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Effects of magnesium and iron on lipid peroxidation in cultured hepatocytes

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Abstract

In primary cultures of rat hepatocytes, the effects of extracellular Mg^{2+} and Fe on lipid peroxidation (LPO) as measured by means of malondialdehyde (MDA) formation were investigated.

Incubation of hepatocytes at decreasing extracellular Mg^{2+} concentration enhanced LPO, depending on extracellular Fe. About 96% of MDA accumulated in the culture medium. Addition of desferrioxamine prevented LPO.

Additionally, the formation of oxygen free radicals was determined by fluorescence reduction of cis-parinaric acid. With this method, an immediate decay of fluorescence was found after addition of Fe^{2+}. Fluorescence reduction was completely prevented by desferrioxamine, indicating the function of extracellular Fe. This mechanism may operate additionally to the increase in intracellular Fe and intracellular formation of oxygen free radicals during Mg deficiency *in vivo*. (Mol Cell Biochem **144:** 141–145, 1995)

Key words: magnesium, iron, lipid peroxidation, hepatocyte

Introduction

During Mg deficiency in rats lipid peroxidation (LPO) is increased in tissues [1–3] and plasma lipoproteins [4]. The mechanism of the Mg deficiency-induced increase in LPO has not yet been defined. LPO is caused by oxygen free radicals which are formed besides other reactions by Fe-dependent Haber-Weiss and Fenton reactions [5]. In Mg-deficient rats Fe content is enhanced in tissues with increased LPO [1–3]. The elevated tissue Fe content in Mg deficiency may be caused by increased disintegration of erythrocytes [6] and consequently increased cellular Fe uptake, particularly in liver cells.

In order to test this mechanism we investigated the effects of extracellular Mg and Fe on malondialdehyde formation in primary cultures of hepatocytes.

Materials and methods

Preparation of hepatocytes

Isolated perfused rat hepatocytes were dispersed by a two-step procedure of Ca^{2+} removal, followed by collagenase treatment (collagenase, type I, Sigma, 75 mg/150 ml) according to Seglen [7]. Collagenase was removed by perfusion with buffer A (in mM): 125 NaCl, 15 $NaHCO_3$, 5 KCl, 1 KH_2PO_4, 5 glucose, 1.2 $CaCl_2$, 0.5 $MgCl_2$, 20 Hepes/NaOH, pH 7.4. The mechanically isolated hepatocytes from one rat were dispersed in culture medium (Instamed Medium 199, Seromed, with the addition of 10 mM Hepes/NaOH, 1.9 g/l bovine serum albumin, 16.7 mM $NaHCO_3$, penicillin 2000 units/l, streptomycin 20 mg/l, insulin 4 units/l, dexamethasone 0.4 mg/l), filtered through two layers of gauze and diluted to 200 ml with culture medium. After sedimentation of the cells, the supernatant was sucked off and the cells were washed twice with 200 ml culture medium by sedimentation.

Cell culture

Plastic plates (diameter 9 cm, Greiner, Berlin) were coated with collagen (Seromed, Berlin). Onto each plate 1 ml fetal calf serum (FCS) (Gibco) and 10 ml hepatocyte suspension were pipetted. The plates were incubated at 37°C for 1 h by gas-

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sing with 95% O_2/5% CO_2. To remove nonparenchymal cells the supernatant was sucked off, 10 ml culture medium (without FCS) were added and incubated for another hour. This incubation was repeated once. After culturing overnight the supernatant was sucked off and the hepatocytes were reincubated at 37°C under gassing in Mg^{2+}-free incubation medium (Medium 199, Gibco, without phenol red, without Mg^{2+}, buffered with 20 mM Hepes/NaOH pH 7.4) with the addition of various Mg^{2+} concentrations, 12.5 µM $FeSO_4$/250 µM ascorbate or 100 µM desferrioxamine (Ciba-Geigy, Basel) as indicated. Fe content of Medium 199 (Gibco) amounted to 1.74 µM. After various periods, supernatant was taken for measurement of malondialdehyde (MDA). The cells were rinsed with 5 ml 150 mM NaCl and taken for measurement of MDA, vitamin E, and protein content.

Measurements

MDA in medium

The incubation medium was centrifuged at 13000 g for 2 sec. To 1.5 ml supernatant 50 µl 75% (w/v) trichloroacetic acid (TCA) was added and centrifuged at 13000 g for 2 min. 500 µl TCA supernatant were taken for measurement of MDA (see below).

MDA in cells

To each rinsed plate 1.5 ml 2.5% (w/v) TCA was added and the cells were scraped off. After centrifugation (13000 g, 2 min), 500 µl of the TCA extract were taken for measurement of MDA. The pellets were taken for measurement of protein with the BCA Protein Assay Reagent (Pierce) [8].

MDA was determined by a variation of the thiobarbituric acid (TBA) method [9, 10]. 500 µl TBA (1%, pH 7) were added to 500 µl TCA supernatant and heated at 95°C for 15 min. After cooling, the probes were extracted with 3 ml 1-butanol by vortexing for 30 sec and centrifugation at 2100 g for 15 min. MDA in the butanol phase was measured fluorometrically (Perkin Elmer LS 50B, excitation: 532 nm, emission: 553 nm, slit width: 5 nm). The calibration curve was prepared with malonaldehyde tetraethylacetal (Sigma), which was treated in the same way.

Vitamin E in cells

To the rinsed plates 1.5 ml 150 mM NaCl was added. The cells were scraped off, centrifuged and the pellet was freeze-dried. The freeze-dried pellets were weighed, suspended in 750 µl 150 mM KCl and vitamin E was determined by its fluorescence in hexane extracts according to Taylor *et al.* [11]. For calibration D,L α-tocopherol (Serva) was used.

Mg and Fe in cells

The plates were rinsed with 5 ml Mg^{2+}-free Medium 199 (Gibco) containing 0.1 mM desferrioxamine. The cells were scraped off, once washed with the same medium, and freeze-dried. The freeze-dried cells were weighed, ashed in the Plasma Processor 200-E (Technics, München, Germany). The ash was dissolved in 0.1 N HCl and Mg and Fe were measured by atomic absorption spectrophotometry (Philips, SP9).

Determination of oxygen free radicals with cis-parinaric acid

To the cells grown in Medium 199 (Seromed) 4 ml of incubation medium (Gibco) with 0.5 mM $MgCl_2$ was added. The cells were scraped off, once washed and resuspended in the same medium. 2 ml cell suspension (containing 1 mg protein) were used for fluorescence measurement at 37°C under slow stirring (Fluorescence Spectrometer Perkin Elmer LS 50B, excitation at 324 nm, slit 2.5 nm, emission at 413 nm, slit 3 nm). After 50 sec 40 µl cis-parinic acid (0.5 mM) (Molecular Probes, Eugene, OR, USA) were added [12]. 50 sec later 40 µl $FeSO_4$/ascorbate or 40 µl desferrioxamine were added as indicated. Fluorescence intensity was measured for 500 sec.

Results

MDA formation

When hepatocytes grown in primary culture were incubated in media with decreasing concentration of extracellular free Mg^{2+} ($[Mg^{2+}]_0$), MDA content of the cells was increased by about 50% (Fig. 1). Incubation of the cells in the presence of 12.5 µM $FeSO_4$ plus 250 µM ascorbate drastically increased MDA, particularly at $[Mg^{2+}]_0$ below the physiological value of 0.5 mM (Fig. 1). The role of Fe in the formation of MDA was additionally shown by the addition of 100 µM desferrioxamine, a specific Fe chelator, which completely inhibited formation of MDA at all $[Mg^{2+}]_0$, indicating that the increase in MDA by reduction of $[Mg^{2+}]_0$ was caused by Fe. The time course of MDA formation is shown in Fig. 2. Without addition of Mg^{2+} and Fe/ascorbate, MDA increased slowly with time.

In the presence of Fe/ascorbate, MDA increased rapidly within 3 h and thereafter at a slower rate. Measuring MDA content in the medium revealed the same time course, indicating that always a constant fraction of formed MDA accumulated in the medium. Each plate with cultured hepatocytes in Fig. 2 contained 10 ml medium and 2.2 mg protein. Hence, from Fig. 2 it can be calculated that after 24 h incubation in the presence of Fe/ascorbate 50 nmol MDA had accumulated in the medium and at the same time 2 nmol MDA had accumulated in the cells. The same quotient for MDA in medium/MDA in cells holds for cell cultures incubated without addi-

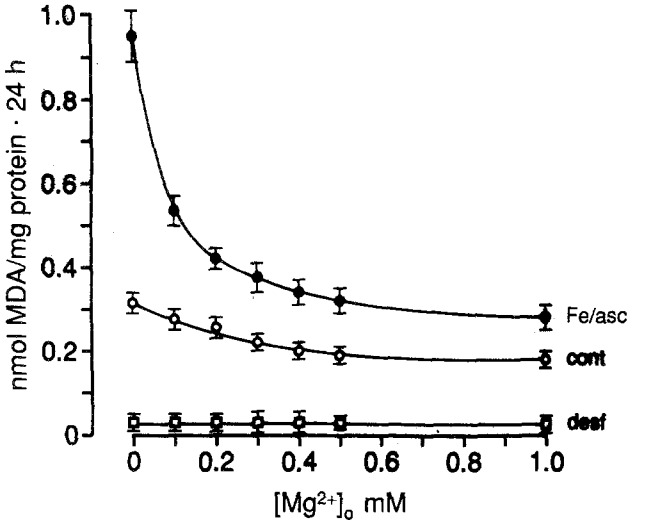

Fig. 1. Malondialdehyde (MDA) content of cultured rat hepatocytes. Primary cultures of rat hepatocytes were incubated for 24 h in incubation medium (Gibco) with various Mg^{2+} concentrations as indicated. Without addition (cont), addition of 12.5 μM $FeSO_4$/250 μM ascorbate (Fe/asc) or 100 μM desferrioxamine (desf). Mean ± SEM of 4 experiments.

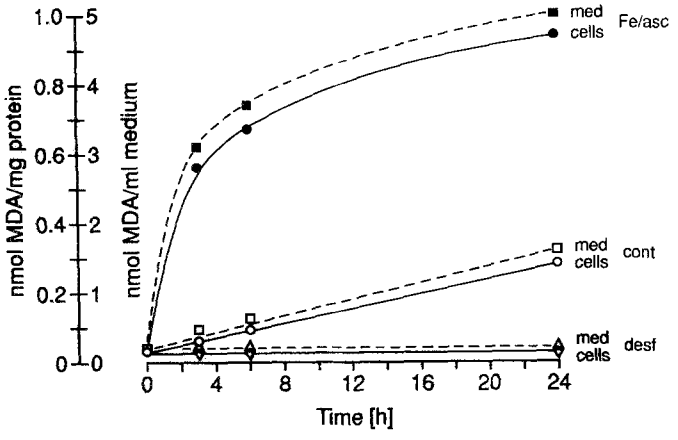

Fig. 2. Malondialdehyde (MDA) formation of cultured rat hepatocytes. Primary cultures of rat hepatocytes were incubated in Mg^{2+}-free incubation medium (Gibco) for various periods as indicated. MDA was determined in the medium (med) and cells (cells). Fe/asc, addition of 12.5 μM $FeSO_4$/ 250 μM ascorbate to the medium; cont, no addition; desf, addition of 100 μM desferrioxamine to the medium. Mean of 3 experiments.

tion of Fe/ascorbate, and for other incubation periods. Thus, under these conditions, 96% of MDA occurred in the medium. With Fe-stimulated cultured chick embryo hepatocytes a similar time course of MDA formation and similarly 90% of total MDA were found in the culture medium [13].

Mg and Fe content

Mg content of the hepatocytes was not significantly reduced by incubation with decreasing $[Mg^{2+}]_o$ (data not shown). In agreement with this result, also total Mg content in rat liver was not significantly reduced by Mg deficiency [14]. Addition of Fe/ascorbate or desferrioxamine did not significantly affect Mg content (data not shown). Fe content of the hepatocytes, incubated at different $[Mg^{2+}]_o$, was not significantly changed amounting to 7.2–7.5 mmol Fe/kg dry weight (Table 1). Addition of Fe/ascorbate increased Fe content by 60%. The increased Fe content of hepatocytes after incubation with Fe may be due to Fe uptake, which can occur without involvement of transferrin [15]. In the presence of 100 μM desferrioxamine, Fe content was not significantly reduced (Table 1).

Table 1. Fe content of cultured rat hepatocytes. Rat hepatocytes grown in primary culture were incubated for 24 h in Medium 199 (Gibco) without or with 0.5 and 1.0 mM $MgCl_2$ and with the addition of 12.5 μM $FeSO_4$/250 μM ascorbate or 100 μM desferrioxamine. Values in mmol/kg dry weight. Mean ± SEM of 4 experiments, significant differences to controls (no addition) by unpaired Student's *t*-test. **, $p < 0.01$; ***, $p < 0.001$.

$[Mg^{2+}]_o$	0 mM	0.5 mM	1.0 mM
No addition	7.5 ± 0.8	7.4 ± 0.6	7.2 ± 0.7
Fe/ascorbate	12.3 ± 0.7**	11.8 ± 0.4***	11.8 ± 0.3***
Desferrioxamine	7.2 ± 0.5	7.0 ± 0.4	7.0 ± 0.6

Vitamin E content

Vitamin E, which was reduced in tissues and plasma of Mg-deficient rats [1–3], was not significantly changed in cultured hepatocytes. It amounted to 0.32 mmol/kg dry weight. In accordance with this result, also in short-time Mg deficiency, vitamin E content of tissues was not reduced [16].

Oxygen radical formation measured by cis-parinaric acid

Oxygen radical formation as measured by MDA required rather long incubation periods. Therefore, during incubation with Fe, some Fe can be taken up and can act intracellularly. Also, desferrioxamine may chelate intracellular Fe after being taken up although its membrane permeability is rather slow [17, 18]. Fe bound to desferrioxamine either intracellularly or extracellularly is inactive in oxygen radical formation [17]. Therefore, in order to differentiate whether Fe is active in LPO either from the intra- and/or extracellular side of the cell membrane, we investigated oxygen radical formation by means of cis-parinaric acid (PnA). PnA (cis-trans-trans-cis-9,11,13,15-octadecatetraenoic acid) is intercalated into the cell membrane and fluoresces in a lipophilic environment. PnA reacts with a variety of free radicals and yields nonfluorescent products [12]. With this method the effects of Fe and desferrioxamine can be measured immediately.

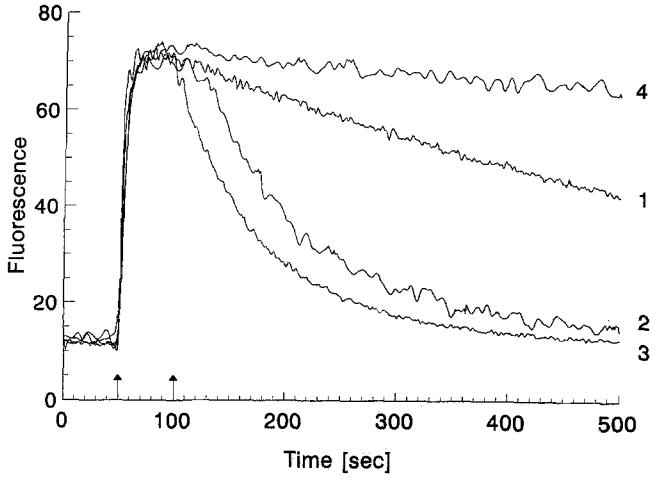

Fig. 3. Fluorescence intensity (arbitrary units) of cis-parinaric acid in cultured hepatocytes. Hepatocytes were grown in culture Medium 199 (Seromed), washed, scraped off and resuspended in incubation medium (Gibco) with 0.5 mM $MgCl_2$ (Fe content: 1.74 μM). After 50 sec 0.5 mM cis-parinaric acid was added. At 100 sec, 3.12 μM $FeSO_4$/250 μM ascorbate (total Fe, 4.86 μM, curve 2), or 6.25 μM $FeSO_4$/250 μM ascorbate (total Fe, 7.99 μM, curve 3) or 100 μM desferrioxamine (curve 4) was added, curve 1 no addition (total Fe, 1.74 μM). A typical experiment of 4 identical experiments was plotted.

Hence, intracellular uptake of Fe or desferrioxamine can be neglected. When primary cultures of hepatocytes were incubated in Medium 199 (Gibco) with Fe/ascorbate, fluorescence was rapidly reduced. This effect occurred immediately after starting the fluorescence measurement (Fig. 3). Addition of 100 μM desferrioxamine prevented fluorescence reduction of cells incubated in Medium 199 (Gibco) with 1.74 μM Fe. These results indicate that oxygen free radicals can be formed by extracellular Fe.

Discussion

The most remarkable result of the present experiments was the increase of LPO in cultured hepatocytes by reduction of $[Mg^{2+}]_o$. Under the experimental conditions only 4% of total MDA was found in the cells, the rest accumulated in the medium. Total MDA formation may still be higher since some MDA may be metabolized by liver mitochondria [19] or may evaporate during incubation. Under *in vivo* conditions with a rather small extracellular volume in animals, low hepatic MDA [1–3] and low extracellular MDA concentrations were found [1, 20]. Therefore, most MDA formed *in vivo* must be metabolised [19] and excreted by the kidneys [21] resulting in a drastic underestimation of LPO. The increase in MDA during incubation with decreasing $[Mg^{2+}]_o$ occurred at constant cellular Fe content. Probably, weakly-bound intracellular Fe, which is active in oxygen radical formation and LPO [22], remained constant during incubation at low $[Mg^{2+}]_o$. Since liver Mg content was not significantly reduced by incubation at low $[Mg^{2+}]_o$ or during Mg deficiency *in vivo* [14] and since $[Mg^{2+}]_i$ is buffered [14], the increase in LPO occurred at almost constant $[Mg^{2+}]_i$. Hence, the increase in LPO by reduction of $[Mg^{2+}]_o$ in the presence of 1.74 μM Fe (no addition of Fe/ascorbate) may be caused by increased binding of Fe at the extracellular side of the membrane due to reduced Mg^{2+}-Fe^{2+} competition. Binding of Fe to the cell membrane, creating 'ill-placed' Fe [22], can increase its catalytic effectiveness. It is well known that Fe has surface binding sites in proteins and binds to the phosphoric acid moieties of phospholipids [22]. At these extracellular binding sites, Mg^{2+} may compete with Fe^{2+}, thus reducing Fe binding and formation of oxygen radicals and LPO. Since this competition is rather unspecific [22], it can be explained that also Zn deficiency [23] and Ca deficiency [24] increased LPO. In the latter experiments [23, 24] the extracellular Zn or Ca concentration were reduced whereas the intracellular concentration of free Zn^{2+} (amounting to 10^{-11} M [25]) and free Ca^{2+} (amounting to 10^{-7} M) were probably unchanged. Therefore, the increase of LPO may be caused by reduction of the extracellular concentration of Zn^{2+} and Ca^{2+}.

After incubating the cultured hepatocytes for 24 h with Fe/ascorbate, cellular Fe content was increased by 60% (Table 1). Therefore, under these conditions Fe may be 'ill placed' at the extra- and intracellular side of the membrane.

The drastic reduction of MDA formation after addition of desferrioxamine, which did not significantly reduce cellular Fe content, may be caused by reduction of 'ill-placed' extracellular Fe. Whether desferrioxamine had also chelated weakly-bound 'ill-placed' intracellular Fe cannot be decided, since this Fe pool is very small.

Oxygen radical formation by 'ill-placed' extracellular Fe was also shown by fluorescence reduction of cis-parinaric acid. In this experiment, fluorescence decay induced by Fe/ascorbate and its prevention by desferrioxamine occurred immediately when there was no significant intracellular uptake of Fe or desferrioxamine, indicating that oxygen free radicals are also formed by extracellular Fe.

During Mg deficiency *in vivo*, Fe content was increased in most tissues, particularly in spleen and liver [3]. Therefore, it is likely that during Mg deficiency *in vivo* the intracellular labile Fe pool, which is active in oxygen radical formation, is increased and may be involved in the production of oxygen radicals. The same mechanism may occur in Zu deficiency *in vivo* which also increased Fe contents in liver [26], probably due to increased osmotic fragility of erythrocytes [27] as in Mg deficiency [6]. Hence, the Fe-induced increase in LPO during Mg deficiency *in vivo* may occur at both sides of the cell membrane. The amount of oxygen radicals formed *in vivo* by Fe at the intracellular or extracellular side of the cell membrane cannot be defined.

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