Glucose fluctuation increased hepatocyte apoptosis under lipotoxicity and the involvement of mitochondrial permeability transition opening

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Abstract

Oxidative stress is considered to be an important factor in producing lethal hepatocyte injury associated with nonalcoholic fatty liver disease (NAFLD). Glucose fluctuation, more pronounced in patients with diabetes, has been recognized as an even stronger oxidative stress inducer than the sustained hyperglycemia. Here, we investigated the role of glucose variability in the development of the NAFLD based on hepatocyte apoptosis and possible mechanisms. To achieve this goal we studied C57BL/6J mice that were maintained on a high fat diet (HFD) and injected with glucose (3 g/kg) twice daily to induce intermittent high glucose (IHG). We also studied hepatic L02 cells incubated with palmitic acid (PA) to induce steatosis. The following experimental groups were compared: normal glucose (NG), sustained high glucose (SHG) and IHG with or without PA. We found that, although hepatic enzyme levels and liver lipid deposition were comparable between HFD mice injected with glucose or saline, the glucose injected mice displayed marked hepatocyte apoptosis and inflammation, accompanied by increased lipid peroxide in liver. *In vitro*, in the presence of PA, IHG increased L02 cell apoptosis and oxidative stress and produced pronounced mitochondrial dysfunction relative to the NG and SHG groups. Furthermore, treatment with the mitochondrial permeability transition (MPT) inhibitor, cyclosporin A (1.5 $\mu\text{mol/l}$), prevented mitochondrial dysfunction, oxidative stress and hepatocyte apoptosis. Our data suggests that IHG under lipotoxicity might contribute to the development of NAFLD by increasing oxidative stress and hepatocyte apoptosis via MPT and its related mitochondrial dysfunction.

Key Words

- ▶ glucose fluctuation
- ▶ nonalcoholic fatty liver disease
- ▶ apoptosis
- ▶ mitochondrial permeability transition

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Introduction

Nonalcoholic fatty liver disease (NAFLD) consists of ectopic fat accumulation in the liver secondary to metabolic factors, mostly obesity and insulin resistance (IR) or diabetes mellitus (Adams & Angulo 2005). The

prevalence of NAFLD has increased dramatically in recent years and affects 15–30% of the general population (Bellentani & Marino 2009). However, in individuals with type 2 diabetes (T2DM), the prevalence of NAFLD is

up to 70% (Targher *et al.* 2007, Leite *et al.* 2009, Prashanth *et al.* 2009).

The spectrum of NAFLD ranges from simple steatosis in its most benign form to cirrhosis on the opposite end of the spectrum where most liver-related morbidity and mortality occur. Nonalcoholic steatohepatitis (NASH) is a lesion of intermediate severity, which is accompanied by hepatocyte injury and death, as well as hepatic infiltration of inflammatory cells. NASH-related liver damage often triggers liver fibrosis. Studies have shown that fibrosis progresses in 38% of patients with NAFLD over a mean follow-up interval of 5.3 years and the presence of necroinflammation on initial biopsy is the strongest predictor of progression (Argo *et al.* 2009). The 'two-hit' hypothesis is a widely accepted paradigm to explain the progression of NAFLD (Day & James 1998). The first hit is the development of hepatic steatosis, and the second hit includes oxidative, metabolic and cytokine stresses that overwhelm hepatocyte survival mechanisms, leading to hepatocyte death. Furthermore, some authors consider hepatocyte apoptosis as a third hit that promotes the development of cirrhosis (Jou *et al.* 2008).

Although the mechanisms underlying NAFLD development have not yet been clearly elucidated, mitochondrial dysfunction has been shown to play a critical role in triggering hepatocyte apoptosis (Malhi & Gores 2008). Mitochondria burns excessive intrahepatic fat by β -oxidation. However, this reaction produces not only ATP but also reactive oxygen species (ROS). An increase in ROS reaching a threshold level can trigger the opening of the mitochondrial permeability transition (MPT) pore, which in turn leads to the simultaneous collapse of mitochondrial membrane potential ($\Delta\psi$) and a massive release of ROS (Zorov *et al.* 2000). The MPT is defined as a rapid increase in the permeability of the mitochondrial membrane to low molecular solutes (Lemasters *et al.* 1998). The opening of the MPT pore can trigger numerous subsequent reactions, for example, mitochondrial release of cytochrome c into the cytosol triggers the assembly of the caspase 9–caspase 3 activation complex (Rusmann *et al.* 2009). It has also been shown that inhibiting the MPT with specific blockers, such as cyclosporin A (CsA), ameliorates caspase activation and apoptosis in several cellular systems (Halestrap *et al.* 1997, Kroemer & Reed 2000, Halestrap & Brenner 2003).

Recently, it has been shown that the glycemic variability is an independent predictive factor for the progression of NAFLD in a diabetic population (Hashiba *et al.* 2013). Mechanistic studies have indicated that chronic sustained hyperglycemia is associated with

increased mitochondrial ROS production, which was further exacerbated by conditions of intermittent high glucose (IHG) (Quagliaro *et al.* 2003, 2005). Clinical studies have also shown that various markers of oxidative damage are significantly related to the mean amplitude of glycemic excursions (Monnier *et al.* 2006, Zheng *et al.* 2010, Wang *et al.* 2011). Despite this association between glycemic variability and ROS production, no systematic experiments have been conducted to elucidate the role of glucose fluctuations in ROS production and apoptosis in the liver. Furthermore, the contribution of glucose fluctuation to the development of NAFLD in T2DM remains to be elucidated. With this in mind, the aim of the current study was to examine the effects of IHG on the development of NAFLD. Moreover, the possible involvement of MPT pore opening was investigated.

Materials and methods

Animal experiments and treatment

Four-week-old male C57BL/6J mice were purchased from the Slack Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China) and fed a standard diet (STD; 70% carbohydrate, 20% protein and 10% fat). After 1 week of habituation, animals were weighed and divided into two groups: the experimental group ($n=24$) was fed a high fat diet (HFD; 35% carbohydrate, 20% protein and 45% fat), and the control group ($n=12$) was maintained on a STD. After 8 weeks, the experimental group was further randomized into two groups of 12 mice. One group (HFD + F group) was treated with 3 g/kg glucose by i.p. injection twice daily (at 0800 and 1600 h), while the remaining experimental animals (HFD + NS) and the control group (STD + NS) were injected with saline. All mice were weighed daily, and their fasting blood glucose levels were measured using One Touch Ultra glucose strips (LifeScan, Milpitas, CA, USA) twice monthly, and fasting insulin levels were measured at 8 and 20 weeks of HFD using an insulin EIA kit (Millipore, Billerica, MA, USA). The animal care committee of Zhejiang University approved all animal experiments.

Biochemical analyses of blood and liver samples

Free fatty acid (FFA) levels and alanine aminotransferase (ALT) activity in the fasted serum were measured using an FFA assay kit (Cayman Chemical, Ann Arbor, MI, USA) and ALT activity assay kit (Bivision, Mountain View, CA, USA) respectively. The concentration of ATP and triglycerides in the liver was determined using an ATP colorimetric assay

kit (Bivision) and a triglyceride quantification colorimetric kit (Bivision) respectively.

Histopathology

Liver tissue was collected from all mice and processed using standard methods. Paraffin-embedded sections of the liver were stained with hematoxylin and eosin (H&E). Frozen liver sections were stained with Oil Red O to assess hepatic lipid content. Immunohistochemical staining was carried out to determine the levels of 4-hydroxy-2-nonenal (HNE; 1:100, rabbit anti-mouse; 1:500; Abcam, Cambridge, MA, USA), malondialdehyde (MDA; 1:250, rabbit anti-mouse; 1:500; Abcam) and α -smooth-muscle-actin (α -SMA; 1:100; Epitomics, Burlingame, CA, USA; rabbit anti-mouse; 1:500) using standard protocols. The extent of hepatocyte apoptosis in the liver was determined using a TUNEL kit (Roche Diagnostics, Indianapolis, IN, USA). To quantify apoptosis, ten visual fields at 400 $\times$ from each section were analyzed for TUNEL-positive cells. A TUNEL index was determined using the following formula: (number of stained cells/total number of cells) $\times$ 100 (Yamada *et al.* 2007).

Western blotting

Western blotting was performed according to standard methods, using equal amounts of protein (50 μ g). Primary antibodies raised against HNE and MDA (Abcam), Bax, cleaved caspase 9, cleaved caspase 3, cleaved PARP, CCAAT/enhancer-binding protein homologous protein (CHOP), p53 up-regulated modulator of apoptosis (PUMA), cytochrome c, β -actin (all 1:1000; Cell Signaling Technology, Beverly, MA, USA) and α -SMA (1:1000; Epitomics) were used. Immunoreactive proteins were detected using a chemiluminescent ECL assay kit (Millipore).

Real-time quantitative RT PCR

A standard real-time quantitative RT PCR (qRT-PCR) was performed. Total RNA was extracted from tissues using TRIzol (Invitrogen, Carlsbad, CA, USA). The mRNA levels were assessed by real-time qRT-PCR using the SYBR Green PCR Master Mix kit (TaKaRa Bio, Inc., Shiga, Japan) with a LightCycler 480 II system (Roche Applied Science). Primer pairs for transcripts of interest are listed in [Supplementary Table S1](#), see section on [supplementary data](#) given at the end of this article. GAPDH was chosen as an invariant standard. All experimental tissues and standard curve samples were run in duplicate in a 96-well reaction plate (MicroAmp Optical, Applied Biosystems). Results are expressed as fold

expression relative to expression in the control group following the $\Delta\Delta$ Ct method (Livak & Schmittgen 2001).

Hepatocyte culture and treatment

L02 cells are a normal hepatocyte cell line derived from adult liver tissue. These cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China) and were maintained in RPMI 1640 medium containing 10% fetal bovine serum. Palmitic acid (PA) conjugated-BSA was prepared as described previously (Shi *et al.* 2006). Cells incubated with or without PA (0.125 mmol/l) were exposed to normal glucose (NG, 5.5 mmol/l), sustained high glucose (SHG, 33.3 mmol/l) or IHG (5.5 and 33.3 mmol/l alternating every 12 h) for 3 days. To assess MPT, cells were pre-incubated overnight with the MPT blocker CsA (1.5 μ mol/l) or the appropriate vehicle (DMSO).

Annexin V staining for apoptosis detection

To assess apoptosis in L02 cells, the level of Annexin V staining was determined by flow cytometry. After incubation, cells were treated according to the manufacturer's protocol (FITC Annexin V Apoptosis Detection Kit, BD Biosciences Pharmingen, San Diego, CA, USA) and analyzed by flow cytometry (FACSort, Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of ROS generation

Aliquots of L02 cells were removed at timed intervals and analyzed for the generation of total ROS using the fluorescent probe dichlorodihydrofluorescein diacetate (DCF/DA, Beyotime, Haimen, Jiangsu, China) by flow cytometry at 480 nm excitation and 540 nm emission settings.

Mitochondrial superoxide assay

Mitochondrial superoxide levels were measured by flow cytometry after staining with the mitochondrial superoxide-specific dye MitoSOX red (Molecular Probes, Eugene, OR, USA) at 396 nm excitation and 580/510 nm emission settings.

Measurement of the mitochondrial membrane potential

The $\Delta\psi$ was estimated by measuring the fluorescence of 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Beyotime) by flow cytometry.

At relatively low concentrations, JC-1 exists as a monomer that fluoresces at 527 nm, and when concentrated by actively respiring mitochondria, JC-1 forms aggregates, which fluoresce at 590 nm (Salvioli *et al.* 1997). The intensity of fluorescence at 590 nm is proportional to the $\Delta\psi$, which indicates a closed MPT pore. On induction of MPT, dissipation of the $\Delta\psi$ prevents the formation of JC-1 aggregates with reduced fluorescence at 590 nm.

Electron microscopy

For electron microscopy, the specimens were fixed and embedded according to standard protocols. Embedded specimens were cut into ultrathin sections of 0.5 μm and stained with uranyl acetate and lead citrate. The sections were then observed under an Hitachi H-7100 transmission electron microscope (Hitachi-High Technologies Co., Shimbashi, Tokyo, Japan).

Statistical analysis

All data have been expressed in terms of means $\pm$ S.E.M. Differences between the means of individual groups were assessed with the independent *t*-test or a one-way ANOVA and false discovery rate (FDR) multiple range tests. All statistical analysis was performed using SPSS 20.0 (IBM, Armonk, NY, USA). A significant difference was defined as $P < 0.05$.

Results

Effect of glucose fluctuation on body weight, metabolic parameters and hepatic function

Blood glucose levels were measured to test the amplitude of fluctuations after a bolus of i.p. glucose injection. The mice fed a HFD and given an i.p. glucose injection showed significant fluctuations as compared to those given an i.p. saline injection and fed on a HFD or STD (Supplementary Figure S1, see section on supplementary data given at the end of this article). Mice fed a HFD were significantly heavier than those fed a STD during the 5-month experimental time period ($P < 0.05$), whereas there was no difference in body weight between HFD mice given glucose or saline injections ($P > 0.05$, Fig. 1A).

Fasting blood glucose levels were significantly higher in mice fed a HFD compared to mice fed a STD at 6 weeks ($P < 0.01$), and the difference was maintained throughout the rest of the experiment (Fig. 1B). There was no difference in fasting blood glucose levels between HFD mice given glucose or saline injections ($P > 0.05$). Similarly, fasting insulin levels were greatly increased in HFD fed mice ($P < 0.05$ at 8 weeks and $P < 0.01$ at 20 weeks), with no differences of the levels between HFD mice receiving glucose or saline injections (Fig. 1C).

Elevated circulating FFA is an established risk factor for NAFLD (Unger & Orci 2002, Feldstein *et al.* 2004). Serum FFA levels in HFD mice were significantly higher than those in STD mice at 8 weeks and were further increased

Figure 1

Effect of glucose fluctuation on body weight, metabolic parameters and hepatic function. Body weight (A) and blood glucose levels (B) were measured throughout the experimental time course. At weeks 8 and 20,

fasting insulin, free fatty acid (FFA) and alanine aminotransferase (ALT) (C, D, E, respectively) levels were measured. Values are means $\pm$ S.E.M.; $n = 10$ –12 mice/group; * $P < 0.05$, ** $P < 0.01$.

at 20 weeks. FFA levels were not different between the HFD mice given saline or glucose injection at 8 or 20 weeks (Fig. 1D). At 8 weeks, the plasma ALT level was similar between STD and HFD mice, whereas a significant increase in plasma ALT was observed at 20 weeks ($P < 0.01$, Fig. 1E). No difference in the ALT level was found between the HFD mice given saline or glucose injection.

Effect of blood glucose fluctuation on hepatic steatosis

H&E staining revealed significant vacuoles present in the liver of mice fed a HFD, which was also evident in Oil Red O stained sections, suggesting these mice had developed hepatic steatosis; however, IHG did not seem to exacerbate this condition (Fig. 2A and B). Consistently, the hepatic triglyceride levels in HFD mice were significantly higher than STD mice ($P < 0.01$), with no difference between HFD mice given saline or glucose injections (Fig. 2C).

Glucose fluctuation increases hepatic apoptosis *in vivo* and *in vitro*

Emerging data has indicated that hepatocyte apoptosis is a histological hallmark that differentiates simple steatosis from NASH; we thus investigated the effect of IHG on hepatocyte apoptosis. The percentage of TUNEL-positive stained cells was low in STD mice; however, it was significantly higher in HFD mice given saline injections and further elevated in HFD mice given glucose injections ($P < 0.01$, Fig. 3A).

To investigate the molecular mechanism through which IHG induces hepatocyte apoptosis, we studied this phenomenon in L02 cells. First we confirmed the effect of IHG on hepatocytes *in vitro*. In the presence of PA, treatment with SHG-induced apoptosis relative to the NG group ($P < 0.05$ vs NG+P group). This effect was more pronounced in cells treated with IHG, which had a higher rate of apoptosis than SHG-treated cells ($P < 0.05$ vs SHG+P group; Fig. 3B). There was no significant effect of SHG or IHG on hepatocyte apoptosis in the absence of PA.

PARP-specific proteolytic cleavage by caspases has been recognized as a characteristic of apoptosis, thus we investigated the cleavage PARP and caspases levels by western blotting. We observed that apoptosis-related proteins were significantly increased when L02 cells were incubated with PA and SHG relative to those with PA and NG treatment and that these proteins were further increased by treatment with PA and IHG (Fig. 3C). Similarly, cleaved caspase 3, cleaved caspase 9, Bax, CHOP and PUMA were also significantly increased in the liver of mice fed a HFD, which was further exacerbated by glucose injections (Fig. 3D).

Glucose fluctuation exacerbates liver inflammation and fibrosis

The intensity of the reparative process generally parallels with the degree of hepatocyte death, resulting in a variable distortion of the hepatic architecture with immune cells infiltration, fibrosis, and epithelial nodules regeneration

Figure 2

Effect of blood glucose fluctuation on hepatic steatosis. Representative images of liver sections stained with hematoxylin and eosin H&E (A) and Oil

Red O (B) are shown. Scale bars, 100 μm. Hepatic triglyceride contents (C) are also shown. Values are means $\pm$ S.E.M.; $n = 10$ –12 mice/group; ** $P < 0.01$.

Figure 3

Effect of glucose fluctuation on hepatocyte apoptosis *in vivo* and *in vitro*. Representative images of liver sections stained with TUNEL are presented and the percentage of TUNEL-positive cells quantified. Scale bars, 100 μ m (A). Green: TUNEL-positive cells; blue: DAPI stained nuclei. Representative data obtained by Annexin V staining and quantification of the number of

apoptotic cells by flow cytometry is presented (B); * P < 0.05, ** P < 0.01. Values are mean $\pm$ S.E.M. of three replicates in three separate experiments. Representative western blots are presented showing the protein expression level of apoptosis-related proteins in L02 cells (C) and mice (D).

(Jou *et al.* 2008). Therefore, we investigated the production of inflammatory markers *in vivo*. Although there was no difference in the hepatic expression of F4/80 (Emr1) mRNA among all groups of mice, we noted a significant increase in the mRNA levels of MCP-1 (P < 0.05) in the liver of mice fed a HFD, with the highest mRNA levels of MCP-1 and TNF- α found in the liver of HFD mice exposed to IHG (Fig. 4A).

We further examined the levels of liver fibrosis. The mRNA levels of ECM components, such as type 1 procollagen α 1 (Col1a1) and type 4 procollagen α 1 (Col4a1), fibrosis markers, such as transforming growth factor beta 1 (TGF β 1), platelet-derived growth factor receptor (PDGFR) and tissue inhibitor of metalloprotease-1 (TIMP1), were

significantly increased in HFD-fed mouse livers, and the mRNA levels of Col1a1, Col4a1, TGF β 1 and TIMP1 were even higher in mice exposed to IHG (Fig. 4B). This is in agreement with those obtained by western blotting (Fig. 4C) and immunohistochemistry (Fig. 4D) of α -SMA expression.

Blood glucose fluctuation promotes hepatic oxidative stress

Several studies on the pathogenesis of NASH have highlighted the central role of excessive activation of mitochondrial oxidative stress. To address this issue, we examined the effect of IHG on oxidative stress *in vivo* and

Figure 4

Effect of glucose fluctuation on liver inflammation and fibrosis. mRNA levels were determined by real-time qRT-PCR, values are means $\pm$ S.E.M.; $n = 10$ –12 mice/group; * $P < 0.05$, ** $P < 0.01$ (A and B). Representative

western blots are presented showing the protein expression level of α -SMA in mice (C). Representative images of immunostained liver sections showing the expression of α -SMA (D) are presented.

in vitro. L02 cells exposed to PA and SHG produced increased levels of ROS and mitochondrial superoxide relative to cells treated with PA and NG ($P < 0.05$), with even higher ROS and mitochondrial superoxide levels detected in cells treated with IHG with PA ($P < 0.05$ vs SHG + P group; Fig. 5A and B). In the absence of PA, SHG or IHG had no effect on oxidative stress in L02 cells.

In vivo, ROS production can increase lipid peroxidation, leading to the production of HNE and MDA. To assess the effect of IHG on oxidative stress in mice fed a HFD, we assessed the abundance of HNE and MDA in the liver. Our immunohistochemistry (Fig. 5C and D) and western blotting data (Fig. 5E) both indicated that after 20 weeks of consuming a HFD, the HNE and MDA levels were significantly increased in the liver and these levels were further increased in mice exposed to IHG.

Blood glucose fluctuation induced mitochondrial dysfunction by affecting the MPT

To assess the effect of IHG on mitochondria, we studied various markers of mitochondrial function. Excess ROS production in mitochondria can trigger the MPT, resulting in impairment of mitochondrial function with effects on cellular metabolism and apoptosis. We therefore examined whether increased ROS production induced by IHG in the presence of PA was associated with mitochondrial

dysfunction. Fluorescence of JC-1 aggregates was significantly reduced in L02 hepatocytes exposed to SHG with PA ($P < 0.05$ vs NG + P group) and was further decreased in cells exposed to IHG and PA ($P < 0.05$ vs SHG + P group; Fig. 6A). In the absence of PA, the different glucose treatments had no effect on mitochondrial $\Delta\psi$. Mitochondrial morphology was subsequently assessed by electron microscopy. This data revealed that the mitochondria of cells treated only with NG had normal cristae, whereas there were reduced cristae and mitochondrial swelling in cells exposed to SHG and IHG, which was exacerbated by exposure to PA. The combined effect of PA and SHG led to more translucent matrix with less cristae formation than PA and NG and cells treated with IHG and PA showing an even greater effect (Fig. 6B). Cytochrome c release from mitochondria involved apoptosis, therefore cytoplasmic cytochrome c levels were assessed. L02 cells exposed to PA and IHG had the highest levels of cytochrome c among all the groups (Fig. 6C). In agreement with this observation, mice fed a HFD and injected with glucose also had the highest release of cytochrome c (Fig. 6D). Finally, we measured hepatic ATP content as another index of mitochondrial function. Here, we found that mice fed a HFD and injected with saline had higher hepatic levels of ATP compared to mice fed a STD ($P < 0.01$); however, mice fed a HFD and injected with glucose had a significantly lower level of ATP ($P < 0.05$ vs HFD + NS group), indicating impaired ATP production (Fig. 6E).

Figure 5

Effect of glucose fluctuation on hepatic oxidative stress. Representative histograms of flow cytometry showing dichlorodihydrofluorescein diacetate (DCF/DA) fluorescence (A) and MitoSOX Red fluorescence (B); * $P < 0.05$, ** $P < 0.01$; values are mean $\pm$ S.E.M. of three replicates from three

separate experiments. Representative images of immunostained liver sections showing the expression of HNE (C) and MDA (D) are presented. Scale bars, 100 μ m. Representative western blots are also presented showing the protein expression level of HNE and MDA (E).

Figure 6

Effect of glucose fluctuation on hepatic mitochondrial dysfunction. Representative data obtained by JC-1 fluorescence and flow cytometry to measure $\Delta\psi$ is presented (A); * $P < 0.05$, ** $P < 0.01$, values are mean $\pm$ s.e.m. of three replicates from three separate experiments. Representative electron micrographs showing mitochondrial ultrastructure are also

presented (B; arrows show mitochondria, $\times 30\,000$ magnification).

Cytosolic cytochrome c expression was determined by western blotting in L02 cells (C) and mice (D) and normalized to the β -actin protein expression level. Hepatic ATP content was also determined in experimental mice (E). Values are means $\pm$ s.e.m.; $n = 10$ –12 mice/group, * $P < 0.05$, ** $P < 0.01$.

MPT inhibition reverses the apoptotic effect of glucose fluctuation

The MPT is a critical factor in mitochondrial dysfunction and excess ROS production. To establish if this process was important in the observed increase in apoptosis following exposure to IHG and PA, we treated L02 cells with the MPT inhibitor CsA. L02 cells pretreated with CsA had a reduced accumulation of JC-1 aggregates and a reduced release of cytochrome c following exposure to SHG and IHG in the presence of PA, compared to untreated cells (Fig. 7A and B). Furthermore, the ultrastructural abnormalities induced by IHG and PA were reversed by pretreatment with CsA (Fig. 7C), as was the increase in oxidative stress and the induction of L02 cell apoptosis by IHG, SHG and NG in the presence of PA (Fig. 7D, E, F and G).

Discussion

The data presented herein provides evidence that IHG induces hepatocyte apoptosis and the corresponding reparative response under lipotoxicity *in vitro* and *in vivo*. Mechanistically, this increase in apoptosis is associated with increased ROS production and mitochondrial dysfunction, secondary to the induction of the MPT. These findings provide a possible mechanistic link between glucose fluctuation and the progression to NAFLD from simple steatosis to NASH.

Data from humans, as well as *in vivo* and *in vitro* experimental models, demonstrate that cell death, particularly apoptosis, is increased in NAFLD and NASH patients, suggesting that it is a crucial factor in disease progression (Feldstein & Gores 2005, Machado &

Figure 7

Effect of CsA on mitochondrial function, oxidative stress and hepatic apoptosis. Representative data showing the effect of CsA treatment on JC-1 fluorescence (A), cytochrome c release (B) mitochondrial ultrastructure (C), ROS production (D), mitochondrial superoxide (E), hepatic apoptosis (F)

and apoptosis-related proteins expression (G) in L02 cells under different experimental conditions are presented. * $P < 0.05$, ** $P < 0.01$, values are mean $\pm$ S.E.M. of three replicates from three separate experiments.

Cortez-Pinto 2011). In our study, although no difference in the ALT level and hepatic lipid content were observed between mice fed a HFD injected with saline and glucose, mice injected with glucose did have evidence of increased

hepatocyte apoptosis. This link between IHG and apoptosis was further confirmed by exposing L02 cells to fluctuating glucose levels. Hepatocyte apoptosis triggers the activation of hepatic stellate cells to myofibroblasts,

causes liver fibrosis and expands hepatic progenitor populations that produce chemoattractants to recruit various types of immune cells into the liver (Jou *et al.* 2008). Patients with T2DM and NAFLD show a significantly higher prevalence of NASH, fibrosis and cirrhosis than non-diabetic subjects with NAFLD (Leite *et al.* 2011, Smith & Adams 2011, Doycheva *et al.* 2013). Furthermore, patients with severe vs mild fibrosis have been reported to have remarkably higher levels of glycemic variability (Hashiba *et al.* 2013). Our data confirms this association and shows that glucose fluctuation and lipotoxicity can significantly induce hepatocyte apoptosis, inflammation and fibrosis. Despite the increased levels of apoptosis observed under conditions of IHG, no differences were observed in markers of glucose levels, lipid deposition and liver enzyme levels in HFD-induced obese mice. Further study will be required to determine if this was due to the relatively short observation period in the current study or to the differences in the frequency and amplitude of glucose fluctuations in animal models vs diabetic patients.

An important factor in the progression of NAFLD is oxidative stress, with NAFLD patients being reported to have increased oxidative stress (Sumida *et al.* 2003, Yesilova *et al.* 2005) and a decrease in antioxidant defenses (Videla *et al.* 2004). T2DM is also characterized by the presence of oxidative stress (Davi *et al.* 1999), and diabetic patients with NAFLD have higher levels of markers of oxidative stress compared to diabetic patients without NAFLD (Narasimhan *et al.* 2010). Recently, several studies have demonstrated that IHG can generate more ROS than SHG in endothelial cells (Quagliaro *et al.* 2003, Piconi *et al.* 2006), islet cell (Del Guerra *et al.* 2007, Kim *et al.* 2012) and Schwann cells (Sun *et al.* 2012a,b). We found that in the presence of lipotoxicity, SHG produced an increase in oxidative stress; moreover, the IHG appeared to further enhance oxidative stress. Previous studies in HepG2 cells demonstrated that ROS generation is increased in cells cultured in 30 mmol/l glucose for 48 h, but with no changes in cell viability until 7 days (Palmeira *et al.* 2007). It was reported that HepG2 cells cultured in 50 mmol/l glucose for 72 h showed a significant increase in apoptosis, accompanied by an increase in ROS levels (Chandrasekaran *et al.* 2010). Different cell models adopted or the shorter incubation period or the lower glucose concentration could possibly explain the discrepancy in these studies. Here, increased oxidative stress and hepatocyte apoptosis was only observed in the context of lipotoxicity. The concern is that lipotoxicity is quite common in T2DM, thus it could not only initiate the development of NAFLD but also promote its progression.

Such an effect would further be enhanced in the presence of high glucose levels, especially IHG. Thus, lipotoxicity and IHG might be an important mechanism mediating the progression of NAFLD to NASH in T2DM.

It is well recognized that mitochondria play a critical role in the induction of cellular oxidative stress and apoptosis, with the most recent attention focused on the role of the MPT in this process (Lee & Wei 2000). It has been proposed that the induction of the MPT itself can increase production of ROS from the respiratory chain (Zamzami *et al.* 1995) through the release of mitochondrial cytochrome c that then impairs transfer of electrons. In turn, this increase may cause oxidation of critical thiol sites in the MPT pore (Lemasters *et al.* 1998) causing further induction of MPT with the subsequent release of cytochrome c and other apoptosis-inducing proteins. Thus, a vicious cycle in the mitochondria originally stimulated by oxidative stress may further perpetuate and magnify the intracellular oxidative stress. Here, the MPT inhibitor CsA prevented PA-induced mitochondrial dysfunction, oxidative stress and hepatocyte apoptosis, indicating that the opening of the MPT pore is an essential prerequisite for triggering the apoptosis. IHG exacerbated this vicious cycle of MPT-ROS production in the mitochondria, leading to excess liver cell apoptosis.

In summary, our findings support the concept that glucose fluctuation accelerates hepatocyte apoptosis, inflammation and fibrosis in the context of lipotoxicity by increasing oxidative stress and mitochondrial dysfunction. Furthermore, the MPT seems to play a critical role in the initiation of hepatocyte apoptosis caused by lipotoxicity in conjunction with SHG and IHG, both of which are common in T2DM. We conclude that a better control of mean glucose levels and glucose fluctuations may be of potential benefit in slowing the progression of NAFLD in T2DM.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-15-0101>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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