) that contains three polypeptides with FMN, some nonheme iron, and labile sulfur [25]. The FP is the smallest fraction known thus far that retains the NADH-oxidase activity with ferricyanide as an electron acceptor. The correlation between activity and the amount of acid-extractable sulfur was interpreted as indicating the involvement of Fe–S clusters in the ferricyanide reduction. However, the rate of ferricyanide reduction calculated per FMN bound is much lower than that of the intact enzyme [26]. Based on these data we can anticipate that the site of action of EQ could be in one of the components of the FP fraction of NADH: ubiquinone oxidoreductase, since a clear inhibitory action was found in the NADH: ferricyanide oxidoreductase activity in bovine heart SMP.

The inhibition of the O_2 consumption in renal SMP by ethoxyquin has not been described previously and may represent the functional site of the mitochondrial degenerative damage after exposure to EQ, observed in morphologic studies [5].

In a previous paper, we reported that EQ inhibits both organic anion and cation transport and Na^+, K^+-ATPase [9]. This inhibitory effect of EQ on NA^+,K^+-ATPase activity only partially explains the degree of inhibition of the anion and cation tubular transport. Inhibition of the oxygen consumption by the mitochondrial chain induced by EQ provides evidence for an additional mechanism that might contribute to the inhibition of the renal transport mechanisms that require energy. We have described a dual inhibitory effect of EQ on the energy pathways of the renal cell: the first was the inhibition of Na^+,K^+-ATPase [9], and the second interference with the mitochondrial electron transport, described for the first time in this work. This mechanism may add an additional explanation for the deleterious effects on the kidney that have been attributed to this antioxidant.

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