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Restoration of the Cellular Thiol Status of Peritoneal Macrophages from CAPD Patients by the Flavonoids Silibinin and Silymarin

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During continuous ambulatory peritoneal dialysis (CAPD) the peritoneal immune cells, mainly macrophages, are highly compromised by multiple factors including oxidative stress, resulting in a loss of functional activity. One reason for the increase of inflammatory reactions could be an imbalance in the thiol-disulfide status. Here, the possible protective effects of the antioxidant flavonoid complex silymarin and its major component silibinin on the cellular thiol status were investigated. Peritoneal macrophages from dialysis fluid of 30 CAPD patients were treated with silymarin or silibinin up to 35 days.

A time-dependent increase of intracellular thiols was observed with a nearly linear increment up to 2.5-fold after 96 hours, reaching a maximum of 3.5-fold after 20 days of culture. Surface-located thiols were also elevated. The stabilization of the cellular thiol status was followed by an improvement of phagocytosis and the degree of maturation as well as significant changes in the synthesis of IL-6 and IL-1ra. Furthermore, the treatment of peritoneal macrophages with flavonoids in combination with cysteine donors resulted in a shortened and more efficient time course of thiol normalization as well as in a further increased phagocytosis. In addition, GSH-depletion in thiol-deficient media simulating CAPD procedures led to intracellular thiol deficiency similar to the *in vivo* situation.

It is concluded that treatment with milk thistle extracts silymarin and silibinin alone or, more effectively in combination with cysteine donors, provide a benefit for peritoneal macrophages of CAPD-patients due to a normalization and activation of the cellular thiol status followed by a restoration of specific functional capabilities.

Keywords: continuous ambulatory peritoneal dialysis, macrophages, thiol status, flavonoids, silibinin

INTRODUCTION

Dialysis patients in general are at increased risk from oxidative stress because of impaired thiol and glutathione pathways. Comparing hemodialysis and continuous ambulatory peritoneal dialysis (CAPD), in the latter procedure a disturbance of antioxidant defence appears to be less [1–3]. However, under conditions of CAPD the peritoneal cells are exposed to an unphysiological environment. Peritoneal macrophages, the

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predominant peritoneal immune cells are intermittently exposed to osmotic and oxidative stress.

A number of studies dealt with peritoneal macrophages from CAPD patients and despite controversial interpretation it becomes evident that this treatment results in a modulation of their functional activities. Besides a different cytokine expression pattern that especially includes increased syntheses of IL-6 and TNF-α [4–7], diminished phagocytosis and bacterial killing was shown [8,9]. Moreover, an increased expression of CD15 in parallel with a decreased expression of CD11c, indicating an increasing immaturity of peritoneal macrophages over the time on CAPD, was demonstrated by McGregor et al. [4]. This impairment of function occurs not instantly but increases progressively. As defined by phagocytosis index, bactericidal activity, H_2O_2 production and cytokine production, the results of a longitudinal 1.5 year follow-up study indicate that peritoneal macrophages of new CAPD patients were more functionally active than those of established patients[10]. By flow cytometric analysis it was demonstrated, that long-term CAPD effects Fc-gamma receptor mediated phagocytosis by reducing the expression of CD16, CD64 and CD11b. Also, the adhesion to mesothelial structures could be impaired by lowered CD11b and CD14 expression [11].

The cellular thiol-disulfide status is defined by intracellular and cell surface bound components. The latter are characterized by SH-bearing membrane proteins and regulated at least in part by surface located oxidoreductases [12,13]. The intracellular thiol status occur in various forms, as protein bound SH-groups (protein thiols) as well as free or unconjugated non-protein thiols. The reduced form of glutathione (GSH) represents the predominant intracellular thiol, rising up to millimolar concentrations, which accounts for about 90% of the non-protein thiols within the cell[14].

Thiols, mainly GSH have been implicated in many cellular functions including detoxification and antioxidant processes, regulation of gene expression, protein synthesis, cell cycle regulation and apoptosis [15,16]. Besides various pathological situations including virus infection, poisoning, acute and chronic inflammations, idiopathic fibrosis, reperfusion syndroms and acute myocardial infarction [17–20], deficiencies of GSH have been documented also in dialysed patients [2,3].

Extracts from milk thistle (silybum marianum) have been used since antiquity. Today the flavonoid complex silimarin and its major pharmacologic active component silibinin were widely used for complementary treatment of acute and chronic liver diseases of toxic, degenerative or inflammatory origin. A number of experimental studies suggested that the main effect of silymarin is to decrease the lipid peroxidation of liver cell membranes[21–24] and to counteract liver fibrosis [25,26] possibly by selective inhibition of NF-kappa B activation [27].

Furthermore, silymarin or its constituents are thought to be useful in selective cytoprotection against nephrotoxic effects of cytostatic (cisplatin, ifosfamide) and immunosuppressive drugs (cyclosporine) [28,29].

In general, one of the main functional properties of silymarin seems to be the antioxidative activity in prevention of lipid peroxidation. As proved for platelets, leukocytes and endothelial cells, silibinin acts as a strong scavenger of free radicals, especially of HOCl from granulocytes and inhibits the formation of leukotrienes via the 5-lipoxygenase pathway [30].

Considering the phenomena that peritoneal macrophages from CAPD are defective in function and that dialysis patients show an impaired GSH metabolism, it is tempting to speculate that the reduced metabolic capacity of peritoneal immune cells could be an essential cause for the increase of inflammatory reactions over the time.

In this study the possible protective effects of the antioxidant flavonoids silymarin and silibinin on the cellular thiol status of peritoneal macrophages from CAPD patients were investigated. We found that the thiol status of

peritoneal macrophages was defective and that this disturbance was associated with a loss of function. The *in vitro* treatment with flavonoids alone and more efficiently in combination with cysteine donors resulted in a restoration of both the cellular thiol status and functional capacity.

METHODS

Materials

5-chloromethylfluorescein diacetate (CMFDA) and 5-(and-6)-(((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine (CMTMR) were obtained from Molecular Probes (Eugene, OR). Silibinin, Silymarin, NAC, BSO, diamide, DMSO and trypan blue were purchased from Sigma (Deisenhofen). IMDM, RPMI 1640 and FCS were supplied by PAA (Cölbe). Trypsin solution, RPMI 1603 and all cell culture supplements were from Life Technologies (Karlsruhe). ELISA specific for human interleukin-1 receptor antagonist (IL-1ra), IL-1β, IL-6 and TNF-α were obtained from R&D Systems (Wiesbaden).

Patients

Peritoneal macrophages from 30 CAPD patients were obtained. Original diseases were as follows: chronic pyelonephritis: 4; polycystic kidney: 2; analgetic nephropathy: 2; cisplatin induced nephropathy: 2; Ig-A-glomerulonephritis (GN): 3; membranoproliferative GN: 6; mesangioproliferative GN: 7; perimembranous GN: 4. One patient with analgetic nephropathy and one patient with membranoproliferative GN were suffered from type II diabetes mellitus. Exclusion criteria were clinical signs of peritonitis, known malignancies and immunosuppressive therapy. Patients characteristics: age 48.7±13.8 years; range: 28–71 years; time on CAPD: 24.2±18.3 months; range: 3–78 months; only two patients had been on CAPD < 9 months.

For comparison of the thiol concentration of peritoneal macrophages, alveolar macrophages and monocytes, bronchalveolary lavage (n=10) and blood samples (n=10) were obtained from apparently healthy subjects.

Cell handling and culture

Peritoneal macrophages were separated after a 120–180 min dwell period from dialysis fluid by shock-cooling the bags at –20°C for 5 min and subsequent centrifugation (300xg, 10 min, 4°C). Peritoneal macrophages counts and viability were defined by trypan blue exclusion test and May-Grünwald cell differential staining. After equilibration in RPMI 1640 supplemented with 10% FCS, streptomycine, penicilline and amphotericine B (Antibiotic-Antimycotic-Solution, Sigma, Deisenhofen) for 2 hours at 37°C, the peritoneal macrophages were washed and seeded into collagen IV coated Biocoat™ 6-well plates (Falcon, Heidelberg) at a density of 10^5 cells/ml. As propagation medium served IMDM supplemented with 20% FCS (pretested charges) and antibiotic-antimycotic solution at 37°C, 6.5% CO_2, humidified atmosphere. Medium changes were performed every two days at a 1:5 ratio. Cells were harvested by standard trypsin treatment. Because the question of alteration of cell surface structures by detachment methods is a common problem, the study protocol was designed in a way that should reduce this risk of methodical errors to a minimum. All handlings, treatments and analyses described in the present study were performed on treated cells (incubation in presence of effectors) and untreated controls. All manipulations including feeding, harvesting by trypsin treatment, staining or functional assays and FACS analyses were identical for treated cells and untreated controls. Therefore, the only difference of handling over the whole culture time was the presence or absence of the effectors (flavonoids, NAC).

Silibinin and Silymarin were dissolved in DMSO, yielding a 10 mg/ml stock solution

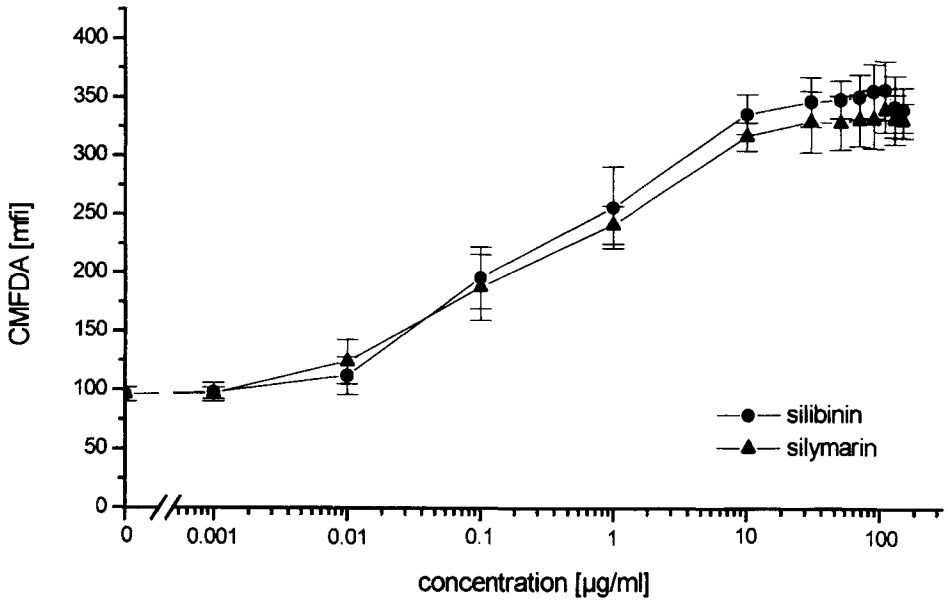

FIGURE 1 Dose response studies for the induction of thiol restoration by flavonoids. Peritoneal macrophages from CAPD patients were cultured for 15 days in presence of silibinin or silymarin. Total intracellular thiol contents were determined by CMFDA staining in flow cytometry. Data are presented as mean fluorescence intensities (mfi). Number of experiments done: n=3

which would be further diluted in IMDM to a final culture concentration of 70 µg/ml silibinin and 84 µg/ml silymarin. These concentrations were used in result of dose response studies (fig. 1), performed to determine maximal thiol reactive concentrations under the condition that these doses are reachable not only after local but also after systemic administration of the flavonoids in man according to pharmacokinetic studies [31–33]. For exclusion of solvent effects, an equal amount of DMSO was added to the untreated controls.

For induction of an artificial thiol deficiency cells were cultured in thiol deficient media (TDM) composed by RPMI 1603 supplemented with 20 mg/l aspartic acid, 20 mg/l L-hydroxyproline and 10% FCS.

Cell viability was determined by trypan blue exclusion test. All cell handling procedures in this study including the thiol deficient cultures ensured a cell viability >90%.

Determination of the cellular thiol status

The intracellular thiol concentration was measured by CMFDA staining in flow cytometry according to Heddley, Chikahisa and Coates [34–36]. Briefly, cell samples were stained with CMFDA at a final concentration of 12.5µM in phosphate buffered saline for 15 min at room temperature. After erythrocytes lysing (only in case of whole blood analysis) and washing, the cells were fixed in 1% paraformaldehyde and analysed within two hours by flow cytometry at λ_{EX}=490nm/ λ_{EM}=520 nm (FACSCalibur, Becton Dickinson, Heidelberg).

The expression of cell surface expressed thiols was determined as described previously [12]. In brief, the cells were washed twice in PBS containing 0.1% NaN_3 and cooled at 4°C. CMTMR at a final concentration of 12.5 µM was added und the samples were stained 3 min on ice. The procedure was stopped by addition of an at least 10-fold volume of icecold PBS containing 0.1%

NaN_3 and centrifugation at 200 xg for 5 minutes at 4°C. Gently, the resulting pellet was resuspended and washed twice again. Within 20 minutes after staining the cells were examined by flow cytometry at λ_{EX}=541nm/ λ_{EM}=565 nm.

The levels of intracellular as well as surface-located thiols were indicated by mean fluorescence intensities [mfi] of stained probes versus negative controls.

Phagocytosis assay

The phagocyting activity of peritoneal macrophages was assayed by determination of phagocytosis of opsonized *E. coli* bacteria using a commercially available flow cytometric test system (Phagotest™ ORPEGEN Pharma, Heidelberg). The recommendations supplied by the manufacturer were slightly modified relating to a prolongation of the incubation time up to 180 minutes.

Cytokines

Cytokine synthesis was determined after a 15 day treatment of peritoneal macrophages with silibinin or silymarin versus negative controls. Cell culture supernatants were stored in aliquots at –70°C. Quantitative analyses were performed using commercially available ELISA kits specific for human sIL-1ra, IL-1β, IL-6 and TNF-α according to the recommendations supplied by the manufacturer (R&D Systems, Wiesbaden) without any modifications.

Cell surface marker analysis

The expression of CD11c, CD15, CD69 and CD71 was analysed by means of double staining experiments in flow cytometry. After a 15 day treatment with silibinin or silymarin, samples of peritoneal macrophages were probed with either CD15-FITC / CD69-PE or CD71-FITC / CD11c-PE mouse monoclonal antibodies or mouse IgG1-FITC / IgG 2a-PE isotype controls (all Becton Dickinson, Heidelberg) according to standard protocols.

Statistics

Flow cytometric data analysis was performed using CellQuest™ software (Becton Dickinson, Heidelberg) on readings from 10.000 cells (monocytes and alveolar macrophages) and 3000 cells (peritoneal macrophages) per sample, respectively.

Data are presented as mean ± standard deviation. Statistical analyses were performed using the Link-Wallace-Test, Wilcoxon signed rank test, paired t-test and linear regression. Significances are indicated by the corresponding p-values.

RESULTS

Intracellular thiol expression of peritoneal macrophages from CAPD

For determination of basal thiol expression, peritoneal macrophages from CAPD fluids were analysed for intracellular thiol content immediately after separation. Here, compared to thiol concentrations of blood monocytes from healthy controls and non-smokers alveolar macrophages the intracellular thiol concentration was found to be significantly decreased. Defining the thiol expression index of blood monocytes as baseline it could be shown that normal alveolar macrophages expressed up to 4-fold higher concentrations of intracellular thiols. In contrast, the thiol expression index of peritoneal macrophages from freshly drawn PD fluids was reduced to 0.18 (tab. I).

TABLE I Total intracellular thiol expression of different macrophages

	blood monocytes (n=10)	*alveolar macrophages (n=10)*	*peritoneal macrophages (n=18)*
thiol expression index[a]	1.0±0.13	3.8±0.3	0.18±0.02

a. Data are indicated as mean ± SD. Thiol expression indices of alveolar macrophages and peritoneal macrophages were defined by designating the intracellular thiol expression of blood monocytes as 1 (baseline). The intracellular thiol content was determined by CMFDA based flow cytometry.

Effects of silibinin and silymarin on the cellular thiol status of peritoneal macrophages

The peritoneal macrophages were divided into control versus treatment groups and long-term treated with either 70 μg/ml silibinin or 84 μg/ml silymarin whereas cultures containing equal amounts of solvent served as control. The cellular thiol status was determined at daily intervals during the first 7 days and then at least every 3 days. As defined by CMFDA mean fluorescence intensity the intracellular thiol concentration increased time-dependently (fig. 2a). After a short lack-phase, within 96 hours a nearly linear increment up to 3-fold (silibinin-mfi_{CMFDA}=305±12; silymarin-mfi_{CMFDA}: 268±29) of the t_0-value (mfi_{CMFDA}=98±6) was observed. The elevation of intracellular thiol content reached a maximum of 3.5-fold compared to non-treated controls after 20 days of culture ($p<0.01$). There were only slight differences between the effects of silibinin and silymarin.

The incubation of peritoneal macrophages in presence of flavonoids resulted also in a strong increase of thiols expressed on the cell surface. In contrast to the effects shown for intracellular thiols, expressions of membrane bound SH-groups were stronger increased by silibinin then by silymarin treatment (fig 2b). Within 5 days after initiation the surface thiol expression (defined by CMTMR mean fluorescence intensity) of silibinin treated peritoneal macrophages was increased by 25% (t_0 mfi_{CMTMR}=56; t_5 mfi_{CMTMR}=75; $p<0.05$). After 4 days, a maximum of expression was reached, which then was sustained. In the case of silymarin only a small rise in surface located thiols was detectable.

GSH dependence of flavonoid effects

To understand the mechanism of the observed effects of silibinin and silymarin, inhibition studies of the glutathione metabolism were performed. After maximum stimulation by flavonoids, GSH synthesis was inhibited by addition of BSO at final concentrations of 10 μM on 5 consecutive days (fig. 3). The inhibition of GSH synthesis by BSO resulted in a significant reduction of the cellular thiol concentration. After 5 days of BSO co-incubation intracellular thiol concentrations of silibinin as well as silymarin treated peritoneal macrophages populations were reduced to 75% (t_{max} mfi_{CMFDA}=340±11; $t_{5\text{-BSO}}$ mfi_{CMFDA}=258±13.5; $p<0.05$) and 72.5% (t_{max} mfi_{CMFDA}=320±26.8; $t_{5\text{-BSO}}$ mfi_{CMFDA}=232±27.5; $p<0.05$), respectively.

Effects of cysteine donors

To test whether there are effects of accessory thiols as cysteine alone or in combination with silibinin and silymarin, N-acetyl-L-cysteine (NAC) at a final concentration of 200 μM was added to the peritoneal macrophages under long-term culture conditions. As expected, a single treatment of peritoneal macrophages with NAC led to an increase of the intracellular thiol concentration up to 2.5-fold after 15 days. These values were stable up to 35 days (fig. 4).

More interestingly, the combined application of the flavonoids and NAC resulted in an amplification of the effects of NAC alone. The nearly linear increment of the thiol concentrations was already complete after 10 days in the presence of silibinin + NAC ($t_0 mfi_{CMFDA}$=106±7; t_{10} mfi_{CMFDA}=354±28.9; $p<0.01$) and silymarin + NAC (t_0

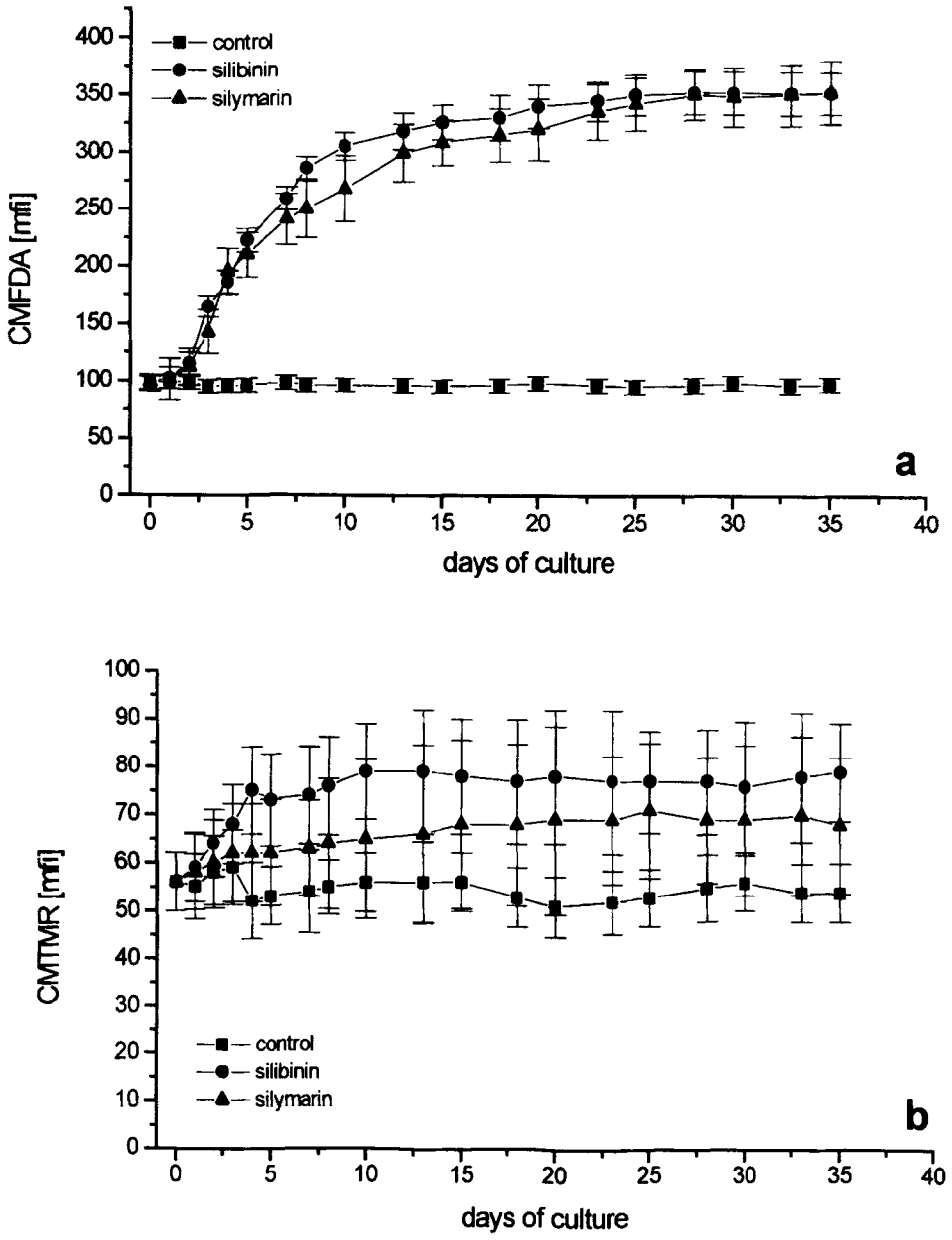

FIGURE 2 Effects of flavonoids on the intracellular thiol status and cell surface expression of thiols. Peritoneal macrophages from peritoneal effluent were separated and cultured as described in methods. At the time points indicated, the intracellular thiol content was determined by CMFDA mean fluorescence intensity (mfi, fig. 2a, n=18) whereas cell surface located thiols were determined by CMTMR mean fluorescence intensity (fig. 2b, n=18). Flavonoid concentrations: silibinin 70 μg/ml; silymarin: 84 μg/ml. Cultures containing equal amounts of solvent (DMSO: 2 μl/ml) served as controls. Number of experiments done: n=18

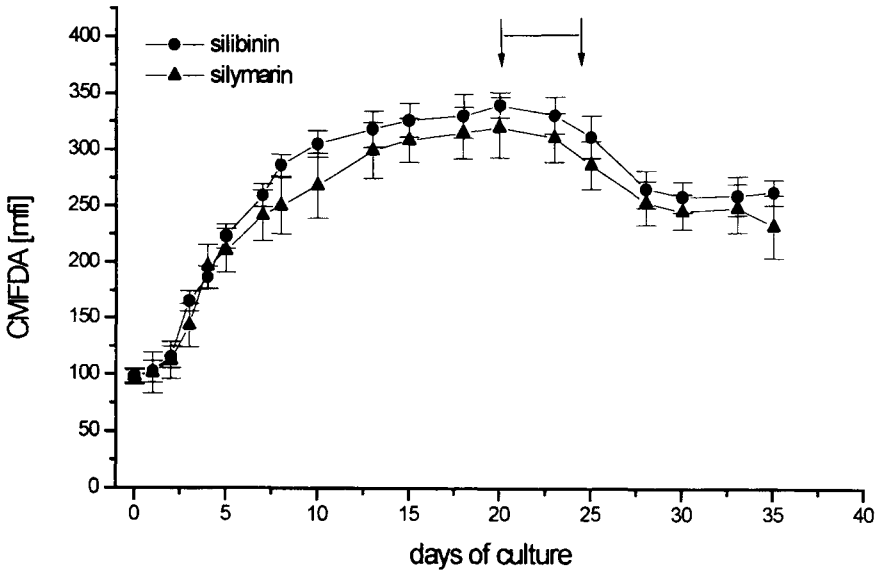

FIGURE 3 GSH dependence of flavonoid induced thiol restoration. Peritoneal macrophages were treated with silibinin (70 μg/ml) and silymarin 84 (μg/ml), resp. Starting at a maximum of thiol induction, 10 μM BSO was added at 5 consecutive days (application indicated by connected arrows). Intracellular thiol expressions were defined by CMFDA mean fluorescence intensity (n=5)

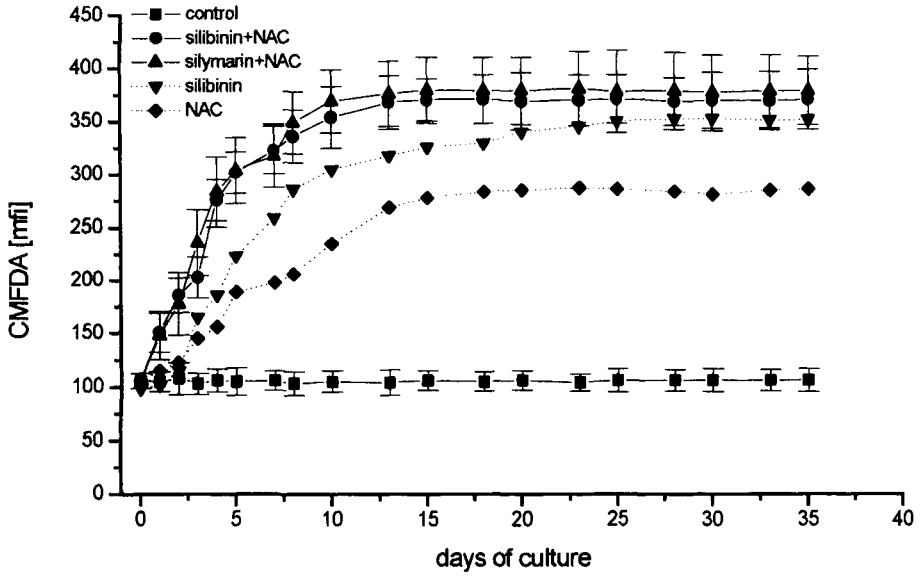

FIGURE 4 Effects of cysteine donors on the flavonoid induced thiol expression. Peritoneal macrophages were cultured either in presence of 70 μg/ml silibinin or 84 μg/ml silymarin each in combination with 200 μM NAC. The pointed lines indicate the single effects of silibinin and NAC, resp. Intracellular thiol expressions were defined by CMFDA mean fluorescence intensity (n=10)

mfi_{CMFDA}=106±7; t_{10} mfi_{CMFDA}= 369±29.5; p<0.01). The maxima of thiol expression reached within the total culture period exceeded the effects of silibinin and silymarin alone (silibinin: t_{max} mfi_{CMFDA}=352±19; silibinin + NAC: t_{max} mfi_{CMFDA}=371±22.8; silymarin: t_{max} mfi_{CMFDA}=352±28; Silymarin + NAC: t_{max} mfi_{CMFDA}=381±35).

Effects of thiol induction on phagocytosis

Several studies indicated that peritoneal macrophages from peritoneal dialysis effluent are functionally impaired [4–9]. These functional defects could be partially due to an altered thiol-disulfide status. Therefore, the influence of the flavonoids alone and in combination with NAC on the phagocytosis rate of peritoneal macrophages after 15 days of culture was investigated. As shown in table II, the treatment of peritoneal macrophages with silibinin, silymarin or NAC revealed a significant and comparable increase of phagocytosis rates (PR).

The application of flavonoids in combination with NAC, at concentrations indicated above, again amplified the PR significantly (PR-control-$mfi/10^4 cells$: 256±69; PR-silibinin+$NAC_{mfi/10^4 cells}$: 1453±169 (p<0.01); PR-$silymarin_{mfi/10^4 cells}$: 1369±209 (p<0.01)). Furthermore, there was a significant correlation of the intracellular thiol content of peritoneal macrophages and the pagocytosis rate (r=0.81, p<0.001).

Effects of thiol induction on the degree of maturity and activation

Peritoneal macrophages are known to become increasingly immature over the time on CAPD. Here, the effects of thiol restoration were tested by flow cytometric analysis of activation- and maturation dependent cell surface markers. After 15 days of flavonoid treatment the number of CD11c expressing peritoneal macrophages was significantly increased (fig. 5a). Compared to controls, after silibinin treatment, the CD11c expression was increased by 83.2 ± 65.7 % and in presence of silymarin by 51.2 ± 41.7 % (p<0.05). The number of CD15 positive peritoneal macrophages was also elevated, but to a lesser extent (silibinin: 23.4 ± 18.5 %; silymarin: 30.9 ± 13.8 %, p<0.05). As indicated by mean fluorescence intensity, the strong rise of the percentage yield of CD11c expressing peritoneal macrophages and the more moderate one for CD15 were accompanied by a significant induction of the respective epitope expression on the single cell level (fig. 5b). Interestingly, the expression of the activation antigen CD69 on CD15 positive cells was increased by about 20 % after flavonoid treatment, whereas no differences were observed for the expression of CD71 on CD11c positive peritoneal macrophages (fig. 5a).

Effects of thiol induction on cytokine synthesis

After a 15 days treatment, the synthesis of IL-6 was significantly lowered in presence of silibinin (27.7 ± 7.8 ng/10^6 cells) and silymarin (29.3 ± 7.6 ng/10^6 cells) compared to untreated controls (45.2 ± 9.1 ng/10^6 cells). In contrast to this, the levels of sIL-1ra were found to be increased for silibinin (135.1 ± 27.1 ng/10^6 cells) and silymarin (137.6 ± 29.1 ng/10^6 cells) in comparison to 110.1 ± 21.2 ng/10^6cells as measured for untreated controls (p<0.05) No differences were detectable between the study groups for levels of IL-1β and TNF-α (tab. III).

TABLE II Effects of flavonoids and NAC on phagocytosis of peritoneal macrophages

	control	*silymarin [84 µg/ml]*	*silibinin [70 µg/ml]*	*NAC [200 µM]*	*silymarin + NAC [84 µg/ml; 200 µM]*	*silibinin + NAC [70 µg/ml; 200 µM]*
phagocytosis rate[a]	256±69	8368±78	869±96	916±102	1.369±209	1.453±196

a. Data are indicated as mean ± SD. Phagocytosis rates were defined after 15 days treatment (either single or combined application of silymarin, silibinin and NAC) by flow cytometric detection of phagocyted opsonized E. coli bacteria of 10^4 cells per sample (n-7).

TABLE III Effects of flavonoids on cytokine synthesis of peritoneal macrophages

	IL-6 [ng/10^6 cells]	*IL-1ra [ng/10^6 cells]*	*IL-1β [pg/10^6 cells]*	*TNF-α [pg/10^6 cells]*
control[a]	45.2±9.1	110.1±21.2	5.8±1.1	381.1±65.0
silibinin [70 μg/ml]	27.7±7.8 ($p<0.05$)	135.1±27.1 ($p<0.05$)	6.1±0.9	384.7±78.8
silymarin [84 μg/ml]	29.3±7.7 ($p<0.05$)	137.6±29.1 ($p<0.05$)	6.0±1.3	379.0±85.0

a. Data are indicated as mean ± SD.

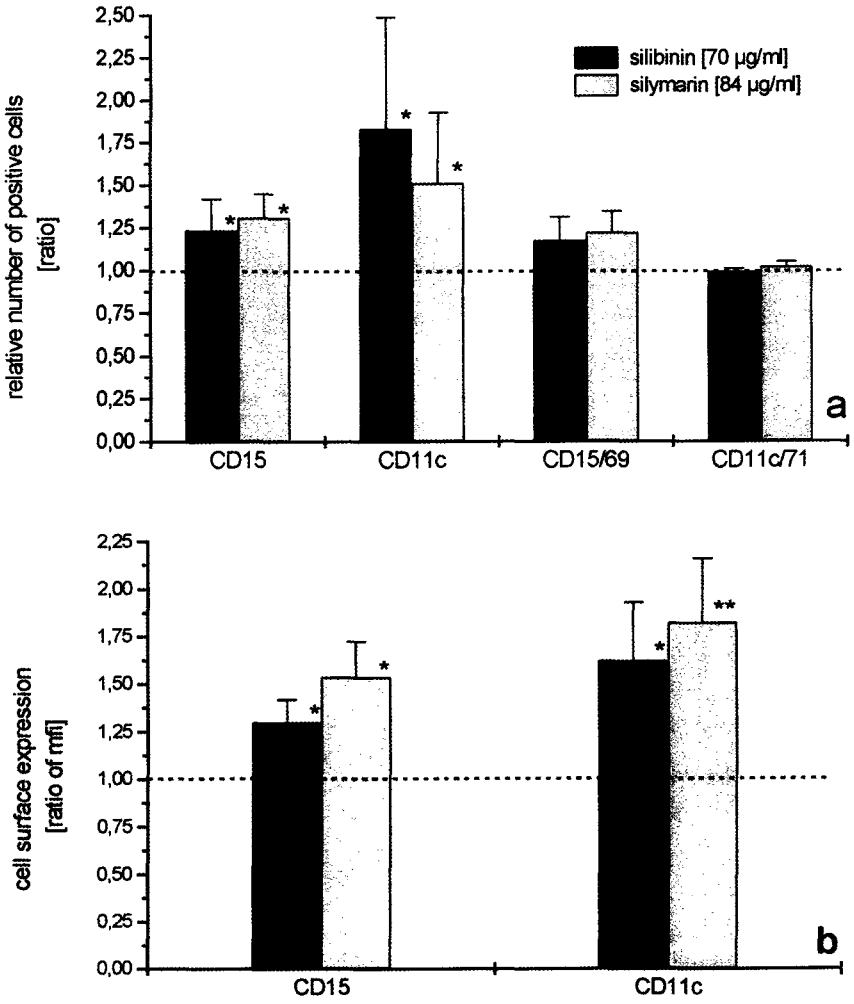

FIGURE 5 Effects of flavonoids on the degree of maturity and activation. Peritoneal macrophages were treated with silibinin or silymarin for 15 days and analysed for cell surface marker expression by double staining in flow cytometry as indicated. Figure 5a displays the ratio of the percentage number of flavonoid treated versus untreated CDx positive peritoneal macrophages. 5b: Ratio of mean fluorescence intensities (mfi) of flavonoid treated versus untreated peritoneal macrophages. Number of experiments done: n=12. (*: $p<0.05$; **: $p<0.01$)

Modulation of the intracellular thiol status by artificially induced thiol deficiency

To test whether the observed defective thiol status of peritoneal macrophages is due to environmental conditions occuring in CAPD and to evaluate the importance of the extracellular redox state we used an experimental model for an artificially induced thiol deficiency. RPMI 1603 supplemented with hydroxyproline and aspartic acid but lacking glutathione, mercaptoethanol, cysteine or any other free thiols served as thiol deficient medium (TDM). Peritoneal macrophages were thiol-restored by treatment with silibinin+NAC for 10 days. After reaching stable thiol expressions, the peritoneal macrophages were subjected to an adaption process. Here, peritoneal macrophages were cultured in RPMI 1640/TDM (v/v:1/1) for 24 hours and subsequently transferred into TDM (each supplemented with 10% FCS). After 36 and 48 hours of thiol deficient culture the intracellular thiol expression of peritoneal macrophages was determined. Figure 6 shows one representative experiment out of 5. Within 36 hours of TDM-culture, the intracellular thiol concentration was found to be decreased to about 60% and after 48 hours to about 10%.

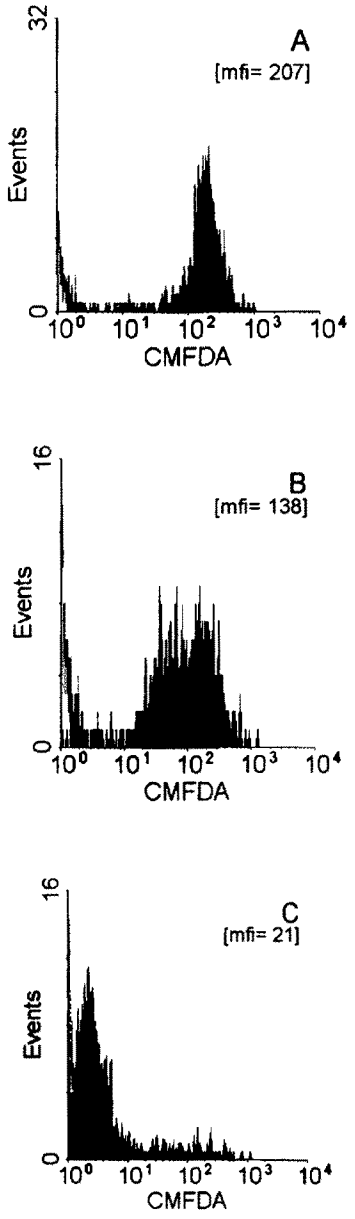

FIGURE 6 Artificially induced thiol deficiency in peritoneal macrophages. Thiol induction in freshly isolated peritoneal macrophages was performed by treatment with 70 μg/ml silibinin in combination with 200 μM NAC for 10 days. Thereafter, the peritoneal macrophages were cultured in thiol deficient media for 48 hours. The thiol concentration after 24 hours (histogram A), 36 hours (histogram B) and 48 hours (histogram C) was measured by CMFDA based flow cytometry (one representative experiment out of 5)

DISCUSSION

Functional properties of peritoneal macrophages from patients with end-stage renal diseases undergoing CAPD have been intensively investigated during the last 15 years. In result the majority of these studies demonstrated several impaired functions of immunocompetent peritoneal cells resulting in episodes of peritonitis. Based on this knowledge various trials were done to normalize special functional parameters. Beside the standard local antibiotic therapy, these trials included the application of anti-inflammatory drugs for modulation of the prostaglandine pathways[37], the substitution of

zink in order to increase stimulated IL-1α/β and TNF-α release[38], intraperitoneal IgG and INFα treatment for prevention of bacterial peritonitis [39,40] as well as the use of tuftsin for prevention of *Candida albicans* infections [41,42].

In the present study it was obvious that the treatment with the flavonoids silibinin and silymarin resulted in a stabilization of the cellular thiol status of peritoneal macrophages which was associated with a restoration of the diminished phagocytosis. The improvement of this global functional capability was accompanied by an increase of CD11c expression and an activation of CD15 expressing peritoneal macrophages, indicating regulatory effects of the flavonoids on macrophages activation and degree of maturity. Furthermore, it was obvious that the restoration of the cellular thiol status was followed by a modulation of cytokine synthesis. This becomes evident by a significant reduction of the known abnormal high IL-6 synthesis and increasing IL-1ra levels during the *in vitro* treatment.

Disturbances of the cellular thiol status have been described for various diseases by investigating changes in levels of glutathione. While playing the predominant key role within the regulation of the thiol status, GSH stands not alone for this fine-tuned mechanism. Therefore, a sensitive determination of the total cellular thiol content should better reflect the cellular defense capacities in a broader range than did the sole measurement of GSH. Caused by these conditions, in this study we determined the cellular thiol status at the single cell level by the use of fluorochrom-conjugated chloromethyl derivatives. The probe of choice for the detection of total intracellular thiols by argon-laser equipped flow cytometry is CMFDA [34–36]. CMFDA does not exclusively react with GSH but also with free thiols within the cell, so that CMFDA released fluorescence is an excellent parameter of the total intracellular thiol content.

The cell surface located part of the cellular thiol status was defined by CMTMR labeling. CMTMR reacts with protein-bound SH-groups on the cell surface exclusively under conditions, which inhibit the penetration of the dye into the cell[12]. The primary targets at the cell surface are mainly proteinogeneic cysteinyl groups. These protein thiols represent the first wall within the selfdefense system of the cell and are of special importence for macrophages and other immune cells that simultaneously need reactive oxygen intermediates and use antioxidants in order to support their function.

Our data show that intracellular thiols of peritoneal macrophages in the peritoneal effluent were extremely low compared to blood monocytes and alveolar macrophages. Considering that a loss of thiols e.g. GSH is associated with a loss of function in several cell systems[15,16], this observation may indicate a local cellular immune defect of the peritoneal barrier during CAPD. Functional impairments of peritoneal macrophages are reported to develop within a few months [10]. As of now it is not known when this process comes to the end. The average on peritoneal dialysis treatment of patients recruited in our study was 26 months.

In the presence of the flavonoids silibinin and silymarin both the cell surface expression of thiols and particularly the intracellular thiol content significantly increased. Silibinin is known as a direct scavenger of reactive oxygen species in several cell systems including human mesangial cells [30,43]. Within the latter it was shown to prevent the high-glucose induced alteration of fibronectin turnover. Our results clearly indicate that silibinin enhances the thiol status by mobilizing the GSH metabolism. This was obvious after inhibition of γ-glutamylcysteine synthetase using BSO at concentrations that do not affect cell viability. The inhibition of GSH synthesis counteracts the silibinin effects resulting in a decrease down to about 75%. Because the flavonoids were still present during BSO treatment it could be suggested that silibinin or silymarin may interfere directly with the GSH metabolism. The target structure of the action of flavonoids

within the GSH system are not known as yet. Protein thiols seem to be resistant to depletion by BSO [44]. Therefore, the favorite candidates might be the γ-glutamylcysteine synthetase and/or the glutathione synthetase which catalyze the reaction of γ-glutamylcysteine with glycine. This hypothesis is supported by our observation that a donor of cysteine which is necessary for the GSH synthesis further amplified the thiol induction by silibinin. Therefore, the principle mechanism of the silibinin action could be based on the activation of the enzymes involved in GSH synthesis which utilizes cellular cysteine stores. NAC, used as cysteine donor in the present study, is widely known as a free radical scavenger itself. Moreover, it was reported to have several functions on activation of immune cells. Beside cytoprotective effects NAC is thought to enhance the random migration of rabbit peritoneal neutrophils [45] and to activate chemotaxis, ingestion and superoxid anion production of murine peritoneal macrophages [46]. These results are in agreement with our data, displaying an increase of phagocytosis after treatment with NAC. The application of NAC and flavonoids in combination remarkably exceeded the enhancing effects of the single substances, indicating a strong correlation of phagocytic activity and activated thiol status.

In the present study a model of artificially induced thiol deficiency was developed to evaluate the influence of a reduced extracellular availablity of thiols on the thiol status of cells like peritoneal macrophages. Such a model was preferred because the common methods for GSH depletion by BSO, diamide, dimethylmaleate or n-ethylmaleimide do not reflect the effects of substrate deficiency occuring under pathological situation. As mentioned above, protein-bound thiols were resistant to BSO depletion [44] and failed to fully deplete GSH levels, due to an alternative pathway that involves ascorbic acid [47–49]. By means of the thiol deficient cell culture model, evidence was provided that an unphysiological change of extracellular thiols as it occurs in CAPD led to a dramatic reduction of the cellular thiol concentration.

Taken together, our data convincingly revealed a defective thiol status of peritoneal macrophages in CAPD that could be normalized by treatment with silibinin / silymarin and NAC. This normalization was associated with restoration of impaired functions. Considering the fact that the defect is caused by increasing oxidative stress and a deficient availability of thiols during CAPD, therapeutical trials should be directed at stabilization of this defective cellular thiol status. Potential candidates are silibinin, silymarin and NAC which do act in scavenging free radicals, inducing thiol synthesis and restoration of functional capabilities in parallel. Glucose containing solutions could further amplify the flavonoid effects by providing limiting substrates for ATP synthesis – necessary for both γ-glutamylcysteine synthetase and glutathione synthetase activity. Ongoing clinical trials concerning systemic and local applications have to substantiate these hypotheses.

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References

[1] S.H. Jacobson and P. Moldéus (1994) Whole blood-, plasma- and red blood cell glutathione and cysteine in patients with kidney disease and during hemodialysis. *Clinical Nephrology*, 42, 189–192.

[2] E.A. Ross, L.C. Koo and J.B. Moberly (1997) Low whole blood and erythrocyte levels of glutathione in hemodialysis and peritoneal dialysis patients. *American Journal of Kidney Diseases*, 30, 489–494.

[3] M.C. Martin-Mateo, E. Del Canto-Jafiez and M.J. Barrero-Martinez (1998) Oxidative stress and enzyme activity in ambulatory renal patients undergoing continuous peritoneal dialysis. *Renal Failure*, 20, 117–124.

[4] S.J. McGregor, N. Topley, A. Jorres, A.B. Speekenbrink, A. Gordon, G.M. Gahl, B.J. Junor, J.D. Briggs and J.H. Brock (1996) Longitudinal evaluation of peritoneal macrophage function and activation during CAPD: maturity, cytokine synthesis and arachidonic acid metabolism. *Kidney International*, 49, 525–533.

[5] M. Sach, P. Loetscher, J.A. Burger, H.P. Knopf, P. Schollmeyer and G.J. Dobos (1995) MCP-1 levels are elevated in peritonitis fluid from CAPD patients due to secretion by peritoneal macrophages. *Advances in Peritoneal Dialysis*, 11, 19–23.

[6] M. Sach, K. Bauermeister, J.A. Burger, P. Loetscher, J. Elsner, P. Schollmeyer and G.J. Dobos (1997) Inverse MCP-1/IL-8 ratio in effluents of CAPD patients with peritonitis and in isolated cultured human peritoneal macrophages. *Nephrology Dialysis Transplantation*, 12, 315–320.

[7] H.B. Steinhauer, U. Brugger, R. Atmanspacher, I. Lubrich-Birkner and P. Schollmeyer (1992) Effect of CAPD dialysate on the release of eicosanoids and cytokines from human peritoneal macrophages. *Advances in Peritoneal Dialysis*, 8, 47–52.

[8] H.M. Alobaidi, G.A. Coles, M. Davies and D. Lloyd (1986) Host defence in continuous ambulatory peritoneal dialysis: the effect of the dialysate on phagocyte function. *Nephrology Dialysis Transplantation*, 1, 16–21.

[9] C.W. de Fijter, H.A. Verbrugh, E.D. Peters, P.L. Oe, J. van der Meulen, J. Verhoef and A.J. Donker (1993) In vivo exposure to the currently available peritoneal dialysis fluids decreases the function of peritoneal macrophages in CAPD. *Clinical Nephrology*, 39, 75–80.

[10] C.Y. Lin, W.L. Ku and T.P. Huang (1990) Serial peritoneal macrophage function studies in new and established continuous ambulatory peritoneal dialysis patients. *American Journal of Nephrology*, 10, 368–373.

[11] C. Carcamo, M. Fernandez-Castro, R. Selgas, C. Jimenez, S. Molina and F. Vara (1996) Long-term continuous ambulatory peritoneal dialysis reduces the expression of CD11b, CD14, CD16, and CD64 on peritoneal macrophages. *Peritoneal Dialysis International*, 16, 582–589.

[12] M. Täger, H. Kröning, U. Thiel and S. Ansorge (1997) Membrane-bound proteindisulfide isomerase (PDI) is involved in regulation of surface expression of thiols and drug sensitivity of B-CLL cells. *Experimental Hematology*, 25, 601–607.

[13] X.M. Jiang, M. Fitzgerald, C.M. Grant and P.J. Hogg (1999) Redox control of exofacial protein/disulfides by protein disulfide isomerase. *Journal of Biological Chemistry*, 247(4), 2416–2423.

[14] A. Meister (1995) Glutathione metabolism. *Methods in Enzymology*, 251, 3–7.

[15] C.K. Sen (1997) Nutritional biochemistry of cellular glutathione. *Journal of Nutritional Biochemistry*, 8, 660–672.

[16] W. Wang and N. Ballatori (1998) Endogenous glutathione conjugates: Occurrence and biological functions. *Pharmacological Reviews*, 50, 335–355.

[17] W. Dröge, K. Schulze-Osthoff, S. Mihm, D. Galter, H. Schenk, H.-P. Eck, S. Roth and H. Gmünder (1994) Functions of glutathione and glutathione disulfide in immunology and immunopathology. *FASEB Journal*, 8, 1131–1138.

[18] J.A. Fields, A. Keshavarzian, G. Eiznhamer, T. Frommel, D. Winship and E.W. Holmes (1994) Low levels of blood and colonic glutathione in ulcerative colitis. *Gastroenterology*, 106, A680.

[19] J.P. Richie, Jr., C.A. Lang and T.S. Chen (1992) Acetaminophen-induced depletion of glutathione and cysteine in the aging mouse kidney. *Biochemical Pharmacology*, 44, 129–135.

[20] A. Usal, E. Acarturk, G.T. Yuregir, I. Unlukurt, C. Demirci, H.I. Kurt and A. Birand (1996) Decreased glutathione levels in acute myocardial infarction. *Japanese Heart*Journal, 37, 177–182.

[21] C. Dehmlow, J. Erhard and H. De Groot (1996) Inhibition of Kupffer cell functions as an explanation for the hepatoprotective properties of silibinin. *Hepatology*, 23, 749–754.

[22] P. Letteron, G. Labbe, C. Degott, A. Berson, B. Fromenty and M. Delaforge (1990) Mechanism for the protective effects of silymarin against carbon tetrachloride-induced peroxidation and hepatotoxicity in mice. *Biochemical Pharmacology*, 39, 2027–2034.

[23] A. Comoglio, A. Tomasi, S. Malandrino, G. Poli and E. Albano (1995) Scavenging effect of silipide, a new silybin-phospholipid complex, on ethanol-derived free radicals. *Biochemical Pharmacology*, 50, 1313–1316.

[24] A. Pietrangelo, F. Borella, G. Casalgrandi, G. Montosi, D. Ceccarelli and D. Gallesi (1995) Antioxidant activity of silybin in vivo during long-term iron-overload in rats. *Gastroenterology*, 109, 1941–1949.

[25] D. Schuppan, T. Lang, G. Gerling, E. Leng-Peschlow, G. Krumbiegel and E.O. Reicken (1994) Antifibrotic effect of silymarin in rat secondary biliary fibrosis induced by duct obliteration with ethibloc. *Zeitschrift für Gastroenterologie*, 32, 45–46.

[26] G. Boigk, L. Stroedter, H. Herbst, J. Waldschmidt, E.O. Riecken and D. Schuppan (1997) Silymarin retards collagen accumulation in early and advanced biliary fibrosis secondary to complete bile duct obliteration in rats. *Hepatology*, 26, 643–649.

[27] C. Saliou, B. Rihn, J. Cillard, T. Okamoto and L. Packer (1998) Selective inhibition of NF-kappaB activation by the flavonoid hepatoprotector silymarin in HepG2 – Evidence for different activating pathways. *FEBS Letters*, 440, 8–12.

[28] C. Bokemeyer, L.M. Fels, T. Dunn, W. Voigt, J. Gaedeke, H.J. Schmoll, H. Stolte and H. Lentzen (1996) Silibinin protects against cisplatin-induced nephrotoxicity without compromising cisplatin or ifosfamide anti-tumour activity. *British Journal of Cancer*, 74, 2036–2041.

[29] T. Zima, L. Kameníkova, M. Janebová, E. Buchar, J. Crkovská and V. Tesar (1998) The effect of silibinin on experimental cyclosporine nephrotoxicity. *Renal Failure*, 20, 471–479.

[30] C. Dehmlow, N. Murawski and H. De Groot (1996) Scavenging of reactive oxygen species and inhibition of arachidonic acid metabolism by silibinin in human cells. *Life Sciences*, 58, 1591–1600.

[31] D. Lorenz, P.W. Lucker, W.H. Mennicke and N. Wetzelsberger (1984) Pharmacokinetic studies with silymarin in human serum and bile. *Methods and Findings in Experimental and Clinical Pharmacology*, 6, 655–661.

[31] R. Schandalik, G. Gatti and E. Perucca (1992) Pharmacokinetics of silybin in bile following administration of silipide and silymarin in cholecystectomy patients. *Arzneimittelforschung*, 42, 964–968.

[33] G. Gatti and E. Perucca (1994) Plasma concentrations of free and conjugated silybin after oral intake of a silybin-phosphatidylcholine complex (silipide) in healthy volunteers. *International Journal of Clinical Pharmacology*, 32, 614–617.

[34] D.W. Hedley and S. Chow (1994) Evaluation of methods for measuring cellular glutathione content using flow cytometry. *Cytometry*, 15, 349–358.

[35] L. Chikahisa, Y. Oyama, E. Okazaki and K. Noda (1996) Fluorescent estimation of H2O2-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. *Japanese Journal of Pharmacology*, 71, 299–305.

[36] A. Coates and E. Tripp (1995) Comparison of two fluorochromes for flow cytometric assay of cellular glutathione content in human malignant melanoma. *Melanoma Research*, 5, 107–111.

[37] S. Shany, J. Rapoport, I. Zuili, A. Gavriel, N. Lavi and C. Chaimovitz (1991) Metabolism of 25-OH-vitamin D3 by peritoneal macrophages from CAPD patients. *Kidney International*, 39, 1005–1011.

[38] P.L. Kimmel, T.M. Phillips, S.Q. Lew and C.B. Langman (1996) Zinc modulates mononuclear cellular calcitriol metabolism in peritoneal dialysis patients. *Kidney International*, 49, 1407–1412.

[39] S. Lamperi, S. Carozzi and M.G. Nasini (1987) Intraperitoneal immunoglobulin (IG) treatment in prophylaxis of bacterial peritonitis in CAPD. *Biomaterials, Artificial Cells, and Artificial Organs*, 15, 151–159.

[40] S. Lamperi and S. Carozzi (1989) Immunological defenses in CAPD. *Blood Purification*, 7, 126–143.

[41] Z. Kain, M. Alkan, C. Chaimovitz, S. Segal, M. Fridkin and R. Levy (1989) Human peritoneal macrophage activity is increased by tuftsin. *Immunological Letters*, 21, 257–261.

[42] R. Levy, Z. Kain, C. Chaimovitz, M. Fridkin, S. Segal and M. Alkan (1989) Potential use of tuftsin in treatment of candida peritonitis in a murine model. *Journal of Biological Regulators and Homeostatic Agents*, 3, 71–78.

[43] S. Wenzel, H. Stolte and M. Soose (1996) Effects of silibinin and antioxidants on high glucose-induced alterations of fibronectin turnover in human mesangial cell cultures. *Journal of Pharmacology and Experimental Therapeutics*, 279, 1520–1526.

[44] D.L. Hamilos and H.J. Wedner (1985) The role of glutathione in lymphocyte activation. I. Comparison of inhibitory effects of buthionine sulfoximine and 2-cyclohexene-1-one by nuclear size transformation. *Journal of Immunology*, 135, 2740–2747.

[45] J.G.R. Elferink and B.M. De Koster (1998) *N*-acetylcysteine causes a transient stimulation of neutrophil migration. *Immunopharmacology*, 38, 229–236.

[46] M. Del Rio, G. Ruedas, S. Medina, V.M. Victor and M. De la Fuente (1998) Improvement by several antioxidants of macrophage function *in vitro*. *Life Sciences*, 63, 871–881.

[47] A. Meister (1994) Glutathione-ascorbic acid antioxidant system in animals. *Journal of Biological Chemistry*, 269, 9397–9400.

[48] J. Martensson and A. Meister (1992) Glutathione deficiency increases hepatic ascorbic acid synthesis in adult mice. *Proceedings of the National Academy of Sciences U S A*, 89, 11566–11568.

[49] A. Meister (1992) Depletion of glutathione in normal and malignant human cells in vivo by L-buthionine sulfoximine: possible interaction with ascorbate. *Journal of the National Cancer Institute*, 84, 1601–1602.