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Palmitate protects hepatocytes from oxidative stress and triacylglyceride accumulation by stimulation of nitric oxide synthesis in the presence of high glucose and insulin concentration

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Abstract

Excessive flux of free fatty acids (FFA) into the liver contributes to liver impairment in non-alcoholic fatty liver disease (NAFLD). It remains unclear how FFA contribute to impairment of hepatocytes. This study treated hepatocytes with linoleic acid and palmitate to investigate the early event triggering FFA-mediated impairment. It determined cell viability, content of nitrite/nitrate and triacylglycerides (TG), inducible nitric oxide synthase (iNOS) protein, oxidation of cardiolipin (CL) as well as formation of F_2-isoprostanes in the presence of insulin and glucose. Linoleic acid caused significant decrease in cell viability. It is shown that palmitate caused induction of iNOS resulting in increased nitrite/nitrate concentration and slight increase in TG content. Linoleic acid led to a decrease in nitrite/nitrate concentration parallelled by massive TG accumulation in combination with increased oxidation of CL and increased F_2-isoprostane levels. It is concluded that nitric oxide (NO) concentration regulates FFA-dependent TG accumulation and oxidative stress in rat hepatocytes.

Keywords: *Adult hepatocyte culture, linoleic acid, palmitate, nitric oxide, lipid accumulation, oxidized cardiolipin, F_2-isoprostanes*

Introduction

Obesity and insulin resistance are associated with the development of NAFLD in men [1]. This disease is characterized by accumulation of triglycerides (TG) within the liver [2,3]. NAFLD has a high potential for liver injury progressing to fibrosis, cirrhosis and hepatocellular carcinoma [4].

There is evidence that elevation of FFA concentration is associated with the pathogenesis of NAFLD [5]. In this context, increased metabolic utilization of FFAs had been derived from plasma lipidomic analysis of patients with NAFLD [6]. Increased levels of FFAs may result from abnormal fat intake, FFA release from adipocytes and reduced β-oxidation [7,8]. After overnight fast, serum concentration of FFAs is below 750 meq/L in humans. There is a tendency that diabetic and obese persons have increased levels up to 1500 meq/L [9]. Higher FFA concentrations are reached post-prandially. Among FFAs, linoleic acid and palmitate had been found in the plasma of NAFLD patients [6]. FFA concentrations in pre-diabetic and diabetic rats are higher than 1 mM [10].

There are controversial reports about the role of free fatty acids in NAFLD. The major part of the studies claims that saturated free fatty acids such as palmitic acid have lipotoxic effects resulting in oxidative stress and apoptosis, whereas unsaturated free fatty acids have no or low lipotoxic effects [11–15]. However, there is an indication that unsaturated FFAs may also have lipotoxic effects. Oral feeding of rats with an iso-caloric fish oil-rich diet caused the

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development of NAFLD involving fatty liver, inflammation, necrosis and increased oxidative stress within 8 weeks [16]. Among unsaturated FFA, linoleic acid seems to play an exceptional role in lipotoxicity. It had been demonstrated that a high linoleic acid diet caused oxidative stress in healthy individuals, as documented by an increase in the concentration of F_2-isoprostanes in the urine of these persons, whereas oleic acid had no such effect [17].

It remains unclear how elevated FFA concentrations causally contribute to impairment of hepatocytes. *In vivo* investigations applying lipid infusion or high fat diet are difficult to interpret because of the complexity of the initiated processes. Effects that are finally induced in hepatocytes may result from changes in glucose and insulin concentrations within the circulation. Therefore, cellular systems are required in order to study causal effects of FFAs. It is known that FFAs may possess lipotoxic potential involving activation of peroxisomes, oxidative stress, mitochondrial dysfunction, and induction of apoptosis [17–19] as well as protective capacity by stimulating the synthesis of diacylglycerides and triacylglycerides [2,17]. However, it is unclear which cellular event triggers lipotoxicity of free fatty acids. One candidate is the cellular nitric oxide (NO) concentration. NO affects cellular concentration of reactive oxygen species (ROS) by interacting with cytochrome c oxidase [20–22], with superoxide anion radicals forming the reactive peoxynitrite and at high concentrations as an antioxidant [23]. Moreover, NO is a regulator of TG synthesis in hepatocytes [24].

To address the early event triggering lipotoxicity of FFAs, we exposed rat hepatocytes of a primary cell culture over 48 h to either linoleic acid or palmitate in the presence of insulin and glucose. Under these conditions, we measured cell viability, cellular nitrite/nitrate content, protein of inducible nitric oxide synthesis and cellular TG content. Additionally, F_2-isoprostanes were determined as a biomarker of oxidative stress and oxidized cardiolipin as an indicator of mitochondrial ROS generation. We found that free fatty acid-dependent NO production triggers accumulation of TG and oxidative stress in hepatocytes.

Materials and methods

Chemicals

Porcine insulin was from Novo Biolabs (Bagsvaerd, Denmark). Collagenase A was from Biochrom AG (Berlin, Germany), M199 medium and bovine serum albumin (BSA) were from Roche Molecular Biochemicals (Mannheim, Germany). Foetal calf serum was obtained from Life Technologies (Karlsruhe, Germany). All other reagents were from Sigma (München, Germany).

Cell culture

Hepatocytes from fed male Wistar rats (180–250 g) were isolated by collagenase perfusion and purified by centrifugation through Percoll [25]; 35 mm dishes were pre-coated with collagen prepared by dissolving rat tail tendons (1 g/300 ml) in 0.1% acetic acid for 24 h. The M199 culture medium additionally contained 15 mM Hepes, 4% new-born calf serum, 10 mM glucose, 0.2% BSA and 10^{-7} M insulin. Each dish contained 10^6 cells/ml and 2 ml of culture medium. The medium was changed after 4 h and 24 h. The hepatocytes were cultured over 48 h in a gas atmosphere containing 5% CO_2, 17% O_2 and 78% N_2 at 37°C. The cells were incubated in the additional presence of (i) 100 µM palmitate, (ii) 100 µM linoleic acid, and (iii) the combination of them.

Determination of reduced MTT

Cells were incubated with 0.15 mg/ml MTT in 2 ml medium for 30 min at 37°C in a CO_2 incubator [26]. At the end of the incubation, the medium was removed and the formazan was extracted with 1 ml dimethylsulphoxide. The quantity of formazan was determined by measuring the absorption at 550 nm in the presence of 20 µl of 0.1 M NaOH to avoid pH-dependent changes of absorption.

Determination of TG content

A sample of 20 µl cell suspension obtained per dish was used for TG determination. TG concentration was determined automatically by using a Hitachi 912 analyser (Bosch, Germany).

NO detection in primary hepatocyte cultures

NO production in hepatocytes was quantified indirectly by using a spectrophotometric method based on the Griess reaction for nitrite detection [27]. The supernatant of the cell culture was mixed with 1 ml of Griess reagent containing one part of 1% NEED solution [N-(naphthyl)ethylenediamine dihydrochloride] and one part of 1% sulphanilamide in 5% aqueous phosphoric acid. After 10 min incubation at room temperature the absorption was measured at the wavelength of 540 nm. The quantitative analysis was undertaken by using nitrite standard solutions.

Western Blot analysis of iNOS

After a cycle of three times freezing and thawing, cells were mixed with protein loading buffer (roti-Load 1, Carl Roth GmbH, Karlsruhe, Germany) according to the manufacturer ´s procedure for immunoblotting. Afterwards, the cells were placed in a heating bath

(95°C) for 5 min. Proteins were separated using SDS-PAGE (gradient gels from 5–25%). The protein amount loaded per lane was 20 µg. After separation the proteins were stained with Comassie brilliant blue or transferred to nitrocellulose paper and unspecific protein binding sites were blocked with blocking buffer (Chemicon International, Hofheim, Germany). The blots were incubated overnight with a primary antibody against iNOS (polyclonal, BD bioscience, Heidelberg, Germany, 1:5000) followed by incubation with a horseradish peroxidase-conjugated secondary antibody (anti rabbit igG, Cell Signalling Technology, Beverly, MA). Additionally, GAPDH detection was used to control equal sample loading.

Determination of F_2-isoprostanes (F_2-IsoPs) in cultured hepatocytes

The determination of the concentrations of F_2-IsoPs (sum of esterified and non-esterified compounds) was carried out as described previously [28,29] with some modifications: To hydrolyse esterified F_2-IsoPs, the hepatocyte samples (150 µl containing ~ 0.5–1 × 10^6 cells/ml and 1.5 µl 10 mM butylated hydroxytoluene to avoid autoxidation) were treated with 125 µl KOH (1 M) at 45°C for 30 min. Thereafter, the samples were neutralized by addition of 4 ml 0.01 M HCl and the pH was adjusted to ~ 2 with 1 M HCl. 9α,11α-$PGF_{2\alpha}$-d_4 (Axxora GmbH, Lörrach, Germany; 0.5 ng in 10 µl ethanol) was added as internal standard. The samples were applied onto a C18-cartridge (Vac 3cc 500 mg, Waters Corporation, Milford, Massachusetts, USA), pre-washed with 5 ml of methanol and 5 ml of water (pH 2). The cartridge was then washed with 10 ml water (pH 2) and 10 ml of acetonitrile/water (15/85, v/v). Isoprostanes were eluted from the column with 5 ml of n-hexane/ethylacetate/2-propanol (30/65/5, v/v/v). The isoprostane containing extract was evaporated to dryness under a stream of argon at 45°C. The residues were reconstituted with 40 µl of pentafluorobenzyl-bromide (10% in acetonitrile) and 20 µl of *N,N*-diisopropylethylamine (10% in acetonitrile) and incubated at 45°C for 30 min. Thereafter, 50 µl of *bis*-(trimethylsilyl) trifluoroacetamide (BSTFA) and 5 µl of *N,N*-diisopropyl-ethylamine were added to the dried sample. The samples were incubated at 45°C for 60 min, the solvents were removed and the samples were reconstituted in 60 µl isooctane containing 0.1% BSTFA. F_2-IsoPs were separated and measured by gas chromatography-mass spectrometry/negative-ion chemical ionization assay (DSQ/Trace GC Ultra, Thermo Fisher Scientific, Dreieich, Germany) using a DB 5-MS column (50 m × 0.25 mm inner diameter; 0.25 µm film thickness; J&W Scientific, Folsom, CA) with the following temperature programme: initial temperature of 80°C for 2 min; with a rate of 30°C/min to a final temperature of 280°C maintained for 19 min; total run time: 27.7 min. Quantitative analysis was performed with ammonia as reagent gas using selected ion monitoring (SIM) of the carboxylate anion $[M\text{-}181]^-$ at *m/z* 569 and 573 for F_2-IsoPs and $9_\alpha,11_\alpha$-$PGF_{2\alpha}$-d_4 (internal standard), respectively. All analyses were performed at least in triplicate, throughout for each sample.

Quantification of cardiolipin species and oxidized cardiolipin by ESI-MS/MS

Extraction. CL was extracted from the samples by a modified Folch extraction procedure [30]. Briefly, 50 ng of tetra-myristoyl-CL [$(C14:0)_4$-CL; Avanti Polar Lipids Inc. Alabaster, AL] were added as the internal standard to 10 µl incubation mixture. The extraction of CL was carried out with chloroform/methanol (2/1) containing 0.05% BHT as antioxidant. Aqueous and lipid phases were separated by adding 0.01 M HCl followed by intensive shaking and subsequent centrifugation. The lower lipid phase was collected, dried under nitrogen atmosphere and acidified as described by Schlame et al. [31]. Ice-cold methanol (2 ml), chloroform (1 ml) and 0.1 M HCl (1 ml) were added to the sample. The solution was intensively mixed and incubated for 5 min on ice. Phase separation was achieved by addition of 1 ml of $CHCl_3$ and 1 ml of 0.1 M HCl. The chloroform phase was recovered as a CL-containing sample. Finally, the sample was dried under nitrogen, dissolved in 0.8 ml $CHCl_3$/CH_3OH/H_2O (50/45/5, v/v/v), mixed and filtered over 0.2 µm PTFE membranes. This sample was ready for immediate ESI-MS/MS analysis according to Valianpour et al. [32].

HPLC-MS/MS. A TSQ Quantum Discovery Max (Thermo Fisher Scientific GmbH, Dreieich, Germany) was used in the negative ion electrospray ionization (ESI) mode. The HPLC system consisted of a Surveyor MS quaternary narrow bore pump with integrated vacuum degasser and a Surveyor auto sampler. The auto sampler tray temperature was maintained at 8°C; 10 µl of the lipid extract dissolved in chloroform/methanol/water (50/45/5) was injected (in partial loop mode) and CL was separated by using a LiChroCart column (125 × 2 mm), LiChrospher Si60 (5 µm particle diameter; Merck, Darmstadt, Germany) and a linear gradient between solution A (chloroform) and solution B (methanol/water, 9:1 (v/v)). Both solutions additionally contained 0.1 ml/l of 25% aqueous ammonia. The gradient was as follows: 0–0.2 min, 92% A, 8% B, 0.2–4.5 min, 92–30% A and 8–70% B, 4.5–6 min, 30% A, 70% B, 6–6.5 min 30–92% A, 70–8% B, 6.5–11 min, 92% A, 8% B. The flow rate was 300 µl/min.

The total time of analysis was 11 min. The eluate between 0.3–6 min was introduced into the mass spectrometer. Nitrogen was used as the nebulizing gas

and argon was used as the collision gas at a pressure of 1.5 mTorr. The spray voltage was 3.5 kV, the ion source capillary temperature was set at 375°C and the cone-voltage was 30 V. Daughter fragments from the doubly charged parent derived from $(C18:2)_4$-CL with *m*/*z* (mass to charge ratio) 723.6 ($(M– 2H)^{2–}/2$) were obtained using a collision energy of 36 eV. This molecular CL species and the internal standard (*m*/*z* 619.6) were analysed by mass transfer reaction monitoring their doubly charged ions and their respective fatty acids linoleic acid *m*/*z* 279.2 and myristic acid *m*/*z* 227.2 using the selected reaction monitoring (SRM) mode. The same approach was used for parent and daughter fragments of other molecular CL species (see Table I). The quantity of these molecular CL species was related to $(C18:2)_4$-CL.

[$(C18:2)_3$-monohydroxylinoleic acid-CL] as oxidized CL species was measured in the SRM mode as a transition from *m*/*z* 731.6 to *m*/*z* 279.2 (linoleic acid) according to [33,34].

To identify CL species in hepatocytes, we first separated CL by thin layer chromatography. In the next step, we used the separated CL to run parent scans for C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:3 and C20:2 quantitatively occurring in hepatocytes. The found parent peaks were used to run product scans to find other fatty acid residues and to characterize CL composition. At the end of this procedure we identified seven CL species listed in Table I.

Determination of protein

The cells were dissolved in 1% dodecylsulphate in the presence of 0.1 M NaOH. The amount of protein was determined by applying the method of Lowry et al. [35] using bovine serum albumin as the standard.

Statistics

Data are presented as mean ± SEM. Statistical analysis is outlined in the legends to the figures. Different conditions were compared with ANOVA for repeated measures or with global non-parametric tests with Friedman test. The global univariate test with Greenhous-Geisser correction was followed by post-hoc pairwise comparisons (unadjusted or with Bonferoni correction). Alternatively, post-comparisons of selected pairs were performed with Wilcoxon matched-pairs signed rank test (unadjusted).

Results

Effect of linoleic acid and palmitate on cell viability

Freshly isolated hepatocytes were cultured over 48 h in the presence of 10 mM glucose and 10^{-7} M insulin. The amount of protein was determined as a parameter of cell quantity. The culture started with 10^6 cells/ml corresponding to 1.23 ± 0.122 mg/ml ($n = 6$). After 4 h, the first change of medium was performed. Only adherent cells remained in the dishes. Thereby, the protein content dropped to 0.47 ± 0.06 mg/ml ($n = 6$). The viability was not affected within the first 24 h of the culture. The free fatty acids palmitate and linoleic acid had no significant effect on the viability within this period of time. For further considerations the protein content after 24 h of cultivation (0.47 ± 0.06 mg/ml) was set to 100% (control). Protein data measured after 48 h are presented in Figure 1A. Without any further addition ('without') the protein content decreased to ~ 91% of control. The presence of palmitate as well as linoleic acid caused a decrease in protein content to ~ 75% of control, indicating loss of cells. In comparison to untreated control, there was the tendency of decreased values (pairwise unadjusted comparison) but no significant difference was found (with Bonferoni correction). The investigation of MTT reduction by hepatocytes revealed that the presence of palmitate as well as linoleic acid in the culture medium tended to increase MTT reduction within the cells by a factor of ~ 1.4 after 48 h of cultivation (Figure 1B). In comparison to control, we did

Table I. Effect of palmitate and linoleic acid on the total CL content and distribution of molecular CL species in hepatocytes.

	Content		
Treatment	Control	Palmitate	Linoleate
Total cardiolipin (μg/mg protein)	3.04 ± 0.21	3.80 ± 0.38	2.95 ± 0.67
Cardiolipin species (% of total)			
$(C18:2)_4$	48.24 ± 1.35	47.20 ± 1.47	46.84 ± 1.53
$(C18:2)_3(C18:1)_1$	29.45 ± 0.37	29.54 ± 0.32	30.05 ± 0.54
$(C18:2)_2(C18:1)_2$	8.93 ± 0.52	9.01 ± 0.46	9.66 ± 0.59
$(C18:2)_3(C16:1)_1$	5.43 ± 0.41	5.66 ± 0.54	5.67 ± 0.50
$(C18:2)_3(C20:3)_1$	3.53 ± 0.08	4.06 ± 0.19	3.27 ± 0.17
$(C18:2)_2(C18:1)_1(C16:1)_1$	3.35 ± 0.31	3.48 ± 0.38	3.23 ± 0.32
$(C18:1)_2(C18:2)_1(C16:1)_1$/ $(C18:2)_2(C18:1)_1(C16:0)_1$	1.06 ± 0.05	1.04 ± 0.05	1.28 ± 0.06

10^6 hepatocytes/ml of a primary culture were exposed to palmitate or linoleic acid at 37°C in a CO_2 incubator. Treatment was as described in the legend of Figure 1. Untreated hepatocytes were considered as control. The data are presented as mean ± SEM ($n = 6$).

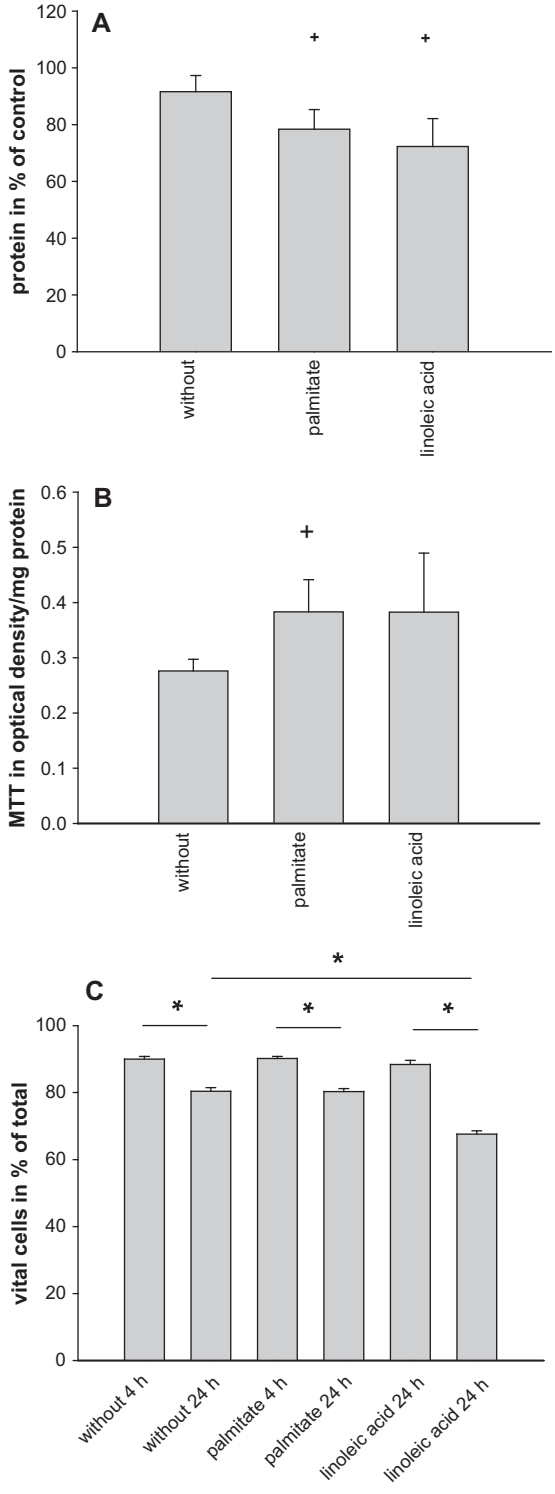

Figure 1. Effect of palmitate and linoleic acid on viability of hepatocytes. 10^6 hepatocytes/ml of a primary culture were exposed to palmitate or linoleic acid at 37°C in a CO_2 incubator. Protein data (A) and MTT values related to protein (B) measured after 48 h of cultivation and cell viability determined by applying the trypan blue exclusion test (C) are shown. Protein values after 24 h of cultivation were set to 100% (control). Treatment: without—without additions; palmitate—100 μM palmitate; linoleic acid—100 μM linoleic acid. Data are presented as mean ± SEM. +, Pairwise unadjusted comparisons showed tendencies in the differences to control for palmitate with $p = 0.019$, linoleic acid with $p = 0.026$ in (A) and palmitate with $p = 0.043$ in (B). *Pairwise comparisons with Bonfereoni correction were significant with $p < 0.05$.

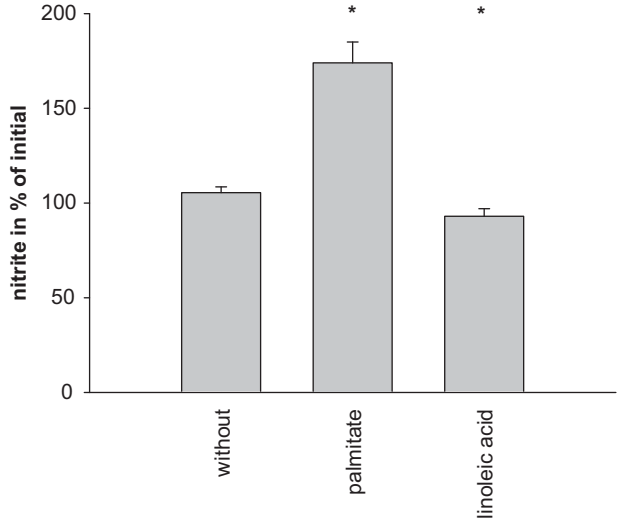

Figure 2. Effect of palmitate and linoleic acid on nitrite content of hepatocytes. 10^6 hepatocytes/ml of a primary culture were exposed to palmitate or linoleic acid at 37°C in a CO_2 incubator. After 48 h of cultivation, nitrite levels were determined as described in Methods. Treatment was as outlined in the legend of Data are presented as mean ± SEM. *Difference to control is significant with $p < 0.05$.

not find any decrease in MTT reduction due to the additions. To test the viability of the hepatocytes in more detail, we applied the trypan blue exclusion test. The percentage of viable cells dropped from ~ 90% to ~ 80% within 24 h of cultivation without treatment and in the presence of 100 μM palmitate. Linoleic acid caused a decrease in viability to 67.6 ± 1.0%, indicating significant cell injury (Figure 1C).

Effect of linoleic acid and palmitate on cellular NO

To evaluate whether free fatty acids influence NO metabolism, we exposed hepatocytes of a primary cell culture to 100 μM palmitate or to 100 μM linoleic acid over 48 h of cultivation and measured nitrite concentration in relation to cellular protein (Figure 2). Selective effects of these free fatty acids were observed. The continuous presence of palmitate caused increase in nitrite content by a factor of 1.65 in comparison to untreated cells ('without'). In contrast, linoleic acid slightly diminished cellular nitrite concentration to ~ 90% of control.

In a separate set of experiments, we studied the expression of iNOS protein in dependence of linoleic acid and palmitate by Western blot analysis. The corresponding data are presented in Figure 3. For better comparison of band intensities of different gels, the band intensity of controls after 48 h of cultivation was set to 100% for each gel. Even under control conditions, iNOS protein significantly increased within 48 h of cultivation. The same tendency was observed in the presence of palmitate and linoleic acid, indicating stimulation of expression of iNOS protein during cultivation. However, palmitate-induced increase in iNOS protein

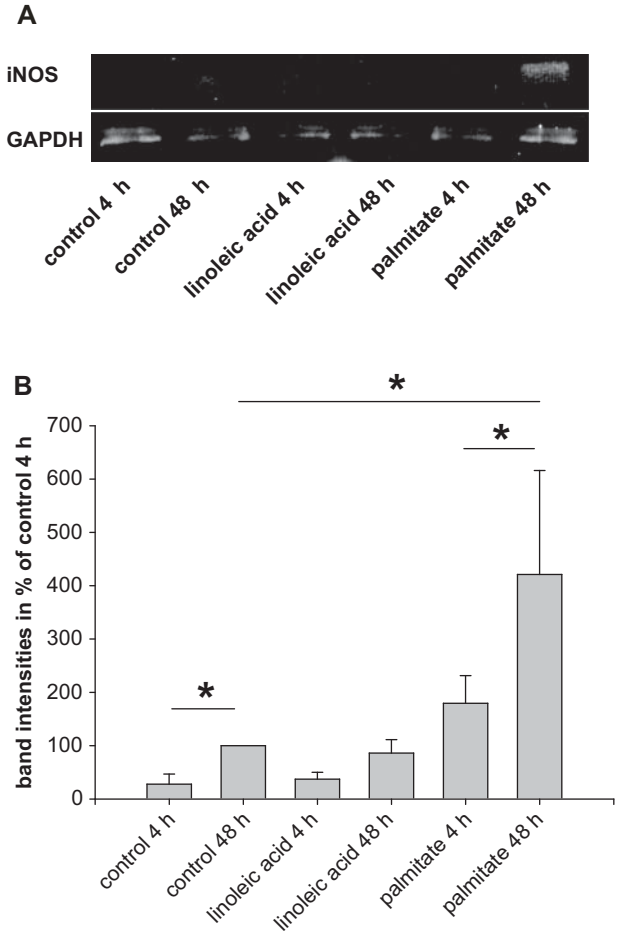

Figure 3. Effect of palmitate and linoleic acid on iNOS expression in hepatocytes. 10^6 hepatocytes/ml of a primary culture were exposed to palmitate or linoleic acid at 37°C in a CO_2 incubator. After 48 h of cultivation, the expression of the iNOS protein was analysed as described in Methods. (A) Western blot of iNOS protein. (B) Relative band intensities. The band intensity without additions after 48 h of cultivation was set to 100% for better comparison. Presented are mean values ± SEM from six cultures. *Difference is significant with $p < 0.05$.

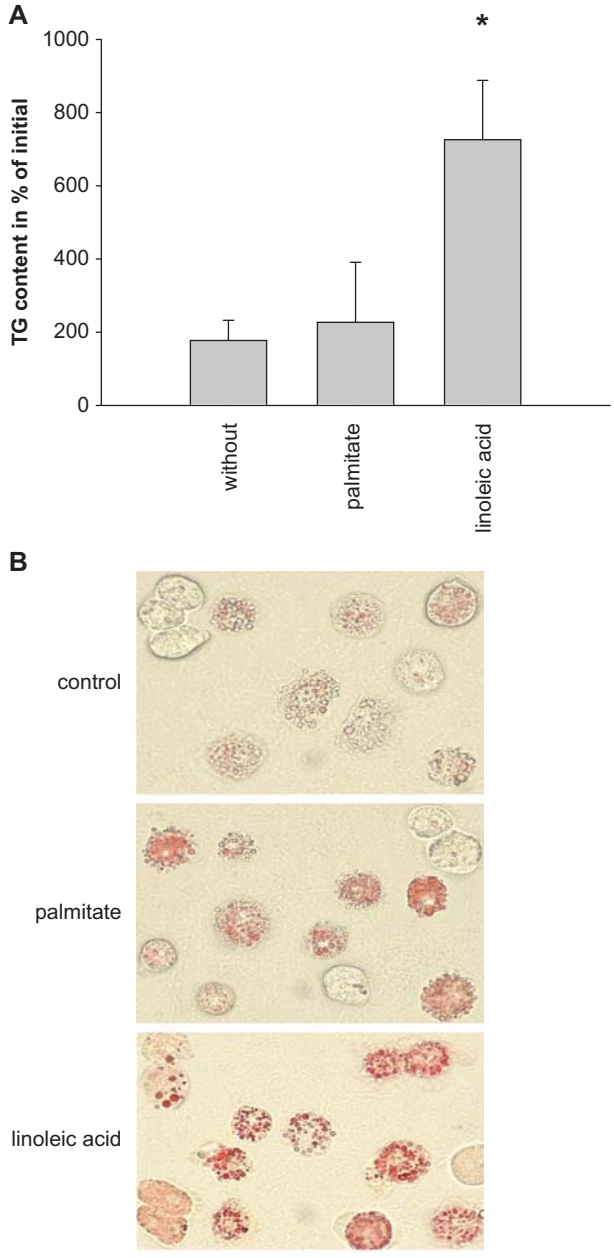

Figure 4. Effect of palmitate and linoleic acid on TG content in hepatocytes. 10^6 hepatocytes/ml of a primary culture were exposed to palmitate or linoleic at 37°C in a CO_2 incubator. The TG content (A) and histological analysis of neutral fat accumulation (B) determined after 48 h of cultivation are presented. The data of TG content are presented as mean ± SEM. *Difference to control is significant with $p < 0.05$. A typical micrograph of a Sudan red staining is shown (magnification ×400). Treatment was as described in the legend of Figure 1.

was ~ 4-fold higher in comparison to control conditions. In contrast, linoleic acid-induced increase in iNOS protein was in tendency lower than in controls.

Effect of linoleic acid and palmitate on cellular TG content

Free fatty acids can be metabolized in hepatocytes in several ways. Among them, they contribute to energy production via *β*-oxidation, to energy storage in form of triglyceride depots, and to phospholipid as well as to cholesterol synthesis. To investigate effects of free fatty acids on the lipid content in hepatocytes, we challenged the cells with 100 µM palmitate or 100 µM linoleic acid and determined after 48 h the TG content in the cells (Figure 4A). The presence of linoleic acid in the culture medium caused significant increases in the cellular TG content to 726 ± 162% of initial. In contrast, palmitate led only to a moderate increase in cellular TG content after 48 h of cultivation to 227 ± 164% of initial. Under control conditions, the content of TGs was 0.197 ± 0.02 mmol/l.

The Sudan red staining of hepatocytes after 48 h of cultivation revealed an accumulation of neutral fat as the result of exposure to 100 µM linoleic acid (Figure 4B, lower part). Much less staining was observed in non-treated cells and in cells exposed to 100 µM palmitate (Figure 4B, upper and middle part).

Exposure of hepatocytes to linoleic acid and palmitate and oxidative stress

Since NO and free fatty acids can affect the generation of reactive oxygen species (ROS), we studied oxidation of arachidonic acid by measuring F_2-isoprostanes and oxidation of cardiolipin (CL) by measuring the content of $[C18:2]_3$-monohydroxylinoleic acid-CL] as a parameter of oxidative stress.

The mitochondrial phospholipid CL is localized in the mitochondrial membrane system. Therefore, oxidation of CL particularly indicates mitochondrially generated ROS. We identified seven individual molecular CL species in hepatocytes by using HPLC-MS/MS. The sum of these molecular CL species as well as the distribution in dependence on the treatment during cultivation is presented in Table I. In hepatocytes, the most abundant molecular CL species are $[(C18:2)_4$-CL] and $[(C18:2)_3(C18:1)_1$-CL], which represent ~ 78% of total CL. In total, we found 3.04 ± 0.21 μg CL/mg protein in hepatocytes. The free fatty acids palmitate and linoleic acid had no significant effect either on the total content of CL or on the distribution of the molecular CL species.

Arachidonic acid is found in nearly all membrane phospholipids of hepatocytes. Therefore, the oxidation of arachidonic acid to F_2-isoprostanes reflects generation of ROS by all cellular sources. The treatment of hepatocytes with palmitate had no effect. However, the presence of linoleic acid in the culture medium specifically caused a significant increase in both F_2-isoprostanes and oxidized CL concentration (Figure 5). The content of F_2-isoprostanes was increased to 486.2 ± 149% of untreated control, whereas oxidized CL increased to 836.4 ± 112% of untreated control. The values of untreated controls were 0.64 ± 0.20 ng/mg protein (F_2-isoprostanes) and 3.04 ± 0.21 μg/mg protein (oxidized CL), respectively.

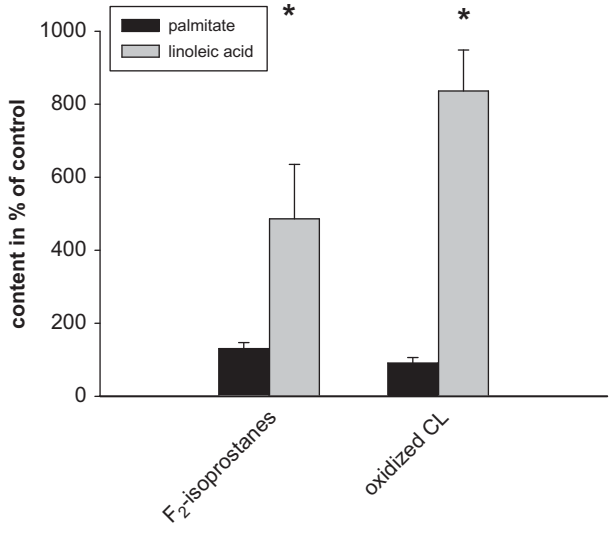

Figure 5. Effect of palmitate and linoleic acid on the oxidation of arachidonic acid and CL in hepatocytes. 10^6 hepatocytes/ml of a primary culture were exposed to palmitate or linoleic acid at 37°C in a CO_2 incubator. Hepatocytes were exposed to either 100 μM palmitate (palmitate) or to 100 μM linoleic acid (linoleic acid). F_2-isoprostanes as oxidation products of arachidonic acid and oxidized CL were determined after 48 h of cultivation. The data are related to untreated hepatocytes (control) and are expressed as mean ± SEM. *Difference to control was significant with $p < 0.05$. For statistical analysis logarithms of the values were used in order to compensate for inhomogeneous variances in the three groups.

Discussion

Primary hepatocyte culture

In this study, primary cultures of rat hepatocytes were used to investigate the capability of palmitate and linoleic acid to cause injury of hepatocytes in the presence of high insulin and glucose concentration. In our experiments, hepatocytes kept viable over 48 h when cultured in the presence of 10 mM glucose and 10^{-7} M insulin (control). Loss of protein, MTT reduction and trypan blue exclusion was less than 5%. It had been shown that under this condition the stimulatory effect of insulin for glycolysis is completely abolished and the stimulation of glycogen synthesis by insulin is restricted to ~ 33% of maximal rate in the absence of dexamethasone [36]. In this context, it had been demonstrated that insulin binding is not affected and glucose transport is not essentially limited in primary hepatocyte cultures. The authors [36] attribute the restrictions in glycolysis and glycogen synthesis to the intra-cellular insulin signal cascade, but not to altered binding of insulin to the receptor. Obviously, alternative pathways are sufficient to ensure cellular energy supply.

Effect of palmitate and linoleic acid on cell viability

Free fatty acids may induce detrimental effects in hepatocytes. It had been reported that exposure of hepatocytes to palmitic acid caused in ~ 40% of the cells apoptosis within 24 h of cultivation [37]. This observation seems to be in contrast to our data showing only a slight decrease in protein content of hepatocytes subjected to palmitic acid over 48 h. Apoptosis-inducing effects of palmitic acid had been also reported by other authors [38,39]. These investigations are common in the fact that cells were cultured in the absence of insulin. In our experiments, however, hepatocytes were cultured in the presence of high insulin concentration. It is known that insulin promotes cell survival, in part, through inhibition of apoptosis [40,41]. It had been demonstrated that insulin inhibited palmitate-induced apoptosis in a dose-dependent manner in H4IIE hepatoma cells [42].

Linoleic acid caused a slight tendency of protein loss. Oxidative stress that was detected during exposure of hepatocytes with linoleic acid may be the reason for the observed cell degradation.

Although we did not observe a rise in cell number during cultivation, palmitic acid and linoleic acid caused an increase in MTT reduction, indicating an increased supply of reducing equivalents to cellular dehydrogenases. It is reasonable to assume that the operation of β-oxidation of fatty acids significantly contributes to the supply of reducing equivalents and concomitantly to MTT reduction.

Effect of palmitate and linoleic acid on cellular NO

It is known that changes in NO concentration may be of impact for cellular function. Besides inhibition of electron transport within the respiratory chain at the level of cytochrome *c* oxidase, regulation of lipid metabolism by NO had been reported. NO limits the expression of citric acid synthase and concomitantly the synthesis of fatty acids in liver [43]. Furthermore, it is known that NO stimulates mitochondrial biogenesis [44]. Further, the reaction of NO with superoxide anion radicals results in decreased concentrations of superoxide radicals anions but increased concentrations of the reactive peroxynitrite [45]. However, NO in excess can act as an antioxidant [46].

Increased expression of the inducible NO synthase (iNOS) in liver had been shown in patients with non-alcoholic steatohepatitis (NASH) [47] and in *ob*/*ob* mice [48]. It had been further demonstrated that nutritional lipids can cause increase in iNOS protein in liver [16,49]. It is shown that iNOS can be expressed in hepatocytes, the main cellular component in the liver. We here report that free fatty acids can modulate NO concentration in primary cultures of hepatocytes. It is, however, important to note that the direction of changes in cellular NO concentration depends on the type of fatty acid. Palmitate caused an increase, whereas linoleic acid slightly decreased NO concentration. In our experiments changes in cellular NO are essentially attributed to iNOS since an increase in NO concentration was accompanied by an increase in iNOS expression. Cellular generated NO reacts with many molecules. Thus, palmitate-mediated induction of iNOS expression may be associated with an increased number of reactants within the cell. Therefore, extracellular measured nitrite must not necessarily quantitatively reflect an increase in iNOS protein.

Effect of palmitate and linoleic acid on cellular lipid content

In liver, fatty acids are used for β-oxidation and lipid synthesis. Both palmitic acid and linoleic acid are metabolized via β-oxidation. We conclude from our observation that palmitic acid and linoleic acid caused an increase in MTT reduction. It is reasonable to assume that, in comparison to linoleic acid, palmitic acid was preferentially metabolized via β-oxidation, since β-oxidation of unsaturated fatty acids is more expensive. Therefore, linoleic acid should contribute more than palmitate to lipid synthesis. The main products of lipid synthesis are TGs. In fact, TG accumulation had been demonstrated in NAFLD [3]. Our histological evaluation of hepatocytes exposed to linoleic acid also revealed TG accumulation. It is known that the main part of fatty acid residues in TGs is composed of unsaturated fatty acids [3]. Therefore, it is likely that linoleic acid is more efficiently used for triacyglyceride synthesis and contributes more than palmitic acid to this type of neutral fat. In line with this view is our finding that linoleic acid caused significant TG accumulation, whereas palmitic acid contributed to TG accumulation only to a minor extent. In our experiments, we did not analyse the fatty acid composition of accumulated TGs.

Besides direct utilization of free fatty acids for lipid synthesis, *de novo* synthesis of fatty acids contributes to lipid synthesis in hepatocytes. A pre-requisite of *de novo* fatty acid synthesis are high NADPH levels. Under our experimental conditions, the operation of the pentose phosphate pathway probably promoted high cellular NADPH concentrations. In this context, a regulatory function of NO had been observed. High concentrations of NO limit fatty acid *de novo* synthesis, whereas low NO concentrations support fatty acid synthesis [50]. Therefore, our findings demonstrating an inverse correlation between nitrite and lipid accumulation support the suggestion that *de novo* fatty acid synthesis significantly contributes to lipid accumulation. In this view, linoleic acid supports FFA synthesis and concomitantly TG synthesis via low NO concentrations.

Effect of palmitate and linoleic acid on oxidative stress

There is consensus about detrimental effects of high cellular ROS concentration. ROS impair biological function of DNA molecules, proteins and lipids by oxidative attack. Mostly, ROS are generated in the neighbourhood of cellular membranes. Important cellular membrane-associated ROS generators are complexes of the mitochondrial electron transport chain and NADPH oxidase. Therefore, membrane lipids are preferred targets of ROS attack. In this context, we investigated the oxidation of arachidonic acid that is mainly bound to phospholipids within all types of cellular membranes and of cardiolipin that is—according to the current view—exclusively localized within mitochondria. The observed oxidation of arachidonic acid and cardiolipin documents oxidative stress when hepatocytes are exposed to high glucose and insulin concentration in combination with linoleic acid. This result is in line with a study demonstrating that a diet with high amounts of linoleic acid causes increased concentrations of F_2-isoprostanes in the urine of healthy subjects [15]. This finding had

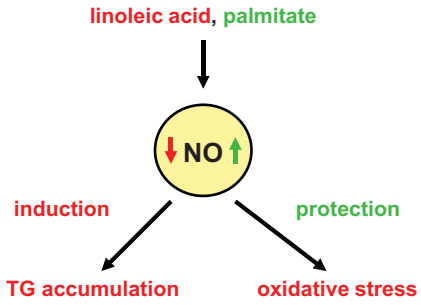

Figure 6. Schematic illustration of the central role of NO in regulating fatty acid induced oxidative stress and TG accumulation in hepatocytes.

been attributed to the precursor function of linoleic acid for arachidonic acid synthesis [15].

In contrast to our previous *in vitro* experiments demonstrating a correlation of cardiolipin oxidation and decrease in cardiolipin content [36], oxidation of cardiolipin in hepatocytes was not accompanied by a decrease in cardiolipin content. High concentrations of extracellular linoleic acid could stimulate CL synthesis, possibly counterbalancing cardiolipin degradation caused by oxidative stress. We interpret increases in F_2-isoprostane concentration and oxidized cardiolipin concentration as indications of oxidative stress with a significant contribution of mitochondria.

Conclusions

Our data document the central role of NO in the regulation of lipid accumulation and oxidative stress in hepatocytes subjected to high glucose and insulin concentration in combination with free fatty acids. Increase in cellular NO concentration protected hepatocytes from oxidative stress and lipid accumulation, whereas a slight decrease caused oxidative stress and lipid accumulation. Therefore, we attribute the protective effect of palmitic acid to the ability to cause an increase in NO concentration. In contrast, linoleic acid caused a slight decrease in NO concentration associated with oxidative stress and lipid accumulation (Figure 6).

Disturbed insulin signalling as well as high extracellular insulin and glucose concentration are hallmarks of diabetes mellitus type II. Our data show that free fatty acids possibly originating from nutrition or from fat depots may essentially contribute to liver impairment. In this context, the type of free fatty acids seems to be of crucial importance. Based on our data, we conclude that an excess of linoleic acid in combination with high glucose and insulin concentration may significantly contribute to the pathogenesis of NAFLD.

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