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Author(s):
Speicher, Tobias; Köhler, Ulrike A.; Chouker, Alexander; Werner, Sabine; Weiland, Timo; Wendel, Albrecht

Publication date:
2012-01-13

Permanent link:
https://doi.org/10.3929/ethz-b-000684610

Originally published in:
Journal of Biological Chemistry 287(3), https://doi.org/10.1074/jbc.M111.266742

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 3, pp. 1837–1846, January 13, 2012

Fructose Protects Murine Hepatocytes from Tumor Necrosis Factor-induced Apoptosis by Modulating JNK Signaling*[S]

Received for publication, June 3, 2011, and in revised form, October 26, 2011 Published, JBC Papers in Press, November 15, 2011, DOI 10.1074/jbc.M111.266742

Tobias Speicher[‡§], **Ulrike A. Köhler**[‡], **Alexander Choukèr**[¶], **Sabine Werner**[§], **Timo Weiland**[‡], **and Albrecht Wendel**[‡1]

From the [‡]*Chair of Biochemical Pharmacology, Faculty of Biology, University of Konstanz, Konstanz D-78457, Germany, the* [¶]*Department of Anesthesiology, Klinikum Grosshadern, Munich D-81377, Germany, and the* [§]*Insitute of Cell Biology, Swiss Federal Institute of Technology, Zurich CH-8093, Switzerland*

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.

Fructose-induced hepatic ATP depletion prevents TNF-induced apoptosis, whereas it contrarily enhances CD95-induced hepatocyte apoptosis *in vitro* and *in vivo*. By contrast, transformed liver cells are not protected against TNF due to metabolic alterations, allowing selective tumor targeting. We analyzed the molecular mechanisms by which fructose modulates cytokine-induced apoptosis. A release of adenosine after fructose-induced ATP depletion, followed by a cAMP response, was demonstrated. Likewise, cAMP and adenosine mimicked *per se* the modulation by fructose of CD95- and TNF-induced apoptosis. The effects of fructose on cytokine-induced apoptosis were sensitive to inhibition of protein kinase A. Fructose prevented the pro-apoptotic, sustained phase of TNF-induced JNK signaling and thereby blocked bid-mediated activation of the intrinsic mitochondrial apoptosis pathway in a PKA-dependent manner. We explain the dichotomal effects of fructose on CD95- and TNF-induced cell death by the selective requirement of JNK signaling for the latter. These findings provide a mechanistic rationale for the protection of hepatocytes from TNF-induced cell death by pharmacological doses of fructose.

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 necrolytic 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)[2]-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

* This study was supported in part by a fellowship of the Deutsche Forschungsgemeinschaft-funded International Research Training Group 1331 (to Marcel Leist and A. W.).
[S] This article contains supplemental Figs. S1–S3.
[1] To whom correspondence should be addressed: Faculty of Biology, University of Konstanz, M667, D-78457, Konstanz, Germany. Tel.: 49-7071-45802; Fax: 49-7071-44464; E-mail: albrecht.wendel@uni-konstanz.de.

[2] The abbreviations used are: ActD, actinomycin D; t-Bid, truncated Bid; CREB, cAMP response element-binding protein; c-FLIP$_L$, long form of cellular FLICE-inhibitory protein; Bt$_2$cAMP, dibutyryl cyclic AMP; LDH, lactate dehydrogenase; MKP-1, mitogen-activated protein kinase phosphatase-1.

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-induced 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 here 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-$FLIP_L$ was from Enzo (New York, NY), HRP-coupled goat anti-rabbit was from Dianova (Hamburg, Germany), agarose-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), Bt_2cAMP 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 (Nürtingen, 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 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 was 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—5×10^6 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 immunoprecipitation overnight. After two washing steps in lysis buffer and two in assay buffer immune-complex kinase assays were carried out as described previously (10, 11). Reactions were performed in a final volume of 30 μl of kinase buffer (20 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 2 mM DTT, 20 mM ATP). Reactions were initiated by addition of 2 μg of c-Jun 1–79 (Calbiochem, Schwalbach, Germany) and 6 μM [γ-^{32}P]ATP (5,000 Ci/mmol). After 30 min, 25 °C, the reactions were terminated by the addition of Laemmli buffer. Phosphorylation of c-Jun was evaluated by SDS-PAGE followed by autoradiography and phosphorimaging analyses (Quantity One software).

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

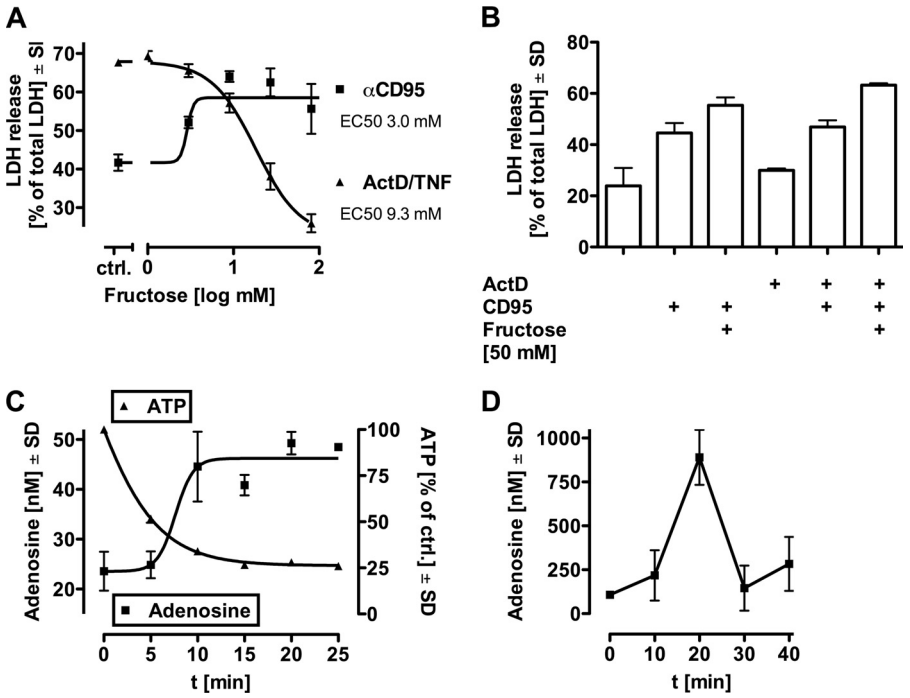

FIGURE 1. **Fructose-induced ATP depletion inversely controls CD95- and TNF-induced cell death and induces adenosine release from hepatocytes *in vitro* and *in vivo*.** *A*, cell death induced by ActD/TNF or by agonistic CD95 antibody in isolated primary murine hepatocytes following incubation with increasing concentrations of fructose for 30 min was assessed by LDH assay after 18 h. Data represent mean ± S.D. of triplicate cultures. *B*, primary murine hepatocytes were pretreated for 30 min with fructose and for 15 min with ActD before agonistic CD95 antibody was added. After 18 h LDH release was determined. Data represent mean ± S.D. of triplicate cultures. *C*, primary murine hepatocytes were treated with 50 mM fructose. Intracellular ATP levels and concentration of adenosine in the supernatant were determined. Data represent mean ± S.D. of triplicate cultures. *D*, fructose (10 g/kg) was injected intraperitoneally into mice. Plasma adenosine was quantified. Values represent mean ± S.D. from two animals per time point.

(12). 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 $MgCl_2$, 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. Supernatant was used for detection of t-Bid translocated to mitochondria. Samples were boiled with Laemmli buffer, and equal amounts were used for SDS-PAGE. Detection of phosphorylated JNK and c-Jun was performed as previously described (13).

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 (EC_{50} 9.3 mM) but enhanced CD95-induced (EC_{50} 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. 1*A*). 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

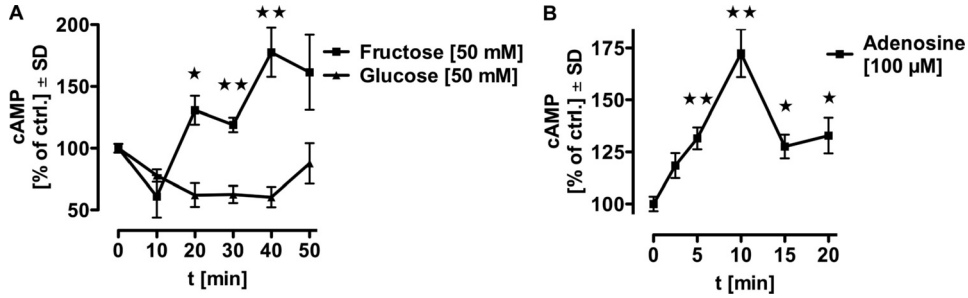

FIGURE 2. **ATP-depleting carbohydrate as well as adenosine induce a cAMP response in primary murine hepatocytes.** *A*, primary murine hepatocytes were incubated with 50 mM of the ATP-depleting carbohydrate fructose or the non-depleting sugar glucose. cAMP content was quantified at the indicated time points. *B*, primary murine hepatocytes were incubated with adenosine (100 μM), and cAMP levels were assessed. Data represent mean ± S.D. of triplicate cultures.

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. 1*B*). 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. 1*C*). 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. 1*D*).

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 adenylate 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. 2*A*). 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. 2*B*).

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 Bt_2cAMP and Br-cAMP protected primary hepatocytes from ActD/TNF-induced cell death (Fig. 3*A*). In analogy, treatment of primary hepatocytes with adenosine or cAMP analogues, respectively, enhanced CD95-induced cell death (Fig. 3*B*). 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, mono-cultured 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 cytoprotective 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 adenylate-cyclase downstream second messenger cAMP, induced pronounced cytoprotection against ActD/TNF-induced cell death in the cell lines used: Hepa 1–6, HepG2, Clone 9, and Huh7. Also, pretreatment 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

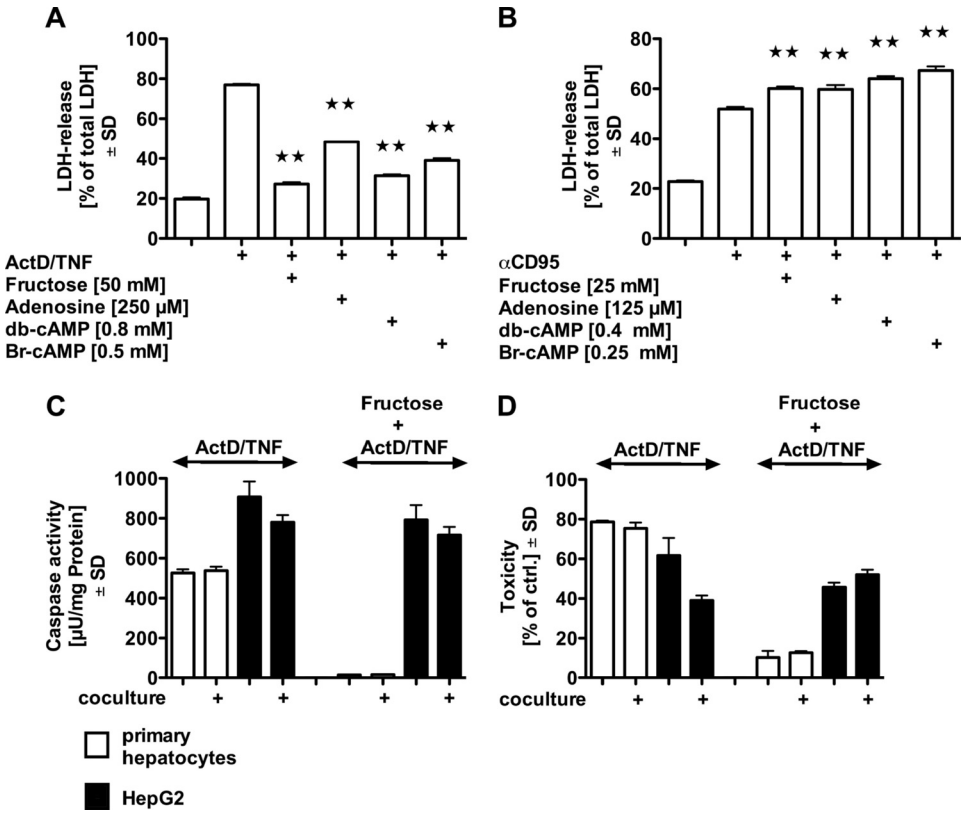

FIGURE 3. **Adenosine and cAMP mimic the effect of fructose on TNF- and CD95-induced cell death.** *A*, primary hepatocytes were pretreated with fructose, adenosine, or one of the cAMP analogues for 15, 5, or 45 min before sensitizing with ActD. After an additional 15 min TNF was added and after 18 h toxicity was analyzed by LDH release. *B*, primary cells were incubated according to setting 3. *A*, cell death was induced with αCD95 antibody without ActD. Data represent mean ± S.D. of triplicate cultures. *, $p < 0.05$ and **, $p < 0.01$ compared with ActD/TNF-/or CD95-induced cell death based on analysis of variance, followed by the Bonferroni multiple comparison test. *C* and *D*, primary murine hepatocytes were cultured in 24-well dishes, whereas HepG2 cells were cultured in co-culture inserts. The effect of fructose on ActD/TNF-induced apoptosis on each cell population in mono-culture and under co-culture conditions was analyzed. Toxicity was examined after 18 h by AlamarBlue assay and, as apoptotic parameter, activities of caspases 3 and 7 were determined after 9 h. Data represent mean ± S.D. of triplicate cultures.

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 Bt_2cAMP, respectively. Primary hepatocytes were treated with KT5720 30 min prior to addition of fructose or Bt_2cAMP, respectively. After another 15 min, alternatively 45 min in the case of Bt_2cAMP, 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. Therefore, primary hepatocytes were pretreated either with KT5720 for 30 min before addition of fructose or alternatively 60 min before addition of Bt_2cAMP, and after an additional 30 min agonistic CD95 antibody was added. Pretreatment with KT5720 also reversed fructose- and Bt_2cAMP-mediated sensitization of CD95-induced caspase activity and LDH release (Fig. 4, *C* and *D*) indicating the involvement of PKA in fructose-mediated regulation of TNF- and CD95-induced apoptosis. Finally, we addressed further downstream mechanisms mediating the cytoprotection against TNF. We assumed differences in the cell death machineries of TNF- and CD95-induced apoptosis to be connected to the inverse PKA-dependent effects of fructose. Schwabe *et al.* (2) recently showed that TNF-induced but not CD95-induced death of hepatocytes requires activation of JNK, which we confirmed by the JNK inhibitor SP600125 (supplemental Fig. S2).

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, of the downstream target c-Jun, 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

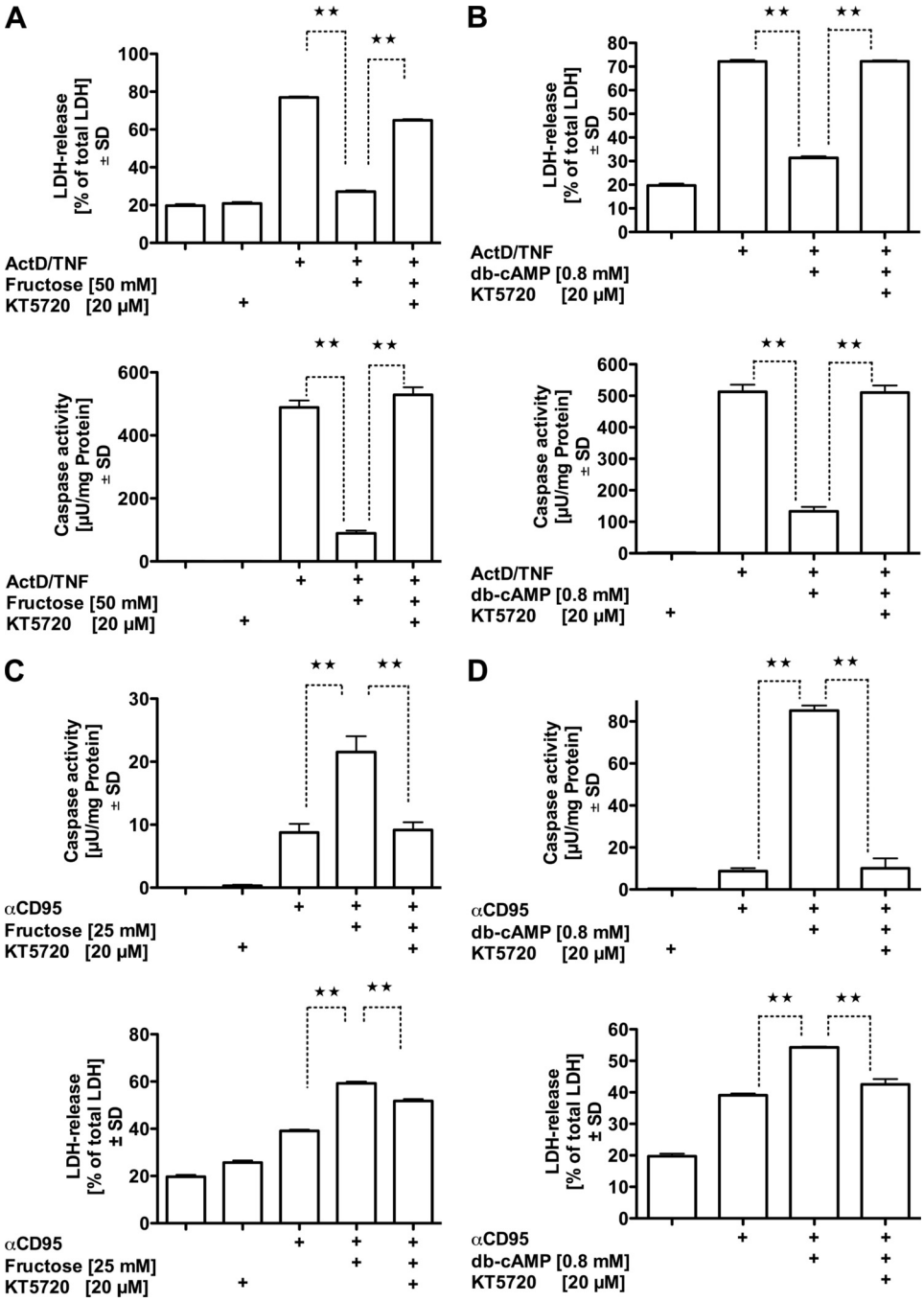

FIGURE 4. **PKA inhibition reverses the inverse effects of fructose or cAMP on CD95- and TNF-induced apoptosis.** *A* and *B*, effect of pretreatment with the PKA inhibitor KT5720 (added 30 min before fructose or cAMP analogue) on fructose- or Bt_2cAMP-mediated protection against ActD/TNF-induced apoptosis 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.

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. 5*B*). Sensitization of hepatocytes by ActD caused sustained TNF-induced JNK activity and cell death in more than 80% of primary hepatocytes (Fig. 5*B*). 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. 5*B*). 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

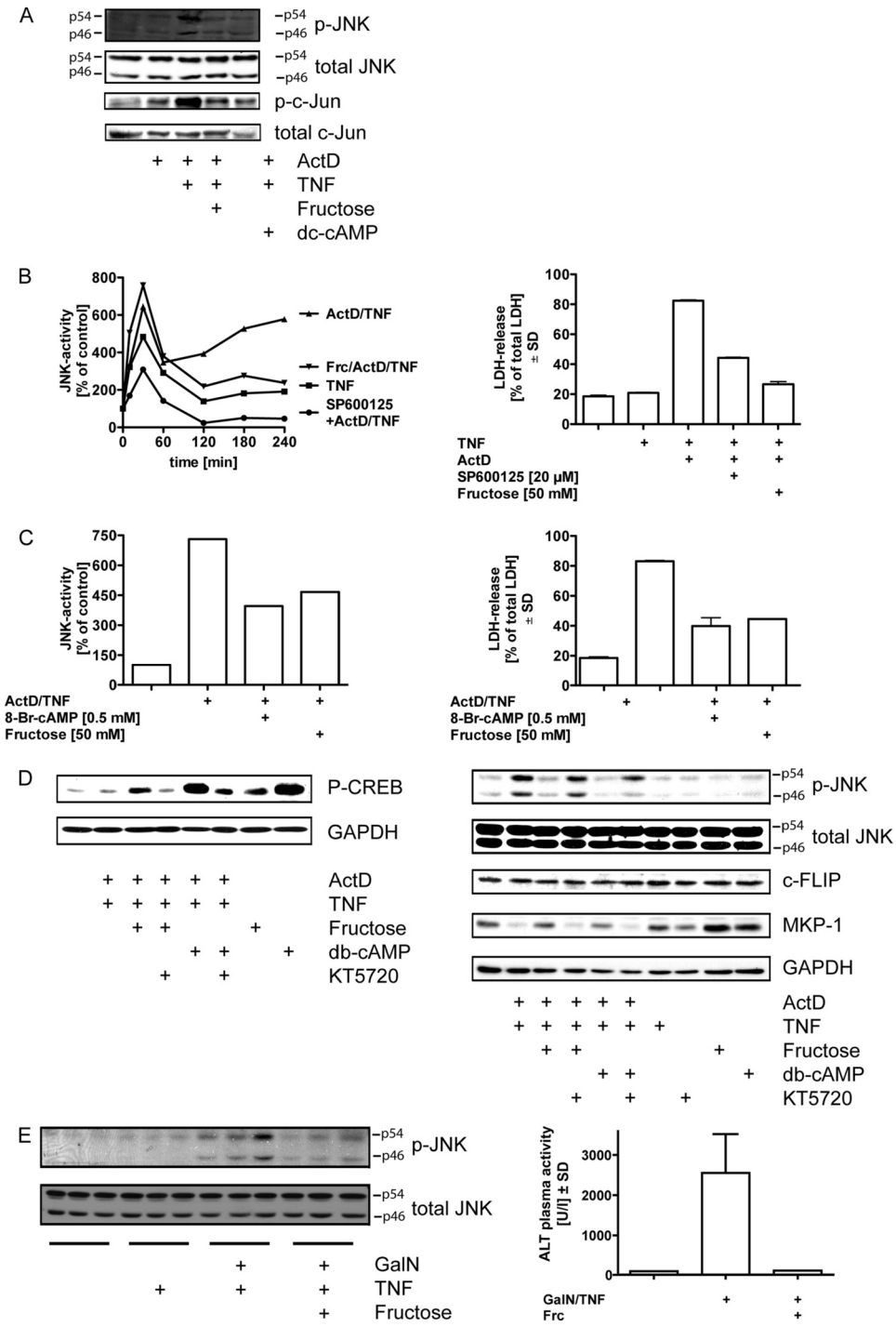

FIGURE 5. **Fructose prevents TNF-induced prolonged JNK activation and toxicity *in vitro* and *in vivo*.** *A*, primary hepatocytes were treated as indicated. Phosphorylation of JNK and c-Jun was analyzed after 3 h. *B*, additionally JNK activity was determined at various time points, and after 18 h toxicity was detected by LDH release. *C*, the effect of Br-cAMP on ActD/TNF-induced JNK activity after 3 h and on LDH release after 18 h was analyzed and compared with the effect of fructose. Toxicity data represent means ± S.D. of triplicate cultures. Primary hepatocytes were treated as indicated with 50 mM fructose, 0.8 mM Bt_2cAMP, and 20 μM KT5720 before induction of apoptosis with ActD/TNF. After 2 h phosphorylation of CREB at Ser-133 was analyzed. *D*, after 3 h samples were analyzed for P-JNK, JNK, c-FLIP, MKP-1, and GAPDH. Mice were pretreated intraperitoneally with fructose (10 g/kg). *d*-Galactosamine (*GalN*) and TNF was injected intraperitoneally/intravenously after 15/30 min, respectively. *E*, phosphorylation of JNK was analyzed after 3 h in liver of three animals per group. Liver injury was quantified by measurement of plasma ALT after 8 h. Values are means ± S.D. of three animals per group.

reduced ActD/TNF-induced prolonged JNK activation and toxicity (Fig. 5*C*) 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. 5*D*). Inhibition of ActD/TNF-induced prolonged

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-$FLIP_L$) 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-$Flip_L$ 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. 5*D*).

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. 5*E*).

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/TND-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. 6*A*).

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. 6*B*). 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.

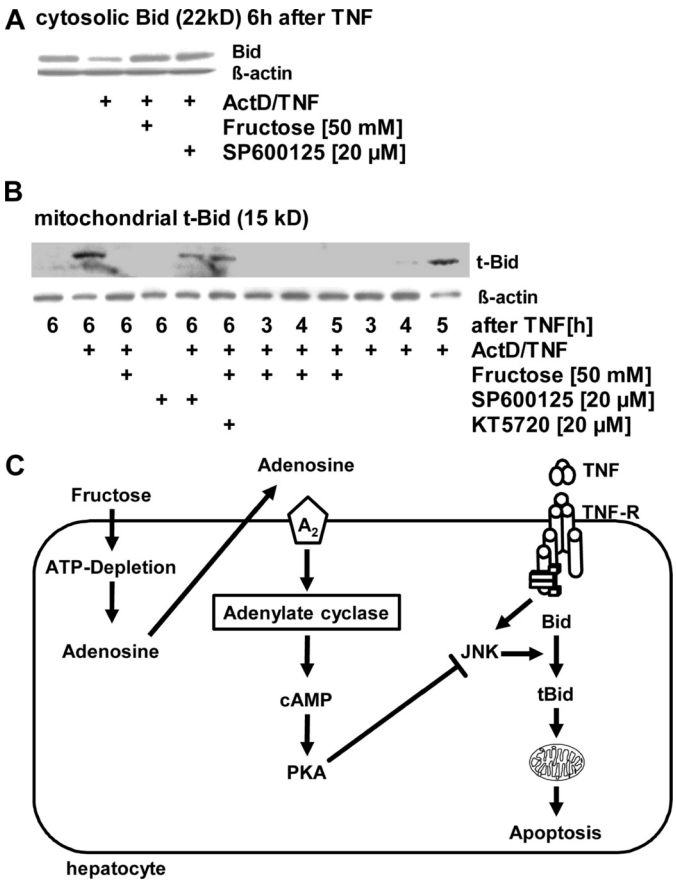

FIGURE 6. **ActD/TNF-induced cleavage of Bid as well as t-Bid translocation to mitochondria is inhibited by fructose in a PKA-dependent manner.** *A*, apoptosis was induced in primary murine hepatocytes by ActD/TNF. Fructose (50 mM) was added 30 min, and SP600125 90 min before TNF stimulus. After 6 h full-length Bid was detected in the cytosolic fraction. *B*, apoptosis was induced in primary murine hepatocytes by ActD/TNF. 30 min before TNF stimulus fructose or 90 min before TNF SP600125 was added. KT5720 was added 30 min before fructose. t-Bid was detected in mitochondrial fraction at the indicated time points. *C*, schematic representation of the proposed molecular mechanism of apoptosis prevention by fructose.

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 (up-regulation of hexokinase 2) in the energy metabolism that prevented fructose-induced ATP depletion and cytoprotection 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 vitro*, 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. 5*E*).

Recently, the JNK pathway was described to be a key regulator of TNF-induced mitochondria-mediated hepatocyte cell death (2). JNK 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. 6*C*). 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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