Free fatty acids shift insulin-induced hepatocyte proliferation towards CD95-dependent apoptosis*

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*Running title: CD95-dependent apoptosis in response to FFA and insulin

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Key words: Epidermal growth factor receptor, CD95, c-Jun-N-terminal kinase, JNK

Background: Hyperinsulinemia and increased blood levels of free fatty acids (FFA) trigger non-alcoholic steatohepatitis (NASH).

Results: Insulin induces EGFR-activation and proliferation. In presence of FFA, however, insulin triggers apoptosis as EGFR triggers CD95-activation JNK-dependently.

Conclusion: Insulin-induced EGFR-activation triggers proliferation, but shifts to CD95-dependent apoptosis when a JNK-signal is provided by FFA.

Significance: The study provides new insights into the pathogenesis of NASH.

ABSTRACT

Insulin is known to induce hepatocyte swelling, which triggers via integrins and c-Src kinase an activation of the epidermal growth factor receptor (EGFR) and subsequent cell proliferation (1). Free fatty acids (FFAs) are known to induce lipoapoptosis in liver cells in a c-Jun-NH₂-terminal kinase (JNK)-dependent, but death receptor-independent way (2). As non-alcoholic steatohepatitis (NASH) is associated with hyperinsulinemia and increased FFA-blood levels, the interplay between insulin and FFA was studied with regard to hepatocyte proliferation and apoptosis in isolated rat and mouse hepatocytes.

Saturated long chain FFAs induced apoptosis and JNK activation in primary rat hepatocytes, but did not activate the CD95 (Fas, APO-1) system, whereas insulin triggered EGFR activation and hepatocyte proliferation. Coincubation of insulin and FFAs, however, abolished hepatocyte proliferation and triggered CD95-dependent apoptosis due to a JNK-dependent association of the activated EGFR with CD95, subsequent CD95 tyrosine phosphorylation and formation of the death-inducing signaling complex (DISC). JNK inhibition restored the proliferative insulin effect in presence of FFAs and prevented EGFR/CD95 association, CD95 tyrosine phosphorylation and DISC formation. Likewise, in presence of FFAs insulin increased apoptosis in hepatocytes from wild type but not from Alb-Cre-FASLox/− mice, which lack functional CD95. It is concluded that FFAs can shift insulin-induced hepatocyte proliferation towards hepatocyte apoptosis by triggering a JNK signal, which allows activated EGFR to associate with CD95 and to trigger CD95-dependent apoptosis. Such phenomena may contribute to the pathogenesis of NASH.

Apart from its metabolic effects, insulin stimulates cell proliferation in the liver and in other organs (for a review, see Refs. 3-7). As shown recently in rat hepatocytes, insulin leads to an activating epidermal growth factor receptor (EGFR) tyrosine phosphorylation at positions Tyr845 and Tyr1173, which is triggered by insulin-induced hepatocyte swelling and activation of a recently described swelling-induced osmosignaling pathway which involves osmosensing by β₁ integrins and c-Src activation (1, 8-9).

Free fatty acids (FFAs) are known to induce lipoapoptosis in hepatocytes (2). The molecular mechanisms leading to hepatocyte lipoapoptosis are not fully defined; however, recent studies suggest that FFAs induce
lipoapoptosis in hepatocytes in a c-Jun NH$_2$-terminal kinase (JNK)-dependent, but death receptor-independent way (2). JNKs have also been implicated in the pathogenesis of non-alcoholic steatohepatitis (NASH) in both, murine models (11, 12) and humans (13, 14). In NASH patients however, upregulation of death receptors, such as TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (15, 16) and CD95 (Fas, APO-1), was also discussed to sensitize hepatocytes towards the extrinsic pathway of apoptosis (17).

JNKs also play a crucial role in CD95-dependent hepatocyte apoptosis triggered by CD95 ligand (CD95L), hydrophobic bile acids or hyperosmolarity (for a review, see Ref. 18). Here, proapoptotic stimuli induce the generation of reactive oxygen species (ROS) leading to both, a Yes kinase-mediated EGFR transactivation and JNK activation. The JNK signal then allows the activated EGFR to associate with CD95 and to tyrosine-phosphorylate CD95, which triggers CD95 oligomerization, translocation of the EGFR/CD95 complex to plasma membrane and formation of the death-inducing signaling complex (DISC) (for a review, see Ref. 18).

The aim of the present study was to elucidate, whether FFAs can shift insulin-induced hepatocyte proliferation to hepatocyte apoptosis in a CD95-dependent manner. This may be relevant in the pathogenesis of non-alcoholic fatty liver disease (NAFLD), such as NASH which is frequently accompanied by peripheral insulin resistance and elevated serum concentrations of FFA (13-23), and hyperinsulinemia (24-26). The present study shows that the FFA-induced JNK-activation directs insulin-induced EGFR-activation from proliferation to EGFR/CD95-association, EGFR-mediated CD95-tyrosine phosphorylation and subsequent activation of the CD95-mediated apoptotic machinery. Thus, FFA-induced JNK-activation provides a switch from insulin-induced hepatocyte proliferation towards CD95-mediated apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials** – The materials used were purchased as follows: William’s Medium E from Biochrom (Berlin, Germany), penicillin/streptomycin and FBS (fetal bovine serum) from Life Technologies GmbH (Darmstadt, Germany); soluble CD95L was obtained from Enzo Life Sciences (Lörrach, Germany) and was always employed with a 10-fold amount of enhancer protein as provided by the supplier. SP600125 was purchased from Tocris/Biozol (Eching, Germany). Collagenase, insulin, FFA-free low endotoxin BSA (bovine serum albumin), caprylate, palmiate, stearate, oleate and linoleate were from Sigma Aldrich (Munich, Germany). 5-(and 6)-Chloromethyl-2,7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA), ProLong® Gold Antifade reagent with DAPI (4′, 6-diamidino-2-phenylindole, dihydrochloride) was from Life Technologies GmbH (Darmstadt, Germany), horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG from Bio-Rad Laboratories (Munich, Germany) and Dako (Hamburg, Germany). The antibodies used were purchased as follows: antibodies recognizing p38 mitogen-activated protein kinase (p38MAPK), caspase 8, Fas-associated death domain (FADD), CD95 and EGFR (immunoprecipitation) were from Santa Cruz Biotechnology (Heidelberg, Germany), extracellular regulated kinase (Erk)-1/-2, EGFR and phospho-tyrosine from Merck-Millipore (Darmstadt, Germany), phospho-JNK-1/-2 from Life Technologies GmbH (Darmstadt, Germany), JNK-1/-2 from BD Bioscience (Heidelberg, Germany), phospho-Erk-1/-2, phospho-p38MAPK, phospho-c-Jun (Ser63) and c-Jun from Cell Signaling Technology, Inc. (Danvers, USA). All other chemicals were from Merck-Millipore (Darmstadt, Germany), at the highest quality available.

**Preparation and culture of primary rat and murine hepatocytes** – Hepatocytes were isolated from livers of male Wistar rats (160-180 g), Alb-Cre control mice (wild type (wt), 8-10 weeks; on C57BL/6 background), or Alb-Cre-FAS$^{fl/fl}$ mice ((8-10 weeks, mice with death domain-encoding exon 9 of Fas, flanked by loxP sites, were generated to allow conditional inactivation of Fas through cell type-specific expression of Cre recombinase) C57BL/6-Fas$^{tm1Cgn/J}$, The Jackson Laboratory, Maine, USA) by a collagenase perfusion technique in an adapted version as described previously (27). Animals were fed ad libitum with a standard diet. Aliquots of murine hepatocytes were plated on collagen-coated culture plates (BD Falcon, Heidelberg, Germany) and maintained in William’s Medium E (Biochrom, Berlin, Germany), supplemented with 5 mmol/l glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 100 nmol/l dexamethasone and
10 % FBS. Cells were incubated in a humidified atmosphere of 5 % CO₂ and 95 % air at 37 °C. Aliquots of rat hepatocytes were plated on collagen-coated culture plates and maintained in bicarbonate-buffered Krebs-Henseleit medium (115 mmol/L NaCl, 25 mmol/l NaHCO₃, 5.9 mmol/l KCl, 1.18 mmol/l MgCl₂, 1.23 mmol/l NaH₂PO₄, 1.2 mmol/l Na₂SO₄, 1.25 mmol/l CaCl₂), supplemented with 6 mmol/l glucose in a humidified atmosphere of 5 % CO₂ and 95 % air at 37 °C. After 2 h, the medium was removed and the cells were washed twice. Subsequently the culture was continued for 24 h in William’s Medium E, supplemented with 2 mmol/l glutamine, 100 mmol/l insulin, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/l glutamine, 100 nmol/l dexamethasone and 5 % FBS. After 24 h experimental treatments were performed using William’s Medium E that contained 2 mmol/l glucose, 100 mmol/l dexamethasone and 1 % FFA-free BSA. The viability of hepatocytes was more than 95 % as assessed by trypan blue exclusion. The experiments were approved by the responsible local authorities.

**Treatment of hepatocytes with FFAs –** Caprylate (C8), palmitate (C16), stearate (C18:0), oleate (C18:1) and linoleate (C18:2) were dissolved each in EtOH at 50 mmol/l and 200 mmol/l. The final working solutions were prepared by diluting stock solutions (1:1000) in culture medium supplemented with FFA-free BSA. Each experimental condition and each control contained 1 % FFA-free BSA and 0.1 % EtOH. Experimental treatment of the cells did not change pH of cell culture media.

**Immunoblot analysis –** At the end of the incubation period, the medium was removed and the cells were immediately lysed at 4 °C by using a lysis buffer containing 20 mmol/l Tris-HCl (pH 7.4), 140 mmol/l NaCl, 10 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 1 % (v/v) Triton X-100, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l sodium vanadate, 20 mmol/l β-glycerophosphate, and protease inhibitor cocktail. The lysates were kept on ice for 10 min and then centrifuged at 8000 rpm for 8 min at 4 °C, and aliquots of the supernatant were taken for protein determination using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Equal amounts of protein were subjected to sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes using a semidyed transfer apparatus (GE Healthcare, Freiburg, Germany). Membranes were blocked for 30 min in 5 % (w/v) BSA containing 20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, and 0.1 % Tween 20 (TBS-T) and exposed to primary antibodies overnight at 4 °C. After washing with TBS-T and incubation at room temperature for 2 h with horseradish peroxidase-coupled anti-mouse or anti-rabbit IgG antibody, respectively (all diluted 1:10 000), the immunoblots were washed extensively and bands were visualized using the FluorChem E detection instrument from ProteinSimple (Santa Clara, CA). Semi-quantitative evaluation was carried out by densitometry using the Alpha View image acquisition and analysis software from ProteinSimple. Protein phosphorylation is given as the ratio of detected phospho-protein/total protein.

**Immunoprecipitation –** Liver samples were harvested in lysis buffer containing 136 mmol/l NaCl, 20 mmol/l Tris-HCl, 10 % (v/v) glycerol, 2 mmol/l EDTA, 50 mmol/l β-glycerophosphate, 20 mmol/l sodium pyrophosphate, 0.2 mmol/l Pefablock, 5 mg/l aprotinin, 5 mg/l leupeptin, 4 mmol/l benzamidine, 1 mmol/l sodium vanadate, supplemented with 1 % (v/v) Triton X-100. The protein amount was determined as described above. Samples containing equal protein amounts were incubated for 2 h at 4 °C with a polyclonal rabbit anti-CD95 or rabbit anti-EGFR antibody to immunoprecipitate CD95 or EGFR, respectively. Then protein A-/G-agarose (Santa Cruz Biotechnology, Heidelberg, Germany) was added and incubated at 4 °C overnight. Immunoprecipitates were washed 3-times with lysis buffer supplemented with 0.1 % (v/v) Triton X-100 and then transferred to Western blot analysis as described above. The anti-phospho-tyrosine antibody was used to detect activating phosphorylation of CD95 or EGFR in the respective immunoprecipitates. Caspase 8 and FADD antibodies were used to detect association to CD95.

**Immunofluorescence staining –** To detect EGFR and CD95 translocation, isolated hepatocytes were cultured for 24 h on collagen-coated glass coverslips (Ø 12 mm) in 24-well culture plates. After treatment, cells were fixed using paraformaldehyde (4 % (w/v), 20 min, 4 °C), permeabilized using Triton X-100 (0.1 % (v/v), 2 min, 4 °C) and blocked with FBS (5 % (w/v), 30 min, room temperature). Then cells were exposed to a mouse anti-EGFR antibody and a rabbit anti-CD95 antibody (1:100 in PBS, overnight, 4 °C), washed off, and stained with an...
RESULTS

Effect of insulin and FFAs on proliferation and apoptosis – In order to analyze the proliferative and apoptotic effects of insulin and FFAs on primary hepatocytes, BrdU-uptake measurements and TUNEL staining were performed. In line with previous data (1), insulin (100 nmol/l) led within 48 h to a 2.4 ± 0.2-fold (n = 6) increase of BrdU-uptake suggestive for hepatocyte proliferation (Fig. 1A). However had no effect on hepatocyte apoptosis as determined by TUNEL-assays [numbers of TUNEL-positive cells after 18 h were 2.3 % ± 0.6 % (n = 6) vs. 0.9 ± 0.1-fold of control (n=6); palmitate 0.8 ± 0.1-fold of control (n=3)] (Fig. 1B). In contrast to insulin, the saturated FFAs caprylate, palmitate and stearate (50 µmol/l each) had no effect on hepatocyte proliferation [caprylate 0.9 ± 0.1-fold of control (n=6); palmitate 0.8 ± 0.1-fold of control (n=6); stearate 0.7 ± 0.1-fold of control (n=3)] (Fig. 1A,C). The number of apoptotic cells measured by TUNEL staining increased within 18 h [caprylate 7.7 % ± 1.5 % (n = 6); palmitate 23.3 % ± 4.1 % (n = 6); stearate 32.0 % ± 10.5 % (n=3)] (Fig. 1B,D). The unsaturated FFAs oleate and linoleate however had no significant effect on apoptosis induction [oleate 4.6 % ± 3.8 %; linoleate 2.0 % ± 1.8 % (n=3)] (Fig. 1D). When hepatocytes were treated with a combination of insulin and saturated FFAs, the insulin-induced proliferation was completely abolished (Fig. 1A), whereas the number of apoptotic cells increased to 21.2 % ± 3.1 % by caprylate plus anti-mouse-FITC and an anti-rabbit Cy3-conjugated antibody (1:500 in PBS, 2 h, room temperature). Following immunofluorescence staining, samples were covered with ProLong® Gold Antifade reagent with DAPI and EGFR, respectively CD95 localization was visualized by confocal laser scanning microscopy using LSM510 META (Zeiss, Oberkochen, Germany).

Statistical analysis – Results from at least three independent experiments are expressed as mean values ± SEM. n refers to the number of independent experiments. For each experimental treatment and time point analyzed, a separate control experiment was carried out. Differences between experimental groups were analyzed by student’s t test or one-way analysis of variance following Dunnett’s multiple comparison post hoc test where appropriate (GraphPad Prism; GraphPad, La Jolla, USA; Microsoft Excel for Windows, Redmond, USA). p < 0.05 was considered statistically significant.
insulin (n=6), 41.9 % ± 5.1 % by palmitate plus insulin (n=6) and 47.1 % ± 10.3 % by stearate plus insulin (n=3) (Fig. 1B,D). In contrast, the combination of insulin and unsaturated FFA induced no enhancement of apoptosis (Fig. 1D); however proliferation increased compared to treatment with the unsaturated FFAs alone [oleate 1.0 ± 0.1-fold of control; olate plus insulin 1.4 ± 0.1-fold of control; linoleate 1.1 ± 0.1-fold of control; linoleate plus insulin 1.5 ± 0.3-fold of control (each n=3)] (Fig 1C). Thus, the proliferative effect of insulin was preserved, but blunted in presence of unsaturated FFAs.

In rat hepatocytes, insulin triggered phosphorylation of the EGFR at positions Tyr^{845} and Tyr^{1173} (Fig. 2A,B), which is known to induce an activation of the EGFR-tyrosine kinase activity (28, 29). No phosphorylation at Tyr^{1045} was observed (Fig. 2A), indicating ligand-independent activation of the EGFR by insulin (30). Insulin-induced EGFR tyrosine phosphorylation was blunted by about 50 % when FFAs were coadministered with insulin, however, EGFR activation was still significantly increased compared to the control. (Fig. 2A,B). No activating EGFR phosphorylation was observed in response to caprylate or palmitate in the absence of insulin (Fig. 2A,B).

The proliferative effect of insulin was preserved, but blunted in presence of unsaturated FFAs. Both, insulin and FFAs induced a transient activation of Erk-1/-2, which was not significantly affected when insulin and FFAs were added together (Fig. 5). There was also no statistically significant difference between insulin and insulin plus FFA with regard to p38MAPK phosphorylation.

A role of JNKs in mediating proapoptotic and antiproliferative signaling following coadministration of insulin and FFAs is also suggested by the finding that two different JNK inhibitors, i.e. SP600125 and L-JNKI-1 could largely restore the proliferative effect of insulin in presence of caprylate [insulin plus caprylate 0.9 ± 0.05-fold of control, in presence of SP600125 2.0 ± 0.4-fold of control; in presence of L-JNKI-1 1.8 ± 0.3-fold of control (n=3 for each condition)] and palmitate [insulin plus palmitate 1.0 ± 0.1-fold of control, in presence of SP600125 1.7 ± 0.1-fold of control; in presence of L-JNKI-1 1.6 ± 0.1-fold of control (n=3 for each condition)] (Fig. 6A).

Activation of mitogen activated protein kinases (MAPK) by FFAs – At concentrations of 200 µmol/l or above, FFAs can induce so-called lipoapoptosis and JNKs were identified as important mediators of FFA-induced lipoapoptosis (1). While FFA concentrations of 200 µmol/l significantly increased JNK-1/-2 phosphorylation after 60 min, FFAs at a concentration of 50 µmol/l induced JNK-1/-2 activation already within the first 20 min of caprylate or palmitate addition (Fig. 3). Caprylate and palmitate (50 µmol/l each) increased within 15 min carboxy-H_{2}-DCFDA fluorescence by 1.6 ± 0.2-fold and 1.6 ± 0.1-fold (n=3), respectively (data not shown), raising the possibility that FFA-induced oxidative stress may trigger activation of JNKs.

Coadministration of insulin had no significant effect on FFA-induced JNK-1/-2 phosphorylation (Fig. 4A,B) and no activation of JNK-1/-2 were observed in response to insulin alone (Fig. 4A).

Compared to saturated FFAs, if at all only a weak JNK-1/-2 activation was induced by unsaturated (oleate and linoleate) FFAs (Fig. 4B).

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type mice [caprylate plus insulin 43.3 ± 2.1 %, palmitate plus insulin 62.3 ± 3.3 % (each n=3)], the proapoptotic effect of insulin in presence of FFA was no longer observed in mice lacking functional CD95 (Fig. 7), indicating an involvement of CD95 during apoptosis induction by coadministered insulin/FFAs. Therefore, the signaling events leading to CD95 activation and DISC formation were studied. 

**CD95 activation and formation of DISC in presence of FFAs and insulin** – Immunoprecipitation studies revealed that in response to insulin or FFA treatment alone, no activation of the CD95 system was detectable with respect to CD95/EGFR association, CD95 tyrosine phosphorylation and DISC formation, *i.e.* association of FADD and caspase 8 to the death receptor (Fig. 8). Only upon coadministration of FFAs with insulin CD95/EGFR association, CD95 tyrosine phosphorylation and DISC formation occurred as it was also observed in response to CD95L (Fig. 8).

In line with this, caspase 8 activity increased in presence of insulin and FFA (Fig. 9) indicating an activation of the CD95 signaling pathway. 

In order to assess the role of JNK-1/-2 in triggering CD95/EGFR association, inhibitor studies were performed. Both, SP600125 and L-JNKI-1, inhibited not only c-Jun phosphorylation, but also largely blocked the association of EGFR with CD95, CD95-tyrosine phosphorylation as well as DISC formation in response to combined treatment with insulin and FFAs (Fig. 8).

The subcellular localization of EGFR and CD95 was studied by immunofluorescence analysis in primary rat hepatocytes. EGFR and CD95 colocalizing pixels were quantified by calculation of the weighted colocalization coefficient according to a preset threshold. As shown in Fig. 10, insulin and EGF, but not FFAs induced within 90 min an internalization of the EGFR. Coadministration of insulin and FFAs for 90 min prevented the insulin-induced EGFR internalization and even triggered an enhancement of EGFR and CD95 immunoreactivity at the plasma membrane (Figs. 10 and 11). Under these conditions the number of colocalizing EGFR- and CD95-positive pixels in the plasma membrane was significantly increased [insulin plus C8 59.2 % ± 7.7 %; insulin plus C16 63.2 % ± 9.6 % (each n=3)] (Fig. 11) in line with the reported translocation of the EGFR/CD95 complex to the plasma membrane following CD95 activation (32). SP600125 and L-JNKI-1 largely inhibited the colocalization of EGFR and CD95 in presence of insulin and FFA [SP600125 23.0 % ± 3.6 % and L-JNKI-1 31.3 % ± 2.0 % in presence of insulin plus C8; SP600125 27.7 % ± 5.7 % and L-JNKI-1 26.4 % ± 4.9 % in presence of insulin plus C16 (n=3 for each condition)]. No significant CD95 translocation to the plasma membrane was observed after treatment with insulin or FFA alone [insulin 8.3 % ± 0.9 %; C8 13.3 % ± 5.2 %; C16 9.7 % ± 3.0 % compared to control 7.7 % ± 1.8 % (each n=3)] (Fig. 11).

**DISCUSSION**

The major finding of the current study is that JNK activation by saturated FFAs not only ameliorates insulin-induced EGFR activation, but also shifts insulin-induced EGFR activation and proliferation towards CD95 activation and apoptosis, as schematically depicted in Fig. 12. Unsaturated fatty acids, however, do not activate JNKS and do not induce apoptosis (Fig. 1D, 4B) (33). Saturated FFAs rapidly trigger generation of ROS, which may explain JNK activation. Mitochondria (34, 35) and NADPH oxidase were discussed as predominant sources of palmitate-induced ROS generation in the liver (36, 37). The FFA-induced JNK signal directs activated EGFR from proliferative signaling towards association with CD95 and proapoptotic signaling.

Insulin induces proliferation of hepatocytes by NKCC1- and Na⁺/H⁺ antiporter-driven cell swelling (38), which induces EGFR activation through an integrin- and c-Src kinase-dependent osmosensing/signaling pathway (1). In line with the literature, insulin caused an activating phosphorylation of the EGFR at positions Tyr845 and Tyr1173 (Fig. 2), thereby inducing hepatocyte proliferation (Fig. 1A). Activation of the insulin receptor leads to tyrosine phosphorylation of the insulin receptor substrate (IRS)-1, thereby triggering downstream signaling pathways such as the phosphatidylinositol 3-kinase/Akt pathway, which mediate metabolic actions of insulin (39) and insulin-induced hepatocyte swelling (40). JNK can induce insulin resistance through serine/threonine phosphorylation of the IRS-1 and -2 and thereby attenuate downstream insulin signaling (41-44). This is also reflected by the
attenuation of insulin-induced EGFR activation in presence of FFA.

Saturated FFAs such as palmitate can induce lipoapoptosis in hepatocytes (2) and other cell types, such as pancreatic β cells (45) or cardiac myocytes (46). Induction of lipoapoptosis involves an endoplasmic reticulum (ER) stress response with activation of inositol-requiring protein-1 and protein kinase-like ER kinase. The resulting activation of JNK and induction of the transcription factor C/EBP-homologous protein, leads to the upregulation of pro-apoptotic proteins such as Bim and PUMA. The following activation of Bax results in activation of effector caspases and death receptor-independent apoptosis (14, 47-49). Whereas recent studies suggest that FFAs induce lipoapoptosis in hepatocytes in a JNK-dependent but death receptor-independent way (2), upregulation of TRAIL receptors 1 and 2 (15, 16) and CD95 were discussed to sensitize hepatocytes to the extrinsic pathway of apoptosis (17).

JNKs also play a crucial role in CD95-mediated hepatocyte apoptosis. Here, pro-apoptotic stimuli, trigger ROS generation via NADPH oxidase(s), which leads to a Yes kinase-mediated EGFR transactivation and JNK activation, mediating the association of the EGFR with CD95, which initiates CD95-dependent apoptotic cell death (for a review, see Ref. 50). A special situation is found in hepatic stellate cells (HSC), which represent a hepatic stem/progenitor cell compartment (51, 52). Here, CD95L triggers in quiescent HSC the activation of the EGFR, stimulates proliferation and induces CD95 tyrosine nitration (53), which leads to apoptosis resistance (54). However, in activated HSC no inhibitory CD95 tyrosine nitration occurs, whereas EGFR activation is preserved. If JNK activation is induced in these cells, EGFR couples to CD95 and the mitogenic signal is shifted to an apoptotic one. Thus JNK activation provides a switch between EGFR-mediated proliferation and CD95-mediated apoptosis (53, 55). A similar phenomenon is shown in the present study in rat hepatocytes: insulin-induced proliferation is shifted towards apoptosis when a JNK signal is provided by FFAs. The mechanism how JNKs promote the association between CD95 and EGFR is still unclear. JNK itself is not found in the protein complex (data not shown) therefore it is likely that JNK phosphorylates a yet unknown protein, such as the receptor interacting protein (RIP), a serine/threonine kinase, that may function as an adaptor protein to CD95, which is required for CD95/EGFR protein assembly (56). The identity of such a hypothetical adapter protein is not yet known and requires further investigation.

This insulin-dependent proapoptotic action of FFAs is almost completely mediated by CD95, as evidenced by our studies with Alb-Cre-FAS<sup>fl/fl</sup> mice (Fig. 7), which lack functional CD95 (31). Due to this result it is unlikely that other FFA-triggered apoptosis pathways are influenced by insulin. In line with this, the palmitate-induced up-regulation and cell surface expression of TRAIL-death receptor R2 (DR5) was not affected by insulin (data not shown).

Taken together, the present study provides new insights into the pathogenesis of hepatocyte apoptosis in response to FFAs and insulin, which may be relevant for the pathogenesis of NASH. Here, insulin resistance, hyperinsulinemia and increased levels of circulating FFAs play an important role in the development and progression of this disease (for a review, see Ref. 57).
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CD95-dependent apoptosis in response to FFA and insulin

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FOOTNOTES

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The abbreviations used are: CD95, CD95 receptor (Fas, APO-1); CD95L, CD95 ligand, CM-H₂DCFDA, 5-(and 6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; DISC, death inducing signaling complex; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; FADD, Fas associated death domain protein; FFA, free fatty acid; HSC, hepatic stellate cell; IP, immunoprecipitation; IRS, insulin receptor substrate; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species; TRAIL, TNF-related apoptosis-inducing ligand

FIGURE LEGENDS

FIGURE 1. Effects of insulin and FFAs on proliferation and apoptosis. (A, C) After a culture period of 24 h, culture medium was removed from rat liver hepatocytes and replaced by medium containing BrdU. Then, hepatocytes were treated with insulin (100 nmol/l), caprylate, palmitate, stearate, oleate or linoleate (50 µmol/l, each) or the combination of insulin and FFA for 48 h and analyzed for BrdU incorporation. BrdU uptake by hepatocytes kept in control medium was set to 1. Statistical analyses of at least three independent experiments for each condition are shown. *, p < 0.05 denotes statistical significance compared to the unstimulated control. (B, D) In another set of experiments, hepatocytes were cultured for 24 h and thereafter stimulated with insulin (100 nmol/l), caprylate, palmitate, stearate, oleate or linoleate (each 50 µmol/l) or a combination of insulin and FFA for 18 h and the number of apoptotic cells was determined using TUNEL technique. Statistical analyses of at least three independent experiments for each condition are shown. *, p < 0.05 versus insulin plus FFA treatment.

FIGURE 2. Inhibition of insulin-induced EGFR activation by FFAs. Rat hepatocytes were cultured for 24 h and thereafter stimulated with caprylate or palmitate (50 µmol/l each), insulin (100 nmol/l) or a combination of insulin and FFA for up to 60 min. Samples were taken at the indicated time points. Activating EGFR-tyrosine phosphorylation was analyzed by Western blotting using phospho-specific antibodies. Total EGFR served as respective loading control. (A) Representative immunoblots of three independent experiments. (B) The values from densitometric analyses of three independent experiments were normalized to the level of total EGFR and expressed as the mean-fold increase over control ± SEM. For the individual time points control was set to 1. Open squares, insulin; closed grey squares, insulin plus C8; closed black squares, insulin plus C16; closed grey triangle, C8; closed black triangle, C16. *, p < 0.05 statistical significance between insulin and insulin plus C16. EGFR activation at positions Tyr⁸⁴⁵ and Tyr¹¹⁷³ was significantly increased by insulin at each analyzed time point. Insulin plus FFAs significantly increased EGFR phosphorylation within the first 20 min at Tyr⁸⁴⁵ and after 5 min at Tyr¹¹⁷³.

FIGURE 3. Concentration-dependent JNK-1/-2 activation by FFAs. Rat hepatocytes were cultured for 24 h and thereafter treated with the indicated concentration of caprylate or palmitate for up to 60 min. Samples were taken at the indicated time points. Phosphorylation of JNK-1/-2 was analyzed by Western blot using specific antibodies and subsequent densitometric analysis. Total JNK-1/-2 served as respective loading control. Representative blots are shown. Closed black squares, 50 µmol/l; closed grey squares, 200 µmol/l. Phosphorylation at t=0 was set to 1. Data represent the mean ± SEM of >three independent experiments, *, p < 0.05 statistical significance compared with the unstimulated control.

FIGURE 4 A,B. Effects of insulin and FFA on JNK-1/-2 activation. Rat hepatocytes were cultured for 24 h and thereafter stimulated with insulin (100 nmol/l), caprylate, palmitate, stearate, oleate or linoleate (50 µmol/l each) or a combination of insulin and FFA for up to 360 min. Samples were taken at the indicated time points. Phosphorylation of JNK-1/-2 was analyzed by Western blot using specific antibodies and subsequent densitometric analysis. Total JNK-1/-2 served as respective loading control.
Closed black squares, FFA; closed grey squares, insulin plus FFA; open squares, insulin. Phosphorylation at $t=0$ was set to 1. Data represent the mean ± SEM of ≥three independent experiments, *, $p < 0.05$ statistical significance compared with the unstimulated control. No statistical significance between FFA alone and insulin plus FFA with regard to JNK activation.

**FIGURE 5 A,B. Effects of insulin and FFA on Erk-1/-2 and p38MAPK activation.** Rat hepatocytes were cultured for 24 h and thereafter stimulated with insulin (100 nmol/l), caprylate, palmitate (50 µmol/l each) or a combination of insulin and FFA for up to 60 min. Samples were taken at the indicated time points. Phosphorylation of Erk-1/-2 and p38MAPK was analyzed by Western blot using specific antibodies and subsequent densitometric analysis. Total Erk-1/-2 and p38MAPK served as respective loading control. Closed black squares, FFA; closed grey squares, insulin plus FFA; open squares, insulin. Phosphorylation at $t=0$ was set to 1. Data represent the mean ± SEM of ≥5 independent experiments, *, $p < 0.05$ statistical significance compared with the unstimulated control. No statistical significance between insulin, FFA and insulin plus FFA with regard to Erk-1/-2 and p38MAPK activation.

**FIGURE 6. Effects of JNK-inhibition on proliferation and apoptosis in isolated rat hepatocytes.** (A) After a culture period of 24 h, culture medium was replaced by medium containing BrdU. Then, hepatocytes were treated with insulin (100 nmol/l), FFAs (each 50 µmol/l) or a combination of both for another 48 h and analyzed for BrdU incorporation. When indicated, cells were pretreated (30 min) with SP600125 (100 µmol/l) or L-JNKI-1 (5 µmol/l). BrdU uptake by hepatocytes kept in control medium was set to 1. Statistical analyses of at least three independent experiments for each condition are shown. *, $p < 0.05$ denotes statistical significance between insulin plus FFA and after administration of JNK inhibitors. (B) In another set of experiments, hepatocytes were stimulated with insulin (100 nmol/l), FFAs (50 µmol/l) or a combination of both for 18 h and the number of apoptotic cells was determined using TUNEL technique. Where indicated, cells were pretreated (30 min) with SP600125 (100 µmol/l) or L-JNKI-1 (5 µmol/l). Statistical analyses of at least three independent experiments for each condition are shown. *, $p < 0.05$ versus insulin plus FFA treatment.

**FIGURE 7. FFA-induced apoptosis in wild type and Alb-Cre-FASfl/fl-mice.** Primary hepatocytes from wild type (Alb-Cre control mice on C57BL/6 background) and Alb-Cre-FASfl/fl-mice were cultured for 24 h and thereafter treated with insulin (100 nmol/l), FFAs (each 50 µmol/l) or a combination of both for 18 h and the number of apoptotic cells was determined using TUNEL technique. Insulin stimulates apoptosis in presence of FFA in wild type, but not in hepatocytes devoid of CD95. Statistical analyses of at least three independent experiments for each condition are shown. *, $p < 0.05$ versus FFA treatment alone. Not significant (n.s.), $p > 0.05$ versus FFA treatment alone.

**FIGURE 8. FFA-induced JNK activation and insulin-induced EGFR activation can trigger CD95 activation and formation of the death-inducing signaling complex in rat hepatocytes.** Rat hepatocytes were cultured for 24 h and subsequently stimulated with caprylate or palmitate (50 µmol/l each), insulin (100 nmol/l) or CD95L (100 ng/ml) for up to 60 min. Where indicated, cells were pretreated for 30 min with SP600125 (100 µmol/l) or L-JNKI-1 (5 µmol/l). Samples were taken at the time points indicated. EGFR and CD95 were immunoprecipitated and analyzed by Western blotting. Activating EGFR-tyrosine phosphorylation (P-EGFR-Y) and JNK-1/-2 activation were detected 15 min after stimulation, CD95-tyrosine phosphorylation (P-CD95-Y) and c-Jun phosphorylation after 30 min, caspase 8/CD95 and FADD/CD95 association (i.e. DISC formation) 60 min after addition of FFAs, insulin or CD95L. Total EGFR, JNK-1/-2, c-Jun and CD95 served as respective loading controls. Representative immunoblots of three independent experiments are depicted.

**FIGURE 9. Effect of FFA on caspase 8 activation.** Rat hepatocytes were cultured for 24 h and subsequently stimulated with caprylate or palmitate (50 µmol/l each), insulin (100 nmol/l) or CD95L (100 ng/ml) for 180 min. Caspase 8 activity was determined as described in the Experimental procedures section and are expressed relative to the activity found in unstimulated controls. Statistical analyses of three independent experiments for each condition are shown. *, $p < 0.05$ denotes statistical
CD95-dependent apoptosis in response to FFA and insulin

significance compared to the unstimulated control. Caspase 8 activation is only found when FFA and insulin were added together, but not when added separately.

FIGURE 10. EGFR expression in primary parenchymal cells. Primary hepatocytes were cultured on glass coverslips for 24 h, stimulated for 90 min with the indicated agents and immunostained for EGFR expression. EGFR localization was visualized by confocal laser scanning microscopy. Insulin (100 nmol/l) and EGF (10 ng/ml) induced internalization of the EGFR, whereas caprylate or palmitate (50 µmol/l each) induced no internalization. The scale bar corresponds to 20 µm. Representative pictures of three independent experiments are depicted.

FIGURE 11. Immunofluorescence staining of CD95 and EGFR in insulin- and FFA-treated primary hepatocytes. (A) Primary hepatocytes were cultured on glass coverslips for 24 h, stimulated with insulin (100 nmol/l), caprylate or palmitate (each 50 µmol/l) or the combination of insulin and FFA for 90 min and immunostained for CD95 and EGFR expression. Localization of both receptors was visualized by confocal laser scanning microscopy. Greyscale images show colocalizing pixels of the red and green channel. The scale bar corresponds to 20 µm. Representative pictures of three independent experiments are depicted. (B) Colocalizing pixels were quantified by calculation of the weighted colocalization coefficient using LSM510 Meta 4.2 Software (Zeiss, Jena, Germany). *, p < 0.05 denotes statistical significance compared to the unstimulated control; #, p < 0.05 versus insulin plus FFA treatment.

FIGURE 12. Schematic presentation of insulin and FFA effects on hepatocyte proliferation and apoptosis in hepatocytes. Insulin induces activation of the EGFR and proliferation, while FFAs induce ROS generation, which triggers death receptor-independent lipoapoptosis and JNK activation. When EGFR is simultaneously activated by insulin, this JNK signal allows for EGFR coupling to CD95 and initiation of CD95-dependent apoptotic cell death.
Figure 1A,B

**A** proliferation

![Graph showing relative BrdU incorporation (x-fold increase) for different treatments.](image)

**B** apoptosis

![Graph showing TUNEL-positive cells (%) for different treatments.](image)
Figure 1C,D

**C**  
Prolineraction

|           | C18:0 | Insulin + C18:0 | C18:1 | Insulin + C18:1 | C18:2 | Insulin + C18:2 |
|-----------|-------|-----------------|-------|-----------------|-------|-----------------|
| Relative BrdU incorporation (x-fold increase) | ![Graph](image) |

**D**  
Apoptosis

|           | C18:0 | Insulin + C18:0 | C18:1 | Insulin + C18:1 | C18:2 | Insulin + C18:2 |
|-----------|-------|-----------------|-------|-----------------|-------|-----------------|
| TUNEL-positive cells (%) | ![Graph](image) |
### Figure 2A

|            | C8                | C16                |
|------------|-------------------|--------------------|
|            | 0 min  | 5 min  | 15 min | 30 min | 60 min | 0 min  | 5 min  | 15 min | 30 min | 60 min |
| P-EGFR-Y^845| ![Image](image1)
| P-EGFR-Y^1045| ![Image](image2)
| P-EGFR-Y^1173| ![Image](image3)
| EGFR       | ![Image](image4) |

|            | Insulin  | Insulin + C8 | Insulin + C16 |
|------------|----------|--------------|---------------|
|            | 0 min  | 5 min  | 15 min | 30 min | 60 min | 0 min  | 5 min  | 15 min | 30 min | 60 min |
| P-EGFR-Y^845| ![Image](image5)
| P-EGFR-Y^1045| ![Image](image6)
| P-EGFR-Y^1173| ![Image](image7)
| EGFR       | ![Image](image8) |
Figure 2B

CD95-dependent apoptosis in response to FFA and insulin

B

![Graphs showing relative P-EGFR-Y1173 protein expression (x-fold increase) over time (min) for different conditions: Insulin, Insulin + C8, and Insulin + C16. The graphs compare different conditions and time points, indicating changes in protein expression.]
Figure 3

|                | Caprylate           | Palmitate          |
|----------------|---------------------|--------------------|
|                | 50 µmol/l           | 50 µmol/l          |
|                | 200 µmol/l          | 200 µmol/l         |
| 0 min          |                     | 0 min              |
| 5 min          |                     | 5 min              |
| 15 min         |                     | 15 min             |
| 30 min         |                     | 30 min             |
| 60 min         |                     | 60 min             |

**P-JNK-1/2**

**JNK-1/2**

The graphs show the relative P-JNK-1/2 protein expression (x-fold increase) over time (min) for both Caprylate and Palmitate at 50 and 200 µmol/l concentrations. The graphs indicate an increase in P-JNK-1/2 protein expression over time, with significant differences marked by asterisks (*) at specific time points.
Figure 4A

CD95-dependent apoptosis in response to FFA and insulin
Figure 4B

CD95-dependent apoptosis in response to FFA and insulin

B

- Relative P-JNK-1/-2 protein expression (x-fold increase)
- Time (min)
- Graphs for different fatty acids:
  - C18:0
  - Insulin + C18:0
  - C18:1
  - Insulin + C18:1
  - C18:2
  - Insulin + C18:2
Figure 5A,B

A

P-Erk-1/-2

relative protein expression
(x-fold increase)

0 0.5 1 1.5 2

0 20 40 60

time (min)

C8  Insulin + C8  Insulin

B

P-p38

relative protein expression
(x-fold increase)

0 0.5 1 1.5 2

0 20 40 60

time (min)

C8  Insulin + C8  Insulin

C16  Insulin + C16  Insulin
CD95-dependent apoptosis in response to FFA and insulin

Figure 6A,B

**A**  
**proliferation**

| Condition               | Relative BrdU Incorporation (x-fold increase) |
|-------------------------|---------------------------------------------|
| Control                 | 1.0                                         |
| Insulin                 | 4.0                                         |
| C8                      | 1.5                                         |
| Insulin + C8            | 3.0                                         |
| Insulin + C8 + SP600125 | 3.5                                         |
| Insulin + C8 + LNKI-1   | 2.5                                         |
| C16                     | 2.0                                         |
| Insulin + C16           | 2.5                                         |
| Insulin + C16 + SP600125| 3.0                                         |
| Insulin + C16 + LNKI-1  | 3.5                                         |

*Significant difference compared to control.

**B**  
**apoptosis**

| Condition               | TUNEL-positive Cells (%) |
|-------------------------|--------------------------|
| Control                 | 5.0                      |
| Insulin                 | 10.0                     |
| C8                      | 15.0                     |
| Insulin + C8            | 20.0                     |
| Insulin + C8 + SP600125 | 25.0                     |
| Insulin + C8 + LNKI-1   | 30.0                     |
| C16                     | 35.0                     |
| Insulin + C16           | 40.0                     |
| Insulin + C16 + SP600125| 45.0                     |
| Insulin + C16 + LNKI-1  | 50.0                     |

*Significant difference compared to control.
Figure 7

[Graph showing TUNEL-positive cell (%) for wild type and Alb-Cre-FAS/yn treated with control, insulin, C8, insulin + C8, C16, and insulin + C16.]

CD95-dependent apoptosis in response to FFA and insulin
CD95-dependent apoptosis in response to FFA and insulin

Figure 8
Figure 9

![Graph showing x-fold increase in caspase 8 activity](http://www.jbc.org/Downloaded.from)
CD95-dependent apoptosis in response to FFA and insulin

Figure 10

EGFR

Control  Insulin  EGF

C8  C16  Insulin + C8  Insulin + C16

90 min

SP600125 + Insulin + C8  L-JNKI-1 + Insulin + C8  SP600125 + Insulin + C16  L-JNKI-1 + Insulin + C16
Figure 11A

CD95-dependent apoptosis in response to FFA and insulin
Figure 11B

CD95-dependent apoptosis in response to FFA and insulin

B

![Graph showing relative number of colocalizing pixels (%)](image-url)
Free Fatty Acids shift Insulin-induced Hepatocyte Proliferation towards CD95-dependent Apoptosis
Annika Sommerfeld, Roland Reinehr and Dieter Häussinger

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Correction: Free fatty acids shift insulin-induced hepatocyte proliferation towards CD95-dependent apoptosis.

Annika Sommerfeld, Roland Reinehr, and Dieter Häussinger

In Fig. 2A, the insulin/P-EGFR-Y1173 and insulin/H11001/P-EGFR-Y845 immunoblots are duplicated. The mishap occurred in 2014, and the reason for this could not be identified due to difficulties in reconstructing the experimental documentation. The authors state that the corresponding statistics for n = 3 are given in Fig. 2B, showing that the result of the experiment is not affected by the incorrect presentation of the exemplary immunoblot. The authors regret any problems that this error may have caused.