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Short communication

Oxidative stress in rheumatoid arthritis leukocytes: suppression by rutin and other antioxidants and chelators

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Abstract

The enhanced production of superoxide ion and peroxynitrite by bloodstream neutrophils and of superoxide ion by monocytes from rheumatoid arthritis (RA) patients was registered. It was suggested that NADPH oxidase together with NO synthase were the major sources of superoxide ion in RA neutrophils, while in RA monocytes superoxide ion was produced by NADPH oxidase and mitochondria. Among the different free radical inhibitors studied (antioxidant enzymes, SOD and catalase; free radical scavengers, bioflavonoid rutin and mannitol; and the iron chelator desferrioxamine), SOD and rutin were the most efficient suppressors of oxygen radical overproduction by RA neutrophils, while mannitol and desferrioxamine were inactive. Thus, in contrast to Fanconi anemia (FA) leukocytes (Korkina LG *et al*., J Leukocyte Biol 1992;52:357–62), iron-catalyzed hydroxyl radical formation was unimportant in RA leukocytes, which mainly produced superoxide ion. Natural non-toxic bioflavonoid rutin (vitamin P) inhibited oxygen radical overproduction in both RA and FA in an equally efficient manner and therefore may be considered as a useful supporting pharmaceutical agent for the treatment of "free radical" pathologies.

Keywords: Rheumatoid arthritis; Neutrophils; Monocytes; Oxidative stress; Rutin

1. Introduction

Free radicals have long been implicated as mediators of tissue damage in RA patients [1]. Correspondingly, it has been shown that RA bloodstream neutrophils and monocytes are, as a rule, characterized by the overproduction of oxygen and nitrogen reactive species [2,3]. Early on, we showed that rutin and some other flavonoids are very effective free radical inhibitors in animal and human pathological states, such as iron overload in rats [4], Fanconi anemia [5], or β-thalassemia [6]). Now, we report the study of oxidative stress in RA leukocytes and the effects of rutin and other antioxidants and chelators on the production of oxygen radicals by RA neutrophils.

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Abbreviations: CL, chemiluminescence; DF, desferrioxamine; DHR, dihydrorhodamine; FA, Fanconi anemia; HBSS, Hanks' balanced salt solution; NMMA, N^G-monomethyl L-arginine; NO, nitric oxide; PMA, 12-*O*-myristate 13-acetate; PMN, polymorphonuclear; RA, rheumatoid arthritis, and SOD, superoxide dismutase.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma Chemical Co. Salts, solvents, and acids applied were of the greatest available chemical purity.

2.2. Patients

Blood was collected from 43 RA patients (American Rheumatism Association criteria, 15 males and 28 females, mean age of 50.4 $\pm$ 10.7 years). Control blood was taken from volunteers (15 persons, 5 males and 10 females, mean age of 40.1 $\pm$ 10.2 years) who had previously given their informed consent.

2.3. Preparation of blood neutrophils and monocytes

PMNs and monocytes were isolated from 10 mL of heparinized venous blood of volunteers and RA patients using Ficoll Hypaque density gradient. The cell pellet con-

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taining erythrocytes and PMNs was suspended in HBSS, the leukocyte-rich supernatant was centrifuged, and the cell pellet mixed with distilled water at 4° to lyse residual erythrocytes. Finally, cells were washed twice, resuspended in HBSS, and stored at 4° during examination. Mononuclear cells were obtained from the Ficoll Hypaque plasma interface, and monocytes were removed from this cell preparation by adherence to glass plates. Cell viability was assessed by exclusion of 0.1% trypan blue and usually exceeded 95%. Final suspensions contained $>$ 95% neutrophils and monocytes (Giemsa staining).

2.4. Measurement of superoxide production and NADPH oxidase activity by the reduction of cytochrome c

Neutrophil suspension (10^6 cells/mL) was added to cytochrome *c* (50 μM) and NaN_3 (2 mM) in the presence or absence of PMA (100 ng/mL) in HBSS (1 mL), and the optical density at 550 nm (the extinction coefficient is 18500 M^{-1} cm^{-1}) was registered after a 1-hr incubation. (In some experiments, NMMA [6 mM] or L-arginine [10 mM] was added before PMA stimulation). The reaction was stopped at a maximal rate by the addition of 0.4% Triton X-100 and restored 45 sec later by the addition of NADPH (0.4 mM) when optical density at 550 nm corresponding to reduced cytochrome was again recorded. The addition of catalase (50 μg/mL) at the beginning of experiment had no effect on the rate of cytochrome *c* reduction, while SOD (20 μg/mL) completely inhibited the cytochrome reduction.

2.5. Measurement of SOD activity in neutrophils

Neutrophil suspension (300 μL) was lysed with 1 mL 0.4% Triton X-100 for 1 hr at room temperature. SOD activity in lysate was determined by the inhibition of cytochrome *c* reduction (25 μM) in the xanthine (100 μM)/xanthine oxidase (1 munits/mL) system in 100 mM phosphate buffer (pH 7.6).

2.6. Chemiluminescent measurement of oxygen radical production

CL was measured on an LKB luminometer (Model 1251). Neutrophil or monocyte suspension (10^6 cells/mL), luminol (500 μM), or lucigenin (80 μM) was incubated in HBSS (pH 7.4) in the presence or absence of PMA (100 ng/mL), rotenone (1 μM), or antimycin A (1 μM) and CL intensity was recorded. In the experiments with antioxidants and chelators, SOD (20 μg/mL), catalase (50 μg/mL), rutin (100 μM), DF (100 μM), or mannitol (10 mM) was added to the incubation mixture, respectively.

2.7. Measurement of peroxynitrite production [7]

Stock 30 mM DHR solution in dimethylformamide was diluted 6 times in phosphate buffer (pH 7.4). Ten microliters of this solution was added to neutrophil suspension (10^6 cells/mL) in HBSS containing 10 μL PMA (10 μg/mL) in the presence or absence of NMMA (6 mM). The formation of rodamine 123 was monitored spectrophotometrically at 500 nm (the extinction coefficient ε = 78000 M^{-1} cm^{-1}).

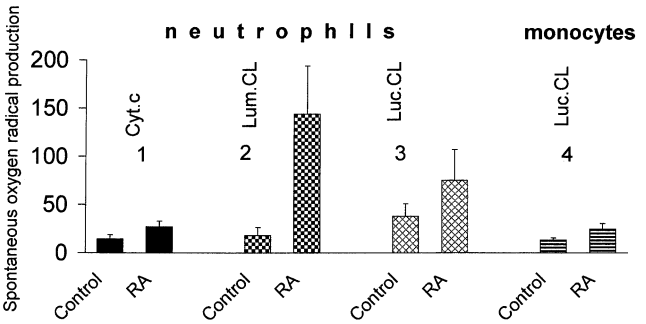

Fig. 1. Spontaneous oxygen radical production by normal (control) and RA neutrophils and monocytes. 1. The rate of cytochrome *c* reduction (nM/hr/10^6 cells), RA neutrophils versus control, $P < 0.05$. 2. The intensity of luminol-amplified CL (mV/10), $P < 0.01$. 3. The intensity of lucigenin-amplified CL (mV), $P < 0.05$. 4. The intensity of lucigenin-amplified CL, RA monocytes versus control (mV $\times$ 10), $P < 0.01$. In 1, 2, and 3, the numbers of patients and controls were 32 and 15, respectively and in 4 the numbers of patients and controls were 10. All data are presented as means $\pm$ SD.

2.8. Measurement of transferrin-bound iron in serum and "loose" iron in leukocytes

Transferrin-bound iron in serum and "loose" iron in the plasmalemma of neutrophils and monocytes were measured by using the ferrozine method (Dr. Lange Test LCN030).

2.9. Statistics

The data were analysed using paired Student's *t*-test except for the measurement of healthy controls against RA patients, where due to unequal numbers of patients and controls the Mann–Whitney U test was applied. All values are expressed as means; the threshold for statistical significance is $P < 0.05$.

3. Results and discussion

3.1. Oxidative stress in RA leukocytes

As is seen from Fig. 1, three assays (SOD-inhibitable cytochrome *c* reduction and luminol- and lucigenin-amplified CL) showed a sharp increase in spontaneous oxygen radical production by RA neutrophils and monocytes compared to normal cells ($P < 0.01$–0.05). There was no significant difference between oxygen radical production by PMA-stimulated RA and normal neutrophils (data not shown), but oxygen radical production by PMA-stimulated

Table 1
Parameters of oxidative stress in RA patients and controls

	RA patients		Controls
NADPH oxidase activity in neutrophils (nM/hr/10^6 cells)	20.4 ± 6.8	$P > 0.05$	17.8 ± 3.6
NADPH oxidase activity in monocytes (nM/hr/10^6 cells)	31.5 ± 8.0	$P < 0.01$	15.9 ± 1.6
NMMA-inhibitable DHR oxidation (nM/hr/10^6 cells)	3.06 ± 1.05	$P < 0.05$	5.47 ± 1.10
SOD activity in neutrophils (units/mg protein)	0.87 ± 0.49	$P < 0.05$	1.67 ± 0.34
Transferrin Fe in serum (μmol/L)	17.5 ± 6.9	$P < 0.01$	39.1 ± 9.4
Neutrophil plasmalemma Fe nmol/μg protein	53.6 ± 12.9	$P < 0.05$	27.5 ± 5.4
Monocyte plasmalemma Fe nmol/μg protein	25.4 ± 18.1	$P > 0.05$	14.0 ± 1.9

RA monocytes was 2.7 times higher. This difference between RA neutrophils and monocytes corresponded well to the difference in their NADPH oxidase activities (Table 1). Therefore, we suggested that another enzyme than NADPH oxidase might contribute to oxygen radical overproduction by non-stimulated RA neutrophils. It has already been shown [8] that NO synthases are able to generate superoxide ion especially under L-arginine-depleted conditions. We found that superoxide production by RA neutrophils decreased by 50% from 21.2 ± 6.0 to 11.6 ± 4.8 nM/h/10^6 cells ($P < 0.05$) in the presence of the NO synthase inhibitor NMMA and slightly enhanced up to 24.5 ± 8.0 nM/h/10^6 cells after the addition of L-arginine. Thus, NO oxidase apparently participates in the production of superoxide ion by RA neutrophils together with NADPH oxidase. We also found out that NO synthase of RA neutrophils (most probably, inducible isoenzyme) generated the enhanced amount of peroxynitrite as it followed from NMMA-inhibitable DHR oxidation (Table 1).

The intensity of lucigenin-amplified CL produced by RA monocytes sharply decreased in the presence of mitochondrial inhibitors rotenone and antimycin A from 5.37 to 1.86 mV and 1.97 mV, respectively ($P < 0.01$). In contrast, rotenone and antimycin A did not at all affect CL produced by normal cells. These findings point to mitochondrial superoxide production [9] as another source of oxygen radicals in RA monocytes. The other parameters of oxidative stress, namely a decrease in neutrophil SOD activity and an increase in the levels of "loose" iron in the plasmalemma of neutrophils and monocytes, were also observed. In contrast, the level of serum transferrin iron was reduced (Table 1).

Thus, on the whole, our data clearly show that both RA neutrophils and monocytes are under oxidative stress characterized by the overproduction of oxygen radicals and peroxynitrite.

3.2. Effects of antioxidants and chelators on oxygen radical overproduction by RA neutrophils

A pattern of the inhibitory activities of antioxidants and chelators in RA neutrophils is shown in Fig. 2. It is seen that the spontaneous and stimulated oxygen radical production by RA neutrophils was strongly inhibited by SOD and rutin, weakly inhibited by the hydroxyl radical scavenger mannitol and iron chelator DF, and weakly stimulated by catalase. These findings show that RA bloodstream leukocytes mainly produce superoxide ion and that the iron-catalyzed formation of hydroxyl radicals is of little importance. This is in contrast to the effects of free radical inhibitors on FA leukocytes where mannitol strongly inhibited oxygen radical production [5]; the stimulating effect of catalase can be explained by its protection of the superoxide-producing NADPH oxidase from inactivation by hydrogen peroxide. Thus, the analysis of antioxidant activities enables one to differentiate between the nature and mechanisms of free

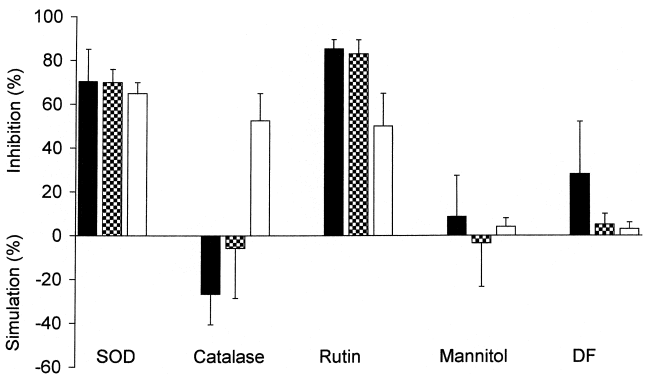

Fig. 2. Effects of antioxidants and chelators on oxygen radical production by the neutrophils from RA patients and control. The number of experiments (N) = 15, all data are presented as means ± SD. ■ RA neutrophils, spontaneous luminol-amplified CL; ▩ RA neutrophils, PMA-stimulated luminol-amplified CL; and □ controls.

radical formation in different pathologies. Comparison of FA and RA shows that although both pathologies are characterized by the overproduction of oxygen radicals, they differ by the nature of the radicals produced: RA leukocytes mainly produce superoxide ion, while FA leukocytes generate the enhanced level of highly reactive hydroxyl radicals due to the iron-catalyzed conversion of superoxide ion into hydroxyl radicals. In conclusion, it should be stressed that natural non-toxic bioflavonoid rutin (vitamin P) was the most effective inhibitor of oxygen radical overproduction in both RA and FA leukocytes and, therefore, may be considered as a useful supporting pharmaceutical agent for the treatment of "free radical" pathologies.

References

[1] Brown KA. The polymorphonuclear cell in rheumatoid arthritis. Br J Rheumatol 1988;27:150–5.
[2] Eggleton P, Wang L, Penhallow J, Crawford N, Brown KA. Differences in oxidative response of subpopulations of neutrophils from healthy subjects and patients with rheumatoid arthritis. Ann Rheum Dis 1995;54:916–23.
[3] Jang D, Murrell AC. Nitric oxide in arthritis. Free Radic Biol Med 1998;24:1511–9.
[4] Afanas'ev IB, Ostrachovitch EA, Abramova NE, Korkina LG. Different antioxidant activities of bioflavonoid rutin in normal and iron-overloading rats. Biochem Pharmacol 1995;50:627–37.
[5] Korkina LG, Samochatova EV, Maschan AA, Suslova TB, Cheremisina ZP, Afanas'ev IB. Release of active oxygen radicals by leukocytes of Fanconi anemia patients. J Leukoc Biol 1992;52:357–62.
[6] Afanas'ev IB, Afanas'ev II, Deeva IB, Korkina LG. Free radical formation and oxyhemoglobin oxidation in β-thalassemia red blood cells in the presence of prooxidants: effects of the free radical scavenger rutin and oral chelator L1. Transfusion Sci 2000;23:237–8.
[7] Kooy NW, Royall JA, Ischiropoulos H, Beckman JS. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radic Biol Med 1996;16:149–56.
[8] Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci USA 1996;93:6770–4.
[9] Rembish SJ, Trush MA. Further evidence that lucigenin-derived chemiluminescence monitors mitochondrial superoxide generation in rat alveolar macrophages. Free Radic Biol Med 1994;17:117–26.