Degradation of cIAPs contributes to hepatocyte lipoapoptosis

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Hepatocyte apoptosis is a hallmark of nonalcoholic steatohepatitis. We have previously observed that the saturated free fatty acids (FFAs) induce hepatocyte apoptosis in part via a death receptor 5 (DR5)-mediated signaling pathway. Cellular inhibitor of apoptosis protein 1 and 2 (cIAP-1 and cIAP-2) proteins are potent inhibitors of death receptor-mediated apoptosis. However, the role of the cIAPs in FFA-mediated hepatocyte apoptosis is unexplored. Our aim was to determine whether cIAPs are dysregulated during hepatocyte lipoapoptosis. cIAP proteins underwent rapid cellular elimination following treatment with the saturated FFAs palmitate (PA) and stearate. In contrast, PA did not decrease cIAP-1 and cIAP-2 mRNA expression in the cells. Degradation of cIAPs was dependent on their E3-ligase activity, suggesting that cIAPs undergo autoubiquitination that leads to proteasomal degradation. Huh-7 cells stably expressing shRNA targeting cIAP-1, but not cIAP-2, displayed enhanced sensitivity to PA-mediated apoptosis. Incubation with the SMAC mimetic JP1584, which induces rapid degradation of cIAPs, also enhanced PA-mediated apoptosis. Hepatocytes isolated from DR5 knockout mice exhibited reduced apoptosis following treatment with PA plus JP1584, implying that degradation of cIAPs sensitizes to DR5-mediated cell death pathways. A decrease of cIAP-1 was also observed in tissue from patients with nonalcoholic steatohepatitis compared with normal obese subjects. Collectively, these results implicate proteasomal degradation of cIAPs by FFA as a mechanism contributing to hepatocyte lipoapoptosis.

free fatty acids; apoptosis; IAPs; SMAC mimetics; DR5

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ulate death receptor-mediated apoptosis, the regulation of cIAP-1 and cIAP-2 during lipoprotein is unknown.

Herein, our data indicate that PA induces degradation of cIAPs by a proteasome-dependent and caspase-independent process. The elimination of cIAPs appears to enhance lipoproteinosis via the DR5-triggered, extrinsic pathway. cIAP-1 was also found to be diminished in a small sample of human NASH patients. We speculate that this dysregulation of cIAPs may be a pathogenic mechanism in human NASH.

MATERIALS AND METHODS

Cells and mice. The human hepatocellular carcinoma cell lines, Huh-7, Hep3B, HepG2 cells, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (25 mM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stable cell lines expressing short hairpin RNA (shRNA) against cIAP-1, cIAP-2, and caspase 8 were generated and cultured as previously described by us (18). C57BL/6 wild-type mice were obtained from Jackson Laboratory (Bar Harbor, ME). TRAIL-R1/2 mice were kindly provided by Dr. El-Deiry (University of Pennsylvania School of Medicine, Philadelphia, PA) (16). Mouse hepatocytes were isolated by collagenase perfusion, purified by Percoll (Sigma-Aldrich, St. Louis, MO) gradient centrifugation, and cultured on collagen-coated plates in Waymouth medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. All animal studies had prior Institutional Animal Care and Use Committee approval. Cryopreserved human primary hepatocytes obtained from Xenotech (Lenexa, KS) were rapidly thawed at 37°C in a water bath and washed and resuspended in DMEM supplemented with 10% FBS, 0.6 μg/ml insulin, 2 μg/ml hydrocortisone, 50 μl penicillin, and 50 μg/ml streptomycin. Cells were plated on collagen-coated cell culture plates and medium was replaced after 4 h with Williams’ E medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% glucose and 2% fetal bovine serum. All experiments were performed in a humidified 5% CO2 atmosphere at 37°C.

Plasmids. pEGFP-N1 was purchased from Clontech (Mountain View, CA). pEBB-HA-cIAP-1H588A, in which His588 in the RING (reverse); human cIAP-1, cIAP-2, and caspase 8 were generated and cultured as previously described by us (18). C57BL/6 wild-type mice were obtained from Jackson Laboratory (Bar Harbor, ME). TRAIL-R1/2 mice were kindly provided by Dr. El-Deiry (University of Pennsylvania School of Medicine, Philadelphia, PA) (16). Mouse hepatocytes were isolated by collagenase perfusion, purified by Percoll (Sigma-Aldrich, St. Louis, MO) gradient centrifugation, and cultured on collagen-coated plates in Waymouth medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. All animal studies had prior Institutional Animal Care and Use Committee approval. Cryopreserved human primary hepatocytes obtained from Xenotech (Lenexa, KS) were rapidly thawed at 37°C in a water bath and washed and resuspended in DMEM supplemented with 10% FBS, 0.6 μg/ml insulin, 2 μg/ml hydrocortisone, 50 μl penicillin, and 50 μg/ml streptomycin. Cells were plated on collagen-coated cell culture plates and medium was replaced after 4 h with Williams’ E medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% glucose and 2% fetal bovine serum. All experiments were performed in a humidified 5% CO2 atmosphere at 37°C.

RESULTS

cIAP-1 and cIAP-2 undergo rapid cellular elimination following treatment with PA. We initially examined cIAP protein levels following FFA treatment. Huh-7, Hep3B, and HepG2 cells were treated with 800 μM of the saturated FFA PA for up...
16 h. In all the cell lines, both cIAP-1 and cIAP-2 underwent relatively rapid cellular elimination (Fig. 1A). Notably, cIAP-2 was almost undetectable by immunoblot analysis in HepG2 cells. The reduction in cIAP-1 and cIAP-2 protein levels was sustained over 16 h. Rapid degradation of cIAP-1 and cIAP-2 was also observed following incubation with SA, another saturated FFA, at the same concentration (Fig. 1B). In contrast, treatment with an identical concentration of the nontoxic unsaturated FFA OA did not result in sustained depletion of either cIAP-1 or cIAP-2 (Fig. 1A). PA-treatment induced also a slight

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**Fig. 1.** Saturated free fatty acids (FFA), but not unsaturated FFA, induce cellular inhibitor of apoptosis 1 and 2 (cIAP-1 and cIAP-2) degradation in hepatocyte cell lines. A: Huh-7, HepG2, and Hep3B cells were treated with 800 μM of palmitate (PA) or oleate (OA) for the indicated time. Whole cell lysates were obtained from each experimental condition and subjected to immunoblot analysis for cIAP-1 and -2. Actin was used as loading control. B: Huh-7 cells were treated with 800 μM of stearate (SA) for the indicated time. Whole cell lysates were subjected to immunoblot analysis for cIAP-1 and -2. Actin was used as loading control. C: Huh-7, HepG2, and Hep3B cells were treated with 800 μM PA for the indicated time. Whole cell lysates were subjected to immunoblot analysis for X-linked inhibitor of apoptosis protein (XIAP). Actin was used as loading control. D: Huh-7, HepG2, and Hep3B cells were treated with 800 μM PA or OA for the indicated time. Total RNA was obtained at 8 h and expression of cIAP-1 and cIAP-2 was quantified by real-time PCR. Fold induction was determined after normalization to 18S. Data represent means ± SE of 3 independent experiments performed in triplicate. Veh, vehicle.
reduction in the protein level of X-linked inhibitor of apoptosis protein (XIAP), the third most abundant member of the mammalian IAP family. However, XIAP decrease occurred significantly later compared with the decrease in cIAP-1 and -2, making it less likely to contribute to the apoptotic signaling (Fig. 1C). Next, we assessed whether the decrease of cIAPs was due to reduced mRNA expression. cIAP-1 and cIAP-2 mRNA expression levels were not significantly altered by PA or OA treatment in any of the cell lines examined (Fig. 1D), suggesting that FFA-mediated cellular elimination of cIAP-1 and cIAP-2 occurs via a posttranslational mechanism. We have previously reported that degradation of cIAP-1 following TRAIL treatment is mediated by caspase 8 cleavage of this protein (18). Interestingly, PA-induced cIAP-1 and cIAP-2 degradation in Huh-7 cells was not affected either by caspase 8 shRNA-targeted knockdown or by the use of a pan-caspase inhibitor (Fig. 2A). In contrast, degradation of cIAP proteins was prevented by the proteasome inhibitor MG132 (Fig. 2B). Similar results were observed in human primary hepatocytes, although cIAP-2 was preferentially degraded compared with cIAP-1 in these cells (Fig. 2C). As expected, cIAP-1 and cIAP-2 expression in human hepatocytes was also significantly lower than in cancer cells. Previous studies have also demonstrated that proteosomal degradation of cIAPs occurs through their autoubiquitination, which is mediated by the E3 ubiquitin ligase activity of their RING domains (15, 26, 30). To test whether FFA-mediated degradation of cIAPs requires their autoubiquitination, cells were transiently transfected with constructs expressing cIAP-1 and cIAP-2 mutants deficient in their E3 ligase activity due to specific replacement of critical histidine residues in their RING domains (H588A in cIAP-1 and H574A in cIAP-2). cIAP-1 and cIAP-2 RING mutants both failed to undergo degradation by FFA treatment and actually accumulated after 16 h of PA treatment (Fig. 2D), consistent with previous finding demonstrating that loss of the E3 ligase activity results in more stable cIAP-1 and cIAP-2 proteins (11). These results imply that cIAPs undergo autoubiquitination followed by proteosomal degradation upon FFA treatment.

cIAP-1 knockdown sensitizes hepatocytes to FFA-mediated apoptosis. The results above suggest that depletion of cIAPs may contribute to efficient FFA-mediated apoptosis. To test this postulate, Hep3B cells were treated with PA (200 μM) with or without the SMAC mimetic JP1584 (500 nM), which causes rapid degradation of cIAPs (18). In the presence of JP1584, PA-mediated apoptosis was significantly enhanced, as demonstrated by increased apoptotic nuclear morphology and

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**Fig. 2. Decrease of cIAPs is caspase independent and proteasome dependent.** A: wild-type (wt) Huh-7 cells were treated with PA (800 μM) in the presence (middle) or absence (left) of the pan-caspase inhibitor Q-VD-OPH (QVD; 5 μM; 30 min preincubation). Right: Huh-7 cells stably transfected with shRNA against caspase 8 (sh casp8) were treated with PA (800 μM). At the indicated time points, cell lysates were obtained and analyzed by immunoblot analysis for cIAP-1 and cIAP-2. Asterisk indicates a nonspecific band. B: Huh-7 cells were treated with 800 μM PA for the indicated time in the presence or absence of the proteasome inhibitor MG132 (MG; 10 μM). Whole cell lysates were obtained and analyzed by immunoblot analysis for cIAP-1 and cIAP-2. C: cryopreserved primary human hepatocytes were cultured for 24 h as described in MATERIALS AND METHODS then treated with 600 μM PA for the indicated time in the presence or absence of MG132 (10 μM). Whole cell lysates were analyzed by immunoblot analysis for cIAP-1 and cIAP-2. D: Huh-7 cells were left untransfected (left) or transiently transfected with plasmids encoding HA-cIAP-1-H588A or HA-cIAP-2-H574A (right) and treated with 800 μM of PA for the indicated times; cell lysates were analyzed by immunoblot for expression of endogenous cIAP-1 and cIAP-2 (left) by using antisera against cIAP-1 and cIAP-2, or HA-cIAP-1-H588A and HA-cIAP-2-H574A (right) with an antibody against the HA tag. Actin was used as loading control.
cleavage of PARP, a well-known substrate for activated caspase 3 (Fig. 3, A and B). In contrast, JP1584 did not sensitize the cells to OA-induced apoptosis (Fig. 3, A and B). To further examine whether one of the cIAPs has a potentially dominant role in protecting against lipoapoptosis, wild-type and Huh-7 cells stably expressing shRNA targeting cIAP-1 or cIAP-2 were treated with a concentration of PA (100 μM) that does not cause significant apoptosis in wild-type cells. Wild-type Huh-7 and cIAP-2 shRNA cells exhibited minimum apoptosis upon treatment with PA, whereas cIAP-1 knockdown sensitized the cells to PA-mediated apoptosis (Fig. 3, C and D). Lipoapoptosis in cIAP-1-deficient cells was blocked by

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**Fig. 3. Knockdown of cIAP-1 sensitizes hepatocyte to PA-mediated apoptosis.** A: Hep3B cells were treated with vehicle or PA (200 μM) or OA (200 μM), in the presence or absence of JP1584 (500 nM) for 16 h. Apoptosis was quantitated by morphological criteria. Data are expressed as means ± SE (*P < 0.05). B: Hep3B cells treated as in A were lysed and the protein extracts were analyzed by immunoblot for poly(ADP-ribose) polymerase (PARP). C and D: Huh-7 cells stably transfected with shRNA against either cIAP-1 or cIAP-2 were treated with vehicle or PA (100 μM) for 16 h. Apoptosis was assessed morphologically after DAPI staining (C) and by measuring caspase 3/7 activation (D). RFU, rhodamine fluorescence units. Data are expressed as means ± SE (*P < 0.01). E: Huh-7 cells were transiently cotransfected with pEGFP-N1 [encoding green fluorescent protein (GFP)] and either HA-cIAP-1-H588A or an empty plasmid (pcDNA3.1) for 48 h, followed by treatment with PA (800 μM) for 16 h. GFP-positive cells were scored as apoptotic on the basis of morphological criteria after staining with DAPI using a fluorescence microscope. Data are expressed as means ± SE (*P < 0.05). F: Huh-7 cells treated as in E were lysed and the protein extracts were analyzed by immunoblot for PARP.
QVD-OPH, a pan-caspase inhibitor, demonstrating that cell death was caspase dependent (Fig. 3D). To confirm that cIAP-1 degradation promotes PA-induced apoptosis, Huh-7 cells were transfected with cIAP-1 H588A (RING mutant) or with an empty vector (pcDNA3.1) as control, along with pEGFP-N1 expressing green fluorescent protein at a ratio of 3:1 to identify the successfully transfected cells. Cells were then treated with PA (800 μM) for 16 h and apoptosis was quantitated by fluorescence microscopy on GFP-positive cells after staining with DAPI, and by immunoblot analysis for cleaved PARP. Indeed, cells expressing the nondegradable cIAP-1 H588A displayed increased resistance to PA-induced apoptosis (Fig. 3, E and F). Finally, to further test this concept in nontransformed cells, we treated isolated primary mouse hepatocytes with PA or OA, in the presence or absence of JP1584. Consistently, JP1584 enhanced PA-mediated apoptosis in isolated mouse hepatocytes but had no effect on OA-mediated cytotoxicity (Fig. 4). Taken together, these results suggest that cellular depletion of cIAP-1 by its E3 ligase activity contributes to PA-mediated hepatocyte apoptosis.

Because DR5 contributes to lipoapoptosis and cIAPs modulate death receptor signaling, we sought to ascertain whether cIAPs inhibited lipoapoptosis by interfering with DR5-mediated signaling pathways. To examine this concept we first verified whether JP1584 enhances transcriptional activation of DR5. As previously reported by us, PA induced a dose-dependent increase in DR5 mRNA expression (Fig. 5A) (8). However, no changes in DR5 mRNA expression were observed in Huh-7 cells treated with 400 μM PA plus JP1584 compared with cells treated with PA alone (Fig. 5A). Next, we assessed whether the sensitizing effect of JP1584 is blocked in hepatocytes isolated from mice deficient in TRAIL-R, the only apoptosis-inducing TRAIL receptor in mice, which resembles human DR5 (32). Notably, JP1584 did not enhance PA-induced apoptosis in isolated hepatocyte from TRAIL-R−/− mice, suggesting that cIAPs exert their antiapoptotic effect downstream of DR5 (Fig. 5B). Previous studies suggested that SMAC mimetic treatment or cIAP-1 depletion induced NF-κB-mediated production of TNF-α and consequent TNF-α-induced apoptosis via an autocrine loop (30). In the same conditions, ubiquitination of the kinase RIP-1 is prevented, sensitizing the cells to RIP-1-dependent necroptosis during death receptor signaling (13, 14). To rule out the possibility that JP1584 enhances FFA toxicity by promoting necroptosis, we assessed whether necrostatin-1, an inhibitor of RIP-1 (14), can prevent the SMAC mimetic-dependent sensitization to PA-mediated cell death. However, necrostatin-1 did not confer significant protection from cell death induced by PA plus JP1584 (Fig. 5C). Similarly, addition of a neutralizing antibody against TNF-α did not attenuate cell death or caspase activation caused by PA plus JP1584 (Fig. 5, D and E), suggesting that SMAC mimetic sensitization to PA-induced cell death is not mediated by TNF-α. Collectively, these results indicate that elimination of cIAPs contributes to PA-induced apoptosis by enhancing a DR5-mediated signaling pathway.

**DISCUSSION**

The present study provides new insights into the mechanisms of FFA-induced hepatocyte injury. The principal findings of this study indicate that 1) cIAP-1 and cIAP-2 undergo proteasomal degradation in hepatocytes following treatment with PA; 2) cellular depletion of cIAPs sensitizes hepatocytes to FFA cytotoxicity; and 3) cIAP-1 is decreased in human liver specimens of NASH. These results are discussed in greater detail below.

CIAp-1 and cIAP-2 are negative regulators of apoptosis (26). Although various studies indicated that cIAPs are overexpressed in malignant cells, including hepatocellular carcinoma, they are also present in nontransformed cells (25, 30, 34). The role of cIAPs in pathological conditions other than malignancies remains largely unexplored. In the present study, we observed that cIAP-1 and cIAP-2 decreased upon treatment with saturated FFA in liver cell lines. In addition, our study revealed that depletion of cIAP-1, but not cIAP-2, contributes to lipoapoptosis in Huh-7 cells. cIAPs are regulated by transcriptional or posttranscriptional mechanisms. For example, cIAP-2 can be transcriptionally regulated by NF-κB (26). However, our results suggested that the decrease of cIAP-1 and cIAP-2 protein levels during PA treatment were not due to a decrease in steady-state mRNA levels, and, therefore, were unlikely to be due to transcriptional repression. Posttranslational degradation of cIAPs can occur in several processes. The carboxy termini of both cIAP-1 and cIAP-2 contain a highly conserved RING domain, which has a key role in posttranscriptional degradation of these proteins (26). cIAPs undergo autoubiquitination and degradation by the 26S proteasome (26). cIAP-1 can also be cleaved by caspase 8 and caspase 3 and subsequently degraded (9, 18). However, in our present observation degradation of cIAPs by FFA was not inhibited either by knockdown of caspase 8 or by a pan-caspase inhib-
itor, suggesting that the degradation of these proteins occurs independent of caspase activity. Instead, FFA-induced cIAP-1 and cIAP-2 degradation was proteasome dependent and required their E3 ligase activity. The precise mechanism by which FFAs induce cIAP-1 and cIAP-2 autoubiquitination remains unclear but merits further investigation. FFAs may promote assembly of protein complexes at the cell membrane either by altering membrane fluidity or via activating kinase signaling pathways (8, 17). Likely one of these mechanisms promotes cIAP-1 and cIAP-2 assembly and conformational changes necessary for their autoubiquitination.

cIAPs are capable of regulating proteins through either degradative K48-linked or nondegradative K63-linked polyubiquitination (20). cIAP-1 and cIAP-2 are known to ubiquitinate RIP-1, resulting in activation of prosurvival pathways through canonical activation of NF-κB (6). However, when cIAP-1 and cIAP-2 are degraded by SMAC mimetics, TNF-α is generated through noncanonical activation of NF-κB, switching the signal to a proapoptotic autocrine loop (30). This mechanism of cytotoxicity was excluded in lipoapoptosis by demonstrating that TNF-α neutralizing antibodies do not protect against PA-mediated cell killing. The proapoptotic effect of cIAP-1 elimination in lipoapoptosis was not apparent in hepatocytes isolated from TRAIL-R−/−/− mice, implying that cIAPs restrain DR5-mediated cytotoxicity during lipoapoptosis. Likely, the depletion of cIAPs by toxic FFA augment DR5 cytotoxic signaling by enhancing formation of a caspase 8-con-
taining protein complex, promoting activation of this proapoptotic protease (6). This interpretation of our data is supported by the observation that FFA cytotoxicity is caspase 8 dependent (8). Although the absence of cIAPs and therefore RIP-1 ubiquitination could also potentially promote RIP-1-dependent necroptosis, the RIP-1 inhibitor necrostatin-1 did not protect from cell death in our study (14). Therefore, necroptosis does not appear to occur during FFA-mediated cell death.

Although both cIAP-1 and cIAP-2 decreased upon PA treatment, our study suggests that depletion of cIAP-1, but not cIAP-2, contributes to FFA-induced apoptosis. In agreement with our findings, Qi and others (25) recently described the importance of cIAP-1 in protecting against FFA-induced cell death in pancreatic cells. Of note, it has been previously shown that elimination of cIAP-2 upon SMAC mimetic treatment is dependent on cIAP-1 E3 ligase activity (12). Thus degradation of cIAP-2 may be downstream of cIAP-1 activation, rendering cIAP-1 the dominant inhibitor in this model. We also note that in isolated, previously cryopreserved human hepatocytes, cIAP-2 was preferentially degraded compared with cIAP-1, and thus the precise cIAP regulating lipopoptosis remains ambiguous. Since expression of IAP proteins seems to vary among the cell types, the relative roles of cIAP-1 and cIAP-2 are likely cell type and stimulus specific (27, 34).

Collectively, our results provide new insights regarding the role of cIAPs in FFA-mediated apoptosis. Our results suggest that elimination of cIAPs by their autoubiquitination and pro-apoptotic protease (6). This interpretation of our data is supported by the observation that FFA cytotoxicity is caspase 8 dependent (8). Although the absence of cIAPs and therefore RIP-1 ubiquitination could also potentially promote RIP-1-dependent necroptosis, the RIP-1 inhibitor necrostatin-1 did not protect from cell death in our study (14). Therefore, necroptosis does not appear to occur during FFA-mediated cell death.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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