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Decrease of Glutathione and Fatty Acids During Iron-Induced Lipid Peroxidation in *Ehrlich* Ascites Tumor Cells**

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In order to investigate a possible relationship between the intensity of lipid peroxidation (LP) in tumor cells and their proliferative activity various methods to quantify LP are desirable. In this study the decrease in the contents of fatty acids and glutathione was measured by established methods in *Ehrlich* ascites tumor (EAT) cells *in vitro,* in which LP was stimulated by the addition of ferrous iron, either as free ion or as histidinate chelate.

When EAT cells were incubated for 30 min at 37 °C in the presence of 5 m*M* $FeSO_4$ the following changes were observed in comparison to appropriate control cells: The content of reduced glutathione (GSH) and total glutathione (GSH + 2 GSSG) decreased significantly by 24 and 30% respectively. The decrease of 4 unsaturated (C 18 : 1; C 18 : 2; C 20 : 4; C 22 : 6) and 2 saturated fatty acids (C 16 : 0; C 18 : 0) by about 15% on the average was statistically significant only for C 16 : 0 and C 20 : 4).

More pronounced effects were observed with 5 m*M* Fe(II)-histidinate. GSH and GSH + 2 GSSG decreased by 54% and 40%, resp. The decrease of fatty acids by about 40% on the average was significant for all of the 6 fatty acids tested. These results are in agreement with previous studies on LP in EAT cells showing Fe(II)-histidinate to be a more powerful promoter of LP compared with free ferrous ion. The observation, that the content not only of GSH but also of total glutathione was decreased in iron-treated tumor cells is in contradiction to the hypothesis that GSH may act as a mere redox mediator of LP under the conditions used and points to a consumption of GSH by several possible pathways. The finding of decreased levels of unsaturated as well as saturated fatty acids in the presence of Fe(II)-histidinate underlines the extraordinary potency of iron as an initiator and catalyst of LP.

(Keywords: Ehrlich ascites tumor; Iron; Lipid peroxidation; Fatty acids; Glutathione; Histidine)

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Abnahme von Glutathion und Fettsäuren während der Eisen-induzierten Lipid-Peroxidation in Ehrlich-Ascites-Tumor-Zellen

Um eine mögliche Beziehung zwischen der Intensität der Lipid-Peroxidation (LP) in Tumorzellen und deren Proliferationsaktivität zu untersuchen, sind mehrere Methoden zur Quantifizierung der LP wünschenswert. In dieser Arbeit wurde die Abnahme von Glutathion und von Fettsäuren mittels etablierter Verfahren in *Ehrlich*-Ascites-Tumor-(EAT)-Zellen *in vitro* gemessen, in denen die LP durch Zugabe von zweiwertigem Eisen, entweder als freies Ion oder als Histidin-Komplex, stimuliert worden war.

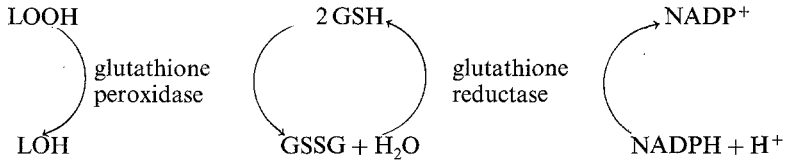
Nach Inkubation von EAT-Zellen 30 min bei 37 °C in Gegenwart von 5 m*M* $FeSO_4$ wurden gegenüber geeigneten Kontrollzellen folgende Änderungen festgestellt: Die Konzentration an reduziertem Glutathion (GSH) und Gesamtglutathion (GSH + 2 GSSG) nahm signifikant um 24 bzw. 30% ab. Die Abnahme von 4 ungesättigten (C 18 : 1; C 18 : 2; C 20 : 4; C 22 : 6) und 2 gesättigten (C 16 : 0; C 18 : 0) Fettsäuren um durchschnittlich ca. 15% war nur im Falle von C 16 : 0 und C 20 : 4 statistisch signifikant.

Ausgeprägtere Effekte wurden mit 5 m*M* Fe(II)-histidinat beobachtet. GSH und GSH + 2 GSSG nahmen um 54 bzw. 40% ab. Die Abnahme der Fettsäuren um durchschnittlich ca. 40% war in allen Fällen signifikant. Diese Ergebnisse stimmen mit vorhergehenden Untersuchungen über die LP in EAT-Zellen überein, wonach Fe(II)-histidinat ein wirksamerer Promotor der LP ist als freie Fe^{2+}-Ionen. Die Beobachtung, daß nicht nur GSH, sondern auch das gesamte Glutathion in den eisen-enthaltenden Tumorzellen abnahm, steht in Widerspruch zur Hypothese, daß GSH unter den gegebenen Bedingungen nur als Redoxvermittler der LP wirkt und deutet auf einen Verbrauch von GSH hin, wofür mehrere Wege möglich sind. Die Feststellung eines erniedrigten Spiegels sowohl der ungesättigten als auch der gesättigten Fettsäuren unterstreicht die außerordentliche Potenz des Eisens als Initiator und Katalysator der LP.

Introduction

In an earlier report [1] it was shown, that ferrous iron, either as free ion or as histidinate chelate can promote lipid peroxidation in native *Ehrlich* ascites tumor (EAT) cells, despite of the high antioxidant potential of these cells. In that study [1] the quantitative estimation of 2-thiobarbituric acid (TBA) reactive substances was used as an indication of the intensity of lipid peroxidation. Since reduced glutathione (GSH) is regarded as one of the main protecting factors against lipid peroxidation [2–5], the influence of iron-induced lipid peroxidation on the GSH content of EAT cells was studied in this paper. In addition to free ferrous ion Fe(II)-histidinate was used as promoter of lipid peroxidation. The latter displays a higher stimulating capability probably because of a more positive redox potential of the chelate compared with ferrous ions [1]. Non-protein thiol groups (nPSH) are assumed to be mainly represented by GSH in some tissues [6]. In this study a comparison was made between the enzymatically determined GSH content and the nPSH content of EAT cells as determined by *Ellman's* method after separation from proteins thiols by ultrafiltration.

It was assumed that the primary protecting action of GSH would be to act as an antioxidant in combination with glutathione peroxidase by this way being converted to oxidized glutathione (GSSG). *Chow* and *Tappel* [7] proposed that GSSG could be recycled to GSH by the action of the NADPH-dependent glutathione reductase



According to this scheme GSH would act as a mediator between lipid hydroperoxides (LOOH) and NADPH, leaving the content of total glutathione (GSH + GSSG) unchanged. In order to test this hypothesis GSSG was also determined by an enzymatic method.

An additional purpose of this investigation was to determine to which extent the process of stimulated lipid peroxidation in EAT cells is reflected in the content of fatty acids. It is known [8] that more than 95% of the total fatty acids are represented by C 16, C 18, C 20 and C 22 acids, therefore concentration changes of these acids were investigated. Four major unsaturated fatty acids and two saturated fatty acids were determined after transformation into methyl esters by gas chromatography.

Material and Methods

Determination of Glutathione

Maintainance and preparation of EAT cells of the hyperdiploid *Heidelberg-Lettrè*-strain were carried out as described previously [1]. Seven to nine days after inoculation the cells were washed with saline solution and resuspended in *Hanks* I solution to a concentration of $60 \cdot 10^6$ cells per ml.

Incubation: 8.0 ml of the cell suspension were incubated for 30 min in a shaking water bath at 37 °C together with 2.0 ml of *Hanks* solution containing $FeSO_4$ with or without *L*-histidine (His). The final concentration were 5 m*M* for Fe(II) and 50 m*M* for His respectively. After termination of incubation the plasma cell membranes were broken up by repeated freezing/thawing (five times) and removed by centrifugation (15.000 g).

Ultrafiltration: The micropartition system MPS-1 (Amicon) with YMB membranes was used. 1 ml samples were ultrafiltrated at 2000 g for 1 h. 0.1 ml aliquots of the filtrate were diluted with 1.0 ml of distilled water. After addition of 0.1 ml of *Ellman's* reagent solution the absorption was read at 412 nm against *Hanks* solution as a blank [9]. In the case of enzymatic determinations of GSH and GSSG by means of glyoxalase I (GI-I) and glutathione reductase (GR) according to *Bernt* and *Bergmeyer* [10] a filtrate aliquot of 0.5 ml was used.

Determination of Fatty Acids

Preparation of EAT cells was carried out as described for glutathione.

Incubation: 3 ml of the cell suspension were incubated for 30 min in a shaking water bath at 37 °C together with 0.75 ml of *Hanks* solution containing $FeSO_4$ with or without His. The final concentrations were 5 m*M* for Fe(II) and 50 m*M* for His, respectively. After incubation the cells were homogenized in a *Potter-Elvejhem*. For the extraction of the fatty acids according to the method of *Folch* et al. [11] 1 ml of the homogenate was mixed with 1 ml H_2O, 40 ml chloroform/methanol (2 : 1), 5 mg butylhydroxytoluene (to prevent oxidation) and 0.5 mg heptadecanoic acid dissolved in chloroform/methanol (2 : 1) (internal standard). The mixture was left for one hour in a shaker at room temperature. The phases were separated in a separatory funnel by the addition of 10–15 ml Na_2SO_4 solution (15% *w/v*). The $CHCl_3$-phase was then brought to dryness in a rotatory evaporator.

For the esterification a modified version of the method of *Morrison* et al. [12] was used. The residue was redissolved in about 4 ml $CHCl_3$ and transferred into a Pyrex-glasstube; 2 ml benzene and 4 ml borontrifluoride-methanol (20%) were added. The mixture was located in a drying cupboard at 110 °C for 90 min. After addition of 10 ml H_2O the mixture was extracted three times with about 5 ml benzene. The combined extracts were then evaporated and the residue was redisolved in 1 ml dichloromethane.

Chromatography: An aliquot of 1 ml of this solution was injected into a gas-chromatograph (DANI gas chromatograph 3800 with PTV-2 CH system; column: 50 m quartz, CP Sil 88; operating conditions: first 5 min at 150 °C, then an increase of 3 °C/min up to 240 °C; injection: programmed temperature vaporization (PTV) with solvent split: 9 s 50 °C, then 250 °C; detection: FID 250 °C.

Results and Discussion

The results of this study support the hypothesis that GSH is involved in the cellular control mechanisms for lipid peroxidation. The enzymatically determined GSH concentration of the control cells (mean $\pm$ standard deviation $= 6.52 \pm 0.71$ nmol/10^6 cells) was at the upper limit of the range mentioned by *Kosower* and *Kosower* [3] for tumor cells and tissue cultures (1–6 nmol/10^6 cells). Lipid peroxidation in EAT cells, when stimulated by ferrous ions (5 m*M*) was associated with a significant decrease by 24% of the GSH level (Table 1; Fig. 1). When Fe(II)-histidinate was applied instead of the free ions the decrease was twice as strong (—54%). These results are in accordance with other parameters of the same experimental system measured under identical conditions: Fe(II)-histidinate is a more powerful prooxidant in EAT cells with respect to the formation of TBA-reactive substances (1) and with respect to the formation of 4-hydroxy-nonenal (HNE), another major break-down product of polyunsaturated fatty acids [13].

nPSH values as determined by the MPS-1/DTNB method were significantly higher than the enzymatically determined GSH values (Table 1; Fig. 1), but the effect of the iron compounds used on the nPSH value

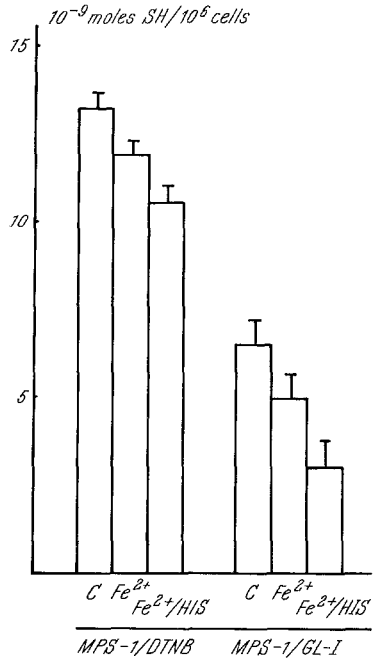


Fig. 1. Comparison of nPSH values as determined by the MPS-1/DTNB method and enzymatically (Gl-I) determined GSH values in peroxidizing EAT cells. Stimulant: either Fe^{2+} (5 m*M*) or Fe(II)/His (Fe^{2+}, 5 m*M*, plus His, 50 m*M*); *C* control, no stimulant; mean values and standard deviations of 4 experiments

was nearly identical to the same effect on the GSH value. We suppose that thiol-containing oligo- and polypeptides which do not react significantly with lipid peroxidation products, can pass through YMB membranes, the permeability limit of which is not specified by the supplier.

The decrease of GSH caused by Fe(II)-ions and Fe(II)-histidinate, respectively, was not accompanied by an equivalent increase in GSSG (Table 1). This was even more significant considering the fact that the method of *Bernt* et al. [10] has been suspected to yield overestimated GSSG values [14]. Therefore, the level of total glutathione is also decreasing in the same order as was found for GSH. This finding is in contradiction to the hypothesis of GSH as a mere redox mediator [7] under the conditions used. In agreement with our results *Rossi* and *Cecchini* [15] observed a decrease of total glutathione during spontaneous lipid peroxidation in homogenates of 4 hepatoma lines with different growth rates. On the basis of increased activities of gamma-glutamyl-transpeptidase compared with liver these authors proposed, that GSH is

Table 1. *Non-protein thiols, reduced, oxidized and total glutathione in peroxidizing EAT cells.* Stimulant: either Fe^{2+} (5 m*M*) or Fe^{2+} (5 m*M*) plus His (50 m*M*); control: no stimulant; mean and standard deviation of 4 experiments (nmol/10^6 cells); values for total glutathione are expressed in "GSH equivalents": GSH + 2 GSSG (note: 2 mol of GSH are formed by reduction of 1 mol of GSSG); percentage values are expressed in relation to control

	control	Fe^{2+}		Fe(II)/His	
non-protein thiols	13.2 +/—0.7	11.8 +/—0.7	—10.6%	10.5 +/—0.8	—20.4%
GSH	6.52 +/—0.71	4.97 +/—0.69	—23.8%	3.00 +/—0.77	—54.0%
GSSG	0.80 +/—0.32	0.24 +/—0.48	—70.1%	0.95 +/—0.29	+18.7%
GSH + 2 GSSG	8.12	5.45	—30.0%	4.90	—39.6%

Table 2. *Fatty acids in both* Fe^{2+}- *and* Fe(II)/*His-treated EAT cells.* Mean and standard deviation of 4 experiments (micrograms fatty acid/10^8 cells); percentage of decrease and statistical significance (2P values according to *Student*'s two-sided paired t-test) relative to untreated controls are also given

	control	Fe^{2+}			Fe(II)/His		
C 16 : 0	127.7 +/—21.2	100.4 +/—27.6	—21.4%	$2P < 0.05$	69.2 +/—28.0	—45.8%	$2P < 0.005$
C 18 : 0	80.5 +/—31.0	68.7 +/—30.4	—14.7%	$2P > 0.05$	52.2 +/—24.0	—35.1%	$2P < 0.01$
C 18 : 1	136.4 +/—78.1	117.7 +/—68.8	—13.7%	$2P > 0.10$	86.6 +/—52.0	—36.5%	$2P < 0.05$
C 18 : 2	104.1 +/—56.6	92.5 +/—53.0	—11.1%	$2P > 0.20$	63.2 +/—31.2	—39.3%	$2P < 0.05$
C 20 : 4	39.0 +/— 9.1	31.5 +/— 9.7	—19.2%	$2P < 0.05$	23.6 +/— 4.3	—39.5%	$2P < 0.01$
C 22 : 6	20.0 +/— 6.2	17.4 +/— 8.0	—12.9%	$2P > 0.10$	10.6 +/— 2.8	—46.7%	$2P < 0.02$

mainly transformed by this pathway. We suppose that a second possibility could be the nonenzymatic reaction of GSH with peroxidation products e.g. reactive aldehydes [16].

A general decrease in the content of fatty acids in EAT cells was observed when either ferrous ion or Fe(II)-histidinate were used as stimulants (Table 2). After an incubation period of 30 min, ferrous ion (5 m*M*) caused a decrease of about 15% compared with the controls. However, only the decrease of arachidonic and palmitic acid turned out to be statistically significant ($2P < 0.05$). Fe(II)-histidinate caused a decrease by an average of about 40%, the significance of which could be proven for all the acids tested.

From the fact that the content of saturated fatty acids was reduced to a similar extend as the unsaturated fatty acids it may be concluded that iron compounds are very effective promoters of lipid peroxidation. According to *Demopoulos* [17] saturated fatty acids can also undergo peroxidation initiated and catalyzed by heavy metals. Differences in the effectiveness of various iron compounds, as found in this study, may be related to differences of the respective redox potentials [1]. For the question of a possible relationship between lipid peroxidation and proliferation it is important to note, that the vitality of the EAT cells was not affected by ferrous ion or Fe(II)-histidinate, as was shown by the trypan exclusion test [18]. Therefore the influence of these iron compounds on the proliferative behavior should be investigated now.

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