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Hydrogen peroxide and hydroxyl radicals mediate palmitate-induced cytotoxicity to hepatoma cells: Relation to mitochondrial permeability transition

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Abstract
We studied the toxicological responses of a human hepatoblastoma cell line (HepG2/C3A) to various free fatty acids (FFA) in order to identify the relation between reactive oxygen species (ROS) production and mitochondrial permeability transition (MPT). Exposure to the saturated FFA, palmitate, led to a time-dependent ROS production and hydrogen peroxide release as well as a loss of mitochondrial potential. The cytotoxicity of palmitate was significantly reduced by treating with scavengers of hydrogen peroxide, hydroxyl radical and the spin trap alpha-(4-pyridyl-1-oxide)-*N-tert*-butyl nitrone (POBN). Superoxide dismutase (SOD) mimics, nitric oxide scavenger, and inhibitor of *de novo* ceramide synthesis had no effect on the toxicity. MPT-inhibitor, cyclosporine, prevented the loss of mitochondrial potential but did not reduce the cytotoxicity. In contrast, inhibiting mitochondrial complexes I and III reduced the early potential loss and the cytotoxicity. These results suggest that palmitate-cytotoxicity to hepatoma cells is mediated through the production of H_2O_2 and *OH and independent of MPT.

Keywords: *Free fatty acids, lipotoxicity, reactive oxygen species, mitochondrial permeability transition, radical scavengers*

Introduction

Elevated plasma free fatty acid (FFA) concentrations are associated with the development of many hepatic disorders. FFAs increase the production of glucose [1], the accumulation of triglycerides [2] and inhibit insulin clearance by hepatocytes [3]. Chronically elevated fatty acid levels are associated with the development of non-alcoholic steatohepatitis (NASH), characterized by extensive hepatocyte cell death. Saturated FFAs cause cell death in a variety of cells including pancreatic beta-cells [4], cardiomyocytes [5] and hepatocytes [6,7]. According to the studies conducted in non-hepatic cells, such as beta-cells and myocytes, the potential mechanisms of cell death by these fatty acids involve increased ceramide production [8], reduced mitochondrial potential [5] and increased production of reactive oxygen species (ROS) [9,10]. Studies in hepatocytes or hepatoma cells have suggested that lysosomal permeabilization [11], release of cathepsin B [12] and reduction of mitochondrial Bcl-2/Bax ratio [6] play important roles in cell death caused by palmitate.

Different molecular species of ROS have been shown to mediate fatty acid toxicity in different types of cells, e.g. superoxide anion is known to mediate fatty acid toxicity in CHO cells [9], while nitric oxide mediates the cell death in pancreatic beta cell line INS-1 [13,14]. However, the type of ROS, which mediate the palmitate-toxicity to hepatoma cells, has not yet been elucidated. Herein, we report the results

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of our investigation towards identifying the ROS molecular species involved in the fatty acid-induced cytotoxicity of hepatoma cells.

Membrane permeability transition (MPT) is considered an important step in cell death [15]. MPT leads to release of pro-apoptotic molecules such as cytochrome *c*, apoptosis inducing factor (AIF) and endonuclease G from the mitochondria [16]. Cell death in response to many stimuli can be abrogated by inhibiting MPT, e.g. inhibiting MPT has been shown to reduce ROS formation and cell death of hepatocytes in response to microcystin treatment [17] and apoptosis of HepG2 cells in response to hypochlorous acid [18]. Saturated fatty acids can cause MPT [19]. MPT mediates the cell death by palmitate in cardiomyocytes [5]. However, it is not clear whether MPT plays a similar role in controlling fatty acid-induced toxicity of hepatocytes.

The objectives of this study were to investigate the mechanisms of fatty acid toxicity to hepatoma cells, identify the molecular species of ROS which mediate(s) the toxicity and study the inter-relationships between ROS production, MPT and cytotoxicity when these cells were exposed to FFAs at an elevated physiological concentration of 0.7 mM [20–23]. Our results suggest that saturated fatty acids can cause lipotoxicity through increased production of H_2O_2 and $^{\star}OH$ radicals. Even though MPT was observed in the cells treated with the saturated fatty acid, the toxicity is not mediated through MPT.

Materials and methods

Cell culture

About 1 million HepG2/C3A cells (American Type Culture Collection, Manassas, VA) were seeded into each well of a 6-well plate. The cells were kept in 2 ml of the medium containing Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (American Type Culture Collection) and 2% penicillin-streptomycin (Invitrogen). Cells were incubated at 37°C and in 10% CO2 atmosphere. After the cells reached confluency, the medium was replaced with 2 ml of the desired medium (Control, BSA, 0.7 mM FFA medium). Palmitate, oleate and linoleate were used as the representative fatty acids for the saturated, monounsaturated and polyunsaturated type of fatty acids. These fatty acids are the most abundant fatty acids of their class in the plasma, e.g. palmitate is the most abundant saturated fatty acid in the plasma. Plasma FFA levels in the obese and type 2 diabetic patients have been reported to be about 0.7 mM in many studies [20–23]. Therefore, this concentration of FFAs was employed in the present study. Media were changed after every 24 h.

Determination of cytotoxicity

Cytotoxicity was measured by measuring the release of lactate dehydrogenase (LDH) into the medium using the cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN). LDH is a cytosolic enzyme, which is released when the membrane integrity of the cells has been compromised. In this assay, the NADH generated during the oxidation of lactate by LDH is utilized as a co-factor by the enzyme diaphorase to reduce tetrazolium salt (yellow) to formazan dye (red). To conduct the assay, the supernatant media was collected after desired exposure times (e.g. 24 and 48 h) and the LDH activity in the medium was determined (called LDH-medium). The cells were washed with phosphate buffered saline (PBS) and lyzed with 1% triton-X-100 in PBS for 24 h at 37°C. The lysate was collected, vortexed for 15 s and centrifuged at 5000*g* for 3 min. LDH activity in the lysate was determined (called LDH-triton). The LDH released into the medium was normalized to total LDH, as shown in the equation below

$$\%\,\mathrm{LDH\,release} = \frac{\mathrm{LDH(medium)}}{\mathrm{LDH(medium)} + \mathrm{LDH(triton)}} \times 100$$

Inhibitor experiments

Fumonisin B1 (FB1) (Sigma-Aldrich, St Louis, MO) and cyclosporine A (CsA) (Calbiochem, San Diego, CA) were employed for inhibiting ceramide synthase and MPT, respectively. A 2 mM stock solution of FB1 was prepared in PBS and a 20 mM stock solution of CsA was prepared in dimethyl sulfoxide (DMSO). The cells were treated with palmitate in the presence of 2–100 μM FB1 and 0.5–5 μM CsA.

Measurement of ceramide

The cells were treated with 0.7 mM palmitate in the presence or absence of 20 μM FB1 (inhibitor of *de novo* ceramide synthesis). After the treatments, the cells were washed with PBS, trypsinized and pelleted down. Lipids were extracted by the method of Bligh and Dyer [24]. Briefly, the pellets were washed once with PBS, resuspended in 3 ml chloroform:methanol solution (1:2 v/v) and lyzed by sonication in this solution for 30 s. 0.8 ml water and 0.5 ml methanol were then added and the solution was kept at room temperature for 15 min. Cell debris was pelleted by centrifugation at 5000*g* for 5 min. The supernatant was decanted into a new tube and 1 ml each of chloroform and water was added. The solution was briefly vortexed and the phases were allowed to separate for 15 min. The tubes were then centrifuged at 3000*g* for 5 min. The aqueous layer was removed and 1 ml of the chloroform solution was transferred to a 1.5 ml microcentrifuge tube and dried in a speedvac. The lipids were resuspended in 80 μl chloroform. The

levels of ceramide were then measured using liquid chromatography-tandem mass spectroscopy (LC–MS), according to a previously published method [25]. Protein levels in the aqueous phase were measured using BCA assay. Ceramide results from LC–MS measurements were normalized to the total protein.

Measurement of ROS

Two different methods were employed to estimate relative ROS levels, as discussed below. In the first method, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (AM-H2-DCFDA) (C-2938, Molecular Probes, Eugene, OR) dye was used to measure the increased oxidized status of the cells in response to palmitate treatment. Stock solution of the dye was prepared in DMSO and diluted to 5 μM in DMEM. Confluent cells were treated with palmitate for different time periods. Measurement of fluorescence was performed by flow cytometry according to a previously published protocol [9].

As a separate measurement, the H_2O_2 released into the medium was measured using a colorimetric hydrogen peroxide assay (Assay Designs, Ann Arbor, MI). This assay is based on the principle of reaction of coordinated iron with peroxide and xylenol orange in acidic solution to produce a purple-colored complex, whose color is proportional to the concentration of H_2O_2 in the sample. Confluent cells were exposed to either HepG2 medium or to palmitate for desired periods. Media were replaced every 24 h. After the treatment periods, media were collected and the H_2O_2 levels in the media were analyzed immediately, according to the manufacturer's instructions. Blanks were generated by incubating the media without cells and subtracting the absorbance of the blanks from that of the samples. The estimated concentrations were multiplied by the volume of the culture to obtain the total amount, which were then normalized to the total protein levels. For estimation of the protein, the cells were washed with PBS and lyzed with 1% triton-X-100 to estimate protein levels by the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL). H_2O_2 levels were then normalized to cellular protein levels.

Radical scavengers and spin trap

1, 3 Dimethyl urea (DMU) (MP Biomedical) and DMSO (Sigma Aldrich) were employed to scavenge hydroxyl radicals. Solutions of these scavengers were prepared in the palmitate medium at 5–50 mM for DMU and 0.2–3% (v/v) for DMSO. The spin trap POBN, 20–100 mM from Sigma Aldrich was dissolved in palmitate medium. Catalase (CAT, 3000 U/ml) was used to scavenge H_2O_2. It was dissolved in the palmitate medium. Cu(II)2(3,5-diisopropylsalicylate)4 (Cu-DIPS) and Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) are the cell permeable mimics of cytosolic (Cu–Zn) and mitochondrial (Mn) superoxide dismutase (SOD), respectively [26]. These SOD-mimics were obtained from Sigma Aldrich and utilized as scavengers for the superoxide anion. Stock solution of Cu-DIPS (5 mM) was prepared in DMSO and used in the concentration range 1–10 μM. Stock solution was prepared by dissolving 50 mM MnTBAP in 0.1 M NaOH, followed by addition of equal volume of 0.1 M HCl. MnTBAP was used in the concentration range 10–100 μM. Pyrrolidone dithiocarbamate (PDTC) and 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron) were obtained from TCI chemicals and employed as scavengers of superoxide anion [9]. These scavengers were dissolved in the medium at concentrations of 0.25–10 and 2–40 mM, respectively. 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (c-PTIO), procured from Axxora Chemicals, was used to scavenge nitric oxide (NO) [27]. A 200 mM stock solution of c-PTIO was prepared in water and used at final concentration of 20–200 μM. Aminoguanidine hydrochloride (AG) and *N*-(3-(aminomethyl)benzyl)acetamidine dihydrochloride (1400 W), both from Axxora Chemicals, were employed to inhibit nitric oxide synthesis [27]. AG was dissolved in PBS at a concentration of 1 M and used at 0.1–1 mM final concentration. A 100 mM stock solution of 1400 W was prepared in water and used in the concentration range 50–500 μM.

Dose-dependence studies

For all the inhibitors and scavengers, dose-dependence studies were performed. That is, the cells were exposed to palmitate in the presence of various concentrations of these scavengers/ inhibitors and the LDH release was measured. The following treatment ranges were studied—FB1 (2–50 uM), CAT (500–3000 U/ml), DMU (2–50 mM), DMSO (0.5–2% v/v), CuDIPS (0.5–10 uM), MnTBAP (25–200 uM), Tiron (2–20 uM), PDTC (0.5–10 uM), cPTIO (25–200 uM), AG (0.1–1 mM), 1400 W (50–500 uM), CsA (0.5–5 uM), POBN (20–100 mM), rotenone (100–2000 nM), antimycin A (50–1000 nM). Results are presented for concentrations which were most effective in reducing LDH.

Measurement of the mitochondrial potential

Mitochondrial potential was measured using JC-1 dye (Calbiochem). Potential-sensitive uptake of this dye leads to its accumulation and aggregate formation in normal, active mitochondria. Aggregates of the dye (mitochondria) fluoresce red, while the dilute solution (cytoplasmic) fluoresces green. The ratio of red to green fluorescence was used to estimate the mitochondrial potential [28]. A 5 mM stock solution of the

dye was prepared in DMSO. After treating the cells for desired times, the media were aspirated and the cells were exposed to 5 μM JC-1 dye in DMEM. Cells were incubated in the dye-containing medium for 30 min. Dye was aspirated and the cells washed twice with 1 ml PBS. About 1 ml PBS was added to each well and the plates were bottom-read in a fluorescence reader (Molecular Probes, Eugene, OR) at excitation of 488 nm and emissions of 527 nm (green) and 590 nm (red). *p*-Trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) is an established mitochondrial uncoupler and abolishes the mitochondrial potential. The mitochondrial uncoupler FCCP (40 μM) was used as a control for totally lost membrane potential.

Statistical analysis

Treatments were analyzed with analysis of variance (ANOVA) followed by Bonferroni's *post hoc* comparison to locate specific differences (Turner and Thayer 2001). Analyses were performed using S-plus software.

Results

Cytotoxicity of different types of fatty acids

To evaluate the toxicity of various fatty acids, the HepG2 cells were exposed to 0.7 mM palmitate, oleate or linoleate—the three primary species of saturated, unsaturated and polyunsaturated fatty acids in the plasma, for two days. Media were changed after 24 h of exposure and the LDH release into the medium was measured. Among the fatty acids studied, only palmitate was found to be toxic to the cells and resulted in a significantly higher LDH release on both days (Figure 1a). The toxicity of palmitate was concentration dependent above 0.2 mM (Figure 1b). When exposed to free fatty acids, the cells metabolized about 0.2 mM of the different free fatty acids (not shown). This suggests that the amount of fatty acid present in excess to the metabolic capacity causes the toxicity, but this toxicity is specific to saturated fatty acid, because the unsaturated fatty acids that were not fully metabolized were non-toxic.

Ceramide metabolism

De novo ceramide synthesis has been shown to be a major factor in palmitate-induced toxicity in a variety of cell types [8]. Among the primary mechanisms of ceramide-induced toxicity is increased ROS production [29–31]. To investigate the role of *de novo* ceramide synthesis in the toxicity of palmitate to HepG2 cells, we studied the effect of inhibiting ceramide synthesis on the cytotoxicity. FB1 inhibits *de novo* ceramide synthesis by inhibiting ceramide synthase, a key enzyme of the pathway. The cells were treated with palmitate in the presence of 2–50 μM FB1 [32]. Similar concentrations of this inhibitor have been

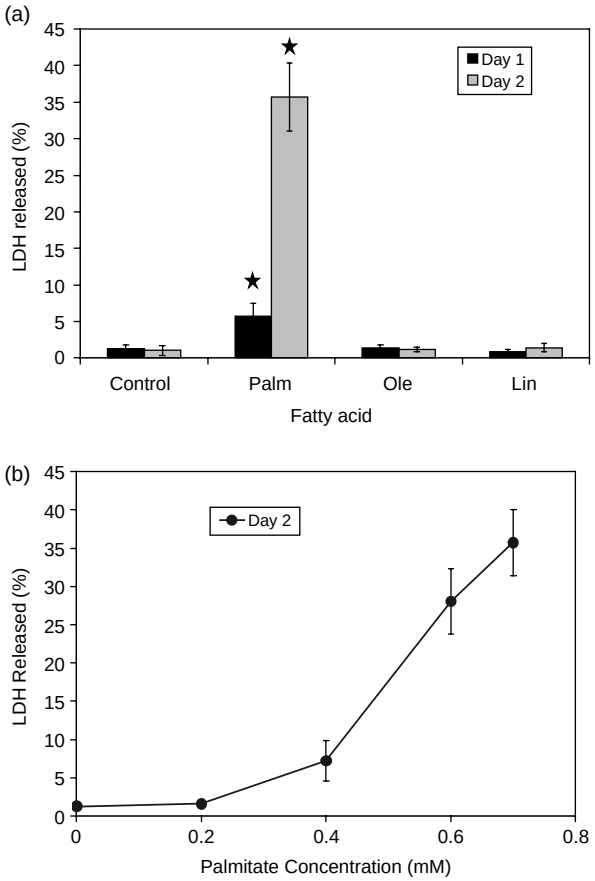

Figure 1. Cytotoxicity of the free fatty acids. (a) Effect of different types of fatty acids on cytotoxicity (LDH release). Confluent HepG2 cells were exposed to different types of fatty acids (0.7 mM, complexed to 4% w/v BSA) for 2 days, with medium-change everyday. LDH released on each day was measured and normalized to total LDH. Total LDH is defined as the sum of the LDH released and the intracellular LDH. The intracellular LDH values were obtained by lyzing the cells with 1% triton (see methods section). (b) Effect of concentration on palmitate-toxicity. The LDH released after exposure to different concentrations of palmitate for two days was measured. Data expressed as averages of nine samples ± SD from three independent experiments. ⋆, significantly higher than control, $p < 0.01$.

shown to inhibit *de novo* ceramide synthesis in HepG2 cells [33]. The cells exposed to palmitate had high levels of C-16 ceramide (Figure 2a) whereas the cells treated with 4% BSA (control) had negligible levels (not shown). Treatment with 20 μM FB1 reduced the ceramide levels significantly (42 and 72% reductions on day 1–2, respectively, Figure 2a). This suggested that the *de novo* ceramide pathway was responsible for the increased ceramide synthesis. However, the reduction in ceramide levels was not associated with a reduction in the toxicity (Figure 2b). Similarly, higher concentrations of the inhibitor (up to 50 μM) were unable to reduce the toxicity. FB1 is a specific inhibitor of ceramide synthase [34,35], the final enzyme of the *de novo* ceramide synthesis pathway. Therefore, the lack of reduction in the toxicity in response to FB1, in spite of a reduction in ceramide levels, suggests that the

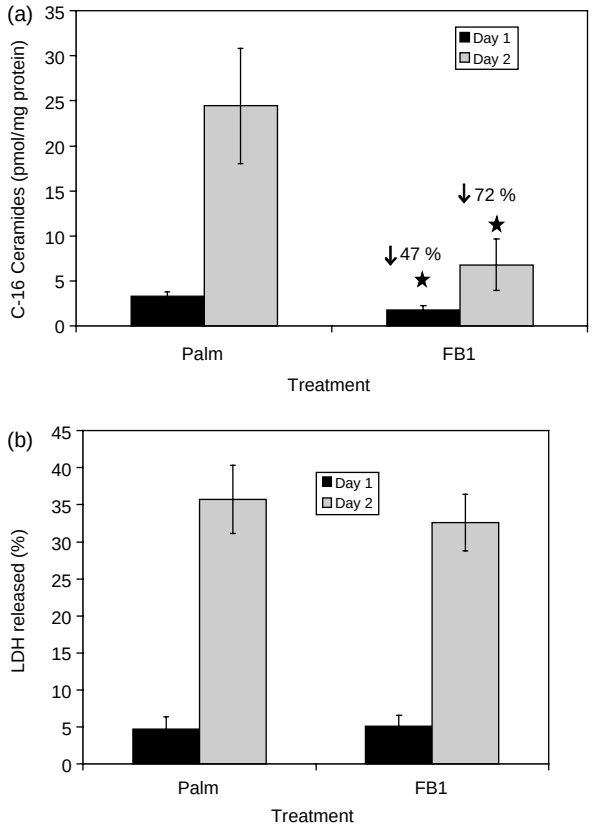

Figure 2. Effect of inhibitors of *de novo* ceramide synthesis. Cells were exposed for two days to 0.7 mM palmitate containing vehicle (PBS), or 20 uM FB1. The media were changed everyday. (a) Effect on C-16 ceramide levels. After treatments, the lipids were extracted. The levels of C-16 ceramide in the cells were measured using LC–MS and normalized to the cellular protein. *, significantly lower than palmitate, $p < 0.01$. (b) Effect on cytotoxicity. The cells were treated with palmitate and FB1 for two days, and the LDH release was measured as described in materials and methods section. Data presented as mean ± SD for nine samples from three independent experiments.

de novo synthesized ceramide does not play an important role in the toxicity. These results are in agreement with other studies on the toxicity of saturated fatty acids to hepatocytes [11,36]. These studies indicate that saturated fatty acid may cause the toxicity to hepatocytes by mechanisms other than ceramide generation, such as lysosomal permeabilization [36], ER stress [11], reductions in the mitochondrial Bcl2/Bax ratio [6] and activating JNK pathway [37].

Role of ROS in the cytotoxicity of palmitate

ROS has been shown to be a major cause of palmitate-induced cytotoxicity in CHO and microvascular endothelial cells [9,10]. To investigate the role of ROS in palmitate-cytotoxicity in HepG2 cells, we employed a two-pronged approach—(a) estimated the oxidized status of the cells using AM-H2-DCFDA dye and measured the release of H_2O_2, (b) measured the effect of species-specific ROS scavengers on the LDH release in response to palmitate. Exposure to palmitate, but not to oleate or linoleate, led to a time-dependent increase in intracellular oxidized status (Figure 3a). A measure of ROS production by the cells is the release of H_2O_2 [38]. The H_2O_2 released by the cells in response to palmitate was measured by a colorimetric assay as described in the methods section. Exposing cells to palmitate led to a time-dependent increase in H_2O_2 release into the medium (Figure 3b). The profile of H_2O_2 release mirrored the observed toxicity, being lower on day 1 and increasing significantly on day 2, suggesting that H_2O_2 may be related to the observed toxicity. H_2O_2 can form *OH radical, a much more reactive and damaging ROS species, in the presence of divalent cations such as Fe^{2+}. To evaluate the role of H_2O_2 and *OH radical in palmitate toxicity, we treated the cells with palmitate in the presence of CAT (3000 U/ml) and *OH radical scavengers DMU, 25 mM and DMSO, 1% v/v and the spin trap POBN, 70 mM. All these scavengers reduced the cellular ROS levels (Figure 3c), as well as the palmitate-toxicity (Figure 3d) significantly. DMU and DMSO are two specific scavengers of hydroxyl radicals [39–42]. Dimethyl thiourea (DMTU) is another commonly used *OH scavenger, but was not employed in the current study because it had been shown to be toxic to rats [43]. The same study showed that DMU was not toxic, and therefore, DMU was used in the present study.

Studies with other cell types have also indicated that superoxide or NO may mediate the toxicity of palmitate [9,13,14]. To evaluate the role of superoxide anion in the toxicity, cells were either pretreated for 2 h or co-treated with palmitate in the presence of cell-permeable SOD mimics CuDIPS (0.5–10 μM) and MnTBAP (25–200 μM), or the superoxide-specific scavengers Tiron, 2–20 uM and PDTC, 0.5–10 uM. However, none of these treatments produced any significant reduction in the toxicity of palmitate, suggesting that the superoxide anion is not the primary toxicity causing species. To evaluate the role of NO in the toxicity, the effect of NO scavengers (cPTIO, 25–200 uM and AG, 0.1–1 mM) or iNOS inhibitor 1400 W (50–500 μM) on the palmitate toxicity was measured. Exposure to palmitate in the presence of these chemicals did not reduce the cytotoxicity, indicating that NO is not involved in the toxicity of palmitate. The efficacy of H_2O_2 and *OH scavengers, but not of superoxide or nitric oxide scavengers or inhibitors, indicated that the former ROS are the likely mediators of the palmitate-toxicity in HepG2 cells.

Exposure to palmitate is associated with the loss of mitochondrial potential

Cytotoxicity is usually associated with the loss of mitochondrial membrane potential. A significant reduction in the mitochondrial potential was observed when the cells were treated with palmitate, but not

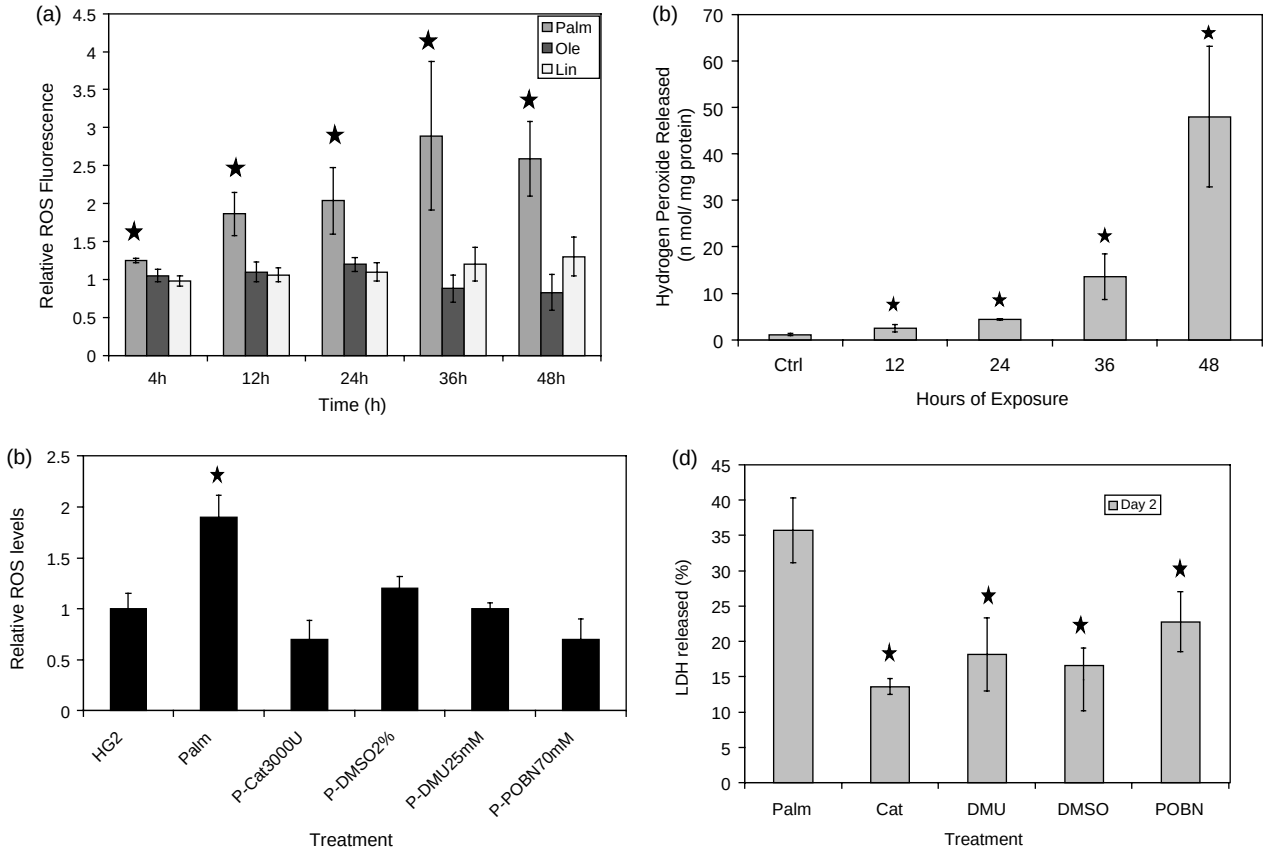

Figure 3. Role of ROS in palmitate-toxicity. (a) Relative oxidized status of the cells after different times of exposure to palmitate. Cells were exposed to 0.7 mM palmitate for different times. Medium was changed after 24 h of exposure. Treatment with palmitate was followed by exposure to 5 uM AM-DCFDA dye for 30 min at 37°C and staining with 2 μg/ml propidium iodide for 10 min. Fluorescence of viable (propidium iodide negative) cells was measured using flow cytometry with excitation at 488 nm and emission at 530 nm. Relative fluorescence values ± SD of six samples from two different experiments are presented. "⋆" denotes significantly different, compared to control, $p < 0.01$. (b) Release of hydrogen peroxide. Cells were treated with 0.7 mM palmitate for various times. The H_2O_2 released into the medium was measured and normalized to total cellular protein. Data presented as mean ± SD for nine samples from three independent experiments. "⋆" denotes significantly different, compared to control, $p < 0.01$. (c) Effects of scavengers of H_2O_2 and hydroxyl radical on the oxidized status of the cells. The cells were treated with palmitate in the presence of 3000 U/ml CAT (scavenger for H_2O_2), or scavengers of ⋆OH radical—DMU (25 m M), DMSO, 1% v/v, or the spin trap POBN, 70 mM for 24 h, followed by treatment with 5 uM AM-DCFDA for 30 min in DMEM. Fluorescence of the cells was measured using a microplate reader with excitation at 488 nm and emission at 530 nm. Relative fluorescence values ± SD of six samples from two independent experiments are presented. "⋆" denotes significantly higher, compared to control, $p < 0.01$. (d) Effects of scavengers of H_2O_2 and hydroxyl radical on palmitate-toxicity. The cells were treated as in Figure 3c for 2 days. LDH released on day two is presented. Data presented as mean ± SD for nine samples from three independent experiments. "⋆" denotes significantly lower, compared to palmitate, $p < 0.01$.

when they were treated with unsaturated fatty acids (Figure 4a). The potential after exposure to palmitate was higher than that observed on treating the cells with 40 μM FCCP (positive control), suggesting a partial loss of potential upon palmitate-exposure. To test the involvement of ROS in the observed reduction of the mitochondrial potential in response to palmitate, the cells were exposed to 100 uM H_2O_2 in the absence of palmitate. This treatment also significantly reduced the mitochondrial potential (Figure 4a), suggesting that the oxidative stress in response to palmitate may play a role in the loss of mitochondrial potential.

MPT has been suggested as a cause of reduced mitochondrial potential and cell death in response to saturated fatty acid in beta cells [5]. To evaluate the involvement of MPT in the loss of mitochondrial potential and toxicity by the palmitate-treatment, we inhibited MPT by treating the cells with 0.5–5 uM CsA in the presence of palmitate. This treatment restored the mitochondrial potential on both days (Figure 4b, data shown for 2 μM). However, the improvement in the mitochondrial potential was not associated with a reduction in the toxicity (Figure 4c), indicating that the toxicity was not dependent on the loss of mitochondrial potential. Similarly, the other concentrations of the inhibitor (0.5–5 μM) were also unable to significantly reduce the toxicity (not shown). In agreement with these observations, treating with palmitate in the presence of DMU, DMSO, CAT and POBN (all of which had reduced the LDH release significantly) did not affect the reduction in mitochondrial potential by palmitate (Figure 4b). Thus, the

treatment with palmitate leads to MPT, but the toxicity of this fatty acid does not depend on MPT.

Role of ROS in MPT: Effect of mitochondrial complex inhibition

ROS and MPT can have a two-way interaction, i.e. some studies have reported that MPT has been associated with increased production of ROS through mitochondrial uncoupling, while ROS can cause oxidation of the thiol-groups on cyclophilin D (Cyp-D) and adenine nucleotide translocator (ANT), causing MPT. Mitochondrial complexes I and III are known to be the primary sites of ROS production [44–46]. The observation of loss of mitochondrial potential also suggested alteration of mitochondrial structure and function. In order to evaluate the role of ROS production at these mitochondrial complexes on the MPT and the LDH release caused by palmitate, we studied the effect of inhibiting mitochondrial complexes I and III. Cells were pretreated for 30 min with various concentrations of rotenone and antimycin A, irreversible inhibitors of complex I and III, respectively. Inhibiting these complexes partially improved the mitochondrial potential (Figure 5) on day 1 and reduced the LDH release (Figure 6) on both days. The partial recovery on day 1 and no effect on day 2 suggested that mechanisms other than ROS generation at complexes I and III are playing roles in the MPT caused by palmitate. Furthermore, co-treatment of the cells with 100 nM rotenone (0.35 pmol/mg protein) or 50 nM antimycin A (0.17 pmol/mg protein), following the pre-treatment of the cells with 500 nM rotenone (1.8 nmole/mg protein) or 200 nM antimycin A (0.7 nmol/mg protein), did not protect against the potential loss. These results show that the ROS production by complexes I and III mediates, partly, the early potential loss and the LDH release in response to palmitate-exposure, but mechanisms other than ROS production at these complexes are also involved in the MPT induced by palmitate. The effect of these inhibitors on the LDH release in the absence of palmitate was also evaluated. Pretreating the cells for 30 min with 500 nM rotenone (1.8 nmole/mg protein) and 200 nM antimycin A (0.7 nmol/mg protein), followed by treatment with control medium, caused a slight increase in toxicity. Nevertheless, even with 1 μM rotenone and 500 nM antimycin A, the LDH release was less than 2% (not shown), much less than that observed with palmitate and the inhibitors. This indicated that the remaining toxicity in the palmitate samples in the presence of these inhibitors is due to some

(c) Effect of the inhibition of MPT on the cytotoxicity. Cells were treated for 2 days with palmitate in the presence of 2 μM CsA. LDH release was measured and normalized to total LDH. Data shown is mean ± SD for nine samples from three independent experiments.

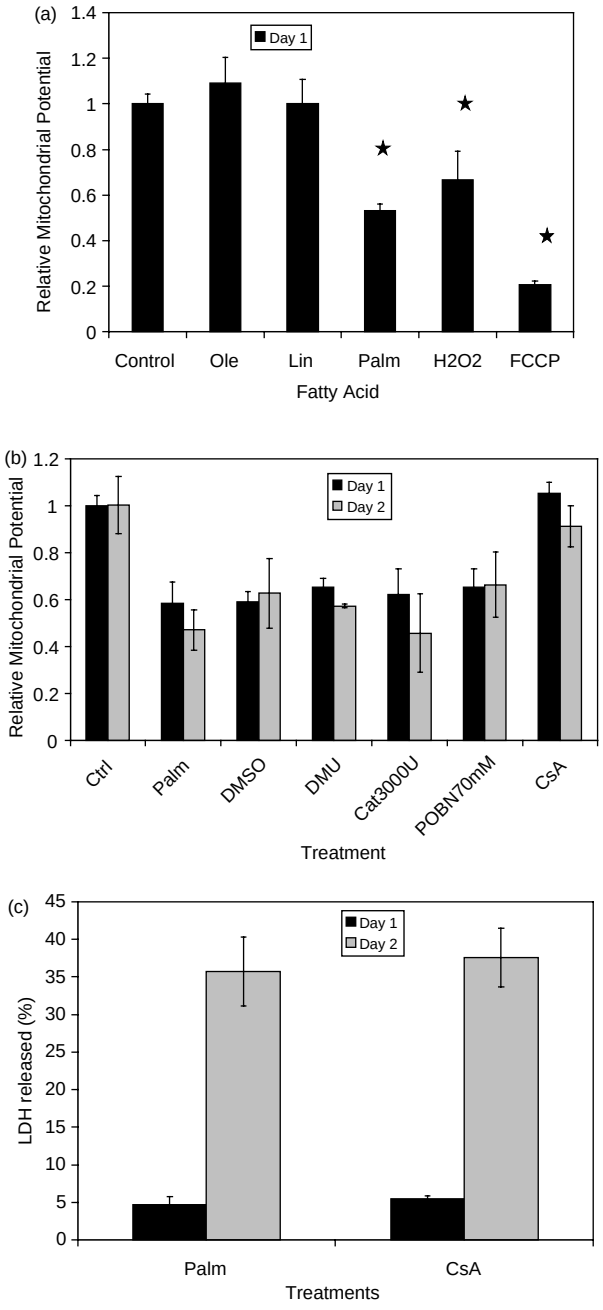

Figure 4. The loss of mitochondrial potential caused by palmitate and its role in the toxicity. (a) Mitochondrial potential after exposure to fatty acids or hydrogen peroxide for 24 h. Cells were exposed to100 μM hydrogen peroxide, to various FFAs (0.7 mM) or to 40 μM FCCP for 24 h. The cells were then incubated in 5 μM JC-1 dye in serum-free medium for 30 min at 37°C, after which the cells were washed with PBS. The fluorescence was measured in a microplate fluorimeter, with excitation at 488 nm and emissions at 530 (green) and 590 nm (red). Data presented as mean ± SD for nine samples from three independent experiments. ⋆, significantly different from control, $p < 0.01$ relative to control. (b) Effect of hydroxyl radical scavengers and the MPT inhibitor on the mitochondrial potential. Cells were treated with 0.7 mM palmitate in the presence of hydroxyl radical scavengers DMSO (1% v/v), DMU (25 mM), or MPT inhibitor CsA (2 uM) for 24 h and the mitochondrial potential was measured, as in "a". Data presented as mean ± SD for nine samples from three independent experiments. "⋆" denotes significantly different from palmitate, $p < 0.01$.

mechanism other than ROS production at these sites (complexes) in response to palmitate.

Discussion

FFAs are known to play important roles in the development of many hepatic complications. Previous studies have shown that saturated fatty acids are important mediators of lipotoxicity. Studies with other cells have implicated *de novo* ceramide synthesis [9,10] in the palmitate-induced cell death. Palmitate is a substrate of ceramide synthesis and has been shown to increase *de novo* synthesis of ceramide [9]. The inhibitor experiments conducted in this study suggest that the palmitate-toxicity to hepatoma cells is not mediated by *de novo* ceramide synthesis. These results are in agreement with another recent study [11], where the authors reported that the cell death caused by saturated fatty acids in murine hepatoma cells is independent of the *de novo* ceramide generation. It is likely that other mechanisms of cell death such as ER stress [11], lysosomal permeabilization [36], JNK activation [37] or reduction in mitochondrial Bcl2/Bax ratio [6] play important roles in the toxicity of the saturated fatty acid to HepG2 cells, as observed in the studies with primary hepatocytes.

Another important mediator of cell death by saturated fatty acids is the increased production of ROS. Mitochondrial complexes I and III are the primary sites of ROS generation by the cells. Inhibiting these complexes reduced the cytotoxicity significantly, suggesting that these complexes are an important source of ROS in the palmitate-toxicity to hepatoma cells. The complex I and III inhibitors employed, rotenone and antimycin A, can themselves cause cell death through increased ROS production and MPT at higher concentrations or prolonged exposures [47,48]. Therefore, we conducted a dose-response study on the toxicity with these compounds in the presence of palmitate to identify the optimal treatment concentrations and times. In agreement with previous studies, we observed an increase in the cytotoxicity when the cells were treated at higher concentrations or longer durations. However, for the optimal treatment conditions shown (pre-treatment for 30 min with 0.5 uM rotenone or 0.2 μM antimycin A), a maximum reduction in the cytotoxicity (55 ± 10% decrease in LDH release for the rotenone pre-treatment compared to palmitate treatment alone) was observed. The cytotoxicity was not completely prevented on treating with mitochondrial complex inhibitors. This suggests that mechanisms other than ROS production in the mitochondria may also contribute to the toxicity of palmitate.

Treatment with palmitate can lead to increased generation of NO in iNOS dependent [49] and independent [13] ways. While increased NO generation has been suggested to play a role in palmitate toxicity to a beta-cell line INS-1 [13], studies in pancreatic islets have indicated no involvement of NO in response to elevated fatty acid exposure [50]. Conflicting roles of NO in mediating the cell death in hepatocytes have been reported. While NO can cause cell death by itself [51], it has been shown to protect against TNF-α-induced cell death [52,53]. It is very likely that NO is non-toxic (and perhaps cytoprotective) at low concentrations, but becomes toxic beyond a certain threshold concentration, which depends on the cell-type. In the present study, either NO does not play a major role in the toxicity, or the NO produced

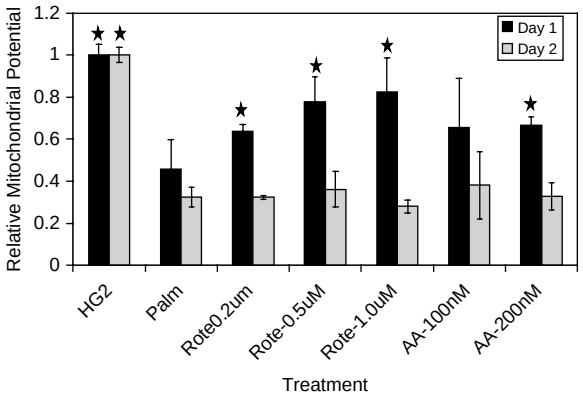

Figure 5. Effects of inhibiting mitochondrial complexes I and III on mitochondrial potential loss. Cells were pre-treated with different concentrations of rotenone or antimycin A for 30 min, followed by the treatment with 0.7 mM palmitate. At the end of 24 h exposure, the media were removed and cells treated with the same concentrations of rotenone and antimycin A for 30 min, followed by exposure to palmitate for another 24 h. Mitochondrial potentials were measured after the 24 and 48 h of exposure to palmitate, using JC-1 dye, as explained in the materials and methods. Data presented as mean ± SD for nine samples from three independent experiments. "⋆" denotes significantly different, compared to palmitate, $p < 0.01$.

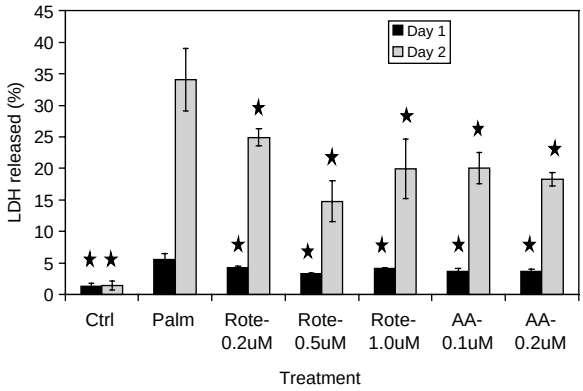

Figure 6. Effects of inhibiting mitochondrial complexes I and III on the cytotoxicity of palmitate. Cells were pretreated with different concentrations of rotenone or antimycin A for 30 min, followed by treatment with 0.7 mM palmitate. After 24 h of exposure, cells were treated again with the inhibitors for 30 min and then exposed to palmitate for another 24 h. LDH release after 24 and 48 h were measured and normalized to the total LDH. "⋆" denotes significantly different, compared to palmitate, $p < 0.01$.

in response to palmitate does not reach toxic levels in these cells.

Hepatocytes are known to have high levels and activities of SOD. Therefore, the superoxide anions generated can be effectively neutralized to H_2O_2. This may explain, in part, why the SOD mimics were not effective in reducing the cell death. The SOD mimics will not reduce the production of H_2O_2—the cytotoxicity mediating species. H_2O_2 is freely diffusible across membranes and thus may cause oxidative stress in neighboring cells as well. Extracellular neutralization of H_2O_2 using a high molecular weight CAT reduced palmitate-toxicity significantly, indicating that H_2O_2 is the likely carrier of oxidative stress to nearby cells. An increased concentration of H_2O_2 was observed in the medium in response to palmitate-exposure, but not in the control medium. However, this increase is not due to an accumulation of H_2O_2, as H_2O_2 is not expected to be stable for hours. While in the absence of cells, H_2O_2 is stable for at least 30 min [54], the typical half-life in the presence of cells is a few minutes [54,55]. Therefore, these values likely indicate an increase in cellular secretion rather than accumulation of H_2O_2. Another variable, which would control the net rate of release by the cells, is the level of peroxide-metabolizing enzymes, such as CAT and glutathione peroxidase. Fatty acids have been shown to alter the expression of these enzymes. For example, a high fat diet rich in saturated fatty acid has been shown to reduce the activities of glutathione peroxidase (GPx) and CAT, while increasing the expression of SOD [56,57]. Thus, the saturated fat may lead to reduced catabolism of the generated H_2O_2, thus increasing the net release.

One of the important mediators of H_2O_2-toxicity is the generation of $^{\star}OH$ in the presence of divalent cations by the Fenton and Haber–Weiss reactions. $^{\star}OH$ is an extremely reactive and damaging radical and can oxidize any lipid, protein or DNA molecule in its vicinity. Treating the cells with palmitate in the presence of $^{\star}OH$-scavengers significantly reduced the toxicity, indicating an involvement of this radical in the cytotoxicity of palmitate.

Mitochondria are the organelles centrally associated with many fundamental cellular processes, such as energy-generation. They are also the reservoirs of many apoptogenic factors, such as cytochrome *c*, AIF and endonuclease G, etc. A loss of structural and functional integrity of these organelles can lead to cell death. Transport of chemicals and proteins to and from these organelles is strictly regulated. The concentration difference of solutes and the resulting potential gradient across mitochondrial inner membrane is crucial for mitochondrial structural and functional integrity. One of the key regulators of transport of low molecular weight solutes across the inner mitochondrial membrane is the mitochondrial permeability pore (MPP) complex. MPP is a protein complex found at the junctions of the inner and outer membranes, which controls the influx of small molecular weight compounds into the mitochondria. MPT refers to the opening of this pore, a process which brings in solutes of up to 1.5 kDa inside the mitochondria, resulting in the loss of mitochondrial potential, swelling of the mitochondria, a reduction in the ATP generation and production of ROS [16]. The swelling leads to the rupture of the outer membrane, releasing intermembrane apoptogenic factors such as cytochrome *c*, AIF and endonuclease G [58]. MPT also causes loss of anti-oxidants such as glutathione from the mitochondria, making them even more susceptible to ROS-induced damage [59]. Two types of MPT have been reported—classical, which is inhibited by CsA and non-classical, which is not inhibited by CsA. According to *in vitro* studies, employing isolated mitochondria or liposomes, palmitate can cause non-classical MPT (NC-MPT) by forming channels in the mitochondrial membrane by complexing with Ca^{2+} [60], or by interacting with and altering mitochondrial membrane permeability [61]. A complete prevention of mitochondrial potential loss upon treatment with CsA suggested that the loss of mitochondrial potential on exposure to palmitate is due to classical MPT. However, the inhibition of MPT had no effect on the cytotoxicity indicating that the cytotoxicity was not dependent on MPT. CsA may also inhibit protein phosphatase 2B (PP2B/calcineurin). Inhibition of PP2B is also associated with a reduction in apoptosis [62]. Among the various organs, the liver isoform of PP2B is least active and is resistant to CsA inhibition [63]. Thus, we presume that the effects of CsA are not likely due to calcineurin inhibition.

Palmitate can also indirectly cause MPT by increasing the generation of ROS. ROS can oxidize the thiol groups on CypD and ANT [64], two important proteins of MPP, causing MPT. Elevated ROS generation also leads to lipid peroxidation, which can also cause MPT [65]. Mitochondrial complexes I and III are the primary sites of ROS generation. Inhibiting these complexes reduced the loss of potential on day 1, albeit incompletely, but had no effect on the mitochondrial potential loss on the second day. These results suggest that (a) generation of ROS at complexes I and III plays an important role in the initial potential loss caused by these fatty acids, and (b) other mechanisms are involved in the MPT by fatty acids. Fatty acids may cause MPT by many mechanisms other than mitochondrial ROS generation. It is likely that the production of H_2O_2 due to oxidation of fatty acids in peroxisomes may, over time, cause MPT. Fatty acids can also cause MPT independently of ROS. Fatty acids are mitochondrial uncouplers, and can reduce mitochondrial potential to below the "gating potential" (mitochondrial potential below which MPT occurs), causing MPT [66]. They can also cause MPT by

binding to and causing structural changes in the ANT [67]. Recently, it was demonstrated that fatty acids can cause endoplasmic reticulum (ER) stress [11]. ER is an important reservoir of cellular Ca^{2+}. ER stress would increase cytosolic and mitochondrial Ca^{2+} levels [68,69], which can cause MPT by itself, or increase the sensitivity of the MPT to oxidative stress [70]. Palmitate can reduce ATP synthesis due to its uncoupling action [71] and reduce cardiolipin synthesis [72]. Because MPT is sensitive to both ATP levels and mitochondrial membrane structure, these effects of palmitate would make the mitochondria more susceptible to MPT.

As both cytotoxicity and MPT were observed in the palmitate-treated cells and were significantly reduced by the reduction in ROS generation, it might be tempting to speculate that the same ROS species is mediating both these effects. However, the inefficacy of the *OH radical scavengers (which reduced the toxicity significantly) on MPT suggests that these radicals are not involved in causing the MPT in response to palmitate.

In summary, hydrogen peroxide and *OH radical, but not *de novo* ceramide synthesis, are the primary mediators of the cytotoxicity of palmitate in hepatoma cells. ROS generation by the mitochondrial complexes I and III are responsible for mediating the toxicity as well as, in part, the early potential loss in response to palmitate. Thus, while MPT and cytotoxicity are related, in that the exposure to palmitate is associated with both MPT and cytotoxicity, the cytotoxicity is not mediated by MPT.

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