

# Metabolic Depletion of ATP by Fructose Inversely Controls CD95- and Tumor Necrosis Factor Receptor 1-mediated Hepatic Apoptosis

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## Abstract

Hepatocyte apoptosis is crucial in several forms of liver disease. Here, we examined in different models of murine liver injury whether and how metabolically induced alterations of hepatocyte ATP levels control receptor-mediated apoptosis. ATP was depleted either in primary hepatocytes or in vivo by various phosphate-trapping carbohydrates such as fructose. After the activation of the tumor necrosis factor (TNF) receptor or CD95, the extent of hepatocyte apoptosis and liver damage was quantified. TNF-induced cell death was completely blocked in ATP-depleted hepatocyte cultures, whereas apoptosis mediated by CD95 was enhanced. Similarly, acute TNF-induced liver injury in mice was entirely inhibited by ATP depletion with ketohexoses, whereas CD95-mediated hepatotoxicity was enhanced. ATP depletion prevented mitochondrial cytochrome c release, loss of mitochondrial membrane potential, activation of type II caspases, DNA fragmentation, and cell lysis after exposure to TNF. The extent of apoptosis inhibition correlated with the severity of ATP depletion, and TNF-induced apoptosis was restored when ATP was repleted by increasing the extracellular phosphate concentration. Our study demonstrates that TNF-induced hepatic apoptosis can be selectively and reversibly blocked upstream of mitochondrial dysfunction by ketohexose-mediated ATP depletion.

Key words: liver injury • hepatocytes • mouse • cytokines • caspases

## Introduction

While a low frequency of apoptosis ensures homeostasis and functional adaptation of liver size (1), increased rates of hepatocyte apoptosis are observed in a plethora of pathological situations. These include ischemia, transplant rejection, intoxications, viral infections, and autoimmune diseases (1–3). Frequently, release of cytokines (3) and death receptor activation are involved in such apoptotic hepatocyte death (2). Data from experimental models of liver failure suggest that distinct sets of cytokines trigger apoptosis in different types of liver disease. The two best characterized death pathways are those triggered via the 55-kD TNF receptor (TNF-R1) (4) or CD95 (5, 6). Apoptotic demise triggered through either of these receptors shares some common elements in hepatocytes, such as signal transduc-

tion via caspases, i.e., unique cysteine proteases involved in the signaling of death receptors and the execution of apoptosis (7). Accordingly, inhibition of caspases prevents in vivo and in vitro hepatocyte apoptosis signaled via either receptor (8, 9). However, death triggered by either CD95 or TNF-R1 also shows some strikingly different features: TNF-induced apoptosis requires sensitization of hepatocytes, e.g., by transcriptional inhibitors (10–12), and is facilitated by high oxygen tension (5), whereas CD95 signaling has no such metabolic prerequisites (5). Conversely,  $\alpha$ 1-antitrypsin prevents TNF-mediated liver apoptosis but has no effect on CD95-triggered hepatotoxicity (13). Consequently, the different apoptotic pathways in the liver may be targeted selectively.

For many therapeutic applications, selective inhibition of apoptosis in the liver only is desirable. A way to achieve such organ specificity is to exploit the metabolic peculiarities of hepatocytes. For instance, depletion of hepatic glutathione prevents CD95- and TNF-R1-mediated liver failure (14, 15). The cellular ATP content is another apop-

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toxicity-related metabolic parameter (16, 17) that may be modified selectively in the liver. ATP depletion affects apoptotic pathways in three fundamentally different ways. First, biochemical evidence suggests that ATP, together with cytochrome c, is needed for the formation of the apoptosome (18), a protein complex involved in the processing of procaspases (19). Accordingly, cellular ATP depletion frequently prevents activation of execution caspases at a step downstream of cytochrome c release (20, 21). Such cells with damaged mitochondria are in most cases still destined to die, but the absence of caspase activity switches the mode of death from apoptosis to delayed necrosis (20–22). Second, in some models, ATP depletion may block apoptotic steps upstream of cytochrome c release from mitochondria (21, 22). In such cases, both caspase activation and mitochondrial damage are prevented, and cells may truly survive an otherwise lethal insult (23). Finally, in cases where CD95 stimulation triggers a cascade of proteolytic events that directly lead to apoptosome-independent activation of effector caspases, ATP depletion may have no effect at all on caspase activation (24) or cell death (25).

It is still unknown which of these roles ATP has in hepatocyte apoptosis. An even more important open issue is the function of ATP for apoptosis in well-defined *in vivo* models of apoptotic organ failure. Such experiments in live animals require metabolic inhibition, which is selective for defined cell populations and thus excludes general inhibitors of oxidative phosphorylation or glycolysis. A well-established method for selective ATP depletion of hepatocytes, *in vivo* and *in vitro*, is exposure to high concentrations of fructose or other sugars that rapidly trap intracellular phosphate (26, 27). This approach has the advantage of ensuring residual ATP levels (>15% of control) in the depleted hepatocytes that are high enough to avoid induction of necrosis (28, 29), which would result from more excessive ATP loss (29–34).

In this study, we examined whether ATP depletion with phosphate-trapping sugars could be used to elucidate the role of ATP in liver cell death. Such an experimental system may open new perspectives on the mechanisms of pathological cell death and offer possibilities for selective therapeutic interventions. Therefore, we examined differential effects of phosphate-trapping sugars on TNF- and CD95-triggered apoptotic pathways in hepatocyte or hepatoma cell cultures, and in models of liver damage in mice.

## Materials and Methods

**Materials.** Recombinant murine TNF was provided by Dr. G.R. Adolf, Bender GmbH (Vienna, Austria). d-galactosamine (GalN)<sup>1</sup> was obtained from Roth Chemicals. Agonistic anti-

CD95 antibody ( $\alpha$ CD95, clone Jo-2) was purchased from BD PharMingen. All enzymes, the DNA fragmentation ELISA, and the ATP test kit were obtained from Boehringer. The caspase inhibitor benzoyloxycarbonyl-val-ala-asp-fluoromethylketone (zVADfmk) was purchased from Bachem. Unless further specified, all other reagents were purchased from Sigma-Aldrich. Cell culture plates were from Greiner. Culture medium was purchased from BioWhittaker, and collagen was obtained from Serva.

**Animals.** Specific pathogen-free male BALB/c mice (25 g) were from the in house Animal Breeding Facility of the University of Konstanz. Animals were held at 22°C and 55% humidity, and given a constant day–night cycle of 12 h. Mice were starved overnight before the *in vivo* experiments started. Experiments generally commenced at 8 AM. All steps of animal handling 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.

**Cell Culture Experiments.** Hepatocytes were isolated from 8-wk-old mice by the two step collagenase perfusion method of Seglen (35) and cultured as described (11). In brief, hepatocytes were plated in 200  $\mu$ l RPMI 1640 medium containing 10% FCS in collagen-coated 24-well plates at a density of  $8 \times 10^4$  hepatocytes per well, or in collagen-coated 6-well plates at a density of  $3.2 \times 10^5$  hepatocytes per well. Cells were allowed to adhere to culture dishes for 5 h before the medium was exchanged to RPMI 1640 without FCS. HepG2 cells were maintained in RPMI 1640 medium containing 10% FCS in 75-cm<sup>2</sup> flasks. The day before experiments were carried out, hepatoma cells were harvested with trypsin/EDTA and plated in 200  $\mu$ l RPMI 1640 medium containing 10% FCS in 24-well plates ( $10^5$  cells/well). Incubation of murine hepatocytes or HepG2 cells with carbohydrates or inhibitors of protein synthesis was started directly after medium exchange for RPMI 1640 without FCS. 30 min later, hepatocyte apoptosis was induced by addition of either  $\alpha$ CD95 (0.37–300 ng/ml) alone or recombinant murine TNF (100 ng/ml) after sensitization with actinomycin D (ActD, 400 ng/ml, 15 min before TNF). Incubations were carried out at 37°C in an atmosphere composed of 5% CO<sub>2</sub>, 40% O<sub>2</sub>, and 55% N<sub>2</sub> for the times indicated. Cytotoxicity was quantitated by measurement of lactate dehydrogenase (LDH) (36) in culture supernatants (S) and in the remaining cell monolayer (C) after lysis with 0.1% Triton X-100, and calculation of the percentage of LDH release from the ratio of S/(S + C). Specific cytotoxicity after incubation with TNF and sugar was defined as: specific cytotoxicity (%) =  $100\% \times (\text{LDH}_{\text{TNF+sugar}} - \text{LDH}_{\text{basal}}) / (\text{LDH}_{\text{TNF}} - \text{LDH}_{\text{basal}})$ .

**In Vivo Experiments.**  $\alpha$ CD95 (2  $\mu$ g/mouse) or TNF (2  $\mu$ g/kg) was injected intravenously in a volume of 300  $\mu$ l saline (containing 0.1% HSA). Mice were sensitized to TNF by intraperitoneal injection of GalN (700 mg/kg) 15 min before the cytokine. 30 min before TNF or anti-CD95, fructose or tagatose were injected at various doses (0.08–10 g/kg) in 300  $\mu$ l pyrogen-free water. Blood and tissue samples were obtained after lethal intravenous anesthesia of mice with 150 mg/kg pentobarbital plus 0.8 mg/kg heparin. After midline laparotomy and opening of the chest, blood was withdrawn by cardiac puncture and immediately centrifuged for 2 min at 4°C at 13,000 *g* to separate the plasma from the cellular fraction. Livers were perfused for 10 s with cold perfusion buffer (PB: 50 mM phosphate, 120 mM NaCl, 10 mM EDTA, pH 7.4), and subsequently excised (3). A slice of the large anterior lobe was immediately fixated in 4% buffered formalin solution for histological studies. A part of the liver was frozen in

<sup>1</sup>Abbreviations used in this paper:  $\alpha$ CD95, agonistic anti-CD95 antibody; ActD, actinomycin D; ALT, alanine aminotransferase; ANOVA, analysis of variance; CHX, cycloheximide; DEVDafc, N-acetyl-asp-glu-val-asp-afc; GalN, d-galactosamine; LDH, lactate dehydrogenase; NF, nuclear factor; zVADfmk, benzoyloxycarbonyl-val-ala-asp-fluoromethylketone.

liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for the determination of caspase-3-like activity. The remaining parts of the liver were disintegrated with three strokes of an Elvehjem-type homogenizer. The 20% homogenate (in PB) was centrifuged at 13,000 *g* for 20 min. The supernatant was diluted 270-fold and used directly in an ELISA designed to detect DNA fragmentation (11).

**Metabolites.** ATP content of cultured hepatocytes or liver tissue was determined luminometrically using a commercially available kit. In brief, hepatocytes ( $8 \times 10^4$ /well, 24-well plates) were incubated with different stimuli and various carbohydrates. At the desired time points, hepatocytes were lysed in 150  $\mu\text{l}$  buffer (Boehringer), immediately frozen at  $-80^{\circ}\text{C}$ , and stored until measurement. Before the assay, frozen samples were thawed on ice and diluted 20-fold. In animal experiments, livers were perfused for 2 s and immediately homogenized. The 10% homogenate (in lysis buffer) was centrifuged at 13,000 *g* for 5 min at  $4^{\circ}\text{C}$ . The supernatants were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Luminescence was measured in 96-well plates using an automated procedure (VICTOR<sup>2</sup> multilabel counter; Wallac Instruments). Data were compared with calibration solutions, and ATP data are expressed as the percentage of untreated control cells per liver. Glutathione was measured by an enzymatic cycling method as described (14). Protein synthesis was measured by [<sup>3</sup>H]leucine incorporation as described (11).

**Caspase-3-like Protease Activity.** Cytosolic extracts of liver tissue were prepared by Dounce homogenization of  $\sim 100$  mg frozen liver sample in hypotonic extraction buffer (25 mM Hepes, pH 7.5, 5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM PEFA block, and 1  $\mu\text{g}/\text{ml}$  each of pepstatin, leupeptin, and aprotinin) and subsequently centrifuged (15 min, 13,000 *g*,  $4^{\circ}\text{C}$ ). In cell culture experiments, cells were washed three times with PBS and lysed with hypotonic extraction buffer plus 0.1% Triton X-100. After centrifugation (15 min, 13,000 *g*,  $4^{\circ}\text{C}$ ) of the lysates, supernatants were immediately frozen at  $-80^{\circ}\text{C}$ .

Generation of free 7-amino-4-trifluoromethylcoumarin (afc) was followed in assay buffer (50 mM Hepes, pH 7.4, 1% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, 50  $\mu\text{M}$  fluorogenic substrate *N*-acetyl-asp-glu-val-asp-afc [DEVDafc; Biomol]) for 30 min at  $37^{\circ}\text{C}$  using a fluorometer plate reader VICTOR<sup>2</sup> (Wallac Instruments) set at an excitation wavelength of 385 nm and an emission wavelength of 505 nm. Protein concentrations of corresponding samples were quantitated with the Pierce Assay (Pierce Chemical Co.), and the specific caspase-3-like protease activity was calculated in picomoles of free afc per minute ( $\mu\text{U}$ ) and milligram of protein using serially diluted standards (0–5  $\mu\text{M}$  afc). Control experiments confirmed that the activity was linear with time and with protein concentration under the conditions described above.

**Western Blot Analysis.** The release of cytochrome *c* from hepatocyte mitochondria was analyzed using a selective digitonin permeabilization method, avoiding artifacts that might be generated by mechanical breakage of the outer mitochondrial membrane of apoptotic cells by Dounce homogenization (37). At the indicated time points, the culture medium of 6-well plates was exchanged for permeabilization buffer (210 mM *d*-mannitol, 70 mM sucrose, 10 mM Hepes, 5 mM succinate, 0.2 mM EGTA, 0.15% BSA, 50  $\mu\text{g}/\text{ml}$  digitonin, and pH 7.2,  $4^{\circ}\text{C}$ ) as described (37). Cell culture plates were shaken gently for 5 min at  $4^{\circ}\text{C}$ , then the permeabilization buffer was removed and centrifuged for 10 min at 13,000 *g*. Protein from the supernatants was separated on 15% polyacrylamide gels. Cytochrome *c* was detected by the enhanced chemiluminescence reaction after blotting on nitrocellulose membranes with an mAb raised against pigeon cytochrome

*c* (clone 7H8.2C12; BD PharMingen). Bid was detected with an anti-murine Bid antibody provided by Dr. B. Antonsson (Serono Pharmaceutical Research Institute, Geneva, Switzerland) (38). Phosphorylated I- $\kappa$ B was analyzed with a commercial antibody (PhosphoPlus<sup>®</sup> I- $\kappa$ B [Ser32] Antibody kit) from New England Biolabs.

**Fluorescence Microscopy.** Chromatin condensation was followed on a fluorescence microscope (Leica DM-IRB equipped with a 40 $\times$ , NA 0.5 long distance lens) in live cells after staining with 1  $\mu\text{g}/\text{ml}$  H-33342 as described (16), or in fixed cells after staining with 0.5  $\mu\text{g}/\text{ml}$  H-33342. Mitochondrial membrane potential was analyzed after staining with 20 ng/ml tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) (20). Cytochrome *c* immunocytochemistry was performed essentially as described (20, 37) in cells grown on collagen-coated glass coverslips and fixed with paraformaldehyde (2%). In brief, hepatocytes were incubated first with an anticytochrome *c* mAb (BD PharMingen) and then with an Alexa 488-coupled secondary antibody plus H-33342, before they were imaged on a confocal microscope (Leica TCS 4D equipped with a 63 $\times$ , NA 1.3 lens).

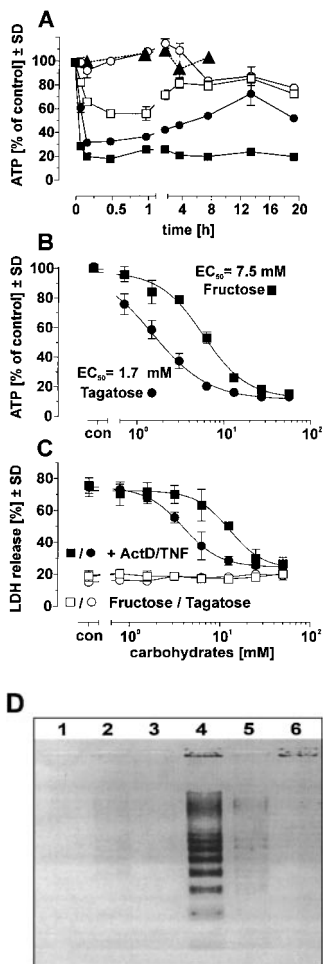
**Statistical Analysis.** Statistical differences were determined with an unpaired *t* test if applicable, or data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. In the case of inhomogenous variances, data were transformed before subtraction to further analysis.

## Results

**ATP Depletion in Murine Hepatocytes by Fructose.** The kinetics and the extent of ATP depletion by fructose were tested in primary murine hepatocyte cultures. High concentrations of the sugar (50 mM) depleted ATP by 70–80% within 5 min, and subsequently cellular steady state ATP levels remained at 15–20% of control for at least 20 h (Fig. 1 A). At lower fructose concentrations (6–25 mM), the depletion was less pronounced (40–70%) and reversible within 5–10 h (Fig. 1 A). The median effective concentration (EC)<sub>50</sub> of fructose for ATP depletion was 7.5 mM (Fig. 1 B), and the sugar alone had no effect on basal hepatocyte viability at any concentration tested (Fig. 1 C).

Hepatic ATP depletion by fructose is due to the preferential metabolism of this sugar via the aldolase pathway (26, 39, 40). In accordance, pronounced ATP depletion (75–85%) was also observed after addition of a panel of other carbohydrates metabolized via the aldolase pathway, such as tagatose, sorbitol, and 2,5-anhydro-mannitol (all 50 mM), whereas no ATP depletion was observed after addition of 50 mM glucose, sorbose, mannitol, or mannose (Fig. 2), which are hardly metabolized via the aldolase pathway.

**Prevention of TNF-induced Hepatocyte Apoptosis by ATP-depleting Carbohydrates.** In ActD-sensitized murine hepatocytes, TNF induces classical apoptosis that can be easily quantified by measuring the LDH release from dying cells at late time points (4, 11). Fructose (50 mM) inhibited TNF-induced cell death by >95% (Fig. 1 C), and this prevention was paralleled by a block of oligonucleosomal DNA fragmentation (Fig. 1 D). All ATP-depleting carbohydrates inhibited apoptosis, whereas the nondepleting sugars had no such effect (Fig. 2). Notably, the potency of

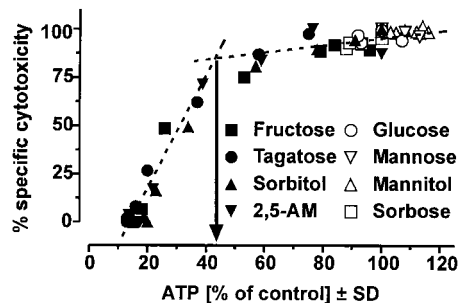


**Figure 1.** Depletion of hepatocyte ATP by different carbohydrates and protection against TNF-induced cell death. (A) Primary murine hepatocyte cultures were incubated with various concentrations of fructose (■, 50 mM; ●, 12.5 mM; □, 6 mM; ○, control [con]). Cellular ATP content was quantified at the time points indicated. In some experiments, phosphate (20 mM) was added to the cultures 2 h before the addition of 12.5 mM fructose (▲). ATP content in untreated cells (30 nmol/10<sup>6</sup> cells) was set as 100%. (B) ATP content in primary cells was quantified 2 h after incubation with various concentrations of fructose or tagatose. (C) Hepatocytes were preincubated for 15 min with various concentrations of fructose or tagatose, then incubated with ActD (400 ng/ml) and 15 min later with TNF (100 ng/ml). LDH release was determined after 18 h. All data are means ± SD from three experiments. (D) After 8 h, DNA fragmentation was analyzed on a 1% agarose gel. The lanes were loaded with samples from the following incubations: (1) control; (2) ActD (400 ng/ml); (3) TNF (100 ng/ml); (4) ActD/TNF; (5) fructose (50 mM) + ActD/TNF; and (6) tagatose (50 mM) + ActD/TNF.

different sugars with regard to ATP depletion correlated with the concentration dependence of their apoptosis inhibition (Fig. 1, B and C). Experiments with eight different carbohydrates showed that TNF-induced cell death was not significantly affected when ATP was maintained above a threshold level of ~40–50% of control. Complete inhibition was observed at ATP levels of 15–20% (Fig. 2).

The correlation of ATP depletion and protection from TNF was further explored by examining the effect of ATP repletion. Supplementation of the culture medium with additional phosphate alleviated ATP depletion by the phosphate-trapping sugar, fructose, and restored ATP levels above the critical threshold of 50% (Fig. 1 A). Restoration of ATP levels correlated with a regain of sensitivity towards TNF-induced apoptosis despite the continued presence of fructose (Fig. 3).

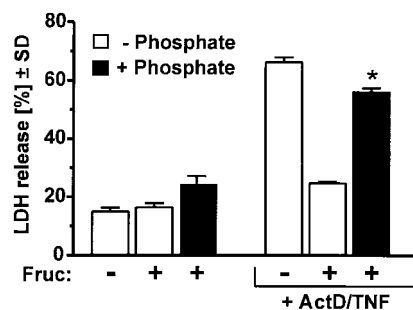
ATP depletion by fructose also correlated with the inhibition of TNF-induced apoptosis in other cell types. For instance, in human HepG2 and murine Hepa1-6 hepatoma cells, fructose did not significantly deplete ATP at concentrations of up to 50 mM, and consequently TNF-induced apoptosis was not affected (change <5%). Similarly, fruc-



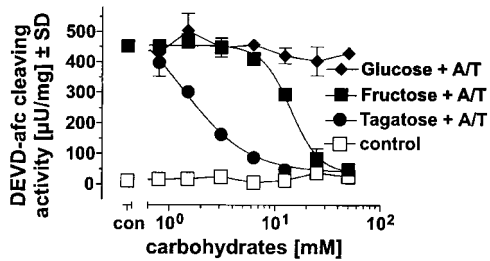
**Figure 2.** Correlation between ATP depletion and prevention of TNF-induced cell death by different carbohydrates. Murine hepatocytes were preincubated with carbohydrates in various concentrations (50, 25, 12, 6, 3, and 1.5 mM) and with ActD (400 ng/ml) for 30 min. Then, 100 ng/ml TNF was added, and specific cytotoxicity was assessed 18 h later by determination of LDH release. In parallel experiments, hepatocytes were lysed after incubation with carbohydrates for 2 h, and the ATP content was quantified. The correlation between LDH release and ATP content is shown. Filled symbols represent ATP-depleting carbohydrates, and open symbols represent other carbohydrates. Each data point represents the mean of a triplicate determination. The arrow indicates the threshold ATP concentration that is required for complete TNF-induced cell death to occur. 0% specific cytotoxicity corresponds to 20 ± 4% basal LDH release, and 100% corresponds to 72 ± 5% absolute LDH release after stimulation with ActD/TNF in the absence of carbohydrates.

tose had no effect on TNF-induced apoptosis in murine fibrosarcoma cells (WEHI-164) lacking the aldolase pathway. In contrast, TNF-induced cell death was inhibited by fructose in primary rat hepatocyte cultures (with active aldolase pathway) just as described for primary murine liver cells (not shown).

**Prevention of TNF-induced Caspase Activation and Cytochrome c Release by Fructose.** Inhibition of caspases by peptide inhibitors correlates with the prevention of TNF-induced hepatic apoptosis (8, 9). Therefore, we investigated whether these proteases were inhibited by addition of fructose or other carbohydrates. The reduction of type II caspase activity (DEVDafc cleavage) correlated closely with the inhibi-



**Figure 3.** ATP repletion through phosphate addition restores ActD/TNF-mediated hepatocyte cell death. Hepatocytes were preincubated for 2 h with combinations of potassium phosphate (20 mM, black bars), fructose (Fruc, 12.5 mM) for 30 min, and ActD (400 ng/ml) for 15 min. Then, 100 ng/ml TNF was added and cytotoxicity was assessed 20 h later by determination of LDH release. Data are means ± SD from three experiments. \**P* < 0.01 compared with ActD/TNF/fructose-treated controls based on ANOVA, followed by the Dunnett's multiple comparison test.



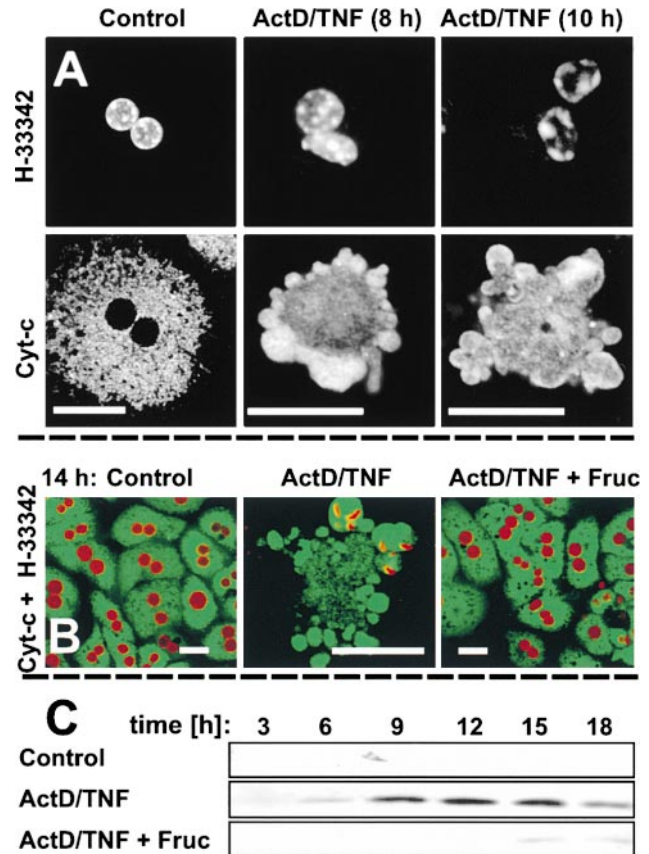
**Figure 4.** Inhibition of ActD/TNF-induced caspase activation by ATP-depleting carbohydrates. Hepatocytes were stimulated with TNF (T, 100 ng/ml) 15 min after incubation with ActD (A, 400 ng/ml), and 30 min after preincubation with various concentrations of fructose, tagatose, or glucose. Caspase-3-like protease activity was measured by quantifications of DEVDafc cleavage after 12 h. All data are means  $\pm$  SD from three experiments.

tion of apoptosis over a wide range of concentrations and sugars (Fig. 4). For instance, the extent of inhibition was  $>95\%$  for 50 mM fructose or 12 mM tagatose.

Even when caspase activity is inhibited, TNF-exposed cells may still have seriously damaged mitochondria and die by delayed necrosis (41). Therefore, we examined cytochrome c release into the cytosol as a parameter for mitochondrial damage. Immunocytochemical analysis showed that cytochrome c was released into the cytosol and the nuclei  $\sim 8$  h after incubation of hepatocytes with TNF. At this stage, chromatin was still noncondensed, but plasma membrane blebs started to form (Fig. 5 A). Chromatin and released cytochrome c were sequestered into apoptotic bodies 2–6 h later. Fructose completely prevented all apoptotic changes, including cytochrome c redistribution (Fig. 5 B). Similar data were obtained when the amount of cytosolic cytochrome c was determined by immunoblot: the mitochondrial intermembrane protein accumulated in the cytosol 6–9 h after stimulation with TNF. Accumulation of cytosolic cytochrome c was blocked by fructose (Fig. 5 C) or tagatose (not shown). Functional imaging of mitochondria with the membrane potential-sensitive dye TMRE yielded essentially similar results: mitochondrial potential remained intact up to 20 h after incubation with ActD/TNF and fructose, whereas it was entirely dissipated in the absence of the sugar (not shown).

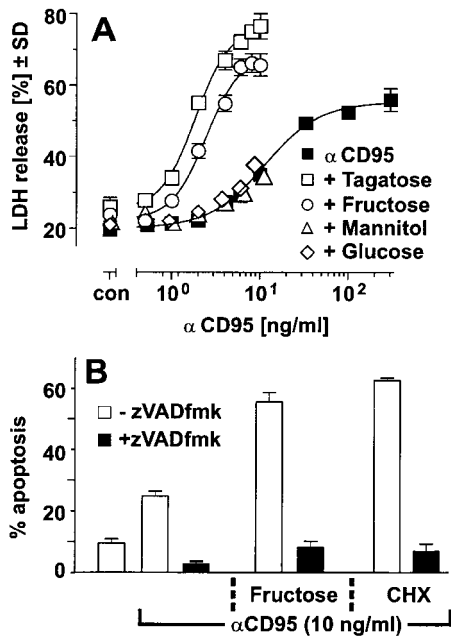
**Enhanced CD95-mediated Apoptosis by Fructose.** When apoptosis was triggered by maximal stimulation of the alternative death receptor CD95 with  $\alpha$ CD95, ATP depletion with different carbohydrates had little effect (Fig. 6 A). Also, CD95-mediated activation of type II caspases ( $1,200 \pm 200\%$  of control) was not inhibited. When suboptimal concentrations of  $\alpha$ CD95 were used, ATP-depleting sugars even increased CD95-mediated apoptosis, whereas non-ATP-depleting sugars did not affect CD95-mediated apoptosis (Fig. 6 A). These experiments show that neither death receptor activation, caspase-8 processing, nor the activity of type II caspases can be directly inhibited by the presence of fructose or by ATP depletion.

Our hypothesis that death receptor signaling per se is not affected by ATP depletion was further corroborated by ex-



**Figure 5.** Prevention of TNF-induced cytochrome c release, membrane blebbing, and apoptotic nuclear changes by fructose. (A) Hepatocytes were fixed at 0, 8, or 10 h after exposure to ActD (400 ng/ml)/TNF (100 ng/ml). After staining for cytochrome c (Cyt-c) and DNA (H-333342), cells were imaged by two-channel confocal microscopy. Chromatin and the corresponding cytochrome c pattern are shown in separate panels for one representative cell per time point. Control hepatocytes showed a typical binuclear phenotype, with cytochrome c immunoreactivity only in mitochondrial structures. The punctuate pattern of cytochrome c corresponded exactly to the stain of mitochondria with MitoTracker Red (not shown). After 8 h, cells started to bleb, and cytochrome c spread all over the cell. Note especially the penetration of the nuclei and the concentration in the blebs. After 10 h, chromatin was strongly margined and formation of apoptotic bodies started. (B) Cytochrome c (green) and chromatin (red) were imaged 14 h after addition of ActD/TNF. Distribution of cytochrome c all over the blebbing hepatocytes was obvious (middle). The chromatin was broken apart to crescent shaped condensed lumps that were packed into blebs and apoptotic bodies. More than 70% of the cells were affected at this stage, and a single representative hepatocyte is shown here. In contrast, hepatocytes exposed to ActD/TNF in the presence of fructose (Fruc, 50 mM) neither released cytochrome c nor displayed chromatin changes or membrane blebs. A larger number of cells is shown to demonstrate the complete protection by fructose. Bars, 15  $\mu$ M. (C) Cytosolic extracts from hepatocytes treated for 3–18 h with ActD/TNF in the presence or absence of fructose (Fruc, 50 mM) were analyzed by Western blot for cytochrome c content. Each lane was loaded with extracts from  $10^5$  hepatocytes, and the 13-kD cytochrome c bands are shown.

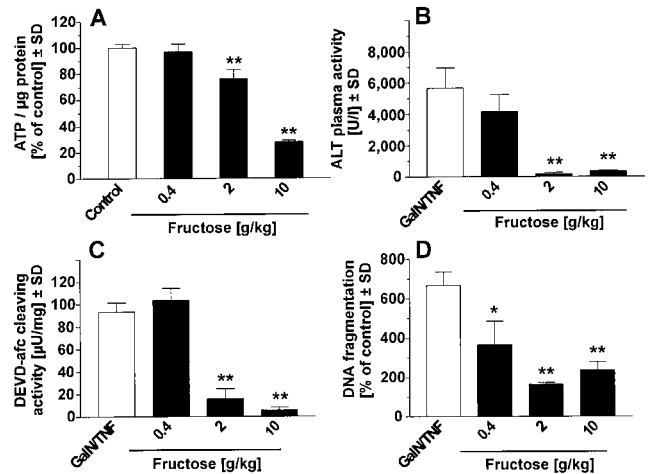
periments where TNF was added before fructose (50 mM) to avoid any effect of the carbohydrate on initial receptor aggregation and signaling events. Protection by fructose was 90, 75, 65, or 55% when it was added after TNF with a delay of 1, 2, 3, or 4 h, respectively. Thus, ATP depletion



**Figure 6.** Exacerbation of CD95-mediated apoptosis by ATP-depleting carbohydrates and inhibitors of protein biosynthesis. (A) Murine hepatocytes were preincubated with various carbohydrates (50 mM each) for 30 min. Then,  $\alpha$ CD95 was added at the concentrations indicated. 18 h later, cytotoxicity was assessed by determination of LDH release. (B) Cells were additionally preincubated with zVADfmk (10  $\mu$ M) and/or CHX for 30 min. The percentage of apoptotic cells was determined after 18 h. Data are means  $\pm$  SD from three experiments.

strongly inhibited apoptosis, also when receptor ligation had already been initiated. Finally, we examined a rapid signaling event triggered by TNF, e.g., I- $\kappa$ B phosphorylation, which leads to NF- $\kappa$ B activation (42). Immunoblotting experiments showed that similar amounts of I- $\kappa$ B were phosphorylated 5–30 min after TNF in the presence or absence of 50 mM fructose (not shown). These findings indicate that apoptosis signals via TNF-R1 and CD95 differ fundamentally: only TNF-triggered pathways involve an ATP-sensitive step located upstream of type II caspase activation and downstream of receptor activation.

**Increased CD95-mediated Apoptosis despite Metabolic Inhibition.** The observed enhancement of CD95-mediated toxicity by ATP depletion (Fig. 6 B) may be due to secondary metabolic consequences of ATP loss, as all the phosphate-trapping carbohydrates also inhibited protein synthesis in a concentration-dependent manner (not shown) (26). In the hepatocyte culture system used here, ATP depletion by 50 mM fructose caused a drop of protein synthesis by >90% as quantitated by measurement of [<sup>3</sup>H]leucine incorporation. Direct inhibition of protein synthesis had a similar effect as ATP depletion: CD95 toxicity was synergistically enhanced by cycloheximide (CHX) (Fig. 6 B) or several other translational inhibitors (not shown). Enhancement of overall cell death by ATP depletion or CHX may have been due to a conversion of CD95-mediated apoptosis into unspecific necrotic cell lysis. However, activation of caspases (not shown) and morphological examination after

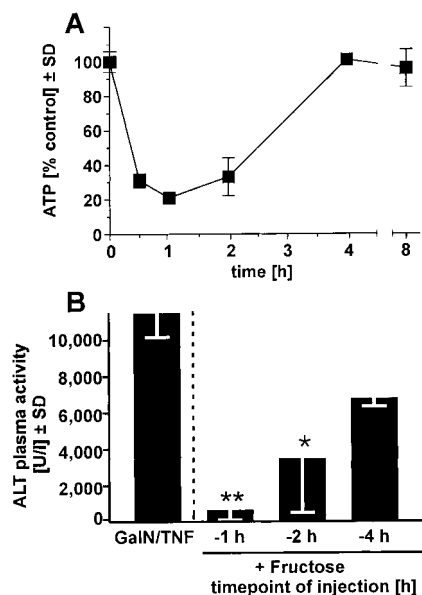


**Figure 7.** Dose-dependent ATP depletion by ketohexoses and protection against GalN/TNF-induced liver injury in mice. (A) The ATP content of the liver was quantified 30 min after application of various doses of fructose. (B) 15 min before application of GalN (700 mg/kg), mice were pretreated with fructose. TNF (2  $\mu$ g/kg) was injected intravenously 15 min after GalN. Liver injury was quantified by measurement of plasma alanine aminotransferase (ALT) after 8 h, (C) by determination of type II caspase activity by DEVDafc cleavage after 6 h, or (D) by measurement of DNA fragmentation after 6 h. Carbohydrates such as glucose and mannitol had no significant effect. Values are means  $\pm$  SD from six animals per group. \* $P$  < 0.05 and \*\* $P$  < 0.01 compared with conditions represented by white bars based on ANOVA, followed by the Dunnett's multiple comparison test.

staining of the chromatin indicated purely apoptotic cell death. Moreover, cell death was entirely prevented under all conditions by the caspase inhibitor zVADfmk (Fig. 6 B). Taken together, these findings show that hepatocyte apoptosis per se requires neither adequate ATP levels nor intact protein synthesis, provided that the caspase cascade is directly triggered, e.g., by CD95 ligation. Alternately, these data indicate that TNF-R1 binding and ligation alone were not sufficient to trigger the caspase cascade and apoptosis, as TNF-mediated apoptosis had to bypass an additional step that was blocked by ATP depletion (Figs. 1 and 4).

**Prevention of TNF-induced Hepatic Apoptosis by ATP Depletion.** Having established the basic experimental system in primary hepatocyte cultures, we were interested in whether fructose had a similar effect on TNF-induced hepatic apoptosis in vivo. For this purpose, we used GalN-pretreated mice, which are highly sensitive to the induction of selective hepatic apoptosis by TNF (4, 12). In agreement with previous studies (26), injection of fructose into mice caused a dose-dependent ATP depletion in the liver (Fig. 7 A). This depletion was associated with a significant protection from TNF-induced liver damage, as assessed by measurement of plasma transaminases (Fig. 7 B), hepatic caspase activity (Fig. 7 C), hepatic DNA fragmentation (Fig. 7 D), and histological examination (not shown). A pronounced protection by fructose was also observed in survival experiments with GalN-sensitized mice: all animals died 8–12 h after injection of TNF (2  $\mu$ g/kg,  $n$  = 8), whereas fructose-pretreated mice (10 g/kg,  $n$  = 8) survived the observation



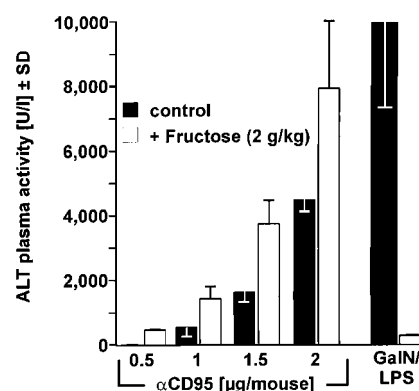


**Figure 8.** Kinetics of ATP depletion in murine livers by fructose and correlation with protection against GalN/TNF-induced liver injury. (A) The ATP content of murine liver homogenates was monitored at different time points after injection of fructose (10 g/kg). (B) Mice were treated with GalN/TNF as described, and liver injury was quantified by measurement of plasma ALT after 8 h. Alternatively, mice were pretreated with fructose (10 g/kg) at different times before challenge with TNF. Values are means  $\pm$  SD from six animals per group. \* $P < 0.05$  and \*\* $P < 0.01$  compared with GalN/TNF-treated controls based on ANOVA, followed by the Dunnett's multiple comparison test.

period of 3 mo. At the highest dose of fructose, liver protection was virtually complete, whereas glucose or mannitol neither depleted ATP nor reduced liver damage.

**Correlation of ATP Depletion and Apoptosis Sensitivity In Vivo.** Analysis of the kinetics of ATP depletion after a bolus injection of fructose showed that ATP levels dropped by 70% within 30 min, reached a minimum (20% of control) after 1 h, and had recovered after 4 h (Fig. 8 A). When TNF was injected 1 h after fructose, maximal protection correlated with the maximum of ATP depletion at that time point. When TNF was injected after 2 or 4 h, protection was decreasing, in parallel with the recovering hepatic ATP levels (Fig. 8 B). This time course suggests a direct link between the block of apoptosis and the metabolic effects of fructose. The data also argue against induction of nonspecific tolerance, e.g., due to endotoxin contamination of the sugar and release of the tolerizing cytokine IL-1 (3, 43).

**Modulation of CD95- and LPS-mediated Apoptosis by ATP Depletion.** In a final set of experiments, we examined whether the opposing effects of ATP depletion on TNF-R1- or CD95-induced hepatocyte death would also hold true in vivo, and which effect would be predominant after stimulation of liver damage with LPS as inflammogen. Mice were injected with increasing doses of anti-CD95 antibody alone or in combination with a strongly ATP-depleting dose of fructose. Under any condition, fructose enhanced CD95-mediated apoptotic liver damage (Fig. 9)



**Figure 9.** Modulation of CD95- and GalN/LPS-mediated liver injury by fructose. Mice were pretreated with fructose (2 g/kg) and 15 min later, different doses of  $\alpha$ CD95 were injected. Alternatively, mice were challenged with LPS (10  $\mu$ g/kg) 15 min after GalN (700 mg/kg) pretreatment. 8 h later, liver injury was quantified by measurement of plasma ALT. Values are means  $\pm$  SD from three animals per group.

similarly to the in vitro experiments. In GalN-sensitized mice injected with LPS, fructose prevented liver damage very potently (Fig. 9). Thus, fructose protected in the more complex GalN/LPS paradigm of hepatic inflammation to a similar extent as in the GalN/TNF apoptosis model.

## Discussion

This study demonstrates that TNF-induced hepatocytotoxicity is prevented in vivo and in vitro after treatment with fructose. This unexpected antiapoptotic action of the sugar was exceptionally potent, as fructose completely abolished liver damage of mice even after exposure to otherwise lethal TNF doses. A similar robustness of fructose-mediated protection was also seen in vitro, because hepatocyte apoptosis was reduced by  $>95\%$  over a wide range of different TNF concentrations. The effect of the sugar was exquisitely specific for nontransformed hepatocytes, which is not the case for caspase inhibitors of similar potency (8, 9). Fructose neither depleted ATP nor did it protect from apoptosis in hepatoma cells (not shown), primary neurons (23), and lymphoid cells (16). Thus, the cell type-selective effects of the sugar may be used to differentially modulate responses of tumor cells and hepatic parenchymal cells in vivo.

A straightforward interpretation of our results is that ATP depletion is the primary metabolic effect of fructose responsible for prevention of TNF- or LPS-induced apoptosis. For instance, the degree of ATP depletion by a large variety of sugars correlated with their inhibition of TNF-triggered apoptosis and with the enhancement of CD95-mediated cytotoxicity. In addition, the time course of ATP depletion in hepatocyte cultures or in murine livers, respectively, correlated with refractoriness to TNF-induced apoptosis. The strongest support for the hypothesis that ATP depletion by fructose is the primary metabolic cause for the selective protection of hepatocytes from apoptosis

comes from ATP repletion experiments. For this line of argument, it is essential to rationalize the mechanism of ATP depletion by fructose. Unlike most other commonly used ATP depleters, fructose neither blocks ATP synthesis nor depletes organic substrates for glycolysis or mitochondrial energy generation. Rather, fructose is itself a substrate for glycolysis that is phosphorylated so rapidly that it acts as a sink for the cellular phosphate pool (39, 44). Cells survive well under such conditions, as ATP is still slowly produced by oxidative phosphorylation or glycolysis (33, 45). This situation differs from ATP depletion with oligomycin or rotenone, which is lethal to hepatocytes (46) unless they are cultured in the additional presence of the alternative metabolic substrates glycine (31) or fructose (33). The particular biochemical mechanism of ATP depletion by fructose explains the fact that ATP could not be repleted by addition of any combination of the energy substrates glucose, glutamate, alanine, glycerol, pyruvate, or lactate (this study). However, supplementation of inorganic phosphate to balance the amount trapped by fructose raised the cellular ATP content. Most importantly, this reconstituted ATP pool made cells sensitive again to TNF, even in the presence of fructose. This strongly supports our view that fructose blocks hepatocyte apoptosis by ATP depletion.

Two further reasons other than lack of ATP as a possible cause for the prevention of cell death after fructose are considered to be unlikely. First, intracellular acidification may result from enhanced glycolysis, and has in fact been demonstrated to protect, e.g., against ischemic hepatocyte necrosis (30, 47–49). However, a decrease in pH increases apoptotic death of hepatocytes (40). In addition, we found no pH dependence *in vitro* of TNF-induced apoptosis between pH 6.0 and 7.6 using various organic buffers (data not shown). Second, an antioxidant function as claimed for fructose (50) is unlikely to account for its prevention of apoptosis, as TNF-induced murine hepatocyte apoptosis is not affected by antioxidants, glutathione precursors, or chelation of iron (11, 12, 14), nor by the strongly antioxidant carbohydrate mannitol (this study).

Modulation of apoptosis and ATP depletion were characterized by a threshold ATP value of 40–50% of control. From this threshold on, any further decrease of cellular ATP was directly proportional to the reduction of TNF-induced apoptosis over a wide range of concentrations with various sugars. A quantitatively and qualitatively similar correlation of ATP with apoptosis susceptibility has been defined earlier in neuronal cultures and lymphoid cells after inhibition of ATP synthesis with oligomycin or nitric oxide (16, 20, 23). Thus, our study suggests the existence of a general threshold level of intracellular ATP required for progression of apoptosis. Notably, absolute ATP concentrations in hepatocytes are in the range of 3–5 mM (39). Thus, the residual intracellular ATP levels under depletion conditions (i.e., ~20% of control) that blocked apoptosis were still in the millimolar range and therefore several orders of magnitude above those required for most kinases to function.

The question arises as to the site of interference of fruc-

tose-induced ATP depletion with the apoptotic program. To date, only one of the ATP-dependent apoptosis signaling steps has been molecularly defined: hydrolysis of deoxy-ATP or millimolar concentrations of ATP are required for conformational changes of apoptotic protease-activating factor 1 (Apaf1) that are essential for formation of the apoptosome (51). Apoptosome formation is generally considered to occur downstream of mitochondrial cytochrome c release. However, there is clear evidence from various experimental systems, including hepatocytes, that the apoptotic cascade may involve feedback loops of downstream effectors (caspase-3) acting back on the activation of molecules that have been considered to be positioned apical in the sequence of death signals (caspase-8, Bid, mitochondrial integrity) (52, 53). The question on whether the apoptosome is involved at all in death receptor-mediated hepatocyte apoptosis would therefore be ideally studied in *apaf1*<sup>-/-</sup> or *caspase-9*<sup>-/-</sup> mice. However, such an approach with knockout hepatocytes is prevented by the embryonal lethality of the homozygote gene-targeted mice. The possibility that ATP depletion may delay or halt the apoptotic cascade at a step upstream of cytochrome c release and apoptosome formation (21–23, 54) is suggested by *in vitro* experiments. Our study provides the evidence that such upstream mechanisms are in fact most relevant for apoptosis block by ATP depletion *in vivo*. Apart from a differential requirement for apoptosome formation, three theoretical explanations remain for the different signaling of the two death receptors, TNF-R1 and CD95. First, Bid or relatives that link death receptors to mitochondria could play a role in TNF-mediated apoptosis only. The finding that Bid-deficient mice are resistant to CD95-induced hepatocellular apoptosis argues against such a rationale (55). Analysis of Bid involvement is complicated by the fact that this molecule may release cytochrome c without being cleaved by caspases (56). In agreement with this, we did not detect any Bid cleavage in our hepatocyte cultures (data not shown). Second, two different types of death receptor-mediated apoptosis signaling have been distinguished on the basis of their different susceptibility to inhibition by Bcl-2: a direct protease cascade (type 1), and a protease cascade controlled by a Bcl-2-inhibitable mitochondrial amplification loop (type 2) (57). As protection of hepatocytes by Bcl-2 from CD95- or TNF-R1-mediated apoptosis has been observed (13, 58, 59), a direct protease cascade triggered by either death receptor seems unlikely in hepatocytes. Third, the fact that both TNF-R1 and CD95 require Fas-associated death domain protein for signal transduction does not exclude that different signaling pathways may be initiated from the two different death-inducing signaling complexes (42). TNF-R1-mediated apoptosis may require two obligatory signal cascades. One of these comprises the caspase cascade common also to CD95 and thus sensitive to caspase inhibitors. The second signal, possibly triggered only by TNF, may require ATP. Two examples may illustrate the possibility of a second signal in TNF-R1 signaling: the necessity of receptor internalization (60), and a caspase-8-independent death pathway that triggers necrosis in L-929



cells independent of the caspase cascade (61).

The most proximal receptor-mediated events are unlikely to be affected by fructose for the following reasons: TNF-R1 has been shown to be solely responsible for apoptosis induction by TNF in hepatocytes (4). After ligand binding, this receptor trimerizes and adapter protein recruitment to the receptor initiates different signaling pathways (42). The most prominent ones are activation of the transcription factor nuclear factor (NF)- $\kappa$ B and activation of apoptosis by processing of caspase-8 (42, 51). Our finding that the NF- $\kappa$ B pathway is also activated in the presence of fructose suggests that receptor aggregation and recruitment of some signaling adapter molecules are not defective in ATP-depleted cells. This is further corroborated by our finding that CD95-mediated apoptosis in hepatocytes is not blocked by ATP depletion. As CD95-mediated apoptosis involves similar initial activation mechanisms for caspase-8 as those triggered via TNF-R1 (42), it is likely that this initial step of cell death signaling was not inhibited by ketoexos.

Typical steps of the apoptotic cascade that can be positioned clearly downstream of the step blocked by ATP depletion are the activation of type II caspases and the release of cytochrome c from mitochondria. The full activity of CD95-triggered type II caspases in the presence of fructose showed further that it is not the enzymatic activity of caspases themselves that is blocked by ATP depletion or fructose, but rather a step leading to their activation by TNF.

Our data provide clear evidence that cell death signaling in hepatocytes by TNF-R1 and CD95 can be differentially regulated. Why is this possible when both death receptors use similar initial signaling pathways? Inhibition of protein synthesis by the metabolic effects of fructose (26) may contribute to the enhancement of CD95-mediated death and protection from TNF. In fact, the general protein synthesis inhibitor CHX has also been shown to enhance CD95 toxicity to hepatocytes (Fig. 6) and to reduce TNF toxicity (11). Fructose has been shown to inhibit protein synthesis selectively in the liver by ATP depletion (26), and we also found in this study that fructose arrests protein synthesis in parallel with its depleting effect on cellular ATP. However, mechanisms triggered by the initial ATP depletion other than translational arrest could also be relevant, as the protection by fructose was consistently more pronounced and robust than the protection we observed (3, 5, 11) with CHX or other inhibitors of protein synthesis. Differences between CD95 and TNF have also been observed in other systems. CD95 cross-linking triggers a proteolytic cascade that in some cells is sufficient alone to execute apoptosis (51, 57). In such a direct chain of proteolytic events, ATP depletion may not have any inhibitory effect. The majority of other known apoptotic signals, including TNF-R1 stimulation, requires enhancing loops and parallel pathways for the full-blown activation of type II caspases and cell death (41, 51, 57). We show here that one of the key regulatory steps in the TNF pathway requires high (>50% of control) ATP levels. Thus, modulation of cellular ATP levels ap-

pears to represent a cellular master switch for TNF-induced hepatocyte apoptosis with a similar potency as Bcl-2 in many other apoptotic model systems.

Our study shows that the decision of a hepatocyte to undergo apoptosis *in vivo* is controlled by basic metabolic parameters in addition to the well-known interactions of specific signaling molecules. For instance, an adequate glutathione status is required in murine livers for TNF- and CD95-mediated apoptosis to occur (14, 15), and excessive reactive oxygen or nitric oxide stress frequently prevents apoptosis signaling (62). We demonstrate here that the cellular ATP level can be an additional key switch controlling apoptosis and organ failure *in vivo*. Exploitation of this refined knowledge base will facilitate the development of improved strategies for chemotherapy, pharmacological interventions, or control of liver failure. In addition, this metabolic control provides an explanation for apoptosis prevention and the occurrence of relatively widespread necrosis (2, 63) in pathological liver failure, where frequently ATP and glutathione are depleted and irreversibly damaged hepatocytes have to die eventually by necrosis.

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