Original Contribution

PROTECTIVE EFFECT OF NATURAL FLAVONOIDS ON RAT PERITONEAL MACROPHAGES INJURY CAUSED BY ASBESTOS FIBERS

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Abstract—Exposure of macrophages to asbestos fibers resulted in enhancement of the production of oxygen radicals, determined by a lucigenin enhanced chemiluminescence (LEG) assay, a formation of thiobarbituric acid reactive substances (TBARS), a LDH release into the incubation mixture, and a rapid lysis of the cells. Rutin (Rut) and quercetin (Qr) were effective in inhibiting LEG, TBARS formation, and reducing peritoneal macrophages injury caused by asbestos. The concentrations of antioxidants that were required to prevent the injury of peritoneal macrophages caused by asbestos by 50% (IC50) were 90 mM and 290 mM for Qr and Rut, respectively. Both flavonoids were found to be oxidized during exposure of peritoneal macrophages to asbestos and the oxidation was SOD sensitive. The efficacy of flavonoids as antioxidant agents as well as superoxide ion scavengers was also evaluated using appropriate model systems, and both quercetin and rutin were found to be effective in scavenging O2. These findings indicate that flavonoids are able to prevent the respiratory burst in rat peritoneal macrophages exposed to asbestos at the stage of activated oxygen species generation, mainly as superoxide scavengers. On the basis of this study it was concluded that natural flavonoids quercetin and rutin would be promising drug candidates for a prophylactic asbestos-induced disease.

Keywords—Flavonoids, Free radicals, Rutin, Quercetin, Oxidative burst, Macrophages, Asbestos

INTRODUCTION

Flavonoids are widespread plant pigments possessing P vitamin activity. In clinical practice, these compounds are used to reduce the permeability of the capillary walls and to decrease their fragility. The opinion prevails that this effect is due to the ability of flavonoids to protect the capillary walls from the damaging action of free radicals. Actually, quercetin and rutin were shown to be capable of inhibiting the enzymatic and ascorbate-dependent lipid peroxidation in liver microsomes and mitochondria,1,2 the lipid peroxidation in human erythrocytes,3 the autoxidation of linoleic acid, and methyl linoleate.4 It has been shown that flavonoids are capable of protecting the lysis of human erythrocyte following photosensitized oxidation.5 Antioxidant action of flavonoids can be due to their ability to scavenge reactive oxygen species (ROS),6,7 free hydroxyl and peroxy radicals2,4 or to chelate iron ions,2,8 which play a vital role in the initiation of free radical reactions. It is common knowledge that ROS and intermediates of free radical reactions normally mediate the bactericidal and tumorcidal activity of immune cells, but under certain circumstances related to ischemia, reperfusion, inflammation, toxemia, and trauma, the overproduction of ROS is described as an important amplifying system, which plays a key role in the pathological processes.9,15 The present work was conducted in order to determine the efficiency of quercetin and rutin against oxidative injury of macrophages. The results obtained are discussed in connection with possible mechanisms of the flavonoids protective effect.

MATERIALS AND METHODS

Chemicals

Quercetin, rutin, riboflavin, 2-thiobarbituric acid, superoxide dismutase, and lucigenin were from Sigma
(St. Louis, MO). Nitro blue tetrazolium and NADPH were from Reanal (Hungary). Asbestos fibers (5-10 mm) were natural chrysotile asbestos (Tuva, Russia).

Macrophages oxidative injury assay

Peritoneal macrophages were isolated from Wistar rats by the slightly modified method described by Kor-kina et al. and washed with an isotonic phosphate buffer (pH 7.3). After washing, the cells were resuspended in the same buffer and used immediately. Macrophages (2 ml) suspension (2 X 10⁶ cells/ml) in the isotonic phosphate buffer (pH 7.3) was preincubated at 37°C for 2 min. At zero time 50 ml of water suspension of asbestos fibers was added (the final concentration was 2 mg/ml). Flavonoids were added as a dimethyl sulfoxide solution (the final concentration of dimethyl sulfoxide not exceeding 2% of the final volume). At various time intervals samples were taken for survival evaluation, determination of TBARS formation, and LDH release. In some experiments the samples (1 ml) were centrifuged at 1000 rpm for 5 min and were used for spectrophotometric evaluation of flavonoids oxidation during exposure of macrophages to asbestos.

Survival evaluation

Live cells were determined by cell viability assay based on the intracellular enzymatic conversion of the virtually nonfluorescent calcein AM to the intensely fluorescent (about 530 nm) polyanionic calcein, using LIVE/DEAD® EukoLight® Viability/Cytotoxicity Kit (Molecular Probes, Inc, USA) and fluorescence microscope.

LDH release assay

A portion (0.5 ml) of incubation mixture containing macrophages was centrifuged at 1000 rpm for 5 min and 0.2 ml of supernatant was measured for LDH content. The assay was based on the measurement of the rate of NADH oxidation in the presence of piruvate at 30°C. LDH values were expressed as changes in absorbance at 340 nm for 2 min.

TBARS formation assay

A portion (0.5 ml) of incubation mixture containing macrophages was mixed with 0.5 ml 30% (w/v) trichloroaeetic acid and 2.5 ml 0.5% (w/v) solution of 2-thiobarbituric acid, and the mixture was heated at 100°C for 15 min. After centrifugation of precipitated proteins, the thiobarbituric acid reactive substances (TBARS) content was determined by measuring the absorbance at 532 nm. An extinction coefficient of 1.56 • 10⁵ M⁻¹ cm⁻¹ was used for quantifying TBARS.

Macrophages respiratory burst assay

Peritoneal macrophages suspension (2 X 10⁶ cells/ml) in the isotonic phosphate buffer (pH 7.3) containing 30 mM lucigenin as an enhancer was added in siliconized counting vial (4 ml capacity) and preincubated at 37°C for 5 min. At zero time 50 ml of water suspension of asbestos fibers was added (the final concentration was 2 mg/ml). Lucigenin-enhanced chemiluminescence (LEG) was evaluated using a single-photon counting technique. The system consisted of a photomultiplier tube and a counter. The data were analyzed by the IBM-type computer. The measured LEC was expressed in terms of total photons counts per 1 s (cps) and as the mean of total photons counts over 5 min.

Superoxide-driven reduction of nitroblue tetrazolium by photochemically reduced riboflavin

Reduction of nitroblue tetrazolium (NBT) was carried out at room temperature (22°C) under fluorescent lighting (20 W, 20 cm). The standard incubation mixture contained 6 mM riboflavin, 0.8 mM of N,N,N',N'-tetramethylylethylenediamine (TMEDA), 0.08 mM EDTA in 0.016 M phosphate buffer (pH 7.8), and 85 mM NBT. Flavonoids were added as a dimethyl sulfoxide solution (the final concentration of dimethyl sulfoxide not exceeding 2% of the final volume). After 5 min incubation, the reaction was stopped by the light switching off and the addition of 0.5 ml 10% (w/v) KI solution and absorbance was measured at 550 nm.

Carbon tetrachloride-dependent lipid peroxidation

Rat liver microsomes (1.2-1.3 mg protein/ml) were incubated at 37°C for 5 min with 1 mM NADPH, 20 mM NaCl, and 0.6 mM EDTA in phosphate buffer (0.05 M, pH 7.4). Carbon tetrachloride was added as an ethanol solution. The final ethanol concentration was 2% (v/v). Lipid peroxidation in the samples (1 ml) was terminated by adding 1.5 ml 10% (w/v) trichloroacetic acid. Then, 2.5 ml 0.5% (w/v) solution of 2-thiobarbituric acid was added and the mixture was heated at 100°C for 15 min. After centrifugation of precipitated proteins, the thiobarbituric acid reactive substances (TBARS) content was determined by measuring the absorbance at 532 nm.

RESULTS

Asbestos-stimulated injury of peritoneal macrophages

Asbestos was shown to enhance the production of oxygen radicals by rat peritoneal macrophages.
our experiments, this enhancement, which was determined by a lucigenin-enhanced chemiluminescence assay, was maximal after 1 min at an asbestos concentration of 2 mg/ml (Fig. 1). When rat peritoneal macrophages were exposed to asbestos, a lysis of the cells, a LDH release into the incubation mixture, and an increase in the TBARS content were also found. The injury of macrophages by asbestos increased incubation time dose dependently. When the cells were exposed to 1 mg/ml asbestos for 20 min only 45 ± 10% of the cells were damaged, in terms of LDH release but nearly 100% of the cells were damaged for 20 min when 2 mg/ml concentration of asbestos fibers was used (Table 1). Five and 10-min exposition of macrophages to 2 mg/ml asbestos resulted in a less injury of the cells (12 ± 4% and 52 ± 8%, respectively). Only 6 ± 4% of rat peritoneal macrophages were damaged, in terms of LDH release, when cells were exposed for 20 min to 2 mg/ml TiO\textsubscript{2} particles used as reference nonpathogenic mineral dust.

\textbf{Effect of flavonoids on injuries caused by asbestos in rat peritoneal macrophages}

The injury caused by asbestos in peritoneal macrophages was used for investigation of the protective action of flavonoids. In these experiments a dose-dependent protection against the asbestos-initiated macrophages lysis and LDH release into the incubation mixture by adding increasing concentrations of quercetin and rutin was found (Table 1). Cell injury was generally evaluated in terms of LDH release. In some experiments, an effect of flavonoids on viability evaluation of macrophages was simultaneously investigated using LIVE/DEAD® Eukolight® Viability/Cytotoxicity Kit and fluorescence microscopy. Similar results were obtained using both approaches to the measurement of the efficiency of protective action of flavonoids (Table 1).

Inhibitory effect of flavonoids on the production of oxygen radicals by rat peritoneal macrophages was also evaluated. Quercetin and rutin were found to inhibit lucigenin-enhanced chemiluminescence emitted from asbestos-stimulated peritoneal macrophages in a dose-related manner (Fig. 1). Treatment with flavonoids also provided a protection against TBARS formation and the protection was generally dose dependent (Table 1). The concentrations of antioxidants, which were required to suppress the effects caused by asbestos in rat peritoneal macrophages by 50%, are shown in Table 2.

### Table 1. Effect of Flavonoids on TBARS Formation and Injury of Macrophages Following Exposure to Asbestos

<table>
<thead>
<tr>
<th>Macrophages Injury (% to Control)\textsuperscript{a}</th>
<th>Macrophages Injury (% to Control)\textsuperscript{a}</th>
</tr>
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<tbody>
<tr>
<td>formation (nmol/ml)</td>
<td>LDH Release</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>- Antioxidants</td>
<td>96 + 4</td>
</tr>
<tr>
<td>+ Qr (15 mM)</td>
<td>84 + 12</td>
</tr>
<tr>
<td>+ Qr (30 mM)</td>
<td>56 + 18\textsuperscript{b}</td>
</tr>
<tr>
<td>+ Qr (60mM)</td>
<td>36 ± 12\textsuperscript{b}</td>
</tr>
<tr>
<td>+ Qr (120 mM)</td>
<td>27 ± 20\textsuperscript{b}</td>
</tr>
<tr>
<td>+ Qr (300 mM)</td>
<td>11 + 21\textsuperscript{b}</td>
</tr>
<tr>
<td>+ Rut (120 mM)</td>
<td>66 + 15\textsuperscript{b}</td>
</tr>
<tr>
<td>+ Rut (240 mM)</td>
<td>52 + 15\textsuperscript{b}</td>
</tr>
<tr>
<td>+ Rut (600 mM)</td>
<td>18 ±4\textsuperscript{c}</td>
</tr>
<tr>
<td>+ Rut (1200 mM)</td>
<td>11 + 12\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} In the control experiments macrophages were incubated at 37°C in the isotonic phosphate buffer (pH 7.3) for 20 min without asbestos.
\textsuperscript{b} \( p < .05 \).
\textsuperscript{c} \( p < .01 \).
\textsuperscript{d} \( p < .001 \) compared to control.

### Table 2. The Concentrations of Flavonoids that Suppressed the Effects Caused by Asbestos in Rat Peritoneal Macrophages by 50% (IC\textsubscript{50} Values)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>LDH release</th>
<th>TBARS Formation</th>
<th>LEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>90</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Rutin</td>
<td>290</td>
<td>80</td>
<td>30</td>
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</table>
The inhibition of lucigenin-enhanced chemiluminescence, TBARS formation, and release of LDH from peritoneal macrophages was accompanied by a flavonoids oxidation. Quercetin oxidation resulted in bleaching at 370 nm and absorption increase at 330 nm (Fig. 2A, trace 3). Rutin oxidation resulted in bleaching at 360 nm (Fig. 2B, trace 3). The oxidation of flavonoids during exposure of macrophages to asbestos was sensitive to SOD, and the maximum inhibition of quercetin oxidation was about 50% (Fig. 2A, trace 4). Rutin oxidation was inhibited by SOD almost completely (Fig. 2B, trace 4). When flavonoids and macrophages were incubated without asbestos, the magnitude of absorbance changes (Fig. 2A and B, traces 1 and 2) was significantly ($p < 0.001$) less than that with asbestos. Absorbance decrease at 370 nm during incubation of quercetin without asbestos (Fig. 2A, trace 1) can be explained by binding drug with cells rather than flavonoid oxidation because quercetin oxidation resulted in not only bleaching at 370 nm but also absorption increase at 330 nm. It is clear from Fig. 2 that there was no marked absorption increase at 330 nm in these conditions. The rates of SOD-sensitive quercetin and rutin oxidation under the conditions in Fig. 2, calculated on the basis of the differential coefficients of extinction between flavonoid reduced (in the presence of 100 mM SOD, Fig. 2A and B, traces 4) and oxidized forms (Fig. 2A and B, traces 3) ($e_{Qr} = 8.7 \times 10^{3} M^{-1} cm^{-1}$; $e_{Rut} = 7.2 \times 10^{3} M^{-1} cm^{-1}$) were $0.07 \pm 0.01$ and $0.15 \pm 0.02$ mM/min, respectively.

### Table 3. Effect of SOD on the Flavonoids Oxidation (DOD$_{406}$ nm) by Photochemically Reduced Riboflavin

<table>
<thead>
<tr>
<th>SOD ng/ml</th>
<th>Quercetin$^a$</th>
<th>Rutin$^b$</th>
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<tbody>
<tr>
<td>4.5</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>11.0</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td>45.0</td>
<td>52</td>
<td>67</td>
</tr>
<tr>
<td>110.0</td>
<td>74</td>
<td>82</td>
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</table>

Reactions were carried out at room temperature (22°C) under fluorescent lighting (20 w, 20 cm). The standard incubation mixture contained 15 mM flavonoid, 6mM riboflavin, 0.8 mM of AWA$^\text{W}$-tetramethylendiamine (TEMED), 0.08 mM EDTA in 0.016 M phosphate buffer (pH 7.8).

$^a$ Photooxidation time 5 min.

$^b$ Photooxidation time 30 min.

**Effect of flavonoids on superoxide-driven reduction of nitroblue tetrazolium by photochemically reduced riboflavin**

There are several ways to inhibit lucigenin-enhanced chemiluminescence emitted from asbestos-stimulated peritoneal macrophages by flavonoids such as scavenging of superoxide ion, inhibition of NADPH-oxidase, and, at last, the second absorption of lucigenin-produced photoemission. In order to elucidate scavenging properties of quercetin and rutin we studied interaction of flavonoids with O$_2^-$, and the illumination of riboflavin in the presence of TEMED was used as a source of superoxide ion. O$_2^-$ generated upon reoxidation in air photochemically reduced flavin was found to oxidize both quercetin and rutin. The reactions were accompanied by the loss of the visible absorption bands of flavonoids at 406 nm. Flavonoids were bleached at 406 nm at the almost same extent when quercetin was photoxidized 5 and rutin was photoxidized 30 min. Su-
peroxide dismutase (SOD) was used to confirm the role of superoxide ion in flavonoids oxidation and an effective inhibition of the oxidation by this enzyme was found (Table 3). The rates of quercetin and rutin oxidation under the conditions in Table 3, calculated on the basis of the differential coefficients of extinction between flavonoid reduced and oxidized forms \( (c_Q = 8.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \text{ mol}^{-1}) \) and \( (c_{Ru} = 7.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \text{ mol}^{-1}) \), were 2.8 and 0.4 mM/min, respectively. Nitro blue tetrazolium (NBT) was also used as an indicator for \( O_2^* \) and the inhibition of superoxide-driven reduction of NBT to formazan by flavonoids as compared with SOD was studied (Table 4). The concentrations of quercetin and rutin that inhibit a superoxide-driven reduction of nitroblue tetrazolium by photochemically reduced riboflavin by 50% \( (IC_{SO}) \) were 6 and 15 mM, respectively.

Effect of flavonoids on carbon tetrachloride-dependent lipid peroxidation

Carbon tetrachloride-dependent lipid peroxidation was used for measurement antioxidant activity of flavonoids because in this system a supplementary mechanism of lipid peroxidation inhibition due to the ability of flavonoids to bind iron ion is not realized. Carbon tetrachloride-dependent lipid peroxidation was inhibited by both flavonoids, but quercetin was a substantially more powerful inhibitor (Table 5). The concentrations of quercetin and rutin that inhibit lipid peroxidation by 50% \( (IC_{SO}) \) were 6 and 120 mM, respectively.

**DISCUSSION**

The addition of asbestos to rat peritoneal macrophages enhances the production of superoxide ion and hydrogen peroxide. It is known that transition metals, mainly iron or their complex, which may be adsorbed by asbestos, can decompose hydrogen peroxide, according to the Fenton mechanism:

\[
\text{HOOH} + \text{Fe}^{2+} \rightarrow \text{HO} + \text{Fe}^{3+} \text{ complex} + \cdot \text{OH}
\]

The hydroxyl radical \( (\cdot \text{OH}) \) can trigger generation of chain-propagating alkoxy radicals from carbon-centered radicals after H \( \cdot \) abstraction from a fatty acid side chain in a membrane lipid or can produce covalently bound protein aggregates and these effects may be a cause of cell oxidative injury. In this study we have showed that natural flavonoids, rutin and quercetin, are very effective in reducing peritoneal macrophages injury caused by asbestos. Several biochemical mechanisms can be likely related to the protective effect of flavonoids: scavenging of superoxide ion; inhibition of NADPH-oxidase and suppression of lipid peroxidation. In the present study for monitoring of ROS production we used chemically enhanced chemiluminescence, and lucigenin was used as the enhancer because this chemical is more specific to superoxide than luminol. The inhibition of chemiluminescence by flavonoids we roughly considered as the inhibition of NADPH-oxidase of macrophages. The inhibition of NADPH-oxidase and respiratory burst in neutrophil by flavonoids has been reported. However, it was also shown that flavonoids are inhibitors of protein-kinase C. This enzyme plays key role in the activation of NADPH-oxidase and the respiratory burst. Therefore, the inhibition of protein-kinase C has been suggested to be the possible mechanism of the inhibition of NADPH-oxidase by quercetin. An alternative mechanism of the inhibition of NADPH-oxidase may be related to the ability of flavonoids to scavenge superoxide ion. To elucidate the detail mechanism of this inhibition the oxidation of flavonoids by superoxide generated in the model photochemical system as well in peritoneal macrophages exposed to asbestos was investigated. The ability of quercetin to interact with superoxide has been previously reported. In the present study, the ability to scavenge superoxide ion was quantitatively evaluated for both rutin and quercetin, and the rate of flavonoids oxidation by superoxide was directly derived from well-known absorbance changes. The specificity of flavonoids oxidation by superoxide was tested by running measurements in...
parallel in the presence of an excess of SOD. In the case of flavonoids oxidation by peritoneal macrophages, we found that about 50% of quercetin oxidation was inhibited by SOD and the oxidation of rutin was inhibited by SOD almost completely (Fig. 2). However, it is possible that in such a heterogeneous system the SOD-insensitive oxidation of quercetin may also be partially related to superoxide. It is clear that the oxidation of quercetin and rutin during exposure of peritoneal macrophages to asbestos is not in agreement with the suggestion that effect of flavonoids on respiratory burst is due to the inhibition of protein-kinase C, and oppositely, the following findings demonstrate that this effect is attributed to the ability flavonoids to scavenge superoxide ion: (1) both rutin and quercetin inhibited the ROS production by rat peritoneal macrophages exposed to asbestos, although only quercetin is an inhibitor of protein-kinase C;20 (2) the inhibition of a respiratory burst in rat peritoneal macrophages exposed to asbestos was accompanied by SOD-sensitive flavonoids oxidation; (3) efficacy of flavonoids as inhibitors of a respiratory burst correlated quite closely with the rate of flavonoids oxidation by superoxide in the model system (photoxidation) and the rate of flavonoids oxidation by macrophages exposed to asbestos.

We also observed the inhibition of lipid peroxidation in rat peritoneal macrophages following exposure to asbestos by quercetin and rutin. An uncontrolled lipid peroxidation is widely discussed as a sufficient cause of cell death.29 Nevertheless, it is questionable whether lipid peroxidation is the main cause for the injury and lysis of peritoneal macrophages by asbestos because we found that quercetin completely inhibited TEARS formation at concentrations in the range in which it only partially protected cells against injury (Tables 1 and 2). Possibly, therefore, another free radical mechanism may play a major role in the macrophages injury by asbestos. It is of interest to note that there is no correlation between the antioxidant efficacy of flavonoids in the model system (IC50Rut/IC50Qr = 20) and the efficacy of protective effect of flavonoids against the injury and lysis of peritoneal macrophages by asbestos. This observation can be explained by assuming that lipid peroxidation is inhibited by flavonoids on the stage of initiation (production of ROS) rather than propagation.

Studies with the cells and the cell-free experimental model indicate that protective action of flavonoids on rat peritoneal macrophages injury caused by asbestos may be due to the scavenging of ROS. At the same time we found that quercetin and rutin inhibited lucigenin-enhanced chemiluminescence of peritoneal macrophages following exposure to asbestos fibers by 50% at concentration 3 and 30 mM, respectively, whereas the values for 50% protection of cells (in terms of LDH release) by quercetin were 90 and 290 mM. This contradiction can be explained by assuming that in addition to the scavenging of superoxide ion there are another effects of flavonoids resulted in the decrease in intensity of lucigenin-enhanced chemiluminescence, for example, the second absorption of lucigenin-produced photoemission by flavonoid. However, further studies are required to elucidate the biochemical mechanism underlying effect of flavonoids on asbestos injury to macrophages.

In conclusion, asbestos-induced disease (asbestosis) and other inflammation-related disorders are likely related to overproduction of ROS and reactive intermediates that normally mediate the bactericidal activity of immune cells and cells injury.16,30 The results of this study demonstrate that natural nontoxic flavonoids quercetin and rutin inhibit these pathological processes and because of their higher efficiency in this respect, would be a promising drug candidates for a prophylactic of asbestosis.

Acknowledgements — This work was supported by grant from the Ministry of Education and Science, Belarus.

REFERENCES


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ABBREVIATIONS
EDTA—ethylenediaminetetraacetic acid
LDH—lactate dehydrogenase
LEC—lucigenin-enhanced Chemiluminescence
NBT—nitro blue tetrazolium
Qr—quercetin
ROS—reactive oxygen species
Rut—rutin
SOD—superoxide dismutase
TBARS—thiobarbituric acid reactive substances