Changes in specific metabolic pathways are essential steps in the early apoptotic process in the liver

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Synopsis
To clarify whether changes in glucose metabolism occur prior to the already known events in the apoptotic process, and whether these can be prevented by the immunosuppressant Cyclosporine A (CsA), we used multinuclear NMR spectroscopy and molecular studies to characterize metabolic pathways in mice liver during anti-Fas-induced apoptosis. An upregulation of specific metabolic pathways of glucose was the earliest indicator of the effect of Fas on the liver. CsA prevented apoptosis and energy failure at late stages, while the reversal of Fas-induced metabolic upregulation at early stages preceded the protective effect of EGF on programmed cell death. These phenomena provide useful hints for the understanding of early mechanisms controlling apoptotic cell death.

Introduction and Aim
Apoptosis is the main mode of cell death in most liver injuries. Mitochondria are not only the key regulatory centers for the genesis of apoptosis, but are also the metabolic centers that provide energy. It is believed that mitochondrial dysfunction, and consequently alterations in energy metabolism, is a late event during hepatocellular apoptosis. In contrast, metabolic events during the early phase of the apoptotic process, and the impact of their reversal, have not yet been investigated. It is noteworthy that mitochondrial involvement in the apoptotic process occurs in viable cells with adequate ATP stores. For example, ATP depletion can prevent activation of executioner caspases at a step upstream of cytochrome c release and mitochondrial permeability transition (MPT). Another intriguing characteristic of the initiation of the apoptotic response is its strong dependence on the cellular redox status. Interestingly, an intriguing observation was made between apoptosis and glucose metabolism: Danial et al. demonstrated that BAD, a pro-apoptotic member of the Bcl-2 family in mitochondria, resided in a functional complex with glucokinase, which catalyzes the first step of glucose metabolism. The immunosuppressant Cyclosporine A (CsA) is known to prevent apoptosis, and has also been shown to impair mitochondrial glucose metabolism. CsA, which is known to prevent the MPT, will be used to test its ability to prevent against apoptosis by changes in hepatocellular metabolism at early stages. We used multinuclear NMR spectroscopy to characterize metabolic pathways in mice liver during anti-Fas-induced apoptosis. We also addressed whether these early changes are associated with the protection against apoptosis by CsA.

Methods
Animal model. 1) To induce hepatocyte apoptosis, BALB/C mice were injected with anti-Fas antibody (10 μg; i.p.). The investigations were done at 7 time-points (15 min - 7.5 h). 2) Protection as well as deterioration of apoptosis was afforded by treatment of Fas-injected mice with CsA (25, 50 or 100 mg/kg; i.p.; 45 min prior to Fas, concomitantly with Fas (t = 0) or 45 min - 4.5 h postinjection). All mice were injected with [U-13C]glucose or [1,2-13C]succinate (2.2 mmol/kg, i.v.). 15-60 min - 7.5 h). 2) Protection as well as deterioration of apoptosis was afforded by treatment of Fas-injected mice with CsA (25, 50 or 100 mg/kg; i.p.; 45 min prior to Fas, concomitantly with Fas (t = 0) or 45 min - 4.5 h postinjection). All mice were injected with [U-13C]glucose or [1,2-13C]succinate (2.2 mmol/kg, i.v.), 15-60 min after administration of the 13C-labelled substrates, the mice were killed by cervical dislocation. The livers were freeze-clamped. Extraction. Tissue samples were powdered over liquid nitrogen and homogenized in 5% perchloric acid (PCA) at 0°C [3]. To obtain lipids extracts from the same tissues, the pellets were extracted with CHCl3/CH3OH. Blood (taken from the carotid artery) was immediately mixed with PCA, dual-extracted, and used for the analysis of serum alanine aminotransferase (ALT) levels and 1H-NMR analysis of the blood.

NMR analysis. After lyophilization, the samples were redissolved in 0.5 ml and centrifuged. 1D 1H, 13C- and 31P-NMR spectra were recorded on Bruker spectrometers DRX 600 or AVANCE-NB/WB 360. Metabolite concentrations were calculated from 1H-NMR spectra; the flux of 13C through metabolic pathways was followed up by 13C-isotomer analysis. Standard enzymatic tests and Western Blot analysis were performed for the analysis of serum ALT, caspase-8 and 3 activities and Bid (tBid) protein expression.

Results
1) Fas treatment time-dependently affected several metabolic pathways during the cell death process in mice liver. Major metabolic changes occurred at 2 time points: 1) at very late stages (7.5 h after Fas injection), when ATP levels and mitochondrial metabolism (flux of glucose through pyruvate dehydrogenase (PDH), the key enzyme for mitochondrial glucose oxidation), and glutathione de novo synthesis declined to 20% of control levels (p<0.001) concomitantly with liver injury (>50-fold elevation in serum ALT and caspase-3 activities), and histological evidence of massive apoptosis. 2) very early (t < 1.5 h), when no liver injury was evident.

2) An upregulation of specific metabolic pathways of glucose was the earliest indicator of the effect of Fas on the liver. In particular, 2-fold increased flux via PDH and via pyruvate carboxylase (PC) were observed during the first 45 min – 1.5 h after apoptotic Fas stimuli (P<0.001). Concomitantly, glutathione synthesis from [U-13C]glucose increased to 190% of controls (P<0.001). 31P-NMR spectra demonstrated constant ATP levels up to the late stages. Serum ALT and caspase-3 activities did not change significantly up to t = 3 h after Fas stimuli. Western Blot analysis demonstrated that caspase-8 activation and cleavage of the pro-apoptotic Bcl-2-family member BID was observed 3 h after Fas injection.

3) CsA can prevent apoptosis energy failure at late stage (t = 7.5 h) by early metabolic changes. When CsA (50 mg/kg) was injected 45 min before anti-Fas, CsA prevented increased ALT values at 7.5 h by 46 ± 10.6% (p<0.001) compared to Fas treatment alone. Concomitantly, impaired glucose oxidation (flux through PDH), energy failure (ATP depletion), and impaired glutathione (GSH) synthesis were attenuated by 49 ± 6.1%, 51 ± 3.5%, and 68 ± 6.0%, respectively. At 1.5 h after Fas stimuli, pre-treatment with CsA either activated (25 mg/kg) or inhibited (50 or 100 mg/kg) mitochondrial glucose metabolism in the liver of FAS-treated mice. In particular, using 160 mg/kg, the flux of glucose through PDH and PC decreased to 27 ± 5.0% and 38 ± 9.3%, respectively.

4) Fas causes metabolic changes in the intermediary stage (t = 5 h) of the apoptotic process, which are different from very early and very late changes. When entry of [U-13C]glucose) into the TCA cycle via PDH and PC was impaired by 50 % (p<0.001), the de novo synthesis of glucose from [2,3-13C]succinate (gluconeogenesis) rose to 203±38% (p<0.01). Furthermore, increased de novo synthesis of lactate (to 262±42%) indicated to a metabolic redirection to pyruvate recycling involving phosphoenolpyruvate carboxykinase (PEPCK) and/or malic enzyme (ME) at the later, but still progressing apoptotic process.

Conclusions
Increased mitochondrial glucose oxidation and glutathione de novo synthesis occur very early and specifically following the initiation of apoptosis. We suggest, that these metabolic events are possibly responsible for maintenance of adequate energy stores and of a reduced cell environment in order for apoptosis to proceed. The Fas-induced upregulation of specific pathways also precede the concentration-dependent protective effect of CsA on the apoptotic process. Therefore, we believe that, in addition to the well-known prevention of the mitochondrial permeability transition (MPT), a later event during the cell death process, CsA causes very early specific metabolic changes in the liver. The reversal of Fas-induced abnormal mitochondrial glucose metabolism at early stages may facilitate its protective effect, which further supports that early manipulations at the metabolic level may become a novel therapeutic strategy in apoptosis-related liver injuries.

References