Pergamon

Biochemical Pharmacology, Vol. 50, No. 5, pp. 627–635, 1995.

0006–2952/95 $9.50 + 0.00

0006-2952(95)00173-5

DIFFERENT ANTIOXIDANT ACTIVITIES OF BIOFLAVONOID RUTIN IN NORMAL AND IRON-OVERLOADING RATS

IGOR B. AFANAS'EV,*† ELENA A. OSTRACHOVITCH,*
NATALIA E. ABRAMOVA,‡ and LUDMILA G. KORKINA‡

Vitamin Research Institute, Nauchny pr.14A, Moscow 117820, Russia; ‡Russian Institute of Paediatric Haematology, Leninskii pr.117, Moscow 117513, Russia

(*Received* 8 *November* 1994; *accepted* 10 *April* 1995)

Abstract—The effects of rutin on liver microsomes, peritoneal macrophages, and blood neutrophils isolated from iron-overloading (IOL) and normal rats were studied. The formation of 2-thiobarbituric acid-reactive products and the level of lucigenin-amplified chemiluminescence (CL) were determined in liver microsomes. Oxygen radical production by phagocytes was measured by luminol- and lucigenin-amplified CL and superoxide dismutase-sensitive cytochrome *c* reduction. These *ex vivo* findings were compared with the *in vitro* effects of rutin on cellular free processes. It was found that rutin administration sharply suppressed free radical production in liver microsomes and by phagocytes of IOL animals and only slightly affected these processes in normal rats. The selective inhibitory effect of rutin under pathologic conditions induced by iron overload is thought to be due to the formation of inactive iron–rutin complexes which are unable to catalyse the conversion of superoxide ion into reactive hydroxyl radicals, a process responsible for the free radical-mediated toxic effects of iron overload. These findings may account for the favourable effects of the treatment of pathologies associated with iron overload with rutin.

Key words: flavonoid rutin; oxygen radicals; iron overload; chemiluminescence

At present, flavonoids continue to draw wide attention as possible very useful therapeutic agents for combating "free radical pathologies", i.e. the pathologic states associated with free radical overproduction (inflammation, ischaemia/reperfusion, environment-associated diseases, etc.). In addition to their well-known inhibitory effects on lipid peroxidation in various model systems [1–5], it has recently been shown that flavonoids are able to suppress oxygen radical production by phagocytes [6–11], inhibit oxygen radical-induced cytotoxicity [12, 13], and exhibit cytoprotective activity in iron-loaded hepatocyte cultures [14].

We have long been interested in the antioxidant and chelating properties of rutin (quercetin-3-rutinoside), a non-toxic bioflavonoid. This flavonoid glycoside is already widely used as a therapeutic agent protecting against vascular disorders, which are probably a consequence of the damaging activity of free radicals, by decreasing the permeability and fragility of capillaries. Being a non-toxic and non-oxidizable compound, rutin may have a certain advantage as a therapeutic agent over other flavonoids such as myricetin, quercetagetin, delphinidin, etc., which occasionally manifest pro-oxidant properties and catalyse oxygen radical production [1, 15]. Earlier, we showed that rutin inhibited lipid peroxidation of lecithin liposomes, bovine heart microsomes [2], and rat brain homogenates [5]. It was proposed that the inhibitory effects of rutin are explained by both its reaction with superoxide ion and other free radicals (the antioxidant effect) and the formation of an inactive iron–rutin complex (the chelating effect). Our interest in studying the mechanisms of the antioxidant and chelating activity of rutin is also supported by the beneficial effects of treating FA§ patients (a hereditary disorder characterized by pro-oxidant/antioxidant disbalance) with rutin [16].

As much evidence of rutin's antioxidant activity has already been obtained in *in vitro* systems, it seems of great importance to study its effects in *ex vivo* models of oxidative stress. Therefore, in this work we have studied the effects of rutin administration to normal and IOL rats on free radical formation in liver microsomes and the release of oxygen radicals by peritoneal macrophages and blood neutrophils. In addition, the results obtained were compared with findings from *in vitro* experiments. The IOL model of oxidative stress was chosen given the important role iron plays in developing the pathologic states initiated by oxygen radicals. Iron-mediated free radical damage is supposedly explained by the conversion of innocuous superoxide ion in reactive hydroxyl radicals via the superoxide-driven Fenton reaction. For example, excess "free" iron is apparently responsible for the overproduction of hydroxyl radicals in sickle erythrocyte membranes [17] and in bile from rats during acute iron poisoning [18]. Similarly, the release of "free" iron is probably a major source of

† Corresponding author: Tel. 095–120–5564, 095–434–4470; FAX 095–434–8678

§ Abbreviations: CL, chemiluminescence; FA, Fanconi anaemia; HBSS, Hanks' balanced salt solution; IOL, iron-overloading; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid.

the increase in luminol-amplified CL (an assay on hydroxyl radical formation [19]) produced by leukocytes from FA patients and children exposed to low-dose irradiation [16, 20]. Our findings suggest that there is a drastic difference between the effects of rutin administration on normal as opposed to IOL animals, probably a consequence of the formation of different free radicals under physiological and pathologic conditions. Rutin's ability to form non-reactive iron–rutin complexes and to be an effective scavenger of hydroxyl radicals make it a prospective pharmacological agent for the treatment of "free radical" pathologies.

MATERIALS AND METHODS

Chemicals. Ferricytochrome *c* (type VIII), NADPH, lucigenin, luminol, PMA, HBSS, phosphate buffer, ADP, and bovine CuZnSOD (EC 1.15.1.1) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rutin was of Russian production. TBA was from Fluka (Buchs, Switzerland).

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

Ex vivo *studies.* Experimental IOL was produced in rats by providing them diets supplemented with elemental (carbonyl) iron [21]. The rats were divided into four groups: group 1 (control, 8 animals) was fed regular chow for 56 days; group 2 (11 animals) was fed a diet supplemented with 2.5% (wt/wt) carbonyl iron for 42 days; group 3 (5 animals), after feeding on regular chow during a 42-day period, received daily intraperitoneal injections of 2 mL 1 mM rutin for the next 10 days; group 4 (6 animals), after feeding on an iron-supplemented diet for 42 days, were administered interperitoneal injections of 2 mL 1 mM rutin daily for the next 10 days. In accordance with protocol, the animals of groups 1 and 2 were killed on days 42, 52, and 56, and the animals of groups 3 and 4 on day 52. In all cases biochemical measurements were carried out after the preparation of liver microsomes and the isolation of peritoneal cells.

Preparation of liver microsomes. The 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 *g* for 20 min, and the supernatant separated and centrifuged at 105,000 *g* for 60 min. After protein analysis by the Lowry method, microsomes were used immediately in the experiments.

Isolation of peritoneal macrophages. Animals were killed under slight ethyl ether narcosis. Peritoneal macrophages were prepared by peritoneal lavage with 2 mL prewarmed saline solution. The lavage fluid was filtered and centrifuged at 300 *g* for 10 min. Cells were resuspended, washed twice, and stored at 4° in HBSS. Macrophage preparations were >90% pure and >95% of the cells excluded trypan blue.

Isolation of blood PMNs. Blood was collected from rat tail vein using heparin (5 U/mL) as an anticoagulant. Rat PMNs were isolated as described previously [22]. Briefly, freshly-obtained whole blood was separated by density gradient centrifugation with Ficoll-Hypaque. A granulocyte-containing pellet was laid on a dextran–metrizoate mixture for erythrocyte sedimentation. The isolated PMNs were washed twice with HBSS. The final leukocyte suspension contained more than 95% PMNs. Cell viability was maintained throughout all experiments as indicated by a greater than 90% trypan blue exclusion.

Lipid peroxidation of rat liver microsomes. Microsomes (0.5 mg protein/mL) were incubated with $FeCl_3$ (50 μM) and ADP (800 μM) in 0.1 M phosphate buffer (pH 7.4) at 37°. 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 heated at 100° for 15 min. After centrifugation of precipitated proteins, the content of TBA reactive products was determined by measuring the absorbance at 535 nm.

Measurement of non-haem iron in rat liver microsomes. In accord with the method described in an earlier study [23], non-haem iron was extracted quantitatively from microsomes with trichloroacetic acid. Microsomes (1.5 mL, 0.5–1.0 mg protein/mL) were incubated with 0.5 mL 20% trichloroacetic acid for 10 min at room temperature and then centrifuged at 5000 *g* for 10 min. Iron content was determined by the phenanthroline method after the reduction of ferric ions with ascorbic acid. For this purpose, the supernatant (0.4 mL) was mixed with 0.15 mL 1 mM *o*-phenanthroline, 0.36 mL water, 0.04 mL saturated ammonium acetate, and 0.05 mL, 60 mM, ascorbic acid. Absorbance of the Fe^{2+} (phenanthroline)$_2$ complex was determined at 510 nm after acidification with 60 mM acetic acid.

Measurement of non-haem iron in peritoneal macrophages. After homogenization of macrophage suspension in HBSS, the homogenate (1.5 mL, 1 mg protein/mL) was mixed with 0.5 mL 20% trichloroacetic acid and, after extraction of non-haem iron, its content determined by the phenanthroline method as described above.

CL assays. CL measurements in a Luminometer model 1251 (LKB, Sweden) were monitored at 37° by continuous mixing on a programmed IBM computer. All experiments were carried out in duplicate. Each point was a mean of three or four independent measurements. Lucigenin-amplified CL was applied as a test for measuring superoxide production [24] while luminol-amplified CL was used as a test on active oxygen species forming during the decomposition of hydrogen peroxide, principally hydroxyl radicals [19].

(a) *Measurement of lucigenin-amplified CL in microsomes.* Lucigenin (40 μM) was added to the incubation mixture containing microsomes (0.5 mg protein/mL), $FeCl_3$ (50 μM) and ADP (800 μM) in 0.1 M phosphate buffer (pH 7.4) at 37°. The basal CL level was first registered for 2 min, then the reaction was started by adding 40 μL (7.5 mM) NADPH in phosphate buffer, and the CL amplitude measured.

(b) *Measurement of lucigenin- and luminol-amplified CL produced by macrophages and neutrophils.* The CL assay with peritoneal macrophages and blood PMNs has been described previously [16]. Macrophage or neutrophil suspension (5.0×10^5 cells) and luminol (20 μM) or lucigenin (400 μM) were incubated in HBSS (pH 7.4, a total volume of 1 mL) in the CL unit of a luminometer for 3 min. After measurement of background for 5 min, CL was activated by adding 10 ng PMA. An amplitude of the CL response to an activator was defined as the difference between the maximal intensity of activated CL and the intensity of spontaneous CL.

Determination of NADPH oxidase activity and the rate of superoxide production by neutrophils. A slightly modified method described by Bellavite [25] was applied. To determine the rate of superoxide production, the rate of cytochrome *c* reduction by PMA-stimulated cells was measured. PMA (100 ng/mL) was added to the incubation mixture containing leukocyte suspension (10^6 cells/mL), cytochrome *c* (50 μM), and NaN_3 (2 mM) in HBSS at 37°, and the absorption at 550 nm was registered continuously. NADPH oxidase activity was determined after stopping the reaction at its maximum rate by adding 0.4% Triton X 100. Thereafter, NADPH (0.05 mM) was added, and a new cytochrome c reduction rate measured.

In vitro *studies.* Lipid peroxidation and lucigenin-amplified CL were measured as described above after adding the appropriate concentrations of rutin to the incubation mixtures containing rat liver microsomes from group 1 rats (control). The rate of NADPH oxidation was measured at 340 nm on a Hitachi 356 spectrophotometer.

Similarly, lucigenin-amplified CL produced by peritoneal macrophages from group 1 rats was measured as in the above experiments after the addition of the appropriate concentrations of rutin to the incubation mixture containing the macrophage suspension.

Statistics. All results are from experiments carried out in duplicate or triplicate and are presented as mean ± SD. Differences were analysed using the Student's *t*-test, the level of significance being set at $P < 0.05$. Each point was a mean of three independent measurements.

RESULTS

The effects of iron overload on non-haem iron content, the formation of TBA-reactive products, and lucigenin-amplified CL in rat liver microsomes

Feeding rats 42 days with carbonyl iron enhanced

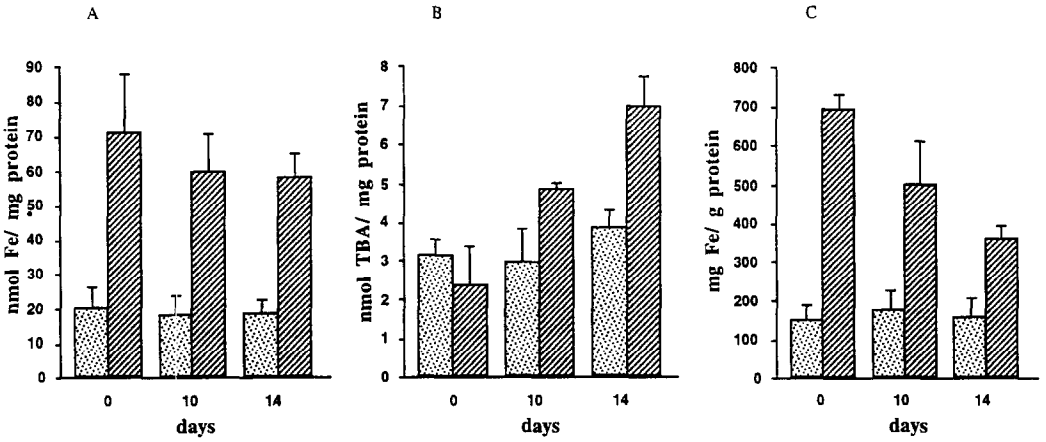

Fig. 1. All measurements were performed on days 0, 10, and 14 after ceasing the iron supplementation. (A) The content of non-haem iron in liver microsomes of normal and IOL rats. Microsomes (1.5 mL, 0.5–1.0 mg protein/mL) were incubated with 20% tricloroacetic acid (0.5 mL) for 10 min at room temperature and then centrifuged at 5000 *g* for 10 min. Iron content was determined by the phenanthroline method after the reduction of ferric ions with ascorbic acid. Values are the means for 5–10 animals and are expressed as mean ± SD. Iron content for normal rats is statistically different from that for IOL rats ($P < 0.01$). (B) The formation of TBA-reactive products in liver microsomes of normal and IOL rats. Microsomes (0.5 mg protein/mL) were incubated with $FeCl_3$ (50 μM) and ADP (800 μM) in 0.1 M phosphate buffer (pH 7.4) at 37°, and the reaction started by adding 40 μL (7.5 mM) NADPH. After 30 min, lipid peroxidation was terminated with 15% trichloroacetic acid and a 10 mM ethanol solution of β-naphthol. Then, the reaction mixture was heated at 100° for 15 min with 0.375% TBA solution, and the content of TBA-reactive products determined. Values are the means for 5–10 animals and are expressed as mean ± SD. The differences between the control rats versus IOL rats are as follows: day 0, $P > 0.05$; the 10th day, $P < 0.05$; and the 14th day, $P < 0.01$. (C) The content of non-haem iron in normal and IOL peritoneal macrophages. Macrophage homogenate in HBSS (1.5 mL, 1 mg protein/mL) was mixed with 20% trichloroacetic acid (0.5 mL) and after extraction, the content of non-haem iron was determined by the phenanthroline method. Values are the means for 5–10 animals and are expressed as mean ± SD. The content of TBA reactive products (nmol/mg protein) for normal rats is statistically different from that for IOL rats ($P < 0.01$). □, Normal rats; □, IOL rats.

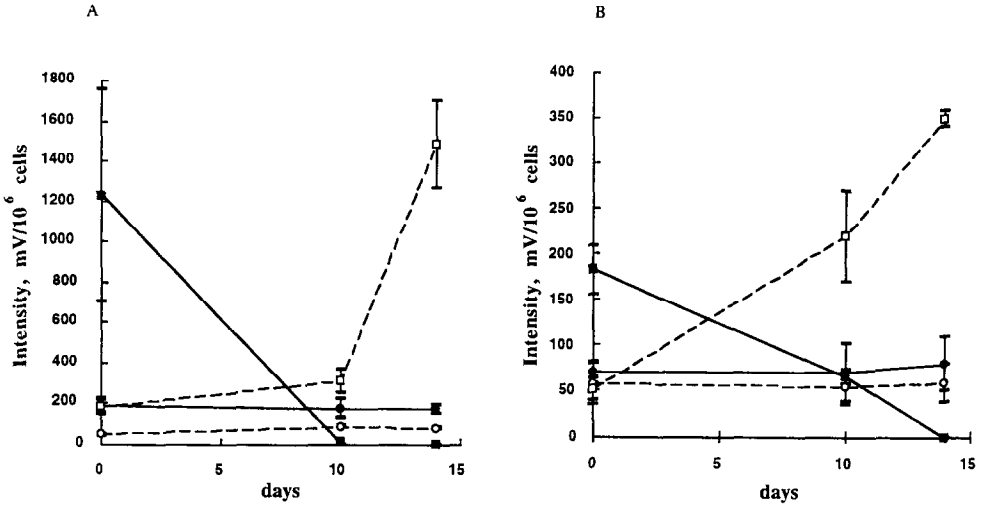

Fig. 2. All measurements were performed on days 0, 10, and 14 after ceasing the iron supplementation. (A) Spontaneous and PMA-stimulated luminol-amplified CL produced by peritoneal macrophages. Macrophage suspension (5.0×10^5 cells) and luminol (20 μM) were incubated in HBSS (pH 7.4) in the CL unit of a luminometer for 3 min. After measurement of background, CL was activated by adding PMA (10 ng), and a new amplitude of the CL response was measured. (B) Spontaneous and PMA-stimulated lucigenin-amplified CL produced by peritoneal macrophages. Experimental conditions are the same as in Panel A, but instead of luminol, lucigenin (400 μM) was added to the incubation mixture. □, Spontaneous CL of IOL macrophages; ■, PMA-stimulated CL of IOL macrophages; ○ Spontaneous CL of normal macrophages; and ●, PMA-stimulated CL of normal macrophages. Values are the means for 5–10 animals and are expressed as mean ± SD.

the content of non-haem iron in liver microsomes 3.5-fold; after stopping the iron administration to the rats, the non-haem iron content slowly decreased over the next 14 days (Fig.1A). The level of TBA reactive products in IOL microsomes after the stopping of iron feeding was close to that in normal microsomes, although it was enhanced approximately two-fold by the end of the experiment (Fig.1B). For the study of microsomal lipid peroxidation we used the traditional technique, in which exogenous components NADPH and Fe^{3+}(ADP) were applied. We fully agree that the results obtained should be referred to as the *ex vivo* data. At the same time, we believe that these data are a good characteristic of the *in vivo* processes taking place in a whole organism. For example, there was a significant growth in TBA reactive products in IOL rats on days 10 and 14 (Fig. 1A) although experimental conditions remained unchanged. Therefore, a given experiment apparently shows an increase in *in vivo* lipid peroxidation due to feeding rats with iron. Lucigenin-amplified CL produced by IOL microsomes was greatly depressed (from 20,000 ± 3000 mV for normal animals to 5500 ± 1000 mV for IOL rats during the whole experiment).

The effects of IOL on oxygen radical production by rat peritoneal macrophages and blood neutrophils

As with liver microsomes, the content of non-haem iron in IOL peritoneal macrophages increased 4.5-fold by the end of iron feeding and then decreased in the subsequent 14 days (Fig.1C). Spontaneous and PMA-stimulated luminol-amplified CL as well as PMA-stimulated lucigenin-amplified CL produced by peritoneal macrophages from IOL rats were enhanced by the end of iron feeding 2.8-, 6.2-, and 3.9-fold, respectively, while spontaneous lucigenin-amplified CL slightly decreased (Fig.2). In the subsequent 14 days the intensity of spontaneous CL sharply increased while that produced by PMA-stimulated cells had already approximated zero on the 10th day.

Superoxide production by PMA-stimulated macrophages from IOL rats measured at the end of iron feeding by the cytochrome *c* reduction method was 2.1-fold greater than that from control animals, whereas the NADPH oxidase activities of IOL and normal macrophages did not differ (data not shown). The non-haem iron content and all types of CL produced by normal macrophages did not change significantly during the experiment (Figs 1 and 2).

Oxygen radical production by neutrophils from IOL rats measured by CL and the cytochrome *c* reduction method at the end of iron feeding followed the same pattern as that of peritoneal macrophages: spontaneous and PMA-stimulated luminol-amplified CL as well as PMA-stimulated lucigenin-amplified CL increased and spontaneous lucigenin-amplified CL was virtually unchanged (Table 1); superoxide production increased and NADPH oxidase activity did not change.

The effects of rutin administration on free radical production in liver microsomes and by peritoneal macrophages and blood neutrophils from IOL and normal rats

Rutin administration had no effect on the content

Table 1. Oxygen radical production by neutrophils from IOL and normal rats*

Groups of animals	Spontaneous luminol-amp. CL	PMA-stimulated luminol-amp. CL	Spontaneous lucigenin-amp. CL	PMA-stimulated lucigenin-amp. CL
Group 1 (Control)	220 ± 86	1270 ± 618	16.0 ± 3.6	93.2 ± 7.3
Group 2 (IOL rats)	311 ± 72	9170 ± 1280	24.0 ± 3.9	299 ± 69

* The amplitudes of the CL response are given as a mean ± SD, N = 5–10. The differences among the groups for control rats versus IOL rats are as follows. spontaneous luminol- and lucigenin-amplified CL, $P > 0.05$; PMA-stimulated luminol- and lucigenin-amplified CL, $P < 0.01$. All measurements were made on day 42 after the beginning of the experiment.

Table 2. Effects of rutin on the contents of non-haem iron and TBA reactive products in rat liver microsomes*

Groups of animals	Non-haem Fe (nmol/mg protein)	TBA reactive products (nmol/mg protein)
Group 1 (Control)	18.4 ± 5.1	3.0 ± 0.9
Group 3 (Control + rutin)	19.6 ± 4.0	4.3 ± 2.7
Group 2 (IOL rats)	60.1 ± 10.4	4.9 ± 0.1
Group 4 (IOL rats + rutin)	67.0 ± 1.5	1.2 ± 0.3

* Rats from Groups 3 and 4 received daily interperitoneal injections of 2 mL 1 mM rutin for 10 days (starting with day 43). All measurements were performed on day 52. All values are given as means ± SD, N = 5–10. There were statistically significant differences among Group 1 versus Group 2 for non-haem Fe, $P < 0.01$ and for TBA reactive products, $P < 0.05$; Group 2 versus Group 4 for TBA reactive products, $P < 0.01$. There is no significant difference between Group 1 and Group 3 for either parameter.

Table 3. Effects of rutin on peritoneal macrophages*

Groups of animals	Non-haem Fe (mg/g protein)	Spontaneous luminol-amp. CL	PMA-stimulated luminol-amp. CL	Spontaneous lucigenin-amp. CL	PMA-stimulated lucigenin-amp. CL
Group 1 (Control)	176 ± 44	87 ± 4.4	175 ± 44	70 ± 32	54 ± 20
Group 3 (Control + rutin)	251 ± 24	167 ± 16	96 ± 12	108 ± 40	24 ± 1
Group 2 (IOL rats)	503 ± 108	309 ± 56		220 ± 50	65 ± 7
Group 4 (IOL rats + rutin)	442 ± 13	92 ± 22	49 ± 1.4	94 ± 11	91 ± 28

* Rats from Groups 3 and 4 were administered interperitoneal injections of 2 mL 1 mM rutin for 10 days (starting with day 43). All measurements were performed on day 52. All values are given as means ± SD, N = 5–10. There were statistically significant differences between Group 2 and Group 4 for both types of spontaneous CL, $P < 0.01$.

of non-haem iron (Table 2) in either IOL or normal liver microsomes. Similarly, rutin did not affect the rate of lipid peroxidation in microsomes of control rats but significantly (four-fold) decreased the level of TBA reactive products in IOL microsomes (Table 2). Rutin barely altered the content of non-haem iron in peritoneal macrophages from both IOL and control rats (Table 3). Although not all differences between the values of CL intensities obtained with and without rutin are significant, there does seem to

Table 4. Effects of rutin on superoxide production by PMA-stimulated neutrophils and on neutrophilic NADPH oxidase activity*

Groups of animals	The rate of cytochrome *c* reduction by PMA-stimulated neutrophils (nmol/min 10^6 cells)	NADPH oxidase activity (nmol/min 10^6 cells)
Group 1 (Control)	0.269 ± 0.010	0.883 ± 0.056
Group 3 (Control + rutin)	0.205 ± 0.004	0.624 ± 0.029
Group 2 (IOL rats)	0.211 ± 0.006	0.659 ± 0.070
Group 4 (IOL rats + rutin)	0	0.876 ± 0.076

* Rats were injected daily with 2 mL 1 mM rutin interperitoneally for 10 days (starting on day 43). All measurements were performed on day 52. All values are given as means ± SD, N = 5–10. There is a statistically significant difference between Group 2 and Group 4 for the rate of cytochrome *c* reduction, $P < 0.01$.

be one basic tendency: rutin increased spontaneous CL and decreased PMA-stimulated CL produced by normal macrophages (Table 3). At the same time, spontaneous luminol- and lucigenin-amplified CL produced by IOL macrophages (which significantly increased within this period) was strongly inhibited by rutin.

It is difficult to estimate the effects of rutin on the PMA-stimulated CL of IOL macrophages due to its sharp decline on the 10th day after the completion of iron feeding. However, we were able to study its effects on superoxide production by PMA-stimulated neutrophils measured via cytochrome *c* reduction. Again, rutin administration to normal rats did not affect the rate of cytochrome *c* reduction by neutrophils, although this rate became equal to zero in the case of IOL neutrophils (Table 4). The effect of rutin administration on NADPH oxidase activity was uncertain.

In vitro *effects of rutin on free radical production in microsomal lipid peroxidation and by peritoneal macrophages*

In vitro experiments were carried out with liver microsomes and peritoneal macrophages prepared and isolated from control animals (group 1). Rutin inhibited the formation of TBA-reactive products and SOD-sensitive lucigenin-amplified CL in lipid peroxidation of rat liver microsomes in a concentration-dependent manner (Fig.3A), with ID_{50} values of 19 μM and 76 μM for TBA-reactive product formation and lucigenin-amplified CL, respectively. Rutin turns out to be a very effective inhibitor of luminol-amplified CL and superoxide ion (measured via cytochrome *c* reduction) produced by rat peritoneal macrophages (Fig.3B), with ID_{50} values of 0.4 μM and 1 μM, respectively. SOD completely inhibited both luminol-amplified CL and cytochrome *c* reduction. Although rutin is able to reduce cytochrome *c* directly, this reduction was insignificant under our experimental conditions given the very small concentrations of rutin administered (Fig.3B).

DISCUSSION

As mentioned above, a major task of this work was to study the *in vivo* effects of rutin on free radical production in normal animals and animals under oxidative stress. Before discussing the results obtained, we need to be certain that the conditions of oxidative stress were indeed achieved in a chosen *in vivo* model. As is seen in Figs 1 and 2, feeding rats with elemental iron did lead to free radical overproduction in liver microsomes and by peritoneal macrophages. The enhancement of TBA reactive products in liver as well as in hepatic mitochondria and microsomes of IOL rats has already been shown [26]. However, to the best of our knowledge, the effect of IOL on oxygen radical production by phagocytes isolated from IOL animals has never been studied. Our results differ somewhat from previous data. Bacon *et al.* [26] have shown that microsomal lipid peroxidation in IOL rats was not observed until hepatic non-haem iron content reached 2000–3500 μg Fe/g liver, while we observed an increase in microsomal lipid peroxidation at a hepatic non-haem iron content of 1430 μg Fe/g liver. However, in our study an increase in TBA reactive products took place only on the 10th day after the stopping of iron administration. As seen in Table 2, the difference between the levels of TBA reactive products in IOL and control rats was significant ($P < 0.05$) and correlated with a strong increase in non-haem iron (more than three-fold, $P < 0.01$). Thus, microsomal lipid peroxidation apparently takes place at iron levels lower than 2000 μg Fe/g liver but only after a lag period which depends on the time needed for the release of iron from iron-transfering proteins, ferritin first of all.

Oxygen radical production by microsomes, macrophages, and neutrophils was measured by luminol- and lucigenin-amplified CL and by the traditional cytochrome *c* reduction method. Lucigenin-amplified CL (as well as a well-known cytochrome *c* assay) is believed to characterize superoxide production [24], while luminol-amplified

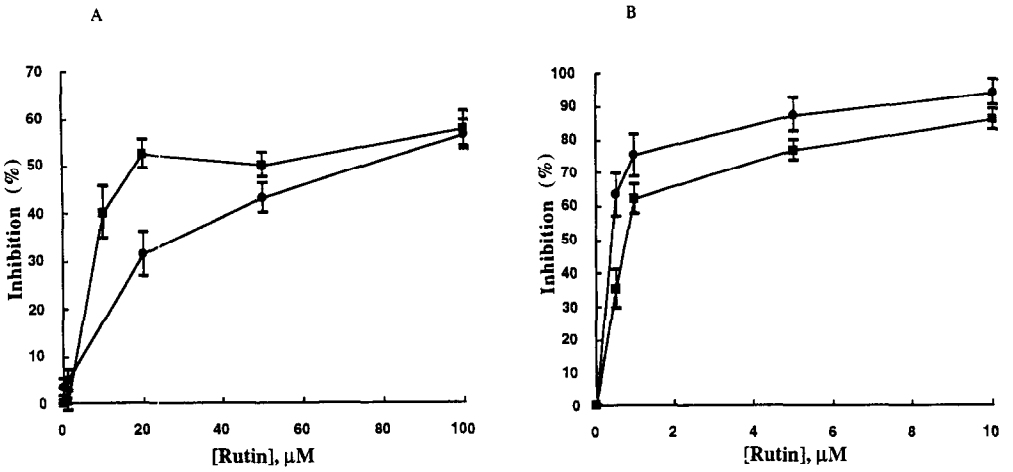

Fig. 3. (A) Inhibition of lipid peroxidation and lucigenin-amplified CL of rat liver microsomes by rutin (the *in vitro* study). Experimental conditions are the same as in the *in vivo* studies (Fig. 1). ■, Inhibition by rutin of the formation of TBA-reactive products; and ●, inhibition by rutin of lucigenin-amplified CL. (B) Inhibition of luminol-amplified CL and cytochrome *c* reduction by PMA-stimulated peritoneal macrophages with rutin (the *in vitro* study). Experimental conditions are the same as in the *in vivo* studies (Fig. 2). ■, Inhibition by rutin of cytochrome *c* reduction; and ●, Inhibition by rutin of luminol-amplified CL. Values are expressed as mean ± SD; N = 5–7.

CL measures hydroxyl radicals and other active oxygen species formed in the decomposition of hydrogen peroxide [19]. In contrast to lipid peroxidation, superoxide production by IOL microsomes sharply decreased on the first day after stopping iron administration. This phenomenon may be due to the interaction of superoxide ion with excess iron ions as we earlier observed in *in vitro* microsomal lipid peroxidation [27].

$$O_2^{\cdot -} + Fe^{3+} \Rightarrow O_2 + Fe^{2+} \quad (1)$$

In this reaction ferric ions are reduced by superoxide into ferrous ions, which participate in the initiation of lipid peroxidation. If this suggestion is valid for *in vivo* lipid peroxidation, then a decrease in the lucigenin-amplified CL observed in IOL microsomes is another evidence of the accumulation in microsomes of active ferrous ions, the initiators of lipid peroxidation.

IOL sharply enhanced oxygen radical production by macrophages and neutrophils (Fig.2, Table 1). These findings confirm previous *in vitro* experiments [28, 29]. It was also found that oxygen radical production by non-stimulated cells sharply increased during the 14 days after stopping iron administration (4.4- and 18-fold when measured by lucigenin- and luminol-amplified CL, respectively). We suggest that CL enhancement by non-stimulated cells is explained by their priming with iron released from iron-transporting proteins in IOL animals. It is interesting that within the same period, CL produced by PMA-stimulated cells dropped to zero. This is probably a consequence of strong priming of cells with iron, making additional stimulation with PMA ineffective.

In the case of neutrophils we measured the superoxide production and the activity of NADPH oxidase after the disruption of cells with Triton X100 monitoring the SOD-sensitive reduction of cytochrome *c*. At the end of iron supplementation, superoxide production by IOL neutrophils was 5.2-fold greater than that by normal neutrophils, but quickly returned to the normal level in the next 14 days. By contrast, there was no significant change in the activity of NADPH oxidase from IOL neutrophils. Thus, some enzyme other than NADPH oxidase (possibly, lipoxygenase) is responsible for the enhancement of superoxide production by IOL neutrophils.

Our findings suggest that IOL in rats induces the oxidative stress that was characterized by oxygen radical overproduction in liver microsomes, peritoneal macrophages, and blood neutrophils. Turning to the *in vivo* effects of rutin on oxygen radical production, the most surprising and unexpected result was the difference between its effects on normal and IOL animals. We believe that this points to the different nature of reactive species mediated damaging processes. Thus, rutin only slightly affected lipid peroxidation in normal microsomes but inhibited it by 75% in IOL microsomes (Table 2). Similarly, rutin was unable to inhibit spontaneous luminol- and lucigenin-amplified CL produced by macrophages or superoxide production by neutrophils from normal animals but strongly inhibited CL (by 2.5–3.5-fold) produced by macrophages and completely suppressed superoxide production by neutrophils in the case of IOL rats (Tables 3 and 4). (It was impossible to estimate the effects of rutin administration on PMA-stimulated IOL macrophages due to a sharp decrease in CL intensities).

When present findings are compared with the *in vitro* data, it is clear that the latter correspond to the effects of rutin on IOL rats. Thus rutin suppressed

the formation of TBA-reactive products and lucigenin-amplified CL in rat liver microsomes (Fig.3A) and was an effective inhibitor of luminol-amplified CL and cytochrome *c* reduction by peritoneal macrophages (Fig.3B). We shall now attempt to account for the diverse effects of rutin administration to normal and IOL rats.

As was mentioned above different *in vivo* effects of rutin on free radical processes in normal animals and in the animals under oxidative stress apparently point to the different nature of the free radicals formed. We found that phagocytes from IOL rats were characterized by the enhanced production of luminol-amplified CL. Thus, for non-stimulated peritoneal macrophages the lucigenin CL/luminol CL ratio decreased from 1.1 ± 0.3 for normal rats to 0.4 ± 0.2 for IOL rats (but returned to a normal value of 1.0 for the IOL rats treated with rutin). In accord with previous data [16, 19, 20, 24], we assume that lucigenin- and luminol-amplified CL characterize superoxide and hydroxyl radical productions. It is possible that excess iron in IOL rats is a major factor responsible for the conversion of superoxide ion in hydroxyl radicals via the superoxide-driven Fenton reaction (reactions 1 and 2).

$$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + HO\cdot + HO^- \qquad (2)$$

We have shown earlier [16] that rutin is an efficient scavenger of hydroxyl and hydroxyl-like radicals. This is thought to be the origin of the beneficial effects of treating FA patients with rutin. Indeed, it was found that the leukocytes isolated from FA patients produced an enhanced level of luminol-amplified CL in comparison with normal leukocytes. We assumed that the prevalence of hydroxyl radical production (measured by luminol-amplified CL) over superoxide production (measured by lucigenin-amplified CL) may be a typical feature of an organism's oxidative stress. Indeed, in addition to FA patients, we observed the enhancement of luminol-amplified CL produced by blood leukocytes from children living in areas contaminated by radioactive materials and thus exposed to low-level, but continuous, irradiation [20].

The inhibitory effects of rutin on *in vivo* free radical production in IOL rats are probably explained by its ability to form inactive iron–rutin complexes [2, 5] and to suppress the conversion of superoxide ion into highly reactive hydroxyl and hydroxyl-like radicals. It is suggested that the enhancement of the formation of highly reactive free radicals is typical of pathologic states mediated by free radicals, which makes rutin possible a very useful medicine for their treatment. Comparison with *in vitro* findings shows that in some cases they may model pathologic conditions, possibly due to the presence of adventitious iron.

Acknowledgements—The research described in this publication was made possible in part by Grant No MUW000 from the "International Science Foundation".

REFERENCES

1. Maridonneau-Parini I, Braquet P and Garay RP, Heterogenous effect of flavonoids on K^+ loss and lipid peroxidation induced by oxygen-free radicals in human red cells. *Pharmacol Res Commun* **18**: 61–72, 1986.
2. Afanas'ev IB, Dorozhko AI, Brodskii AV, Kostyuk VA and Potapovitch AI, Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* **38**: 1763–1769, 1989.
3. Negre-Salvayre A, Affany A, Hariton C and Salvayre R, Additional antilipoperoxidant activities of alpha-tocopherol and ascorbic acid on membrane-like systems are potentiated by rutin. *Pharmacology* **42**: 262–272, 1991.
4. Decharneux T, Dubois F, Beauloye C, Wattiaux-De Coninck S and Wattiaux R, Effect of various flavonoids on lysosomes subjected to an oxidative or osmotic stress. *Biochem Pharmacol* **44**: 1243–1248, 1992.
5. Kozlov AV, Ostrachovitch EA and Afanas'ev IB, Mechanism of inhibitory effects of chelating drugs on lipid peroxidation in rat brain homogenates. *Biochem Pharmacol* **47**: 795–799, 1994.
6. Blackburn WD Jr, Heck LW and Wallace RW, The bioflavonoid quercetin inhibits neutrophil degranulation, superoxide production, and the phosphorylation of specific neutrophils proteins. *Biochem Biophys Res Commun* **144**: 1229–1236, 1987.
7. Robak J and Gryglewski RJ, Flavonoids are scavengers of superoxide anions. *Biochem Pharmacol* **37**: 837–841, 1988.
8. Lonchampt M, Guardiola B, Sicot N, Bertrand M, Pedrix L and Duhault J, Protective role of a purified flavonoid fraction against reactive oxygen radicals. *Arzneim-Forsch* **39**: 882–885, 1989.
9. Cypriani B, Limasset B, Carrie M-L, Le Doucen C, Roussie M, de Paulet AC and Damon M, Antioxidant activity of micronized diosmin on oxygen species from stimulated human neutrophils. *Biochem Pharmacol* **45**: 1531–1535, 1993.
10. Limasset B, le Doucen C, Dore JC, Ojasoo T, Damon M and de Paulet AC, Effects of flavonoids on the release of reactive oxygen species by stimulated human neutrophils. Multivariate analysis of structure-activity relationships (SAR). *Biochem Pharmacol* **46**: 1257–71, 1993.
11. Ursini F, Maiorino M, Morazzoni P, Roveri A and Piffeni G, A novel antioxidant flavonoid (IdB 1031) affecting molecular mechanisms of cellular activation. *Free Radical Biol Med* **16**: 547–555, 1994.
12. Nakayama T, Yamada M, Ozawa T and Kawakishi S, Suppression of oxygen-induced cytotoxicity by flavonoids. *Biochem Pharmacol* **45**: 265–267, 1993.
13. Ramanathan R, Das NP and Tan CH, Effect of γ-linolenic acid, flavonoids, and vitamins on cytotoxicity and lipid peroxidation. *Free Radical Biol Med* **16**: 43–48, 1994.
14. Morel I, Lescoat G, Cogrel P, Sergent O, Pasdeloup N, Cillard P and Cillard J, Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. *Biochem Pharmacol* **45**: 13–19, 1993.
15. Hodnick WF, Kung FS, Roettger WJ, Bohmont CW and Pardini RS, Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. A structure-activity study. *Biochem Pharmacol* **35**: 2345–2357, 1986.
16. Korkina LG, Samochatova EV, Maschan AA, Suslova TB, Cheremisina ZP and Afanas'ev IB, Release of active oxygen radicals by leukocytes of Fanconi anemia patients. *J Leukocyte Biol* **52**: 357–262, 1992.
17. Repka T and Hebbel RP, Hydroxyl radical formation by sickle erythrocyte membranes: Role of pathologic iron deposits and cytoplasmic reducing agents. *Blood* **78**: 2753–2758, 1991.
18. Burkitt MJ, Kadiiska, Hanna PM, Jordan SJ and

Mason RP, Electron spin resonance spin-trapping investigation into the effects of paraquat and desferrioxamine on hydroxyl radical generation during acute iron poisoning. *Molec Pharmacol* **43**: 257–263, 1993.

19. Allen RC and Loose LD, Phagocytic activation of a luminol dependent chemiluminescence in rabbit peritoneal macrophages. *Biochem Biophys Res Commun* **69**: 245–254, 1976.
20. Korkina L, Afanas'ev IB and Diplock AT, Antioxidant therapy in children affected by irradiation from the Chernobyl nuclear accident. *Biochem Soc Trans* **21**: 314S, 1993.
21. Fletcher LM, Roberts FD, Itving MG and Powell LW, Effect of iron loading on free radical scavenging enzymes and lipid peroxidation in rat liver. *Gastroenterology* **97**: 1011–1018, 1989.
22. Boyum A, Separation of white blood cells. *Nature* **204**: 793–794, 1964.
23. Brumby PE and Massey V, Interference is also caused by groups which form complexes with iron, i.e. phosphate and pyrophosphate. In: *Methods in Enzymology* (Eds. Estabrook RW and Pullman ME), Vol 10, pp. 463–474. Academic Press, New York.
24. Gyllenhammar H, Lucigenin chemiluminescence in the assessment of neutrophil superoxide production. *J Immunol Meth* **97**: 209–214, 1987.
25. Bellavite P, Superoxide-forming enzymatic system of phagocytes. *Free Radical Biol Med* **4**: 225–261, 1988.
26. Bacon BR, Tavill AS, Brittenham GM, Park CH and Recknagel RO, Hepatotoxicity of chronic iron overload: role of lipid peroxidation. In: *Free Radicals in Liver Injury* (Eds. Poli G, Cheeseman KH, Dianzani MU and Slater TF), pp. 49–57. IRL Press, Oxford, 1985.
27. Afanas'ev IB, Dorozhko AI, Polozova NI, Kuprianova NS, Brodskii AV, Ostrachovitch, EA and Korkina LG, Is superoxide an initiator of microsomal lipid peroxidation? *Arch Biochem Biophys* **302**: 200–205, 1993.
28. Hoepelman IM, Jaarsma EY, Verhoef J and Marx JJM, Influence of iron (II) and polynuclear and mononuclear iron (III) complexes on polymorphonuclear leukocytes (PMN) function. In: *Free Radicals, Cell Damage and Disease* (Ed. Rice-Evans C), pp. 45–54. Richelieu Press, London, 1986.
29. Takahushi M, Ikeda H, Sato EF, Akimari K, Edamatsu R, Inoue M and Utsumi K, Stimulus-specific enhancement of luminol chemiluminescence in neutrophils by phosphatidylserine liposomes. *Arch Biochem Biophys* **298**: 43–48, 1992.