INTRODUCTION

Acetaminophen (APAP), also known as paracetamol, is a widely used analgesic and antipyretic. APAP is safe when administered in modest doses; however, high doses can lead to liver injury or even liver failure (1-3). Apart from damage to hepatocytes, also the involvement of nonparenchymal liver cells contributes to the injury (4). Patients abusing alcohol are at high risk of developing liver failure (1, 5). Similarly, non-alcoholic fatty liver disease (NAFLD) seems to predispose liver to the toxicity of APAP and other toxicants (6-9).

Early administration of N-acetylcysteine remains the main therapeutic option for APAP overdose (1). In experimental studies, several other strategies were suggested to ameliorate the APAP-induced liver injury: inhibition of 5-lipoxygenase (10), inhibition of protein tyrosine phosphatase 1B (11), activation of autophagy (12), inhibition of c-Jun N-terminal kinase (13), free radical scavenging (14) and activation of Nrf2 (15).

The nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor important for combating electrophilic and oxidative stress in the liver and other organs. This encompasses detoxification of hepatotoxic drugs, including acetaminophen (APAP). Recently, an association between apolipoprotein E (ApoE) genotype and Nrf2 expression was described. We compared the toxicity of APAP on primary culture hepatocytes isolated from transgenic mice carrying two different human ApoE alleles and wild-type controls. The cells were exposed to APAP in concentrations from 0.5 to 4 mM for up to 24 hours. APAP led to a dose-dependent hepatotoxicity from 1 mM after 16 h exposure in all mice tested. The toxicity was higher in hepatocytes isolated from both transgenic strains than in wild-type controls and most pronounced in ApoE3 mice. Concurrently, there was a decline in mitochondrial membrane potential, especially in ApoE3 hepatocytes. The formation of reactive oxygen species was increased after 24 hours with 2.5 mM APAP in hepatocytes of all strains tested, with the highest increase being in the ApoE3 genotype. The activity of caspases 3 and 7 did not differ among groups and was minimal after 24 hour incubation with 4 mM APAP. We observed higher lipid accumulation in hepatocytes isolated from both transgenic strains than in wild-type controls. The expression of Nrf2-dependent genes was higher in ApoE3 than in ApoE4 hepatocytes and some of these genes were induced by APAP treatment. In conclusion, transgenic mice with ApoE4 and ApoE3 alleles displayed higher susceptibility to acute APAP toxicity in vitro than wild-type mice. Of the two transgenic genotypes tested, ApoE3 allele carriers were more prone to injury.

**Key words:** acetaminophen, apolipoprotein E, hepatocytes, nuclear factor erythroid 2-related factor 2, oxidative stress, primary cell culture, caspase, reactive oxygen species

COMPARISON OF ACETAMINOPHEN TOXICITY IN PRIMARY HEPATOCYTES ISOLATED FROM TRANSGENIC MICE WITH DIFFERENT APOLIPOPROTEIN E ALLELES

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The nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor, important for combating electrophilic and oxidative stress in the liver and other organs. This encompasses detoxification of hepatotoxic drugs, including acetaminophen (APAP). Recently, an association between apolipoprotein E (ApoE) genotype and Nrf2 expression was described. We compared the toxicity of APAP on primary culture hepatocytes isolated from transgenic mice carrying two different human ApoE alleles and wild-type controls. The cells were exposed to APAP in concentrations from 0.5 to 4 mM for up to 24 hours. APAP led to a dose-dependent hepatotoxicity from 1 mM after 16 h exposure in all mice tested. The toxicity was higher in hepatocytes isolated from both transgenic strains than in wild-type controls and most pronounced in ApoE3 mice. Concurrently, there was a decline in mitochondrial membrane potential, especially in ApoE3 hepatocytes. The formation of reactive oxygen species was increased after 24 hours with 2.5 mM APAP in hepatocytes of all strains tested, with the highest increase being in the ApoE3 genotype. The activity of caspases 3 and 7 did not differ among groups and was minimal after 24 hour incubation with 4 mM APAP. We observed higher lipid accumulation in hepatocytes isolated from both transgenic strains than in wild-type controls. The expression of Nrf2-dependent genes was higher in ApoE3 than in ApoE4 hepatocytes and some of these genes were induced by APAP treatment. In conclusion, transgenic mice with ApoE4 and ApoE3 alleles displayed higher susceptibility to acute APAP toxicity in vitro than wild-type mice. Of the two transgenic genotypes tested, ApoE3 allele carriers were more prone to injury.

**Key words:** acetaminophen, apolipoprotein E, hepatocytes, nuclear factor erythroid 2-related factor 2, oxidative stress, primary cell culture, caspase, reactive oxygen species
underwent repeated centrifugation in a cold centrifuge at 28 g. Afterwards, the suspension was diluted in Williams E medium (in all isolations > 80%). Cell density was counted using a Cellometer (Nexcelom Bioscience, Lawrence, MA, USA).

In the present study, we compared the toxicity of APAP on primary culture hepatocytes isolated from transgenic mice carrying different human ApoE alleles (ApoE3 or ApoE4) and from wild-type mice.

MATERIAL AND METHODS

Chemicals

Collagenase (lot 120296, 0.12 PZU/mg) was purchased from Seva (Heidelberg, Germany). The anesthetic ketamine (Narketan) was obtained from Vetoquinol (Lure Cedex, France) and the xylazine (Rometar) from Bioveta (Ivanovice na Hane, Czech Republic). Reagents and kits are described in the respective paragraphs. All other chemicals were in analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Female mice with targeted replacement of the endogenous mouse ApoE gene with human ApoE3 or ApoE4 gene on a C57Bl/6 background (age 7–9 weeks, body weight 20.2 ± 1.3 g) were purchased from Taconic (Hudson, NY, USA). Female C57Bl/6 age-matched mice, obtained from Velaz (Unetice, Czech Republic), were used as wild-type (WT) controls. The mice were housed at 23 ± 1°C, 55 ± 10% humidity, with air exchange 12–14 times/h and a 12 h light-dark cycle. All animals had free access to tap water and were fed a standard ST-1 pelleted diet (Velas, Lysa nad Labem, Czech Republic). All animal care was in accordance with Czech legislation. The animal study was approved by the Animal Welfare Body of the Faculty of Medicine in Hradec Kralove and by the Czech Ministry of the Environment.

Hepatocyte preparation

Before the isolation, the mice were pre-anesthetized with ether and then injected intraperitoneally with a mixture of ketamine (100 µg/g body weight) and xylazine (10 µg/g body weight) dissolved in water for injections. Hepatocytes were isolated by two-step collagenase perfusion as described by Bajt et al. (32) with some modifications. Before opening the abdominal cavity, heparin was injected intraperitoneally (0.5 IU/g body weight) to prevent blood clotting throughout the subsequent steps. After opening the abdominal cavity, the falciform ligament was deranged and the gallbladder excised. The portal vein was first with a calcium-free solution for 3 minutes, then with a solution containing collagenase (1 mg/ml). When the liver took up a sponge-like appearance (8–10 minutes), it was excised and underwent repeated centrifugation in a cold centrifuge at 28 g.

Cell viability was determined by a Trypan blue exclusion test (in all isolations > 80%). Cell density was counted using a Cellometer (Nexcelom Bioscience, Lawrence, MA, USA). Afterwards, the suspension was diluted in Williams E medium enriched with 6% fetal bovine serum (Merek Millipore, Berlin, Germany), glutamine (2 mmol/l), penicillin (100 IU/ml), streptomycin (10 µg/ml), insulin (0.08 IU/ml), dexamethasone (0.12 µg/ml) and glucagon (0.008 µg/ml) to a density of 4 × 10⁶ cells/ml. After seeding on collagen-coated culturing 6-well (2 ml per well; for qRT-PCR), 12-well (1 ml per well; for phase contrast imaging and protein isolation), 24-well (0.3 ml; for LDH leakage, glutathione levels and Oil Red O staining) and 96-well (0.1 ml; for WST-1 test, reactive oxygen species measurements and caspase activities) NUNC plates (Thermo Scientific, Waltham, MA, USA) for 2 hours, the medium was replaced and the serum-free Williams E medium with concentrations of APAP ranging from 0.5 mmol/l to 4 mmol/l was added. The cells were incubated at 37°C in an atmosphere with 5% CO₂ for up to 24 hours.

RNA isolation and quantitative real-time PCR

The total RNA from hepatocyte suspensions (leftover not used for seeding) and from the 6-well plates treated for 24 hours was isolated by phenol-chloroform extraction using RNA Blue (Top-Bio, Prague, Czech Republic). Afterwards, the total RNA of each sample was reverse transcribed using oligo-dT primers (Generi Biotech, Hradec Kralove, Czech Republic) and M-MLV Reverse Transcriptase (Top-Bio) according to manufacturers’ instructions. Genes of interest were Nrf2-dependent genes (24) glutathione S-transferase 2 (Gstα2), heme oxygenase-1 (Hmox1) and NAD(P)H dehydrogenase, quinone 1 (Nqo1). A primer design summary is provided in Table 1. To avoid genomic DNA contamination, the primers were designed to span exon boundaries. The hydrolyzation probes were labeled with FAM fluorescent reporter dye. The selection of the two housekeeping genes was based on experimental validation of their expression stability: beta 2-microglobulin (β2m) and polypeptide A RNA-Polymerase II (Polr2a). Real-time quantitative RT-PCR was performed on a CFX96 Touch cycler (Bio-Rad Laboratories, Hercules, CA, USA) with amplification conditions: 95°C for 3 min, and 50 cycles of 95°C for 10 s and 60°C for 20 s. Each cDNA sample was analyzed in PCR triplicates.

REST 2009 software V2.0.13 (QIAGEN GmbH, Hilden, Germany) was applied to determine statistically significant expression differences and relative fold changes (33).

Microscopic evaluation

All images were taken using an Olympus IX51 microscope with an E-600 Digital Camera (Olympus Imaging Corp., Tokyo, Japan) and Quick Photo Camera 3.0 software (Promicra, Prague, Czech Republic). Scale bars were added using the Quick Photo Camera software; no other image editing was performed. The objective magnifications used was × 40. All the types of staining described below were performed at room temperature. Phase contrast images were taken at 4 h and 24 h after isolation.

To semi-quantify the presence of lipids, we used a Steatosis Colorimetric Assay Kit (Cayman Chemical Comp., Ann Arbor, MI, USA) based on Oil Red O dye; haematoxylin was used for nuclear counter-staining. We checked for Oil Red O positive cells, i.e. cells with orange-red cytoplasm in the bright field mode. The positive cells were then counted in at least 10 visual fields; at least 500 cells were included. The percentage of positivity was then calculated.

The mitochondrial membrane potential was evaluated using JC-1 Dye (Life Technologies, Carlsbad, CA, USA) as described previously (34). Briefly, the cells were incubated for 45 minutes at 37°C with 10 µM JC-1 dye dissolved in the medium. After washing, the cells were visualized in fluorescence mode. The percentage of cells with high mitochondrial membrane potential was counted; at least 230 cells per group were included.

Viability tests: WST-1, lactate dehydrogenase activity and leakage

The activity of intracellular dehydrogenases was determined using Cell Proliferation Reagent WST-1 (Roche, Penzberg, Germany), based on Oil Red O (2,3,5-triphenyltetrazolium), haematoxylin and the xylazine (Rometar) from Bioveta (Ivanovice na Hane, Czech Republic). Reagents and kits are described in the respective paragraphs. All other chemicals were in analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).
GSH in the supernatant were measured using a modified conjugate of APAP with glutathione (APAP-SG). The levels of the samples were centrifuged (20,000 g, 4°C). The levels of life Technologies, as described previously (34) with the diacetate (DCFDA) and malondialdehyde (MDA) determined using fluorescence probe DCFDA obtained from water. Afterwards, 5% cold metaphosphoric acid was added and the medium was removed and the cells were lysed in distilled media, added Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) and scraped the cells. The LDH leakage was calculated as a ratio of extracellular and total LDH activity.

Markers of oxidative stress: dichlorodihydrofluorescein diacetate (DCFDA) and malondialdehyde (MDA)

The production of reactive oxygen species (ROS) was determined using fluorescence probe DCFDA obtained from Life Technologies, as described previously (34) with the exception that the cells were incubated for 55 minutes with the DCFDA probe. The presence of MDA, a marker of lipid peroxidation, was determined by assessing the thiobarbituric acid reactive substances in the medium (36, 37).

Reduced glutathione and acetaminophen-glutathione conjugates

The content of reduced glutathione (GSH) was determined after 4 h and 24 h of incubation with acetaminophen (APAP). The medium was removed and the cells were lysed in distilled water. Afterwards, 5% cold metaphosphoric acid was added and the samples were centrifuged (20,000 g, 4°C). The levels of GSH in the supernatant were measured using a modified fluorimetric method (38).

Sample preparation was identical for measurement of conjugate of APAP with glutathione (APAP-SG). The levels of APAP-SG were measured by mass spectrometry as described previously (39).

Caspase 3/7 activities

The activity of executioner caspases 3 and 7 was assessed at 4 h and 24 h after isolation using Caspase Glo 3/7 from Promega (Madison, WI, USA). The total luminescence was measured at time 0 min, 60 min and 120 min.

Protein isolation and Western blot analysis

The levels of antiapoptotic proteins Bcl-2 and Mcl-1 (11), phosphorylated 4E-BP1, an mTOR downstream target (12) and the 2E1 isomorph of cytochrome P450, which is able to form a reactive APAP metabolite N-acetyl-p-benzoquinone imine (40, 41) were assessed by means of Western blot. After removing the media, the cells were lysed in Cell Lysis Buffer (Cell Signaling) supplemented with a cOmplete Mini protease inhibitor and a PhosSTOP phosphatase inhibitor (Roche Applied Science, Penzberg, Germany). The total protein content of lysates was assessed using the Bradford method. The samples were then adjusted to contain the same amount of protein (30 µg per lane) and subjected to SDS-PAGE using the gradient gels NuPAGE® Novex® Bis-Tris Mini 4 – 12% (Life Technologies). Afterwards, the proteins were transferred to a cellulose nitrate membrane (Bio-Rad Laboratories, Hercules, CA, USA) and exposed to the following antibodies: mouse Bcl-2 antibody, rabbit anti-Mcl-1, rabbit anti-CYP2E1, rabbit anti-phospho-4E-BP1, all of which were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse anti-β-actin was obtained from Sigma-Aldrich. Secondary goat anti-rabbit and goat anti-mouse IgG were obtained from Santa Cruz Biotechnology. Proteins were visualized using a chemiluminescence detection kit (Roche, Penzberg, Germany) and autoradiographic film (Foma, Hradec Kralove, Czech Republic). To confirm equal protein loading, each membrane was reprobed and reincubated to detect β-actin. Band density was evaluated using the Quantity One 4.6 software (Bio-Rad Laboratories, Hercules, CA, USA). The ratio of band densities of protein of interest and β-actin is referred to as relative density.

Statistical analysis

Hepatocytes were isolated repeatedly three times for ApoE4 and wild-type controls and twice for ApoE3. The results are expressed as means ± S.D.; n values are depicted in tables and figure legends. All statistical analyses were performed using GraphPad Prism 6.01 software (La Jolla, CA, USA). The numerical data were first tested for normality by means of a Kolmogorov-Smirnoff test. Data with Gaussian distribution were analyzed with ANOVA and Tukey’s post-test. Data with non-Gaussian distribution were analyzed by Kruskal-Wallis test and Dunn’s post-test. P < 0.05 was considered statistically significant.

Table 1. Quantitative RT-PCR primer design data.

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<th>qPCR assay code</th>
<th>Reference sequence</th>
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RESULTS

Gene expression of Nrf2-dependent genes Nqo1, Hmox1, Gsta2

In the suspension of hepatocytes, there was a lower expression of Gsta2 and higher expression of Hmox1 in hepatocytes from ApoE4 mice when compared to wild-type hepatocytes (P < 0.05). A similar but non-significant trend was observed when comparing ApoE3 versus WT. There were no significant differences between the transgenic strains (not shown).

In hepatocytes cultured for 24 hours, the yield of RNA was sufficient only in cells treated with medium or 0.5 mmol/l APAP; we were not able to analyze the RNA from cells exposed to 1 mmol/l APAP and higher. The expression of Gsta2 and Nqo1 was lower in control ApoE4 hepatocytes when compared to control WT hepatocytes (P < 0.001). The expression of Gsta2 was also lower in control ApoE3 hepatocytes when compared to control WT hepatocytes (P < 0.05). All three genes displayed a higher expression in control ApoE3 hepatocytes when compared to control ApoE4 hepatocytes (P < 0.05 for Gsta2 and P < 0.01 for Nqo1 and Hmox1) (Fig. 1A).

In cells exposed to 0.5 mmol/l APAP, there was a lower expression of Gsta2 (P < 0.001) and Nqo1 (P < 0.05) when comparing ApoE4 and WT hepatocytes. The expression of Gsta2 was downregulated (P < 0.05) and Hmox1 upregulated (P < 0.01) when comparing ApoE3 and WT hepatocytes. Again, all three genes of interest were higher in ApoE3 when compared to ApoE4 hepatocytes (P < 0.01 for Gsta2 and Nqo1 and P < 0.001 for Hmox1) (Fig. 1B).

When comparing medium-treated cells with cells treated with 0.5 mmol/l APAP, there was an increase in Gsta2 and/or Hmox1 in all genotypes tested: increase in Gsta2 in WT hepatocytes (P < 0.05), Hmox1 in ApoE4 hepatocytes (P < 0.01), Gsta2 and Hmox1 in ApoE3 hepatocytes (P < 0.001) (not depicted).

Cell viability

As shown by the activity of extracellular lactate dehydrogenase, APAP led to a dose- and time-dependent toxicity. After 16 hours of exposure to 1 mM APAP, the apparent (twice or higher) increase in extracellular LDH activity was significant in all strains tested (P < 0.01 for ApoE4, P < 0.001 for WT and ApoE3) (Fig. 2A). After 20 h, the increase was most pronounced in ApoE3 hepatocytes. The damage after 24 h incubation identified by LDH leakage tended to be worst in hepatocytes from ApoE3 mice in concentrations of 0.75 mmol/l and 1 mmol/l APAP. When exposed to 4 mmol/l APAP, the LDH leakage reached 90% and was similar in all groups, suggesting necrosis in most of the cells (Fig. 2B).

In the WST-1 test, the activity of cellular dehydrogenases was significantly lower in hepatocytes treated with 4 mmol/l APAP for 24 h when compared to untreated controls (P < 0.001 in all genotypes). This decrease was more pronounced in both transgenic strains, most in ApoE3 mice (Fig. 2C).

Incubation of hepatocytes of all genotypes with 1 mmol/l APAP led to signs of cellular damage visible in phase contrast microscopy: detachment of cells from collagen, spherical shape and plasma membrane blebbing, highly granular cytoplasm, loss of nuclear visibility. The loss of nuclear visibility was most pronounced in hepatocytes from ApoE3 mice. We also observed distinct intracellular vacuoli resembling lipid droplets in untreated controls of both transgenic strains (not shown).

Caspase activities

The activity of executioner caspases 3 and 7 did not differ among groups at the 4-hour interval (not shown). When exposed to 4 mmol/l APAP for 24 hours, there was a distinct decrease in all genotypes, most visible in ApoE3 (Fig. 2D). Similar results were obtained when recalculating the values to the relative luminiscence per µg of protein (not shown).
Oxidative stress

The levels of MDA in the culture medium did not differ among groups and were not significantly affected by APAP treatment. Only a non-significant trend to higher MDA production at 1 mmol/l and 4 mmol/l APAP was observed in all genotypes (Fig. 2E).
The ROS production displayed a non-significant increase at 2.5 mmol/l APAP in hepatocytes from WT mice and significant increases in both transgenic strains (P < 0.01 for ApoE4 and P < 0.001 for ApoE3) after 24 hour cultivation (Fig. 2F).

Lipid accumulation

The percentage of Oil Red O positive cells was higher in both untreated controls of ApoE4 and ApoE3 mice when compared to wild-type controls after 24 h cultivation (P < 0.05 for ApoE4 and P < 0.001 for ApoE3). The percentage of Oil Red O positive medium-treated cells did not differ significantly among transgenic mice (Fig. 3A-3D).

Mitochondrial membrane potential

After the 4 h incubation, there was a significant decrease in the percentage of cells with high mitochondrial membrane potential (MMP) in samples from ApoE3 mice exposed to 4 mmol/l APAP when compared to untreated controls and corresponding wild-type hepatocytes (P < 0.001 for both comparisons). The potential was higher in untreated cells from ApoE3 mice when compared to wild-type mice (P < 0.05) (Fig. 4G).

After 24 h, there was a significant (P < 0.01) decrease in MMP in cells exposed to the concentrations of APAP from 1 mmol/l in all genotypes tested (Fig. 4D-4F). The MMP was completely lost in hepatocytes from ApoE3 mice exposed to 1 mmol/l APAP for 24 hours while in hepatocytes from WT and ApoE4 mice after exposure to 2 mmol/l APAP. Concentrations of 0.5 mmol/l APAP did not affect the mitochondrial membrane potential in any group (Fig. 4H).

Western blot analysis

The levels of antiapoptotic protein Bcl-2 were downregulated in hepatocytes treated with 4 mmol/l APAP for 4 hours. This decline was more pronounced in WT and ApoE4 than in ApoE3 cells (Fig. 5B). We did not observe major differences in the expression of another antiapoptotic protein, Mcl-1 (Fig. 5A).

The expression of phosphorylated 4E-BP1 displayed an increase after 4 hours of treatment with 1 and 4 mmol/l APAP in wild-type hepatocytes. In ApoE4 cells, there was a decline in phospho-4E-BP1, especially in cells exposed to 4 mmol/l APAP. Only a mild decrease after exposure to 1 and 4 mmol/l APAP was observed in ApoE3 cells (Fig. 5C).

The levels of the 2E1 isoform of cytochrome P450 showed a mild decrease when treated with 4 mmol/l APAP in both transgenic strains and negligible effect in WT hepatocytes (Fig. 5D).

Glutathione and acetaminophen-glutathione conjugates

A dose-dependent decline in GSH levels was observed in all APAP-exposed cells. At the highest dose of APAP (4 mmol/l), the GSH levels were below the detection limit in both transgenic strains. After 24 h treatment with 1 mmol/l APAP, the levels were lowest in hepatocytes from ApoE3 mice (non-significant difference between genotypes) (Fig. 5E).
Fig. 4. Mitochondrial membrane potential: hepatocytes from wild-type (A, D), ApoE4 (B, E) and ApoE3 mice (C, F) after 24 hour cultivation: (A), (B), (C) - hepatocytes treated with medium only; (D), (E), (F) - hepatocytes treated with 1 mmol/l APAP, note cells with high mitochondrial potential (orange) and low potential (green). Percentage of cells with high potential (G) after 4 hours and (H) after 24 hours.

†, ††, ††† versus control WT; ‡, ‡‡, ‡‡‡ versus control ApoE4; #, ##, ### versus control ApoE3; * *, ** versus corresponding WT; P < 0.05, P < 0.01, P < 0.001 respectively. JC-1 staining, objective magnification × 40, bar 50 µm.
Fig. 5. Protein expression and APAP metabolism: (A) Western blot after 4 h cultivation. Densitometry analysis of (B) Bcl-2, (C) phosphorylated 4E-BP1, (D) cytochrome P450 isoform 2E1. (E) levels of reduced glutathione, (F) levels of conjugate APAP-SG. ‡‡ versus control ApoE4, ** versus corresponding WT (P < 0.01).
DISCUSSION

In the present study, APAP led to a dose-dependent hepatocyte toxicity in all genotypes tested. The toxicity was higher in both transgenic strains than in wild-type controls and most pronounced in ApoE3 mice. When exposed to 4 mmol/l APAP for 24 hours, nearly all cells displayed necrotic cell death.

We were able to reproduce the main findings of Graeser et al. (24), i.e. higher expression of Nr2f2-dependent genes in cultivated hepatocytes from ApoE3 mice when compared to ApoE4 mice. The exposure to APAP led to the activation of some of the Nr2f2-dependent genes in all genotypes tested, which is in accordance with other authors (11). However, the activation of Nr2f is not always beneficial: a constitutive activation of Nr2f led to a delay in liver regeneration after partial hepatectomy via changes in expression of cell-cycle and apoptosis regulating proteins (42).

Our findings of a decline in mitochondrial membrane potential after the treatment with APAP are in agreement with the findings of Xie et al. (13). This decline was most pronounced in hepatocytes from ApoE3 mice.

The activity of executioner caspases 3 and 7 was not increased upon APAP treatment, despite downregulation of the antiapoptotic protein Bcl-2. In light of other methods used, this is probably caused by a decrease in the total cell count due to necrosis. However, even when related to protein amount per sample, the activity was still lowest in hepatocytes treated for 24 hours with 4 mmol/l APAP. Our results are in accordance with Ni et al. (12), who reported cell death by necrosis only and in contrast to the findings of Mobasher et al. (11), who noticed activation of apoptosis. Inconsistency may be caused by different times and conditions of cultivation, doses of APAP and methods used for quantifying apoptosis.

The higher toxicity of APAP in both transgenic strains, when compared to wild-type controls, may be partly explained by lipid accumulation. In previous studies, hepatocytes and/or liver affected by NAFLD were more susceptible to APAP toxicity than in wild-type controls (P < 0.01 for ApoE4, non-significant for ApoE3). There was no significant difference between the transgenic strains (Fig. 5F).

CONFLICT OF INTERESTS: None declared.

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