Fructose Protects Murine Hepatocytes from Tumor Necrosis Factor-induced Apoptosis by Modulating JNK Signaling

Tobias Speicher, Ulrike A. Köhler, Alexander Choukeř, Sabine Werner, Timo Weiland, and Albrecht Wendel

Background: Fructose-induced ATP depletion selectively protects hepatocytes but not hepatoma cell lines from actinomycin D (ActD)/TNF-induced apoptosis.

Results: Fructose induces a cAMP response that via PKA prevents sustained activation of ActD/TNF-induced pro-apoptotic JNK activation, thereby Bid cleavage and apoptosis.

Conclusion: These findings explain the hepatocytic mechanism of fructose-mediated cytoprotection against ActD/TNF-induced apoptosis.

Significance: These findings explain selective cytoprotection of hepatocytes, potentially enabling selective tumor targeting.

Selective and organ-specific tumor therapy has been partially reached in a variety of malignant diseases. However, in solid tissue cancer of the liver, in particular in hepatocellular carcinoma, such a therapeutic concept is currently not available. TNF was discovered by its tumor necrotic activity and was the first cytokine to be used for cancer therapy. TNF induces apoptotic cell death in hepatocytes via TNF receptor 1, most efficiently concomitantly with inhibition of NF-κB. Inhibition of NF-κB-induced pro-survival gene expression can be achieved under experimental conditions by actinomycin D (ActD)-mediated global transcriptional inhibition (1). Inhibition of NF-κB signaling was shown to sustain TNF-induced JNK activation, resulting in activation of the intrinsic apoptosis pathway via cleavage of Bid (2). Truncated Bid (t-Bid) translocates to mitochondria and induces pore formation, cytochrome c release, and subsequent activation of the caspase cascade, which finally leads to execution of ActD/TNF-induced apoptosis.

Recombinant TNFα is a potent antineoplastic drug, but treatment of primary or secondary tumors of the liver is limited due to its profound systemic as well as hepatic toxicity. To avoid such toxicity, locoregional drug delivery systems such as isolated hepatic or isolated limb perfusion were applied (3). Although combination of TNF with antineoplastic agents such as melphalan in isolated limb perfusion allowed impressive clinical success in the remission of advanced non-resectable soft tissue sarcomas, selectivity for tumor cells remains a largely unsolved problem in liver cancer (4). As an alternative approach to a selective tumor cell hit, we propose and demonstrate the feasibility of a selective protection of healthy hepatocytes against death receptor ligand-induced killing based on the differences of the liver-specific fructose metabolism between healthy and transformed hepatocytes.

We previously showed that fructose-induced hepatic ATP depletion inversely controls CD95- and ActD/TNF-induced apoptosis (5). The phenomenon of fructose-induced ATP depletion only occurs in the liver due to the unique fructose metabolism in this organ. Within hepatocytes, ketohexoses are phosphorylated via fructokinase very rapidly to fructose 1-phosphate and then cleaved with a much slower rate by aldolase b. As a consequence, high doses of fructose lead to an accumulation of fructose 1-phosphate at the expense of ATP. In other words, fructose functions as a phosphate trap and as a consequence the ATP stores are transiently depleted in rodents...
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and in humans without inducing detectable toxicity within this time (5, 6).

Although ATP-depleted hepatocytes are fully protected against TNF-induced apoptosis in vitro and in vivo, the sensitivity toward CD95-induced cell death is shown to be increased (5). However, malignantly transformed hepatoma cell lines are not depleted of ATP by fructose and hence fail to be protected against ActD/TNF-induced toxicity upon fructose treatment. As the biochemical rationale, we found type II hexokinase expression to be up-regulated in malignant hepatocytes, which in turn induces a switch of the hepatocyte-specific fructose metabolism via fructose 1-phosphate to the ubiquitous metabolism via fructose 6-phosphate. This explains why fructose induces neither ATP depletion nor cytoprotection against TNF-induced apoptosis in transformed cells (7). The most interesting clinical correlate is that increased expression of type II hexokinase was shown to be characteristic for advanced tumor stage in human hepatocellular carcinomas (8), as analyzed by uptake of 18-fluorine-fluorodeoxyglucose via PET imaging (9).

To understand the molecular mechanisms of fructose to conversely modulate TNF or CD95-induced apoptosis, we investigated downstream events following fructose-induced ATP depletion in healthy hepatocytes. We show that massive ATP depletion results in a transient accumulation and release of the degradation product adenosine from hepatocytes in vitro and in vivo. This in turn leads to increased levels of intracellular cAMP and cytoprotection. We show that adenosine and cAMP analogues mimic the dichotomous effects of fructose-induced ATP depletion on ActD/TNF- and CD95-induced apoptosis and that inhibition of cAMP-dependent protein kinase A abolishes them. To explain the dichotomy between enhanced CD95-induced toxicity and suppressed ActD/TNF-induced toxicity under these conditions, we addressed the role of JNK activation in signal mediation. Fructose as well as cAMP analogues prevented the sustained, pro-apoptotic part of TNF-inhibited JNK activation in vitro and in vivo. Finally, we show that fructose-induced JNK-inhibition prevents the cleavage-mediated activation and translocation of the pro-apoptotic protein Bid to mitochondria. Fructose thereby prevents activation of the intrinsic apoptotic pathway in response to TNF receptor activation, in agreement with our previous report of fructose-mediated inhibition of TNF-induced cytochrome c release from mitochondria in primary hepatocytes (5). The mechanistic evidence presented herein lends molecular support for the feasibility of a strategy to selectively protect healthy hepatocytes from TNF receptor-induced cell death by non-toxic doses of fructose.

EXPERIMENTAL PROCEDURES

Agonistic CD95 antibody (clone Jo-2) was purchased from BD Pharmingen (San Diego, CA), Bid antibody, p-JNK, and p-CREB were from Cell Signaling (Boston, MA), HRP-coupled rabbit anti-goat was from Dako (Glostrup, Denmark), c-FILIP, was from Enzo (New York, NY), HRP-coupled goat anti-rabbit was from Dianova (Hamburg, Germany), agarse-coupled JNK1/JNK2, total JNK, p-c-jun, c-jun, and MKP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA), murine TNF was from Innogenetics (Ghent, Belgium), KT 5720 was from Alexis (Lausen, CH), Bt2cAMP and ZM241385 were from Tocris Cookson (Bristol, UK), and 8-Br-cAMP-AM was from Biolog Life Science (Bremen, Germany). Cell culture plates and inserts were from Greiner (Nütingen, Germany), culture medium, FCS, penicillin, and streptomycin were from PAA (Pasching, Germany), collagen was from Serva (Heidelberg, Germany), and Percoll was from Pharmacia Biotech (Uppsala, Sweden). Unless further specified, other reagents were purchased from Sigma-Aldrich.

Cell Cultures—Isolation, incubation, and culture of primary hepatocytes and cell lines were performed as previously described (5). Apoptosis was induced by using agonistic CD95 antibody (100 ng/ml), ActD (400 ng/ml for primary cells and 1 μg/ml for cell lines) and TNF (100 ng/ml). Cytotoxicity was quantified by measurement of lactate dehydrogenase release. LDH was analyzed in the culture supernatant (S) and in the remaining cell monolayer (C) following cellular lysis with PBS/0.1% Triton X-100. The percentage of LDH release was calculated as the ratio S/(S + C). In coculture experiments cytotoxicity was measured by AlamarBlue assay according to the manufacturer’s description (BioSource, Solingen, Germany). ATP and specific caspase-3/-7-like protease activity were determined as described previously (5). Intracellular cAMP was determined by using the Direct cAMP Enzyme Immunoassay Kit according to the manufacturer’s instructions (Assay Designs, Ann Arbor, MI).

JNK Activity—5 × 10⁶ primary hepatocytes were washed (2 mM sodium vanadate in PBS), lysed (10 mM Tris, pH 7.6, 1% Triton X-100, 0.05 M NaCl, 5 mM EDTA, 2 mM sodium vanadate, 20 mg/ml aprotinin), centrifuged (15 min, 4 °C, 14,000 × g). 100 μg of protein was incubated for incubation with 10 nM antibody (100 ng/ml), ActD (400 ng/ml for primary cells and 1 μg/ml for cell lines) and TNF (100 ng/ml). Cytotoxicity was quantified by measurement of lactate dehydrogenase release. LDH was analyzed in the culture supernatant (S) and in the remaining cell monolayer (C) following cellular lysis with PBS/0.1% Triton X-100. The percentage of LDH release was calculated as the ratio S/(S + C). In co-culture experiments cytotoxicity was measured by AlamarBlue assay according to the manufacturer’s description (BioSource, Solingen, Germany). ATP and specific caspase-3/-7-like protease activity were determined as described previously (5). Intracellular cAMP was determined by using the Direct cAMP Enzyme Immunoassay Kit according to the manufacturer’s instructions (Assay Designs, Ann Arbor, MI).

In Vivo Experiments—Pathogen-free male C57Bl6 mice (25 g) from the Animal Breeding Facility of the University of Konstanz were held at 22 °C and 55% humidity at a constant day-night cycle of 12 h. Mice were starved overnight before the in vivo experiments at 8:00 a.m. All steps were performed according to the guidelines of the European Council (directive 86/609/EEC) and the national German authorities and followed the directives of the University of Konstanz Ethical Committee.

Fructose (10 g/kg) and d-galactosamine (700 mg/kg) were injected intraperitoneally in 300 μl of water. TNF (2 μg/kg) was injected intravenously in 300 μl of saline (0.1% human serum albumin). Blood samples were obtained after lethal intravenous anesthesia with 150 mg/kg pentobarbital, 0.8 mg/kg heparin. Plasma concentrations of adenosine were determined by high performance liquid chromatography as described previously.
Alanine aminotransferase content was determined by using an enzymatic/colorimetric kit (Sigma-Aldrich).

Immunoblotting—Translocation of Bid from cytosol to mitochondria was analyzed by digitonin permeabilization method. To separate cytosol- and mitochondria-containing fractions for Western blotting, 5 × 10^6 cells per six wells were used. After washing the cells with PBS, the cell membrane was permeabilized in buffer A (10 mM Hepes, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, pH 7.4) containing 0.05% digitonin by gentle shaking for 8 min. Cells were scraped, centrifuged for 10 min at 10,000 × g, 23 °C. Protein from the supernatants was separated on 15% polyacrylamide gels for cytosolic detection of Bid. Integrity of the mitochondria membrane was confirmed by cytochrome c Western blotting. The pellet was resuspended in buffer A containing 2% CHAPS, incubated for 30 min on ice, and centrifuged for 15 min at 10,000 × g, 23 °C. Protein from the supernatants was separated on 15% polyacrylamide gels for cytosolic detection of Bid. Integrity of the mitochondria membrane was confirmed by cytochrome c Western blotting.

Statistical Analysis—Statistical differences were determined with an unpaired t test if applicable, or analyzed by one-way analysis of variance followed by Dunnett’s multiple comparison test.

RESULTS

Fructose-induced Hepatic ATP Depletion Leads to Release of Adenosine in Vitro and in Vivo—Our previous study has shown that fructose-induced ATP depletion protects hepatocytes against TNF-induced apoptosis and, in contrast, enhances CD95-induced apoptosis (5). Further examination showed that cytoprotection against TNF-induced cell death is lost in malignantly transformed hepatocytes due to up-regulation of hexokinase 2. Increased expression of this enzyme is described as a typical feature of fast growing tumors but prevents fructose-induced ATP depletion and subsequently cytoprotection from TNF-induced cell death in malignantly transformed hepatocytes (7). These findings suggest the combination of fructose and TNF to achieve specific tumor targeting by selective cytoprotection against TNF-induced apoptosis in healthy hepatocytes.

To confirm our previous findings, primary murine hepatocytes were treated with various concentrations of fructose for 30 min before addition of TNF or αCD95 antibody. For TNF-induced apoptosis cells were sensitized with ActD 15 min before TNF application. Toxicity was assessed by LDH release from dying cells after 18 h. Fructose prevented ActD/TNF-induced (EC50 9.3 mM) but enhanced CD95-induced (EC50 3.0 mM) cell death in primary hepatocytes in a concentration-dependent manner, whereas fructose alone did not affect basal hepatocyte viability at any concentration tested (Fig. 1A). The fact that induction of hepatocyte apoptosis via the TNF pathway in contrast to the CD95 pathway requires sensitization is a crucial difference between these two cell death machineries studied here. We compared the inverse effect of fructose-mediated ATP depletion on these two apoptotic stimuli to identify molecular mechanisms preventing TNF-induced apoptosis and...
to include potential harmful action of fructose treatment. We showed that the enhancing effect of fructose on CD95-induced apoptosis is independent on pretreatment with ActD (Fig. 1B). Therefore, induction of apoptosis by CD95 was performed in the absence of ActD in the following experiments.

Next, we focused on the secondary effects of fructose-induced hepatic ATP depletion. Fructose-induced transient, non-toxic ATP depletion is due to the liver-specific fructose metabolism via fructokinase and aldolase B (14), which has been described in mice and humans (5, 6). A general loss of adenosine nucleotides has been described during fructose-induced ATP depletion (14). We addressed the metabolic consequences on production and release of adenosine as a potential degradation product during ATP depletion from primary hepatocytes in vitro after fructose exposure. 50 mM fructose depleted hepatocyte ATP by >70% within 20 min. Concomitantly, hepatocytes released adenosine into the supernatant, where the concentration doubled from 24 to 49 nM within 20 min (Fig. 1C). To extend these in vitro findings to the in vivo situation, we examined whether injection of fructose into mice leads to the accumulation of plasma adenosine. We administered 10 g/kg fructose intraperitoneally into mice, a dose that we previously showed to induce hepatic ATP depletion and protection against TNF-induced hepatotoxicity (5). Although adenosine has a very short half-life and will be severely diluted in the total plasma volume after release from the liver, we detected a transient 9-fold increase in plasma adenosine with a maximum adenosine plasma concentration of 900 nM after fructose treatment (Fig. 1D).

**ATP-depleting Carbohydrates and Adenosine Induce a cAMP Response in Primary Murine Hepatocytes**—Further mechanistic details were studied in vitro in isolated primary murine hepatocytes. Others had observed that adenosine induces cytoprotection during cell stress or injury via adenylyl cyclase-activating receptors (15). Therefore, we checked the intracellular cAMP response upon treatment with fructose as an ATP-depleting ketohexose, glucose as a non-depleting control, or adenosine. Although 50 mM fructose induced a cAMP response to 180% of control cells within 40 min, the non-ATP-depleting sugar glucose did not increase the cAMP level (Fig. 2A). Adenosine as such (100 μM) also induced a time-dependent cAMP response in primary murine hepatocytes. Within 10 min after adenosine administration the level of cAMP reached a maximum of 170% compared with controls (Fig. 2B).

**Adenosine and cAMP Mimic the Effects of Fructose on Cytokine-induced Apoptosis in Primary Hepatocytes but Not in Cell Lines**—We next compared adenosine or cAMP with fructose to modulate cytokine-induced apoptosis by pretreating hepatocytes with fructose, adenosine, or a cAMP analogue (for 15, 5, 45 min, respectively), before sensitization with ActD. After an additional 15 min, cell death was induced with TNF. Similar to fructose, treatment with adenosine or one of the two cAMP analogues Bt2cAMP and Br-cAMP protected primary hepatocytes from ActD/TNF-induced cell death (Fig. 3A). In analogy, treatment of primary hepatocytes with adenosine or cAMP analogues, respectively, enhanced CD95-induced cell death (Fig. 3B). This was associated with increased activation of the effector caspases 3 and 7 (data not shown).

Pursuing our clinical aim to selectively protect primordial hepatocytes while killing cancer cells, we determined whether selective protection by fructose holds true in co-cultures of primary hepatocytes with malignantly transformed hepatocytes. Cytotoxicity and activation of effector caspases was assessed in primary murine hepatocytes co-cultured with HepG2 cells in permeable inserts and compared with monoculture conditions. Fructose-mediated inhibition of Act/TNF-induced caspase activation and cell death in healthy hepatocytes was independent of the presence of HepG2 cells. In contrast, monocultured HepG2 cells were sensitive to ActD/TNF-induced apoptosis even in the presence of fructose, which was also not affected by co-culture. Thus, fructose allowed the selective killing of HepG2 cells by ActD/TNF in this setting (Fig. 3, C and D).

We previously showed that in addition to HepG2 other hepatic cell lines are also not protected by fructose against ActD/TNF-induced cell death (7). After we showed the cytotoxic effect of adenosine and cAMP in primary cells we wondered whether this mechanism is restricted to normal hepatocytes. We tested whether adenosine, cAMP, or a JNK inhibitor protects hepatic cell lines against ActD/TNF-induced cell death. Neither adenosine, nor the adenylyl-cyclase down-stream second messenger cAMP, induced pronounced cytokine protection against ActD/TNF-induced cell death in the cell lines used: Hepa 1–6, HepG2, Clone 9, and Huh7. Also, pre-treatment with the JNK inhibitor SP600125 did not prevent cell death in this setting (supplemental Fig. S1).

**Fructose- and cAMP-mediated Modulation of CD95- and TNF-induced Apoptosis Is Sensitive to PKA Inhibition**—Because we had shown that fructose treatment induces a cAMP
response and that cAMP analogues mimic the effects of fructose on cytokine-induced cell death, we investigated whether PKA is a downstream effector of fructose-induced cAMP signaling, modulating receptor-mediated apoptosis. We analyzed the effects of the PKA inhibitor KT5720 on ActD/TNF-induced cytotoxicity modulated by fructose or Bt2cAMP, respectively. Primary hepatocytes were treated with KT5720 30 min prior to addition of fructose or Bt2cAMP, respectively. After another 15 min, alternatively 45 min in the case of Bt2cAMP, ActD was added, and 15 min later death receptor activation was induced by TNF. After 18 h, toxicity and activity of effector caspases was assessed. Fructose- and cAMP-mediated inhibition of ActD/TNF-induced caspase activity and LDH release were completely reversed by KT5720 (Fig. 4, A and B), as well as by the structurally unrelated PKA inhibitor H-89 (data not shown). In the following experiment we analyzed whether PKA inhibition also modulates the sensitizing effects of fructose and cAMP on CD95-induced cell death. Primary hepatocytes were pretreated with KT5720 30 min prior to addition of fructose or Bt2cAMP, respectively. After another 15 min, alternatively 45 min in the case of Bt2cAMP, ActD was added, and 15 min later death receptor activation was induced by TNF. After 18 h, toxicity and activity of effector caspases was assessed. Fructose- and cAMP-mediated inhibition of ActD/TNF-induced caspase activity and LDH release were completely reversed by KT5720 (Fig. 4, A and B), as well as by the structurally unrelated PKA inhibitor H-89 (data not shown).

Fructose and cAMP Analogues Prevent ActD/TNF-induced Prolonged JNK Activity and Toxicity—TNF-induced JNK activity occurs in two phases. The early transient phase (≤1 h) mediates cell survival, whereas the sustained phase (>1 h) mediates pro-apoptotic signaling (16). TNF-induced NF-κB activation is described to prevent apoptosis by inhibiting prolonged JNK activity (17). We analyzed the JNK activation pattern in ActD/TNF-induced signaling modulated by fructose or the JNK inhibitor SP600125 by phospho-specific Western blotting for JNK, c-Jun after 3 h, and by in vitro kinase assay in primary murine hepatocytes. TNF-induced phosphorylation of the p46 and p54 JNK isoform, and of subsequent cytotoxicity only occurred when cells were sensitized with ActD. Pretreatment with 50 mM fructose 30 min before TNF stimulus prevented phosphorylation of both JNK isoforms, phosphorylation of c-Jun, and cytotoxicity (Fig. 5, A and B). Additionally, in vitro kinase activity of JNK was analyzed...
over time. TNF alone induced a maximum JNK activity after 30 min, followed by a continuous reduction, reaching the basal level after 120 min without inducing toxicity (Fig. 5B). Sensitization of hepatocytes by ActD caused sustained TNF-induced JNK activity and cell death in more than 80% of primary hepatocytes (Fig. 5B). Pretreatment with SP600125 attenuated the early (anti-apoptotic) and completely blocked the late (pro-apoptotic) phases of ActD/TNF-induced JNK activity and reduced cell death to 44%. Fructose pretreatment strongly suppressed ActD/TNF-induced prolonged JNK activity and subsequent toxicity (Fig. 5B). Thereby, fructose restricted the course of JNK activation to the early, transient phase, which, like in case of TNF alone, does not induce cell death. Fructose pretreatment inhibited ActD/TNF-induced sustained JNK activation in a concentration- and time-dependent manner, which correlates with the cytoprotective effects (supplemental Fig. S3). Because we claimed the involvement of cAMP as a downstream effect of fructose-induced ATP depletion, we examined the effect of the stable cAMP analogue Br-cAMP on ActD/TNF-induced JNK activity. In analogy to fructose, Br-cAMP

FIGURE 4. PKA inhibition reverses the inverse effects of fructose or cAMP on CD95- and TNF-induced apoptosis. A and B, effect of pretreatment with the PKA inhibitor KT5720 (added 30 min before fructose or cAMP analogue) on fructose- or Bt2cAMP-mediated protection against ActD/TNF-induced apoptosis and on sensitization against CD95-induced apoptosis (C and D) was analyzed in primary murine hepatocytes. Activity of effector caspases and LDH release was determined after 9 and 18 h, respectively. Data represent mean ± S.D. of triplicate cultures.
reduced ActD/TNF-induced prolonged JNK activation and toxicity (Fig. 5C) in a concentration- and time-dependent manner (supplemental Fig. S3). Subsequently, we tested whether or not fructose- and cAMP-mediated modulation of JNK activity is PKA-dependent. We analyzed the effect of fructose and cAMP on phosphorylation of cAMP response element-binding protein (CREB) as a downstream effect of PKA activity. Fructose and cAMP increased phosphorylation of CREB at Ser-133 after 2 h, which was reversed by pretreatment with the PKA inhibitor (Fig. 5D). Inhibition of ActD/TNF-induced prolonged...
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JNK activation by fructose- and cAMP was also reversed by PKA inhibition.

cAMP-mediated inhibition of UV-induced JNK activity and apoptosis in Rat-1 and NIH 3T3 cells has been shown to be dependent on CREB-mediated induction of cellular FLICE-inhibitory protein (c-FLIPL) and mitogen-activated protein kinase phosphatase-1 (MKP-1), a negative regulator of JNK (18). We tested whether a similar mechanism is involved in PKA-mediated inhibition of JNK by fructose and cAMP in primary murine hepatocytes during TNF-induced apoptosis. c-FlipL levels were not affected by fructose or cAMP. In contrast, levels of MKP-1 were affected by fructose or cAMP. 3 h after treatment with ActD/TNF the level of MKP-1 was reduced and phosphorylation of JNK was detected. Both effects were prevented by cAMP and fructose in a PKA-dependent manner (Fig. 5D).

After showing the potential of fructose to inhibit TNF-induced JNK activation and toxicity in, we were interested in whether fructose had a similar effect on TNF-induced hepatic JNK activation in vivo. Because murine apoptosis induced in vitro and in vivo by TNF requires sensitization, e.g. by transcriptional arrest, the mice were sensitized with d-galactosamine. According to the results in primary hepatocytes, TNF induced activation of the p46/p54 JNK isoforms only in sensitized mice, when transcription was blocked. According to the in vitro data the phosphorylation of both JNK isoforms was reduced by fructose pretreatment, which also prevented liver damage determined by alanine aminotransferase release (Fig. 5E).

Fructose Prevents ActD/TNF-induced Cleavage of Bid and Translocation of t-Bid to Mitochondria in a PKA-dependent Manner—JNK was shown to be essential for TNF-induced apoptosis in hepatocytes by inducing cleavage of the "BH3-domain only protein" Bid to truncated Bid (t-Bid). Truncated Bid translocates to the nucleus, induces release of mitochondrial cytochrome c, and thereby mediates amplification of the caspase cascade (19). Bid-deficient primary hepatocytes have been shown to be resistant against ActD/TNF-induced apoptosis, and our previous data showed that fructose-induced ATP depletion prevents TNF-induced cytochrome c release (5). We tested whether pharmacological or fructose-mediated inhibition of JNK prevents ActD/TNF-induced activation and mitochondrial translocation of Bid. The amount of full-length Bid in the cytosol was reduced by apoptosis induction by ActD/TNF, analyzed after 6 h. This was most likely due to translocation of activated Bid to mitochondria. Pretreatment with fructose or SP600125 prevented ActD/TNF-induced reduction of full-length Bid in the cytosol (Fig. 6A).

In addition we analyzed the amount of truncated, activated Bid in the mitochondrial fraction after treatment of hepatocytes with ActD/TNF. After 4 h we detected t-Bid at mitochondria, and most t-Bid was detected after 6 h. Fructose pretreatment completely prevented ActD/TNF-induced t-Bid translocation to mitochondria between 3 and 6 h after TNF stimulation (Fig. 6B). The effects of SP600125 and the PKA inhibitor KT5720 were analyzed as well in this setting. Complete fructose-induced inhibition of t-Bid translocation to mitochondria by fructose after 6 h of TNF stimulation was reversed by the PKA inhibitor KT5720. Additionally, inhibition of JNK activity by SP600125 strongly reduced ActD/TNF-induced t-Bid translocation to mitochondria.

DISCUSSION

Despite its failure in systemic cancer therapy, the locoregional treatment of solid limb tumors with combinations of TNF and melphalan achieved outstanding response rates at low adverse reactions (20–22). This encouraged us to provide molecular evidence for a better understanding of our previous findings that (i) fructose-induced ATP depletion prevents TNF-induced hepatocyte apoptosis in healthy murine and human hepatocytes in vitro and in mice in vivo (5, 7) and (ii), in contrast, various malignantly transformed hepatoma cell lines where not protected due to tumor-specific alterations in the energy metabolism (up-regulation of hexokinase 2) in the energy metabolism that prevented fructose-induced ATP depletion and cytotoxicity against TNF. Up-regulation of hexokinase 2 was described by others as being characteristic of highly malignant cells such as advanced stage hepatocellular carcinoma (8). Here, we comparatively studied the previously described dichotomous effects of fructose on cytokine-induced hepatotoxicity.
Our crucial finding is that fructose-induced ATP depletion leads to the systemic release and transient extracellular accumulation of the ATP degradation product adenosine from murine hepatocytes in vitro and in vivo (Fig. 1, C and D). Three arguable experimental issues need to be discussed in detail: 1) The time course of this adenosine release matches well with the decline of intracellular hepatocyte ATP levels, indicating that these processes follow a cause and effect relationship. 2) At the first glance, the concentration of adenosine found in vivo (i.e. ~1 μM) seems to be different from the ones used for the in vitro experiments (i.e. 100 μM). However, considering that the systemic half-life of adenosine in men is below 2 s (23), we have good reasons to assume that the increased plasma adenosine, which we measured 20 min after fructose injection, was due to (i) a continuous production and release and (ii) a concentration at the site of production, which was orders of magnitude higher than the 1 μM we measured in the steady state. From these kinetics, we conclude that the concentration of adenosine used in vitro is comparable to that found in vivo, if not underestimating the latter ones. 3) The concentrations of fructose we used are transiently reached within the liver after intake of fructose-sweetened commercially ubiquitous beverages in humans (24, 25). This might explain why they were not acutely toxic in any of our experiments, suggesting that we are dealing with non-toxic pharmacological doses of a naturally occurring sugar within a short exposure time toward hepatocytes.

Further experimental considerations to be discussed include: by co-culturing healthy hepatocytes and malignantly transformed cells we could show that the cytoprotective effect of fructose is restricted to healthy hepatocytes even under co-culture conditions (Fig. 3, C and D). It was shown that adenosine has the potential to protect the liver during ischemia/reperfusion injury adenosine receptor 2-mediated activation of adenylate cyclase resulting in the formation of cAMP (26). In agreement with these observations we demonstrated that fructose, as well as adenosine, lead to increased levels of intracellular cAMP in primary murine hepatocytes (Fig. 2). Furthermore, we provide evidence that both, adenosine and cAMP, as putative downstream mediators of fructose-induced ATP depletion, mimic the dichotomous effects of fructose on CD95- and TNF-induced apoptosis (Fig. 3, A and B). In addition we observed that two structurally unrelated PKA inhibitors reversed fructose- and cAMP-mediated effects on cytokine-induced cell death (Fig. 4). We showed that fructose and cAMP enhance phosphorylation of CREB, which was blocked by a PKA inhibitor. Our data indicate the involvement of PKA downstream of fructose-induced production of cAMP in the apoptosis-modulating effects. Thus, our data strongly suggest the involvement of adenosine/cAMP/PKA signaling in the apoptosis-modulating property of hepatic fructose metabolism. As a novel insight, we propose to localize the bifurcation of the fructose-mediated signaling mechanism between TNF- and CD95-induced apoptosis downstream of the level of PKA.

Because JNK has been described to be only required for TNF-induced but not for CD95-induced hepatocyte apoptosis, we extended our fructose studies on TNF-induced JNK signaling. In primary murine hepatocytes TNF induced a non-toxic, transient activity of JNK, which was sustained by sensitization via ActD-mediated transcriptional arrest; i.e. when the apoptotic power of TNF was facilitated. We found that fructose or cAMP prevented the late and sustained part of ActD/TNF-induced JNK activation as well as toxicity in a concentration- and time-dependent manner (Fig. 5). In addition to the in vitro kinase assay, Western blotting revealed that fructose prevented ActD/TNF-induced phosphorylation of both JNK isoforms in a PKA-dependent manner and of the downstream target c-Jun in primary hepatocytes (Fig. 5, A and D). cAMP has been reported to reduce phosphorylation of JNK via the phosphatase MKP-1 during apoptosis (18). We demonstrated that fructose and cAMP also increase MKP-1 levels in ActD/TNF-treated hepatocytes and correspondingly reduce phosphorylation of JNK in a PKA-dependent manner in primary murine hepatocytes. The finding of such a key selected point does of course not exclude further regulators of JNK activity under our conditions. Importantly, we showed that the potential of fructose to block TNF-induced JNK signaling also holds true for the in vivo situation, because fructose also reduced d-galactosamine/TNF-induced activation of JNK and toxicity in vivo (Fig. 5E).

Recently, the TNF pathway was described to be a key regulator of TNF-induced mitochondria-mediated hepatocyte cell death (2). TNF was shown to activate the pro-apoptotic protein Bid, which leads to amplification of the caspase cascade via the intrinsic pathway of TNF-induced hepatocyte apoptosis. In our experiments, the presence of fructose completely prevented ActD/TNF-induced Bid cleavage and t-Bid translocation to mitochondria in a PKA-dependent manner, i.e. activation of the intrinsic apoptotic pathway was blocked (Fig. 6, A and B). This is in line with our previous report on fructose-mediated inhibition of TNF-induced cytochrome c release from mitochondria in primary hepatocytes (5), which is the main result of mitochondrial activation leading to activation of caspases.

In conclusion, our findings are consistent with the uniform conception that fructose-induced ATP depletion leads to adenosine release, which in turn induces cAMP signaling in an autocrine manner. Subsequent PKA activation inversely modulates CD95- and TNF-induced apoptosis. We propose that the anti-apoptotic effect of fructose on TNF-induced apoptosis is due to the inhibition of the sustained phase of ActD/TNF-induced JNK signaling, finally preventing formation of t-Bid and mitochondrial amplification of the caspase cascade (Fig. 6C). These results provide a plausible mechanistic rationale for the strategy to selectively protect healthy hepatocytes from death receptor-induced cell death by pharmacological and non-toxic doses of fructose.

Acknowledgments—We thank Dr. T. Dunker, Nycomed, Konstanz, for parallel independent measurements of cAMP. We thank Prof. Dr. Franz Weber for providing MKP-1 antibody.

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