An *In vitro* Study of the Protective Effect of the Flavonoid Silydianin against Reactive Oxygen Species

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The inhibitory effect of silydianin, an active constituent of *Silybum marianum*, on the *in vitro* production and release of oxidative products has been examined. Polymorphonuclear neutrophils (PMNs) play a primary role in the initiation and propagation of inflammatory responses. Their apoptosis is a major mechanism associated with the resolution of inflammatory reactions. Neutrophils were assessed for caspase-3 activity, the first step in the execution phase of apoptosis. When cells were cultured with 100 µM silydianin for 24 h, caspase-3 was activated. Induction of apoptosis by silydianin was accompanied by a decrease in luminol-enhanced chemiluminescence as well as superoxide radical (O₂•−) release in freshly isolated cells and lipid peroxidation in mouse spleen microsomes. No significant effect of silydianin on PMN hydrogen peroxide production evaluated by a flow cytometric dichlorofluorescin oxidation assay was found. Such results indicate a possible antiinflammatory activity for silydianin, which regulates caspase-3 activation, affects cell membranes and acts as a free radical scavenger. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: flavonoid; silydianin; oxidative stress; apoptosis; polymorphonuclear neutrophils.

INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are the primary effector cells in host responses to injury and infection. Activated neutrophils generate extremely high amounts of reactive oxygen species (ROS), but these are normally targeted at pathogens inside intracellular phagosomes. These same beneficial antimicrobial functions can cause significant local tissue injury and lead to the development of pathologic systemic inflammatory conditions (Edwards, 1994).

The apoptotic death of activated neutrophils is a major mechanism associated with the resolution of the inflammatory process. Caspase-3 activation is the first step in the execution phase of apoptosis (Sweeney et al., 2001). The precise role of reactive oxygen species in the modulation of caspase activity remains unresolved. The understanding of these different mechanisms and the discovery of substances capable of regulating them therefore constitute a future direction of therapy for these pathologies (Fadeel et al., 1998).

Various flavonoid compounds have been noted to have antiinflammatory properties, and several modes of action have been demonstrated for these, including inactivation of free radicals (Cotelle, 2001; Rice-Evans et al., 1997; Aherne and O’Brien, 2002; Zielińska et al., 2003). As free radicals are possibly involved in DNA damage and tumour promotion, the antioxidative activity may contribute to a cancer-protective effect (Russo et al., 2000). Evidence for the potential role of oxidative stress in various diseases and pathophysiological processes suggests that the dietary intake and the therapeutic use of flavonoids may have positive health effects (Halliwell, 1996; Hollman and Katan, 1999).

Thus, the goal of the present study was to investigate the ability of silydianin (Fig. 1), a flavonoid isolated from *Silybum marianum* (L) Gaertn., to protect *in vitro* human neutrophils and mouse spleen microsomes from oxidative damage.

MATERIALS AND METHODS

Plant material. Silydianin was isolated from *Silybum marianum* Gaertn., using HPLC (Kvasnička et al., 2003) and identified by UV method and ¹³C NMR spectroscopy (Markham et al., 1982). This compound
was a generous gift from Professor P. Górecki of the Institute of Medical Plants and Herbal Drugs in Poznań, Poland. A stock solution of silydianin was prepared in dimethyl sulfoxide (DMSO) and diluted in phosphate buffered saline (PBS). The final concentration of dimethyl sulfoxide was always 0.2% (v/v).

Reagents. Ac (N-acetyl)-DEVD (Asp-Glu-Val-Asp)-AMC (7-aminoc- methylcoumarin) was obtained from BD Biosciences (Heidelberg, Germany). 2,7'-dichlorofluorescin diacetate (DCFH-DA) was purchased from Acros Organics (New Jersey, USA). Diphenylene iodonium (DPI), ferriytochrome c, luminal, n-2-hydroxyethylperazyne-N'-2-ethanesulfonic acid (HEPES), phorbol 12-myristate 13-acetate (PMA), RPMI 1640 medium, DMSO, trypan blue and zymosan-A were provided by Sigma Chemical Co. (St Louis, MO, USA). Other reagents were provided by standard chemical suppliers.

Isolation and culture of human neutrophils (PMNs). Peripheral venous blood from healthy adult volunteers was collected in sterile heparinized tubes (10 U/mL blood). Neutrophils were isolated by Gradisol G (d 1.115 g/mL; Aqua-Medica s.c., Poland) density gradient centrifugation (Ferrante and Thong, 1978). The cell viability was greater than 97% as assessed by the trypan blue exclusion test. Isolated PMNs (2 × 10^6 cells) were maintained in RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin and 5 mM HEPES) in 40 mm dishes. Cells were preincubated for 1 h with PBS alone or different concentrations of silydianin and then with PMA (200 nM) and DPI (10 µM), an inhibitor of the NADPH oxidase, at 37 °C in a humidified atmosphere containing 5% CO₂.

Cytotoxicity. Cell suspensions were incubated with different concentrations of silydianin (up to a final concentration of 1–100 µM) for 30 min at 37 °C in a dark incubator, together with untreated control samples. The samples were then centrifuged at 800–900 rpm, PMNs were resuspended in RPMI 1640 and 0.4% trypan blue, and the viable and dead cells scored.

Preparation of microsomes. Five mice (female Swiss mice, 7–9 weeks old, 25 g) were used for each experimental group. The spleen homogenates were prepared according to established procedure (Gnojkowski et al., 1984) and the microsomes were prepared by differential centrifugation. Microsomal pellets were suspended in 0.01 M phosphate buffer (pH 7.4) containing 20% (v/v glycerol). Protein concentrations were determined by the method of Lowry et al. (1951).

Flow cytometric assay of dichlorofluorescin diacetate (DCFH-DA) oxidation. The intracellular production of ROS in stimulated neutrophils was quantified in individual cells by flow cytometry using the procedures described by Bass et al. (1983). Fifty milliliters of heparinized whole blood was incubated with 15 µL of 0.3 mM DCFH-DA in PBS for 30 min. After that PMA (10 µg/mL) as a neutrophil-stimulating agent was added. The erythrocytes were removed by lysis with 1 mL of Ortho-mune solution. Intracellular DCF fluorescence of PMNs was determined by flow cytometry (Cytoron Absolute, Ortho, USA) using 488 nm excitation wavelength. The DCF fluorescence was measured with the green filter (515–548 nm) using the linear amplification of the signal. The fluorescence intensity was expressed as the value of the ‘mean channel’, calculated by ImmunoCount 2 software (Ortho).

Chemiluminescence assay (CL). Luminol dependent CL was measured according to Allen (1986) using an LKB Wallac 1250 luminometer. The method is based on the oxidative degradation of luminol by the released hydrogen peroxide; when reactive oxygen species are obtained: O₂•−, •OH, O₂. Incubation mixture in total volume of 1 mL contained luminol (10 µM), freshly isolated neutrophils (1 × 10^6 PMNs) and opsonized zymosan (1 mg/mL). The reaction was started by adding the ligand at 37 °C and light emission was recorded for 15 min. After approx. 10 min of ‘oxidative burst’ reactions, the characteristic maximum appeared. Chemiluminescence intensity was determined by measuring its peak value (mV). Zymosan alone in the presence of luminol (blank) and background activity without PMNs but in the presence of silydianin produced only negligible emission of light.

Measurement of O₂•− formation. The superoxide formation was determined according to the method of Cohen and Chovaniec (1978). Reduction of ferricytochrome c by O₂•−, produced by 1 × 10^6 freshly isolated PMNs stimulated by opsonized zymosan (1 mg/mL) at 37 °C was assayed by measuring the absorbance at 550 nm.

All assays were performed after 30 min incubation of PMNs with silydianin. Control samples contained PBS only.

Lipid peroxidation. The complete incubation mixture (1.5 mL total volume) consisted of a suspension of microsomes isolated from the mouse spleen in 0.15 M Tris-HCl 1 mM KH₂PO₄ buffer (pH 7.4) in a final concentration of 1 mg protein and various concentrations of the tested compound. Lipid peroxidation was induced by addition of 0.01 mM FeSO₄ and 0.05 mM ascorbic acid (non-enzymatic lipid peroxidation). The products of lipid peroxidation were measured by the thiobarbituric acid method (Ohkawa et al., 1979) and expressed as µmol of malondialdehyde (MDA) equivalents formed per mg protein.

Caspase-3 activity. The measurement of AC-DEVD-AMC cleavage was performed in a fluorometric assay modified from Fadel et al. (1998). DEVD-AMC is a specific substrate for caspase 3-like proteases and represents the common cleavage site for this class of enzymes. Neutrophils were lysed in 0.5 mL of lysis buffer (10 mM Tris-HCl; 10 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7.5); 130 mM NaCl; 1% Triton X-100: 10 mM NaPPi). The supernatant (containing approximately 100 µg of protein) was diluted to 1 mL of HEPES buffer (20 mM HEPES pH 7.5; 10% glycerol; 2 mM DTT) containing 20 µM Ac-DEVD-AMC. After a 1 h incubation at 37 °C, fluorescence of AMC (excitation 380 nm and emission 450 nm; spectrofluorimeter Hitachi F-2500) was
Table 1. Effect of silydianin on oxidative metabolism of human PMNs and lipid peroxidation in mouse spleen microsomes

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Control</th>
<th>Silydianin 10 µM</th>
<th>Silydianin 100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometry H₂O₂ production</td>
<td>78.74 ± 12.14 (100%)</td>
<td>88.37 ± 14.27 (112.2%)</td>
<td>85.36 ± 11.70 (108.4%)</td>
</tr>
<tr>
<td>[mean peak channel]</td>
<td></td>
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</tr>
<tr>
<td>Chemiluminescence maximum light intensity [mV]</td>
<td>221.62 ± 17.28 (100%)</td>
<td>179.51 ± 10.91* (81.0%)</td>
<td>88.20 ± 14.91* (39.8%)</td>
</tr>
<tr>
<td>Reduction of cytochrome c</td>
<td>10.39 ± 0.21 (100%)</td>
<td>6.92 ± 0.16* (66.6%)</td>
<td>3.88 ± 0.2* (37.5%)</td>
</tr>
<tr>
<td>[nmol O₂•⁻/1 × 10⁶ PMNs]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation in microsomes</td>
<td>84.40 ± 5.00 (100%)</td>
<td>68.20 ± 4.10* (80.8%)</td>
<td>47.00 ± 13.10* (55.7%)</td>
</tr>
<tr>
<td>[µmol MDA equivalents/mg protein]</td>
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</tbody>
</table>

Values are mean ± SE of 4–19 experiments. The percentage of respective control is shown in parentheses. Significantly different from untreated control: * p < 0.05, paired t-test.

determined. Blanks in the absence of cell lysates were carried out to determine background fluorescence.

Presentation of the results. Data are given as mean ± SE (if n ≥ 3) with n referring to the number of independent experiments. Statistical analyses were performed by means of two-tailed t-test.

RESULTS

The original measurements are expressed as the appropriate concentration units and as the percent of remaining metabolic activity after silydianin treatment (Tables 1 and 2). The control group (no flavonoid added) is then considered as 100%. This method enables a comparison of results from various methods and studies.

The cell viability was 94.8 ± 3.8% for silydianin at concentrations of 1–100 µM, indicating no cytotoxicity toward neutrophils.

The PMN oxidant production in response to PMA or opsonized zymosan, was characterized by the production of hydrogen peroxide, superoxide radical (O₂•⁻) and chemiluminescence intensity. Pretreatment of PMNs with silydianin was associated with a significant decrease in PMN superoxide radical production and CL intensity compared with control (Table 1). The antioxidative activity of silydianin is concentration dependent, being the most marked for 100 µM.

No significant effect of silydianin on PMN hydrogen peroxide production evaluated by flow cytometric dichlorofluorescin (DCFH)-oxidation assay was found. Figures 2, 3, 4 present examples of histograms of resting, stimulated and stimulated + silydianin treated PMNs, respectively.

Membrane oxidation was studied by the thiobarbituric acid method in mouse spleen microsomes. The tested flavonoid was effective in reducing the extent of lipid peroxidation. This decrease was statistically dose-dependent (p < 0.05) (Table 1).

Cultured in vitro, neutrophils isolated from the blood undergo apoptosis spontaneously. There was a time dependent induction of caspase activity. As shown in Table 2 silydianin in a concentration of 100 µM slightly induced apoptosis of stimulated and unstimulated cells during 24 h of incubation. Lower concentrations of silydianin did not affect or inhibit the level of tested enzyme.
Table 2. Effect of silydianin on caspase-3 activity in neutrophils

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>0.1 µM</th>
<th>1 µM</th>
<th>10 µM</th>
<th>100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMNs + DPI + PMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Unstimulated PMNs</td>
<td>543.20 ± 11.68</td>
<td>546.47 ± 9.12</td>
<td>545.95 ± 4.85</td>
<td>544.3 ± 3.11</td>
<td></td>
</tr>
<tr>
<td>Silydianin</td>
<td>541.63 ± 4.85</td>
<td>541.63 ± 4.85</td>
<td>545.25 ± 0.45</td>
<td>545.25 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>PMNs</td>
<td>527.07 ± 3.84</td>
<td>548.25 ± 0.45</td>
<td>545.96 ± 4.85</td>
<td>544.3 ± 3.11</td>
<td></td>
</tr>
<tr>
<td>Silydianin</td>
<td>527.07 ± 3.84</td>
<td>548.25 ± 0.45</td>
<td>545.96 ± 4.85</td>
<td>544.3 ± 3.11</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE from 3 independent experiments, as fluorescence of AMC.

DISCUSSION

Recent evidence suggests that cellular stress induced by reactive oxygen species is responsible for a large number of pathophysiological processes such as cancer, ageing, inflammation and ischemia-reperfusion damage (Halliwell, 1996; Hollman and Katan, 1999).

Oxidative injury leads to lipid peroxidation, DNA breakage and enzyme inactivation, including free radical scavenging enzymes. Antioxidants are capable of interfering with the processes involved in oxidative stress. Plant flavonoids are phenolic substances which contain a number of hydroxyl groups attached to the ring structure. The redox properties of polyphenols allow them to act as reducing agents, hydrogen-donating antioxidants and singlet oxygen quenchers; in some cases they also chelate transition metal ions (Rice-Evans et al., 1997; Catapano, 1997; Basaga et al., 1997; De La Puerta et al., 1996).

Our findings indicate that silydianin diminished the oxidative metabolism of human polymorphonuclear neutrophils (Table 1) measured either by enhanced chemiluminescence (CL) or the reduction of cytochrome c (O$_2^-$ determination). The ROS formation in both methods was inhibited in a dose-dependent manner.

Neutrophil oxidative bursts are accompanied by intracellular and extracellular CL, which depends on the hydrogen peroxide concentration in the assay. Hydrogen peroxide is generated by different enzymes and it may cause cellular and nuclear damage that leads to apoptotic cell death. The autooxidation of membrane polyunsaturated fatty acids (called also lipid peroxidation) is induced by active oxygen species including hydrogen peroxide (Edwards, 1994). An inhibitor of lipid peroxidation can protect cells against H$_2$O$_2$.

Hydrogen peroxide production was examined by the flow cytometry dichlorofluorescin (DCFH)-oxidation assay. In this method H$_2$O$_2$ is the main factor oxidizing DCFH-DA. Silydianin at the concentrations of 10 and 100 µM did not affect the intensity of DCF fluorescence.
Additionally, the extent of lipid peroxidation diminishment under the influence of silydianin was measured in mouse spleen microsomes. The observed reduction of the MDA formation depended on the silydianin concentration. These results suggest that silydianin at the concentrations 10 and 100 µM affected mainly cell membranes, which is in agreement with the observation of Despace et al. (1975). The luminol-enhanced CL reaction depends on oxygen radicals and myeloperoxidase generated or present in different subcellular compartments. As the fusion of these compartments seems to be a prerequisite for the induction of CL (Dahlgren, 1989), the stabilization of cytoplasmic membranes should decrease the intensity of this reaction. The same mechanism can be responsible for the inhibition of free radicals release, as indicated in the standard assay method (cytochrome c reduction).

Flavonoids were reported to inhibit the activity of several enzymes, among them the human neutrophil NADPH-oxidase (Tauber et al., 1984). The results of our study suggest that silydianin at the concentrations used (10 and 100 µM) cannot inhibit this enzyme. This effect may be explained either by a low affinity of this flavonoid to the enzyme, or by its lower accessibility to the cell compartments containing the oxidase. This last supposition is in agreement with the postulated action of flavonoid on cell membrane fluidity (Muriel and Mourelle, 1990; Bosisio et al., 1992; Buzzelli et al., 1993).

The interesting finding was that no caspase activation in neutrophils stimulated with PMA was detected. Pretreatment with the NADPH oxidase inhibitor, DPI, allowed the caspases to function in PMA-stimulated neutrophils, indicating that caspase is suppressed by NADPH-oxidase-derived reactive oxygen species. These preliminary results are therefore suggestive of a specialized caspase-independent pathway of cell death in activated neutrophils. Treatment of cells with silydianin, at a concentration of 100 µM, slightly induced the level of caspase-3 (Table 2). This effect is important, because induction and control of neutrophil apoptosis appears to be central to the resolution of an inflammatory state.

Generally, the results presented here demonstrate that silydianin is an effective inhibitor of free radicals production and release. This partly can explain the therapeutic use of silydianin as an antiinflammatory drug.

REFERENCES


