Effect of Magnesium Deficiency and Salicylate on Lipid Peroxidation in vivo

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Zusammenfassung

Normal- und Mg-arm-ernährte Ratten erhielten 700 mg/kg Salizylsäure als Na-Salizylat per os. 24 Stunden später wurden Blut, Leber und die Lungen in Nembutalnarkose entnommen. Nur in den Lebern von den Mg-Mangelratten und Salizylat-behandelten Ratten war die Lipidperoxidation (LPO), gemessen an Hand der Bildung von Malondialdehyd, signifikant erhöht. Die erhöhte LPO kann mit der Zunahme des Fe-Gehaltes in den Lebern im Mg-Mangel und nach Applikation von Salizylat erklärt werden.

Summary

Normally fed and Mg-deficient rats received one oral dose of 700 mg/kg salicylic acid, given as Na salicylate. Twenty-four hours later, blood, liver and lung were removed under Nembutal anesthesia. Only in liver was lipid peroxidation (LPO), as indicated by the formation of malondialdehyde and measured by the thiobarbituric acid method, significantly increased by Mg deficiency and application of salicylate. The increase of Fe content in liver due to Mg deficiency and salicylate is the most probable cause for the increase in LPO.

Résumé

Des rats sous alimentation normale ou carencée en Mg ont reçu une dose orale unique de 700 mg/kg d'acide salicylique sodique. Des prélèvements sanguins, hépatiques et pulmonaires ont été effectués vingt quatre heures plus tard, sous anesthésie par le Nembutal. La peroxydation des lipides (POL), révélée par la formation de dialdéhyde malonique et confirmée quantitativement par la méthode à l'acide thiobarbiturique, n'a été significativement augmentée par la carence en Mg et l'administration d'un dérivé salicyclé qu'au niveau hépatique. L'augmentation de la teneur hépatique en Fe résultant de la carence en Mg et de l'ingestion d'un dérivé salicylé est la cause la plus probable de l'accroissement de la POL.

Introduction

In isolated liver mitochondria from Mg-deficient rats, lipid peroxidation (LPO) as measured by formation of MDA was increased by 80 % [13].
Possible mechanisms for increased LPO in Mg deficiency are

1. increased Fe content in liver [13, 28]
2. changed composition of unsaturated fatty acids in the phospholipids of cell membranes [30]
3. increased mitochondrial Ca concentration.

However, in these experiments, LPO was investigated in vitro under artificial conditions, such as the addition of NADPH, Fe and ADP [20], or salicylate [14].

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Radical scavengers and protective substances such as Zn or Zn-metallothionein (ZnMT) or glutathione (GSH), GSH-peroxidase, catalase, and superoxide dismutase [5, 22, 23, 38, 39, 40], which are present in vivo, were absent in these experiments.
In preceding experiments we found that salicylate enhanced LPO in isolated mitochondria by increasing Fe content and by forming Fe-salicylate complexes, which can induce LPO [11, 14]. Moreover, ototoxicity [17] and teratogenicity [10] of salicylate were enhanced by Mg deficiency. In order to investigate whether LPO may play a role in Mg deficiency and salicylate toxicity, we measured LPO in serum and tissues of Mg-deficient rats either untreated or treated with salicylate. To receive some information on possible LPO affecting mechanisms, we also measured LPO-enhancing substances such as Fe and protective substances such as Zn and MT.

Materials and Methods

Female Wistar rats, weighing 103 ± 3 g, were fet a control diet (Mg content: 41 mmol/kg, Ca content 250 mmol/kg) or an Mg-deficient diet (Mg content: 3 mmol/kg, Ca content 250 mmol/kg, Ssniff, Soest, FRG) and distilled water ad libitum. After 28 days, half of the control and Mg-deficient rats received one oral dose of 700 mg/kg salicylic acid, given as Na salicylate. Twenty-four hours later, blood, liver and lung were taken under Nembutal anesthesia (50 mg/kg i.p.).
The livers and lungs were rinsed in 0.9 % NaCl. The major part was frozen at —20 °C and the rest was freeze-dried, powdered and taken for measuring Fe and Zn content.
Blood was centrifuged at 1000 g for 5 min.
The concentrations of Mg, Ca, Zn, and Fe in serum were measured by atomic absorption spectrophotome-

try (AAS) (Philips, SP 9). For determination of Fe and Zn in liver, freeze-dried, powdered liver and lung were ashed overnight in the Plasma Processor 200-E (Technics, Munich). The ash was dissolved in 0.1 N HCl, and Fe and Zn were measured by AAS.

For measurement of salicylate content, serum and a 20 % liver homogenate in 10 mol/l Tris, pH 7.4, were deproteinized by 10 % trichloroacetic acid (TCA). Salicylate was measured fluorometrically in the supernatants [36].

Metallothionein (MT) was determined in the 100,000 g supernatants of the 20 % liver homogenates by the ^{109}Cd-hemoglobin assay, as already described in detail [18].

For determination of malondialdehyde (MDA), serum and aliquots of the 20 % liver homogenates were deproteinized with 10 % cold TCA and centrifuged. 0.5 ml 1 % thiobarbituric acid (TBA), adjusted to pH 7, was added to 0.5 ml aliquots of the supernatants and heated for 10 min at 95 °C. After cooling, extinction was measured at 531 nm. The calibration curve was prepared with malonaldehyde tetraethylacetal (Sigma) which was treated in the same way.

Histochemistry

Another part of the livers was placed on cork plates carrying wet filter paper, wrapped with plastic foil, frozen in liquid nitrogen and stored in sealed plastic bags at —25 °C until use. 10 µm thick sections were cut at —25 °C using a cryostat (model 2800 N, Reichert-Jung, Nußloch, FRG), mounted on non-precooled glass slides and air-dried to demonstrate lipids with the Sudan Black method according to *Romeis* [35] or freeze-dried in a Leybold-Heraeus freeze-dryer (Cologne, FRG) equipped with an EM 12 vacuum pump (Edwards, Crawley, UK) and mounted with 0.5 % celloidin according to Lojda et al. [29] for the visualization of iron with the Prussian Blue reaction as given by Romeis [35].

Results and Discussion

During the Mg-deficient period serum Mg concentration was reduced by 80 % and increase of body weight was reduced by 40 % (tab. 1), indicating drastic Mg deficiency.

Salicylate toxicity can be judged by the increase in serum Mg and decrease in serum Ca concentration, which is in agreement with preceding results [15].

Lipid peroxidation

Mg deficiency caused a 40 % increase in MDA in liver (tab. 1). The same increase in MDA in liver was produced by salicylate. When salicylate was given to Mg-deficient rats, MDA content in liver seemed to be more elevated. However, the difference was not significant.

In lung and serum, there were only minor insignificant increases in MDA content due to Mg deficiency and salicylate. Lung was included because in similar experiments with Zn-deficient rats, LPO was increased in lung [3].

The increase in MDA content in liver is in agreement with preceding results on LPO in vitro, in which the rate of LPO was enhanced in liver mitochondria by 80 % [13]. The smaller increase in MDA in vivo than in vitro particularly after application of salicylate may be due to the presence of protective substances or due to restricted availability of Fe. Moreover, MDA formed by LPO is rapidly metabolized by mitochondrial aldehyde dehydrogenase and MDA is rapidly excreted by the kidneys [6, 33].

Our results on MDA in serum do not agree with the results of *Mahfouz* and *Kummerow* [30], who found a 68 % increase of MDA (from 5.59 to 9.35 µmol/l) in the serum of Mg-deficient rats. These values are 10 times higher than in our measurements. Very different values for TBA-positive substances in serum, which are assumed to represent MDA, were reported. In rat and mouse serum, an MDA concentration of 3.6 µmol/l [4, 31] was found. As shown for human plasma, 80 % of TBA-positive material was

Tab. 1: Mg, Ca, Zn, Fe, malondialdehyde (MDA) and salicylate (SA) contents in serum and tissues and metallothionein (MT) content in liver of normally fed or Mg-deficient rats, either untreated or treated with one oral dose of 700 mg/kg salicylic acid, given as Na salicylate. Mean± SEM of 7 rats in each group. Significant differences to untreated controls as revealed by Student's unpaired t-test. d.w., dry weight, w.w., wet weight.
a) $p<0.05$; b) $p<0.01$; c) $p<0.001$.

	Control	Mg-def.	Control + SA	Mg-def. + SA
Body weight [g]	191 ± 7	152 ± 4[c]	189 ± 3	150 ± 5[c]
$[Mg]_{scr.}$ mmol/l	0.91 ± 0.03	0.17 ± 0.04[c]	1.23 ± 0.06[c]	0.29 ± 0.05[c]
$[Ca]_{scr.}$ mmol/l	2.29 ± 0.02	2.34 ± 0.06	1.87 ± 0.07[c]	1.81 ± 0.12[b]
$[Zn]_{scr.}$ µmol/l	15.1 ± 0.2	14.5 ± 0.5	13.7 ± 0.4[b]	13.9 ± 0.5[a]
$[Fe]_{scr.}$ µmol/l	60.8 ± 2.1	53.7 ± 2.6	44.2 ± 5.9[a]	38.5 ± 5.3[b]
$[Fe]_{liver}$ mmol/kg d.w.	10.3 ± 0.6	13.3 ± 0.6[b]	17.1 ± 1.2[c]	15.6 ± 1.7[b]
$[Fe]_{lung}$ mmol/kg d.w.	4.8 ± 0.1	4.4 ± 0.2	5.5 ± 0.1[c]	5.5 ± 0.2[b]
$[Zn]_{liver}$ mmol/kg d.w.	1.14 ± 0.03	1.34 ± 0.04[b]	1.79 ± 0.09[c]	1.81 ± 0.08[c]
$[MDA]_{ser.}$ µmol/l	0.89 ± 0.02	0.91 ± 0.06	0.94 ± 0.06	1.04 ± 0.10
$[MDA]_{liver}$ µmol/kg w.w.	9.0 ± 0.5	12.5 ± 0.4[c]	13.0 ± 1.6[a]	15.2 ± 2.1[a]
$[MDA]_{lung}$ µmol/kg w.w.	7.8 ± 0.4	8.8 ± 1.0	8.7 ± 1.1	9.7 ± 1.2
$[MT]_{liver}$ µmol/kg w.w.	2.07 ± 0.26	5.04 ± 0.33[c]	16.7 ± 1.35[c]	21.42 ± 1.17[c]
$[SA]_{liver}$ mmol/kg w.w.	–	–	0.91 ± 0.07	0.90 ± 0.09
$[SA]_{scr.}$ mmol/l	–	–	3.61 ± 0.22	3.36 ± 0.28

found not to have originated from lipid hydroperoxide conversion to MDA [27]. Plasma contains many substances that react in the TBA assay, e.g. bile pigments which produce a different chromogen [20]. So, in our measurements the TBA assay with serum gave a product with a different absorption spectrum compared to MDA from liver and pure MDA (not shown). Based on these findings and due to the rapid elimination of MDA from serum [6, 33], MDA in serum cannot be taken as an indicator for LPO.

However, from the corresponding increase of LPO due to Mg deficiency and salicylate in isolated liver mitochondria and liver in vivo, it can be concluded that MDA measurements in liver indicate an increase of LPO in vivo.

Lipid peroxidation-affecting substances

LPO can be increased by various LPO-affecting substances:

Iron

Liver plays a major role in Fe metabolism [26]. Fe content in liver was increased by Mg deficiency and after application of salicylate. In lung, salicylate only caused a small increase in Fe (tab. 1). Also, in preceding experiments an increase of Fe in liver by Mg deficiency [13, 28] and salicylate [11] was found. Two mechanisms may exist by which liver Fe was increased by Mg deficiency:

1. Mg-deficient feeding and thus low intestinal Mg concentration can increase intestinal Fe resorption because ^{59}Fe uptake by duodenal fragments was inhibited by Mg in the incubation medium [34].
2. Mg deficiency increased hemolysis [7], thus increasing liberation of Fe from hemoglobin.

The increase of liver Fe due to salicylate may be caused by chelation of Fe^{3+} to salicylate, K_A amounting to 10^{17} $(mol/l)^{-1}$ [37]. The Fe-salicylate complex, being more lipophilic, may transport Fe into the liver cells. The increase in liver Fe can be correlated with the increase of MDA in liver.

Fe, bound in active form, is involved in the formation of oxygen free radicals:

1. by reduction of O_2 to superoxide anion radicals ($O_2^{\cdot-}$),
2. by formation of high toxic hydroxyl radicals ($OH^{\cdot}$) according to the Haber-Weiss reaction: $O_2^{\cdot-}+H_2O_2 \rightarrow O_2+OH^{\cdot}+OH^-$, or according to the Fenton reaction: $H_2O_2 \rightarrow OH^{\cdot}+OH^-$.

Oxygen radicals formed by these Fe-dependent reactions induce LPO and, besides other products of LPO, the formation of MDA.

Calcium

In Mg deficiency, intracellular Ca concentration is elevated [8] and already a small increase in Ca can enhance LPO [1].

Catecholamines

In Mg deficiency more catecholamines are released [16], and during oxidation of catecholamines to adrenochromes, oxygen radicals are formed [21, 32], which can induce LPO [22, 23, 38].

Polyunsaturated fatty acids

Lipid peroxidation depends on the content of polyunsaturated fatty acids [20, 38, 41]. As another possible reason for increased LPO in Mg-deficient rats the spectrum of fatty acids in the phospholipids of liver microsomes was changed [30].

Zinc and metallothionein

Metabolism of Zn and ZnMT are most expressed in liver [24]. Zn and ZnMT can protect against LPO [2, 39, 40]. The following mechanisms may be responsible:

1. Zn may competitively inhibit binding of Fe to a low-affinity ligand, thus preventing the formation of oxygen radicals, and
2. oxygen radicals are scavenged by MT. By this reaction, Zn may be liberated from ZnMT, which in turn may compete with Fe.

However, Zn and ZnMT seem to play no role in the present experiment. Zn and ZnMT were increased in liver in Mg deficiency and more expressed by application of salicylate. Mg deficiency-increased MT content in liver can be explained by the increased release of catecholamines in Mg deficiency, which induce the synthesis of MT [24]. Also after application of salicylate, the synthesis of MT is induced [18]. The newly-synthesised MT in turn can bind Zn from the extracellular fluid, thus increasing liver Zn and decreasing serum Zn (tab. 1). Since cellular Zn and ZnMT were increased in parallel to LPO, cellular Zn and ZnMT may have no protective effect on LPO due to Mg deficiency and salicylate toxicity under these experimental conditions. In agreement with this result, pretreatment of rats with two i.p. injections of $ZnCl_2$ 24 and 48 h before stress by immobilization and food and water deprivation, which increased liver Zn and ZnMT, did not affect stress-increased LPO [25]. So, the increase of LPO in vivo in Mg deficiency and after application of salicylate is best correlated to the increase in liver Fe (see above).

Histochemistry

Fe-staining was increased after application of salicylate (not shown) in parallel to the Fe content, as measured by AAS (tab. 1). The same was found in preceding experiments [11]. In order to see whether application of salicylate caused hepatotoxicity, livers were inspected histologically. Liver cell necroses were not observed. As a reversible injury by salicylate, staining of lipids in liver with Sudan Black was increased, as found in preceding experiments [12].

Pathological role of lipid peroxidation

Many papers have discussed the role of LPO in cytotoxicity. However, there was no correlation between cytotoxicity and LPO. For review see [41]. Therefore, LPO may only be a concomitant event or an initial trigger in cytotoxicity.

Cell injury depends on the consecutive cascade of reactions, such as increased cell membrane permeability, increase of intracellular Ca^{2+}, and consequently activation of Ca-dependent

phospholipase A_2, protease and nuclease.

In the present experiment, there was only an increase in liver lipid droplets and no irreversible hepatotoxicity because irreversible hepatotoxicity would have produced liver cell necrosis within 18–24 hours after application of the toxic substance [41].

Thus, chronically increased lipid peroxidation due to Mg deficiency and particularly in combination with stress [25] and other LPO-favouring and low-toxic substances (e.g. Fe, salicylate) did not produce irreversible damage of liver cells, but increased LPO and may induce precocious aging of liver and other susceptible organs [9].

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