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Effect of rutin and its copper complex on superoxide formation and lipid peroxidation in rat liver microsomes

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Abstract Two free radical scavengers, bioflavonoid rutin and the copper-rutin complex $Cu(Rut)Cl_2$, inhibited lucigenin-amplified chemiluminescence and lipid peroxidation in rat liver microsomes, $Cu(Rut)Cl_2$ being a 5–9 times more efficient inhibitor than rutin. The enhanced inhibitory activity of $Cu(Rut)Cl_2$ was due to the presence of the additional superoxide-dismutating center (Cu), as follows from the comparison of its effects on microsomal chemiluminescence and cytochrome *c* reduction by xanthine oxidase. Similar effects of both inhibitors on superoxide production and lipid peroxidation as well as the elevated activity of $Cu(Rut)Cl_2$ indicate an important role of superoxide ion in the initiation of microsomal lipid peroxidation.

Key words: Superoxide; Chemiluminescence; Microsomal lipid peroxidation

1. Introduction

Superoxide is a ubiquitous radical anion produced inside the cells by mitochondria and microsomes and released outside the cells by leukocytes, macrophages, and other phagocytosing cells upon their activation with various stimuli. It has been proposed that in mitochondria superoxide ion may be produced by the one-electron transfer oxidation of electron carriers (ubiquinones, cytochromes *b*, and non-heme iron proteins [1,2] or even primary dehydrogenases [3]). Superoxide production sharply increases in the resting state of mitochondria, when energy consumption is low and the concentration of oxygen is high [4]. In microsomes there are two superoxide-producing centers: NADPH-cytochrome P-450 reductase and cytochrome P-450 [5]. Numerous data have been obtained concerning the superoxide production by phagocytes [6]. It is believed that superoxide is a rather innocuous free radical, however, being a precursor of very active hydroxyl radicals (through the Fenton reaction), superoxide may play an important role in the initiation of various free radical-mediated damaging processes [7].

A role of superoxide in lipid peroxidation had long been discussed ([6] Chapter 5). The ability of superoxide to initiate lipid peroxidation of microsomes, heavy mitochondrial fractions, and liposomes has been shown [8–10]. Later on, Morehouse et al. [5] doubted the importance of superoxide participation in the initiation of microsomal lipid peroxidation because the superoxide production measured via the reduction of acetylated cytochrome *c* was only about 1.5% of the total microsomal NADPH-dependent reduction activity. However, the use of much more sensitive and specific assays of superoxide production such as the spin-trapping technique [11] and lucigenin-amplified chemiluminescence (CL) [12] supported the earlier data indicating the participation of superoxide in lipid peroxidation. The latest data confirm an important role of superoxide in the initiation of in vivo lipid peroxidation [13–16].

In this work we compared the effects of the bioflavonoid rutin and its copper complex $Cu(Rut)Cl_2$ (two free radical scavengers with different superoxide-dismutating activity) on superoxide production by xanthine oxidase, lucigenin-amplified CL produced by rat liver microsomes, and microsomal lipid peroxidation. Our findings suggest that the inhibitory effects of both scavengers on lipid peroxidation depend mainly on their superoxide-dismutating activities.

2. Materials and methods

Rutin, xanthine, cytochrome *c*, thiobarbituric acid (TBA), the chemiluminescent probe lucigenin, the enzymes bovine erythrocyte CuZnSOD (EC 1.15.1.1) and xanthine oxidase (EC 1.1.3.22) were purchased from Sigma Chemical (St. Louis, MO, USA). The other reagents were of the highest available chemical purity. $Cu(Rut)Cl_2$ was obtained by mixing aqueous solutions of $CuCl_2$ and rutin.

Male Wistar rats weighing 150–200 g were maintained under standard laboratory conditions with chow diet and water ad libitum.

2.1. Xanthine-xanthine oxidase system

Xanthine oxidase (1.0 U/ml), catalase (500 U/ml), cytochrome *c* (25 μM), and rutin or $Cu(Rut)Cl_2$ (1–100 μM) were incubated in 0.1 phosphate buffer (7.8 pH) at 25°C. After starting the reaction with xanthine (100 μM), the reduction of cytochrome *c* was monitored spectrophotometrically at 550 nm on an Ultraspec spectrophotometer (LKB, Sweden).

2.2. Preparation of liver microsomes

The rat liver was perfused with 0.9% NaCl solution and homogenized with 1.12% KCl solution (1:3 v/v). The homogenate was centrifuged at $10\,000\times g$ for 20 min, and then the supernatant was separated and centrifuged at $105\,000\times g$ for 60 min. After protein analysis by the Lowry method, microsomes were immediately used in the experiments.

2.3. Lipid peroxidation of rat liver microsomes

Microsomes (0.5 mg protein/ml) were incubated with $FeCl_3$ (50 μM) and ADP (800 μM) with or without rutin and $Cu(Rut)Cl_2$ in 0.1 M phosphate buffer (pH 7.4) at 37°C. The reaction was started by adding 40 μl (7.5 mM) NADPH in phosphate buffer. The total volume of incubation mixture was 1 ml. After 30 min incubation, lipid peroxidation was terminated by adding 15% trichloroacetic acid (1 ml) and 10 mM ethanol solution of β-naphthol (0.1 ml). Then, 0.375% TBA solution (1 ml) was added, and the reaction mixture was heated at 100°C for 15 min. After centrifugation of precipitated proteins, the

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Abbreviations: CL, chemiluminescence; CuZnSOD, copper, zinc superoxide dismutase; TBA, thiobarbituric acid; TBAR products, thiobarbituric acid reaction products

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content of TBA reactive products was determined by measuring the absorbance at 535 nm.

2.4. Measurement of lucigenin-amplified CL in microsomes

CL measurements on a Luminometer model 1251 (LKB, Sweden) were monitored at 37°C and under continuous mixing. Lucigenin (40 μM) was added to the incubation mixture containing microsomes (0.5 mg protein/ml), $FeCl_3$ (50 μM), ADP (800 μM), and rutin or $Cu(Rut)Cl_2$ at corresponding concentrations in 0.1 M phosphate buffer (pH 7.4) at 37°C. At the beginning, the basal CL level was registered for 2 min, then the reaction was started by adding 40 μl (7.5 mM) NADPH in phosphate buffer, and the CL amplitude was measured.

3. Results and discussion

It has been shown earlier by us [12] and others [17] that microsomes generate intensive lucigenin-amplified CL. As this type of chemiluminescence is considered to be a specific and sensitive test for superoxide production [18], it might be suggested that superoxide ion participates in the initiation of microsomal lipid peroxidation. Unfortunately, it is quite difficult to measure superoxide production in microsomes by the traditional method of cytochrome *c* reduction due to the direct reduction of cytochrome *c* by cytochrome P-450 reductase. To confirm the possibility of using the lucigenin-amplified CL as an assay of superoxide production in microsomes, we compared the effects of free radical scavengers with different superoxide-dismutating activities on microsomal CL and cytochrome *c* reduction by xanthine oxidase. (Both processes were inhibited by SOD, and scavengers did not affect the activity of xanthine oxidase, data not shown.) It was found that rutin and its copper complex $Cu(Rut)Cl_2$ inhibited these processes in a similar concentration-dependent manner (Fig. 1), the inhibitory effects of each scavenger on cytochrome *c* reduction and lucigenin-amplified CL being practically identical. On these grounds, we assumed that microsomal lucigenin-amplified CL could be applied for the estimate of superoxide production in microsomes.

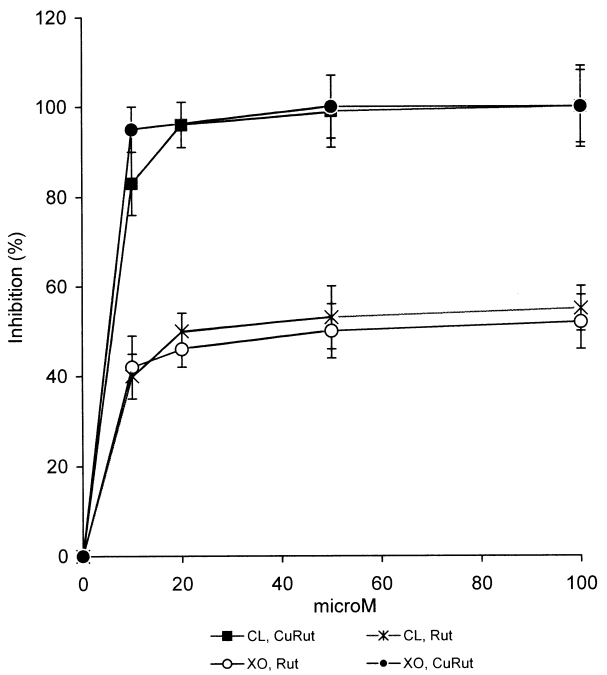

Fig. 1. Inhibition by rutin and $Cu(Rut)Cl_2$ of cytochrome *c* reduction by xanthine oxidase and lucigenin-amplified CL in rat liver microsomes. Xanthine was incubated with the mixture of xanthine oxidase, catalase, cytochrome *c*, and rutin or $Cu(Rut)Cl_2$ in 0.1 M phosphate buffer at 25°C, and then the reduction of cytochrome *c* was monitored spectrophometrically. Microsomal CL was measured after the addition of lucigenin to the incubation mixture containing rat liver microsomes, $FeCl_3$, ADP, and rutin or $Cu(Rut)Cl_2$ in 0.1 M phosphate buffer at 37°C. Each point is the mean of triplicate measurements.

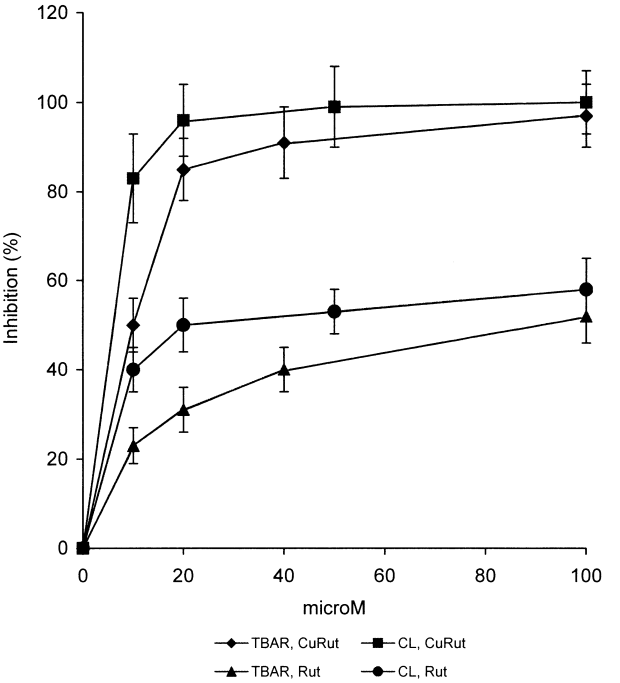

Fig. 2. Inhibition by rutin and $Cu(Rut)Cl_2$ microsomal lipid peroxidation and lucigenin-amplified CL. Lipid peroxidation was started by the addition of NADPH to rat liver microsomes incubated with $FeCl_3$, ADP, and rutin or $Cu(Rut)Cl_2$ in 0.1 M phosphate buffer at 37°C. Each point is the mean of triplicate measurements.

We also found that $Cu(Rut)Cl_2$ was a much more effective inhibitor of superoxide production than rutin (Table 1), which may be explained by $Cu(Rut)Cl_2$ having an additional superoxide-dismutating center, a copper ion. Therefore, we proposed that if superoxide ion is an important initiation species of microsomal lipid peroxidation, then the inhibitory effect of the copper-rutin complex must significantly exceed that of rutin. As is seen from Table 1, this is exactly the case because an I_{50} value for $Cu(rUt)Cl_2$ is 9 times smaller than that for rutin. It is also important that the concentration curves describing the effects of scavengers on TBAR formation are similar to the curves obtained for lucigenin-amplified CL (Fig. 2). It supports the proposal that superoxide ion plays an important role in the initiation of microsomal lipid peroxidation.

Table 1
IC_{50} values (μM) for the inhibition of cytochrome *c* reduction, lucigenin-amplified CL, and TBAR products formation by rutin and $Cu(Rut)Cl_2$

	Cytochrome *c* reduction by xanthine oxidase	Lucigenin-amplified CL in rat liver microsomes	TBAR formation in rat liver microsomes
Rutin	35 ± 0.3	20 ± 0.3	90 ± 10
$Cu(Rut)Cl_2$	2.5 ± 0.2	4.5 ± 0.1	10 ± 2

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