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Silybin Reduces Lipid Peroxidation of Rat Hepatocyte Membrane Caused by Cyclosporin A

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Abstract—An effect of cyclosporin A on lipid peroxidation in isolated rat hepatocytes was tested. A significant increase in lipid peroxidation marker (the concentration of lipofuscin-like pigments) was observed in samples incubated with cyclosporin A in comparison with the control. When hepatoprotective flavonoid silybin was added, the production of lipofuscin-like pigments decreased significantly. This result indicates a potential positive role of silybin in lowering of cyclosporin A side effects associated with the production of reactive oxygen species and plasma membrane damage.

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Key words: cyclosporin A, silybin, hepatocytes, lipid peroxidation, lipofuscin-like pigments

Cyclosporin A (CsA) is a potent immunosuppressive drug that is widely used in transplant medicine [1] and in the treatment of some autoimmune disorders [2]. However, CsA therapy is accompanied by several side effects (e.g., nephrotoxicity and hepatotoxicity). It is assumed that some of these effects are facilitated by the oxidative stress induced by CsA. For example, it was reported that CsA could participate in the production of reactive oxygen species (ROS) due to its metabolism via cytochrome P450 [3]; the involvement of NADPH oxidase activity [4] and xanthine oxidase activity [5] was also described. CsA has been shown to alter the expression levels of antioxidant enzymes. It reduced the activities of catalase and glutathione peroxidase [6]. In addition, one reactive form of another cyclosporin, cyclosporin D hydroperoxide, was described [7]. Therefore, the possibility of transformation of CsA to a hydroperoxide and direct oxidative cell damage cannot be excluded.

Lipofuscin-like pigments (LFP) are created as final products of lipid peroxidation [8]. These pigments have been implicated in the pathogenesis of numerous diseases including atherosclerosis, diabetes, and aging, and due to their fluorescence properties they are used as markers for encrypting of this negative process in cells [9]. Precursors of these fluorescent pigments are aldehydes, e.g., malonyl dialdehyde, which is also used as a lipid peroxidation marker [10, 11].

Although exact structures of LFP are not known so far, the estimation of their relative concentrations by fluorimetric or immunochemical detection was proved to be useful method for the evaluation of oxidative injury to the lipid part of cell membranes [12]. The main advantage of this method is the chemical stability of LFP — they do not decompose to other products, and they accumulate in cells during aging [13].

Silymarin is a mixture of hepatoprotective flavonoids obtained from the milk thistle (*Silybum marianum*). Silybin (Fig. 1) is the main component of silymarin and it is used either alone or in the mixture with other flavonoids. The silymarin complex protects liver cells both by its antioxidative properties and by the stabilizing of the cell membranes [14]. Therefore, it is used in the treatment of various liver diseases [15]. Silymarin is able to scavenge free radicals [16] and protects cells against

Abbreviations: CsA) cyclosporin A; LFP) lipofuscin-like pigments; ROS) reactive oxygen species.

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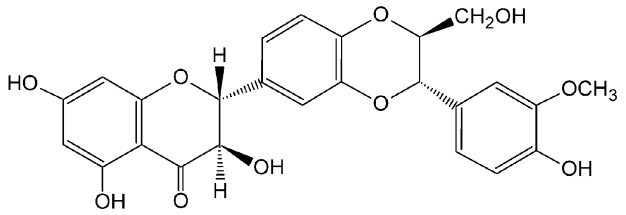

Fig. 1. Structure of silybin.

damage by lipid peroxidation in acute intoxications, e.g., with carbon tetrachloride, ethanol, or paracetamol [17-19]. At the same time silymarin inhibits activity of some key enzymes producing free radicals and ROS: activity of xanthine oxidase decreased in the presence of silymarin [20], and blocking of cytochrome P450 activity was also observed [21, 22].

We supposed that silybin might protect against oxidative stress damage caused by CsA in liver cells. Therefore, the aim of this study was to detect some lipid peroxidation markers generated during the incubation of isolated rat hepatocytes with CsA and to test if silybin is able to decrease the concentration of these markers, and in such a way to protect cells against oxidative injury by CsA.

MATERIALS AND METHODS

Chemicals and compounds tested. Chemicals were obtained from Penta (Czech Republic); HEPES, bovine albumin, and fetal calf serum were from MP Biomedicals, Inc. (formerly ICN Biomedicals, Inc.); EGTA, dimethylsulfoxide (DMSO), Williams' medium E, and trypan blue were from Sigma (Czech Republic). Collagenase was obtained from Sevapharma a.s. (Czech Republic). Cyclosporin A and silybin were from IVAX Pharmaceuticals s.r.o. (Czech Republic). Ketamine (Narkamon®) and xylazine (Rometar®) were obtained from Leciva, a.s. (Czech Republic).

Animals. Adult male Wistar/Han rats (*Rattus norvegicus*) weighing 150-200 g were obtained from BioTest s.r.o. (Czech Republic). The animals were kept in standard laboratory conditions (12 h light/dark cycle) with free access to food and water until the start of the experiments. Before cell isolation, the rats were anesthetized by i.p. administration of ketamine (100 mg/kg of body weight) in combination with xylazine (16 mg/kg of body weight). All procedures with animals were approved by the Ethics Committee, Ministry of Education, Czech Republic.

Primary rat hepatocyte cultures. Rat hepatocytes were isolated by *in vitro* two-step collagenase perfusion of rat liver [23]. The cells were collected in phosphate buffer saline, filtered, and washed several times by low-speed centrifugation (50*g*), and then were washed with Williams' medium E with $NaHCO_3$ (without L-glutamine and phenol red) [24]. Freshly prepared hepatocytes were resuspended in Williams' medium E supplemented with 0.5% fetal calf serum. The viability of the cells was estimated using the trypan blue exclusion test. The gently stirred cell suspensions were incubated for 1 h in sterile tubes under air at 6°C. Cell viability was roughly 85-95% in all experiments. The oxidative experiments began after controlling cell viability. The Lowry protein assay was used for protein content determination [25].

Oxidative experiments. Incubations of hepatocytes with the tested compounds were performed in heart jars at 20°C. Incubation suspensions contained 25 μM CsA, then 25 μM CsA and 100 μM silybin (added together), and control. Final cell concentration was $8 \cdot 10^5$ cells/ml (3.3 mg protein/ml). Fetal calf serum (0.5%) and 0.5% DMSO as a solvent for the studied chemicals were always present in the incubation suspension. Samples were collected after 0, 3, 6, 9, 12, 24, and 48 h incubation from each suspension, immediately frozen, and stored under liquid nitrogen for the evaluation of LFP concentration.

Fluorescence measurements. LFP in hepatocytes were determined according Goldstein and McDonagh [26] with some modifications as used in previous studies [27]. Hepatocyte suspension (500 μl) was added to 5 ml of chloroform–methanol mixture (2 : 1) and extracted for 1 h on a shaker. After extraction, 2 ml of distilled water was added, mixed, and the mixture was centrifuged (2000*g*, 10 min). After centrifugation, the lower chloroform phase was separated and used for measurements. Fluorescence excitation spectra of chloroform extracts were measured with an AMINCO-Bowman Series 2 spectrofluorometer. The excitation spectra were measured in the range of 250-400 nm for emission adjusted between 300-500 nm with a step of 10 nm. For more detailed characterization of LFP, synchronous spectra were measured with a constant difference 10 nm (excitation – emission). The quantitative estimation of LFP was based on the excitation and emission maxima found in 3D spectral arrays. The fluorimeter was calibrated with the standard No. 5 of the instrument manufacturer. LFP concentrations were expressed in relative fluorescence units (rfu) per mg of protein.

Data analysis. The data were processed by spectrofluorometer software and by SigmaPlot software. Experiments were made in triplicates; results are presented as mean ± S.E.M.

RESULTS

Excitation spectra of oxidized membrane samples showed peaks in the area of excitation wavelength between 350 and 390 nm and emission wavelength between 450 and 470 nm, which confirmed the presence of some kind of lipofuscin-like pigments. These pigments

are considered as the final products and markers of lipid peroxidation of membranes and were found in all samples. Samples with CsA showed a significant increase in LFP concentration for all incubation times compared to control samples. This increase was most pronounced after 12 h incubation. Excitation spectra of samples with CsA and silybin had much less increase in LFP amount; in fact, there were less amounts of LFP than in control samples. A comparison of excitation spectra of samples after 12 h incubation is in Fig. 2 (see color insert).

We compared the synchronous spectra of samples with CsA, with CsA and silybin, and control during experiments. We observed that LFP of the same kind were formed in all samples — either in the presence of silybin or without. LFP in samples differ in their quantities only. Fluorophores with emission wavelength 350 and 405 nm were produced throughout the experiments. It is evident that even the silybin addition was not able to eliminate LFP formation completely. Synchronous spectra showed that also later — 24 h after starting of the experiment — there were no qualitative changes in LFP fluorophore composition (Fig. 3, see color insert), although the onset of LFP formation already started after 12 h incubation (Fig. 4). Quantitative measuring of LFP concentration in rat hepatocytes was performed during 48 h experiments. LFP amounts in relative fluorescence units (rfu) were estimated from the height of the main peak in 3D spectral arrays, where the excitation and emission wavelengths were set at 352 and 430 nm, respectively. After application of 25 μM CsA at the beginning of the experiment, LFP concentrations increased until 12 h, and the amount of LFP was 4.43 ± 0.37 rfu/mg of protein maximally. Concentration of LFP in samples after application of 25 μM CsA with 100 μM silybin also increased until 12 h but much less — maximal LFP amount was only 2.47 ± 0.19 rfu/mg of protein. In control samples, LFP concentration was 3.07 ± 0.13 rfu/mg of protein after 12 h incubation. Differences between LFP amounts in samples with CsA and in samples with CsA and silybin were statistically significant at incubation times 6, 9, 12, 24, and 48 h. In comparison with LFP amount in control (3.07 ± 0.13 rfu/mg of protein after 12 h of incubation), samples with CsA and silybin had lower LFP concentrations, which were statistically significant at incubation times 3, 6, 9, and 12 h. LFP concentrations during experiments are shown in Fig. 4.

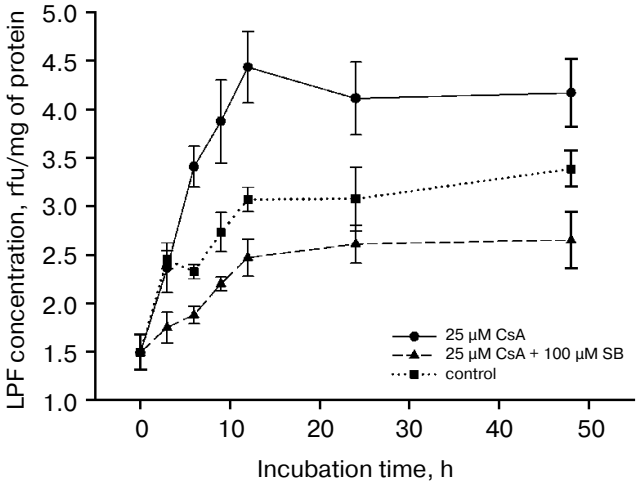

Fig. 4. Quantitative evaluation of LFP concentrations during 48-h experiments with 25 μM CsA (circles), with 25 μM CsA and 100 μM silybin (SB) (triangles), and control (squares); LFP concentrations are expressed in relative fluorescence units (rfu) per mg of protein.

DISCUSSION

There are several studies investigating LFP production as a marker of oxidative damage in liver of various biological models: Guerre et al. and Tuan et al. observed lipofuscin after aflatoxin B-1 intoxication [29, 30], Hassan et al. and Nowak et al. investigated the influence of the pesticides Endrin and Thiodan [31, 32], Antosiewicz et al. measured oxidative damage caused by hydrazine [33], Loumbourdis et al. detected lipofuscin in liver after $CdCl_2$ exposure [34]. All these studies were focused on liver damage caused by evident toxins or environmental pollutants. Our work shows that liver LFP can be increased also by CsA. Our study is apparently the first one showing that the liver LFP can be increased also by CsA. We have detected oxidative damage of membranes in isolated hepatocytes by LFP fluorescence measurement after a simple dose of CsA. As far as we know there is only one report about LFP production associated with CsA: lipofuscin nephropathy was detected after CsA application during renal transplantation [35]. This observation is consistent with our results indicating that CsA is able to enhance the risk of cell oxidative damage.

The concentration of CsA used in the experiments (i.e., 25 μM) is about one-two orders of magnitude higher than the usual concentration of CsA in the blood of transplant patients (about 0.1-1 μM, average levels; 1-2.5 μM, c_{max}, maximal levels) [28]. This higher concentration was chosen with respect to the much smaller duration of the experiment with hepatocytes and with regard to the differences in mechanisms of CsA transport to the cells *in vivo* and *in vitro* (CsA in artificial medium is not bound to suitable transport proteins, e.g., cyclophilin).

LFP in cells can be detected either by microscopy of stained samples or by fluorescence measurements. Microscopy is very often used in histochemical analysis, where LFP granules are stained by various methods: hematoxylin-eosin staining [36], periodic acid-Schiff reaction [30], bleaching with H_2O_2 [34], Kinyoun's carbol

fuchsin staining [37], etc. Where the quantification of LFP is necessary, more or less complicated digital processing is needed. Fluorescence measurements give a very fast and simple answer, how much LFP is in the sample. Despite that, this method is not so often used. LFP formation and accumulation by fluorimetric assay has been studied most extensively in the retinal pigment epithelium of the eye, but the autofluorescent age pigments are apparently produced in most postmitotic cells [13]. Additional non-physiological oxidative stress could enhance the production of these pigments to detectable concentrations. LFP as products of lipid peroxidation after various stimuli were detected in human serum [38], brain [39], erythrocytes [40], and in proximal tubules of renal cells [41]. As we know, in liver LFP were evaluated only in two works: one study showed an increase in LFP in rat liver homogenate after γ-irradiation [42]; blue fluorescence was observed in rat liver microsomes during chemical induced oxidation [11]. Our findings in isolated rat hepatocytes correspond with these two works in the sense that LFP fluorescent measurement is a useful test system for CsA oxidative damage in liver. It suggests that this method would be applicable also for testing of other drugs supposed to be hepatotoxic due to oxidative stress. In addition, liver is a very important organ from the point of view of drug metabolic pathways; therefore, this model of oxidative stress evaluation seems to be very relevant.

We have found that silybin was able to act as a protective antioxidative agent against lipid peroxidation caused by CsA in liver cells. However, other properties of silybin besides this antioxidative effect cannot be discarded for their hepatoprotective actions, e.g., interactions of silybin and CsA with plasma membrane proteins [43]. This inhibitory effect of silybin on LFP production might help decrease CsA toxicity in various cases. On the other side, the mutual interactions of silybin and CsA cannot be extrapolated to the steady state effect during the long-term treatment. Also, whether this provides efficient protection against oxidative damage *in vivo* remains to be confirmed. Hence, additional research based on monitoring of LFP concentrations during CsA application seems to be very important.

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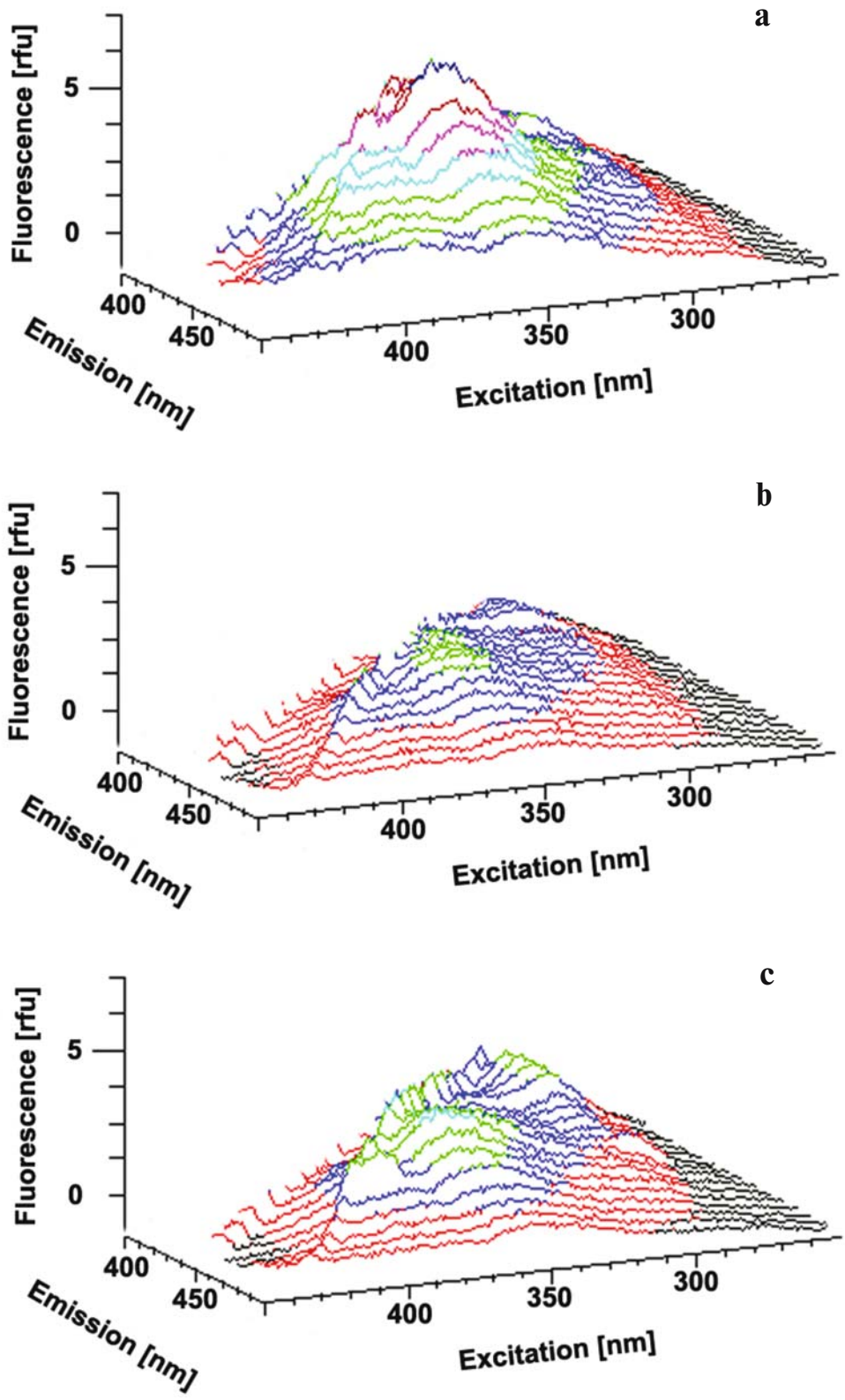

Fig. 2. (V. Moulisova et al.) Excitation spectra of samples after 12 h incubation: the peak of LFP (excitation/emission: 352 nm/430 nm) in sample with 25 μM CsA (a), 25 μM CsA and 100 μM silybin (b), and control (c); fluorescence is expressed in relative fluorescence units (rfu).

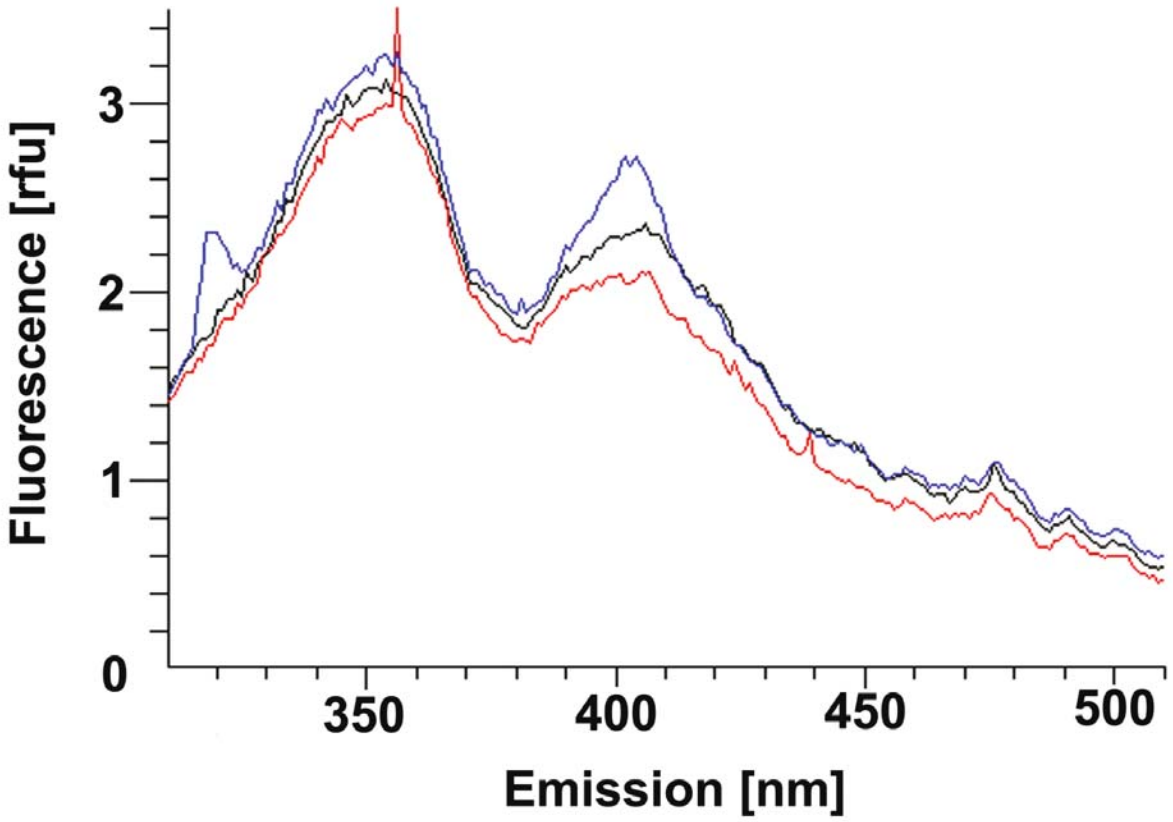

Fig. 3. (V. Moulisova et al.) An example of synchronous spectra: samples with 25 μM CsA (blue), 25 μM CsA and 100 μM silybin (red), and control (black) after 24 h incubation; two peaks for fluorophores (350 and 405 nm) are present in all samples; fluorescence is expressed in relative fluorescence units (rfu).