Overexpression of Aldose Reductase Render Mouse Hepatocytes More Sensitive to Acetaminophen Induced Oxidative Stress and Cell Death

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Abstract Acetaminophen (APAP) a commonly used drug for decrease the fever and pain but is capable to induced hepatotoxicity at over dose. This study was carried out to investigate the effect of APAP on the expression of anti-apoptotic and antioxidative defense genes, and whether aldose reductase over-expressing plasmid capable to protect against APAP-induced oxidative stress and cell death. APAP treatment induced oxidative stress and hepatotoxicity, and significantly increased aldose reductase mRNA and protein expression in mouse hepatocyte (AML-12).

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Unexpectedly, AML-12 cells over-expressing aldose reductase augmented APAP-induced reduction in cell viability, reactive oxygen species (ROS) production, glutathione (GSH) depletion and glutathione *S*-transferase A2 expression. Moreover, over-expression of aldose reductase potentiated APAP induced reduction on proliferating cell nuclear antigen, B cell lymphoma-extra large (bcl-x_L), catalase, glutathione peroxidase-1 (GPx-1) and abolished APAP-induced B-cell lymphoma 2 (bcl-2) inductions. Further, over-expression of aldose reductase significantly abolished AMP activated protein kinase (AMPK) activity in APAP-treated cells and induced p53 expression. This results demonstrate that APAP induced toxicity in AML-12, increased aldose reductase expression, and over-expression of aldose reductase render this cell more susceptible to APAP induced oxidative stress and cell death, this probably due to inhibition AMPK or bcl-2 activity, or may due to competition between aldose reductase and glutathione reductase for NADPH.

Keywords Aldose reductase · Acetaminophen · APAP · Oxidative stress · ROS · AML-12

Abbreviations

APAP	Acetaminophen
AMPK	AMP activated protein kinase
AR	Aldose reductase
CAT	Catalase
DCFH-DA	2,7 Dichlorofluorescein diacetate
Gpx-1	Glutathione peroxidase-1
GSH	Glutathione
GSTA2	Glutathione <i>S</i> -transferase A2
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAPQI	<i>N</i> -acetyl- <i>p</i> -benzoquinoneimine

Nrf2	Nuclear factor erythroid 2 p45-related factor 2
ROS	Reactive oxygen species
PBS	Phosphate buffered saline
bcl-2	B-cell lymphoma
bcl-x _L	B-cell lymphoma-extra large
PCNA	Proliferating cell nuclear antigen
4HNE	4-Hydroxy-2-nonenal
SOD1	Copper zinc superoxide dismutase 1

Background

Acetaminophen (APAP; paracetamol; *N*-acetyl-*p*-aminophenol) is a widely used drug with analgesic and antipyretic activity. When consumed in large doses, it is known to cause severe centrilobular hepatic toxicity [1–3]. But at therapeutic doses is rapidly metabolized in the liver principally through glucuronidation and sulfation, and only a small portion is oxidized by cytochrome P-450 2E1 to generate a highly reactive and cytotoxic intermediate, *N*-acetyl-*p*-benzoquinoneimine (NAPQI) [4, 5]. This metabolite is efficiently detoxified by being reduced back to APAP or covalently linked to GSH to form a 3-glutathione-*S*-yl-APAP conjugate [6]. After an overdose of APAP, the glucuronidation and sulfation routes become saturated and more extensive bioactivation of APAP occurs within 1 to 2 h, leading to rapid depletion of hepatic GSH levels. Subsequently, covalent binding of NAPQI to cellular macromolecules, membrane lipid peroxidation, disturbance of intracellular calcium balance, oxidative stress and reactive oxygen formation [7].

Aldose reductase (AR, AKR1B1, EC1.1.1.21) is an NADPH dependent aldo-keto-reductase, first found to be implicated in the etiology of the diabetic complications. This enzyme catalyzes the first and rate limiting step in the polyol pathway, in which the reduction of aldehyde from glucose to sorbitol, which is subsequently oxidized to fructose by NAD dependent sorbitol dehydrogenase [8], this reaction could trigger a sequence of metabolic changes resulting in osmotic stress, cell death, disturbances in signal transduction, and oxidative stress [9]. Oxidative stress and ROS production resulting from decreased availability of reduced glutathione due to competition between aldose reductase and glutathione reductase for NADPH [10]. In the other hand, aldose reductase is a detoxification enzyme that is transcriptionally regulated by a variety of stimuli or substances, including ROS [11]. It is now widely accepted that induction of aldose reductase expression represents an adaptive response that increases cellular resistance to toxic injury. Previous studies demonstrated the importance of aldose reductase in detoxification of 4-hydroxy-2-nonenal

(4HNE), and inhibition of aldose reductase activity sensitizes cells to the cytotoxicity of 4HNE [12, 13]. Furthermore, previous studies mentioned that aldose reductase has a very close association with ROS, and the behaviors of AR remain underestimations, and cellular mechanisms that regulate its expression in non-diabetic tissues are poorly understood. Therefore, considering that aldose reductase is detoxifying enzyme under normal condition, regulated by Nrf2 transcription factor and play an important role in oxidative stress, this study was carried out to study the effects of APAP on aldose reductase expression, and whether aldose reductase over-expressing plasmid pFLAG-AR can protect against APAP-induced oxidative stress and cell death. We found that, APAP increased aldose reductase expression, and unexpectedly and surprising finding, aldose reductase over-expressing plasmid augmented APAP induced oxidative stress and cell death.

Materials and Methods

Chemicals

DMEM-F12, fetal bovine serum (FBS), APAP, DCFH-DA (2,7 dichlorofluorescein diacetate), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich. Aldose reductase, PCNA, p53, Catalase Rabbit polyclonal antibodies, and beta-Actin from Santa Cruz. Phospho-AMPK from Millipore. Bcl-2 from cell signaling. GSH kit from Jianchen, Nanjing, P.R. China. Protease inhibitor cocktail was purchased from KeyGen, China. BCA protein assay kit and enhanced chemiluminescent substrate were obtained from Pierce (Thermo Scientific, U.S.A).

Plasmid Construction

Mouse aldose reductase cDNA was PCR-amplified from pBluescriptmAR, a plasmid containing a cDNA fragment for mouse aldose reductase, using the following primers: ATATGCGGCCGCGATGGCCAGCCATCTGGAA and TGCTCTAGATCAGACTTCGGCGTGGAA, carrying a *NotI* site and an *XbaI* site, respectively. The PCR products were cut with *NotI* and *XbaI* and cloned into the corresponding sites of pFLAG-CMV2 (Sigma) to obtain plasmid pFLAG-mAR.

Cell Culture, Transfection and Experimental Design

Mouse hepatocyte cells were obtained from ATCC. Cells were maintained in DMEM-F12 medium supplemented with 10 % fetal bovine serum and incubated in a humidified incubator at 37 °C in 5 % CO₂ until semi-confluent.

Different concentrations of APAP were dissolved in serum free culture medium.

Twenty-four hours after cell plating, the plasmid pFLAG-mAR or the empty vector pFLAG-CMV were transfected into AML-12 cells using the lipofectamine 2000 transfection reagent (invitrogen) according to the instructions provided by the manufacturer. Then after 24 h the culture media were replaced with a serum-free medium containing or not 10 mM APAP. After incubation for 24 h with APAP or not, the cells were washed twice with phosphate-buffered saline (PBS), and whole-cell lysates were prepared for western blot, real time PCR, GSH, ROS, MTT analysis. For time course study cells were treated with APAP 10 mM for different time points.

Assessment of Cytotoxicity

Cell viability was determined by measuring the ability of cells to transform MTT to a purple formazan dye. Cells were seeded in 96-well tissue culture plates at 1×10^4 - cells/well for 24 h. The cells were then incubated with APAP at 10 mM for different periods of time. For transfection experiment, the cells transfected with pFLAG-AR or control vector pFLAG-CMV for 24 h followed by incubation with APAP 10 mM for 24 h. After incubation, 20 μ l/well of MTT solution (5 mg/ml phosphate buffered saline) was added and incubated for 4 h in incubator. The medium was aspirated and replaced with 200 μ l/well of DMSO to dissolve the formazan salt formed. The plates were then shaken for 1 h to extract the blue products. And the color intensity of the formazan solution, which reflects the cell growth condition, was measured at 490 nm using a microplate spectrophotometer.

Western Blotting

Cell lysates were prepared by scraping the cells by PBS followed by lysis the cell into lysis buffer, followed by centrifugation. The protein concentration of the supernatant was determined by the BCA protein assay kit and then 40 μ g of denatured protein was resolved on 12 % SDS-PAGE and electroblotted onto nitrocellulose membranes (Amersham). After blocking, membranes were incubated with following antibodies: AR, PCNA, Catalase, p53, bcl-2, phospho-AMPK at a dilution of (1:1000 or 1:2000) at 4 °C overnight followed by further incubation with secondary antibody (1:4000). After washing with Tris-Buffered Saline containing 0.05 % Tween 20 (TBST), the blots were detected by the chemiluminescence. Followed by exposure to Kodak-X-Omat x-ray film.

Quantitative Real Time PCR Analysis

Total RNA was isolated from AML-12 using TRIzol reagent according to manufacturer's instructions. Then the total RNA was reverse transcribed to cDNA using Rev-erTra Ace (TOYOBO, Tokyo, Japan) according to the manufacturer's instruction.

Real-time PCR was performed with the SYBR Green assay. The condition of cycles consisted of a denaturation step at 95 °C for 5 min, 40 cycles at 95 °C for 15 s, annealing step between 58 and 60 °C for 30 s, and finally a holding temperature at 72 °C for 30 s. Quantitative results of real-time fluorescence PCR were assessed by a cycle threshold (Ct) value. The relative gene expression was determined by calculated Δ Ct values by subtraction of the Ct value from control primer. The primers and genes name listed on Supplementary Table S1. 18S rRNA served as a control.

Flow Cytometry Measurement of Intracellular ROS

DCFH-DA is a cell-permeable compound. When it enters the cell its acetate group is cleaved by cellular esterases and nonfluorescent DCFH is trapped inside. Subsequent oxidation by ROS DCFH yields the fluorescent product DCF and upon excitation at 488 nm emits green fluorescence, proportional to the intracellular level of ROS. Therefore DCFH-DA is an ROS-sensitive probe that can be used to detect oxidative activity in living cells.

In the present experimental, AML-12 cells were grown in 6-well plates in triplicate for 24 h to reach 90 % confluence. Following transfection and APAP treatment, cells were washed and resuspended in PBS. DCFH-DA was then added to the resuspended cells at a final concentration of 20 μ mol/L in the dark in an incubator for 30 min and immediately used for ROS detection by flow cytometry.

Quantification of Intracellular GSH Levels

The GSH in cells was measured using a GSH quantification kit. Briefly, after treatment, cells were scraped and centrifuged at 2500 rpm for 5 min at 4 °C, and the cell pellets were resuspended, sonicated in 1 ml PBS containing 1 mM EDTA and centrifuged at 14,000 rpm for 15 min at 4 °C. Then removed the supernatant and stored on ice, and measured the GSH concentration according to the manufacturer's instructions. Then the concentrations of GSH were calculated. Cellular protein was determined by BCA assay kit.

Statistical Analysis

All values reported in the text are mean $\pm$ standard error mean. Comparisons between multiple groups were performed with one-way ANOVA followed by Student's *t* test (two-tailed) for multiple statistical comparisons. A *P* value <0.05 was determined to be significant. All statistical analyses were performed with statistical GraphPad Prism software.

Results

APAP Induced Hepatotoxicity in AML-12

In order to evaluate the effect of APAP on cell viability, hepatic expression of xenobiotic detoxification, anti-apoptotic, and oxidative stress related genes, western blotting and qPCR analysis performed in the AML-12 mouse hepatocytes. We investigated the time-course studies of APAP-induced toxicity in AML-12. The viability of AML-12 was inhibited after treatment with 10 mM APAP in time dependent manner. There is significant cell damage was evidenced in AML-12 when compared with the control as indicated by cell viability assay (MTT assay) and PCNA expression (Fig. 1a, b).

Furthermore, the effect of APAP on anti-apoptotic and antioxidative genes were investigated, APAP significant induced reduction of bcl-x_L mRNA expression between 6 and 48 h. Conversely APAP have different effect on bcl-2. APAP at 6 and 12 h decrease bcl-2 protein expression, then bcl-2 showed significant increases compared to the control at 24 h then slightly decrease at 36, and 48 h, where as bcl-2 mRNA expression were increased at 24 h then significantly decreased at 36 and 48 h (Fig. 1c, d).

Moreover, APAP down-regulated GPx-1 mRNA expression between 6 and 48 h, where as catalase slightly decreased at 6 h then showed significant increases at 12 h then return back to basal levels at 24 and 36 h then showed significant decreases at 48 h compared to the control (Supplementary Fig. 1A, B). The Cu, Zn-SOD and GSTA2 showed significant increases at 6 h and peaked at 12 h, which then slightly return back to basal level, where as GSTA2 significantly down-regulate at 48 h (Supplementary Fig. 1C, D).

Interestingly, in order to evaluate the effect of APAP on xenobiotic detoxification genes western blotting and qPCR analysis performed for aldose reductase. At 6 h APAP treated AML-12 showed there is no significant different of aldose reductase mRNA and protein expression. At 12, 18, 24 h treatment with APAP 10 mM western blot and qPCR showed significant increases in the aldose reductase protein and mRNA expression (Fig. 2a, b).

Overexpression of Aldose Reductase Augmented APAP-Induced Oxidative Stress

To determine the effects of aldose reductase on the oxidative stress of APAP-treated AML-12 cells marked by ROS level, GSH, and antioxidative and detoxification genes, we generated a AR-overexpressing plasmid (pFlag-AR) to overexpress AR in AML-12 or HepG2 cells (Supplementary Fig. 2).

Cells were transfected with pFLAG-AR or control vector pFLAG-CMV for 24 h, then exposed to 10 mM APAP for additional 24 h. APAP at 10 mM treatment caused a significant increase in ROS production, GSH depletion and reduction of GPx-1, catalase and induction of GSTA2 mRNA compared to control, which was augmented by overexpression of the cells with aldose reductase (Fig. 3a–f). These results indicate that overexpression of aldose reductase potentiated APAP-induced oxidative stress.

Overexpression of Aldose Reductase Enhanced APAP-Induced Apoptosis and Cell Death

To directly demonstrate the role of bcl-x_L, bcl-2, p53, PCNA, cell viability in APAP-induced apoptosis, and cells death western blot, qPCR and MTT assay were performed in AML-12. AML-12 transfected with pFLAG-AR or control vector pFLAG-CMV for 24 h, followed by incubation with APAP 10 mM for additional 24 h. Overexpression of aldose reductase significant induced p53 and reduced bcl-2, PCNA and phospho-AMPK at protein level (Fig. 4c). Moreover, MTT assay and qPCR analysis demonstrated that overexpression of aldose reductase significantly induced reduction on cell viability and on anti-apoptotic protein bcl-x_L respectively (Fig. 4a, b). This result indicates that overexpression of aldose reductase caused apoptosis and cell death in AML-12.

Furthermore, overexpression of aldose reductase decreased APAP-induced p53, abolished APAP-induced bcl-2 induction, and potentiated APAP-induced reduction on PCNA, cell viability, and bcl-x_L.

Discussion

We previously demonstrated in APAP-induced hepatotoxicity that overexpression of aldo-keto reductase-7a (AKR7A) in HepG2 caused significant hepatoprotection against APAP-induced oxidative stress and cell death [14]. In the present study, we have shown that APAP-induced hepatotoxicity and increased expression levels of aldose reductase in AML-12, and plasmid overexpressing aldose reductase augmented APAP induced oxidative stress and cell death.

Fig. 1 AML-12 cells are sensitive to APAP-induced hepatotoxicity. **a** APAP reduced cells viability. Cells were seeded in 96-well plates and exposed to 10 mM APAP for different durations. Cell viability was determined by MTT assays. APAP at 10 mM significantly reduced viability of AML-12 cells. **b** Time course of PCNA mRNA and protein following APAP treatment. Cells were exposed to APAP 10 mM for different durations. APAP treatment caused time dependent significantly PCNA reduction. **c** Time course of bcl-2 mRNA and protein following APAP treatment. Cells were exposed to APAP 10 mM for different durations. APAP treatment caused significant changes on bcl-2 expression. **d** Time course of bcl-x_L mRNA following APAP treatment. Cells were exposed to APAP 10 mM for different durations. APAP treatment caused significantly reduction on bcl-x_L mRNA expression. Statistical comparisons were made between the APAP-treated groups vs vehicle control. Values are expressed as the mean $\pm$ SE (n = 3/group). NS not significant, *P < 0.05; **P < 0.01; ***P < 0.001

APAP a commonly used drug for decrease the fever and pain but is capable to induced hepatotoxicity at over dose. Oxidative stress and production of reactive oxygen species, GSH depletion, and apoptosis is the major mechanism of APAP induced hepatotoxicity [15]. The results obtained from the present study agreed with those of the previous studies. Furthermore we have demonstrated that APAP up-regulate aldose reductase at mRNA and protein levels. Aldose reductase is a detoxify enzyme that is transcriptionally regulated by Nrf2, an increased expression of aldose reductase by curcumin prevent rat aortic VSMC against oxidative stress [16]. Moreover, inhibition of aldose reductase renders J774A.1 macrophage cell line more susceptible to acrolein or hydrogen peroxide-induced cell death [17]. Also we previously demonstrated that increased AKR7A3 in HepG2 cells was associated with the up-regulation of oxidative stress-related enzymes to enhance cellular antioxidant defense, which appeared to contribute

significantly to protection against APAP-induced toxicity [14]. Therefore, we suggest that aldose reductase may provide a mechanism for the cellular detoxification of APAP. We transiently transfected AML-12 cells with plasmid overexpressing aldose reductase and then we assessed the oxidative stress and cell death related markers.

Unexpectedly AML-12 overexpressing aldose reductase augmented APAP induced ROS production, potentiated APAP induced GSH depletion, decreased the expression of antioxidative defense, catalase, GPx-1 and increased GSTA2. It is well known that excess ROS induce oxidative modification of cellular macromolecules, inhibit protein function, promote apoptosis and cell death, and this is associated with decreased cellular GSH levels and the loss of cellular redox balance [18]. Time course studies of APAP shown that APAP up-regulate GSTA2 mRNA, and overexpression of aldose reductase potentiated APAP-induced GSTA2 induction. The elevation mRNA expression

Fig. 2 Effects of APAP on aldose reductase expression in AML-12 cells. Cells were treated with APAP 10 mM for different time points. **a** APAP increased AR protein expression. Aliquots of 40 μ g of protein extracts were resolved by 10 % SDS-PAGE gel, transferred onto the PVDF membrane, and immunoblotted with the AR and beta-actin antibodies. APAP at 10 mM significantly increased AR protein expression. **b** APAP increased AR gene expression. Total RNA

isolated using TRIzol reagent, converted to cDNA, and then were quantified using quantitative real-time polymerase chain reaction (RT-PCR) analysis and normalized to 18S rRNA. APAP at 10 mM significantly increased mRNA expression of AR. Values are expressed as the mean $\pm$ SE ($n = 3$ /group), *** $P < 0.05$, compared with vehicle control

of GSTA2 may contribute to the enhancement of APAP-GSH conjugation, but may also augment the toxicity rather than protection. There are many conjugations and detoxifications enzymes especially that under control of Nrf2, may involved in protection against APAP-induced toxicity, although some of this enzymes can enhanced APAP toxicity, for example, glutathione *S*-transferase Pi knockout mice were much less sensitive to acetaminophen-induced hepatotoxicity than the wild-type mice [19]. We therefore hypothesized that up-regulation of GSTA2 by APAP or by overexpression of aldose reductase would be make the cells more sensitive to APAP induced toxicity, because neither protection against ROS production nor inhibition cell death induced by APAP or potentiated by overexpression of aldose reductase. Consequently, to elucidate clearly the role of GSTA2 during APAP-induced hepatotoxicity, further studies appear to be required.

In subsequence to oxidative damage caused by APAP and augmented by aldose reductase overexpression, we investigated the effect of overexpression of aldose reductase on anti-apoptotic and cell proliferation markers. Interestingly, we found that overexpression of aldose reductase induced apoptosis and cell death by decreasing anti-apoptotic protein bcl-2, bcl-x_L, PCNA, cell viability,

and increasing p53 expression. Moreover, overexpression of aldose reductase potentiated APAP induced reduction on bcl-x_L, cell viability, and PCNA.

The anti-apoptotic oncogene bcl-2, bcl-x_L, the proapoptotic oncogene p53, and PCNA are key regulators of cell cycle progression and/or apoptosis subsequent to DNA damage in vitro and in vivo. In the present study APAP-induced p53 expression may be to prolong cell cycle and allow DNA repair to prevent excessively damaged by APAP, but overexpression of aldose reductase lead to degradation of p53 and may prevent DNA repair which indicated by decreasing PCNA, cell viability, and anti-apoptotic protein.

Time course studies, indicate that APAP down-regulate bcl-2 at protein level not mRNA at 6 and 12 h then unexpected at 24 h up-regulate this gene at mRNA and protein levels. Furthermore, and very interestingly overexpression of aldose reductase down-regulates bcl-2 and abolished APAP-induced bcl-2 induction. On the other hand bcl-x_L another antiapoptotic gene more affected by APAP than bcl-2 in this cell, the mRNA of bcl-x_L decreased after 6 h and dramatically decreased until 48 h, and over-expression of aldose reductase augmented APAP-induced reduction on bcl-x_L. In consistent with that,

Fig. 3 Overexpression of aldose reductase renders AML-12 more susceptible to APAP-induced oxidative stress. Cells were transfected with pFLAG-AR or pFLAG-CMV for 24 h, and then treated with APAP 10 mM for additional 24 h. **a** The levels of intracellular H_2O_2 were assayed by flow cytometry using DCFH-DA as the fluorescent probe. Overexpression of AR significantly augmented H_2O_2 production after APAP treated cells. **b** Intracellular GSH was measured using a GSH quantification kit according to manufactures instruction, the absorbance was read at 420 nm. Overexpression of AR significantly augmented APAP-induced GSH depletion. **c–f** Western blot, qPCR were performed in AML-12. Overexpression of AR significantly altered the effect of APAP on expression of catalase, GPx-1, and GSTA2. Values are expressed as the mean $\pm$ SE ($n = 3/\text{group}$), *** $P < 0.05$, comparison between cells treated with APAP alone versus treated with APAP + pFLAG-AR

previous studies demonstrated that transgenic mice overexpressing bcl-2 is more susceptible to APAP induced hepatotoxicity with massive panlobular necrosis by 24 h [20]. Moreover, administration of APAP to BALB/c mice caused extensive centrilobular apoptosis and necrosis, accompanied by up-regulation of anti-apoptotic protein bcl-2 after 24 h [21]. Furthermore, APAP-induced hepatocellular apoptosis is associated with a dramatic decrease in the expression of bcl-x_L [22]. These findings suggest that a decreases in bcl-x_L levels is a common event in AML-12 cell death induced by APAP, therefore, we suggest that bcl-x_L may more affected as anti-apoptotic gene than bcl-2 in mouse liver, but as the same time we believe that,

decreased expression of bcl-2 contribute also to apoptosis caused by APAP or over-expression of aldose reductase.

Importantly, the present study showed over-expression of aldose reductase deactivated AMPK by decreasing its phosphorylation, therefore, we suggest that might suppressing activity of AMPK is the mechanism of aldose reductase induced ROS production and subsequence apoptosis and cell death. AMPK, an important energy sensor in cells, also plays an important role in cell survival/death by regulating H_2O_2 , previous studies demonstrated that activation of AMPK significantly reduced hydrogen peroxide induced necrosis and cell death in mouse primary culture hepatocyte and H9c2 cells respectively, and

Fig. 4 Overexpression of aldose reductase renders AML-12 more susceptible to APAP-induced apoptosis and cell death. Cells were transfected with pFLAG-AR or pFLAG-CMV for 24 h, and then treated with APAP 10 mM for additional 24 h. **a** Cell viability was determined by MTT assay. Overexpression of AR significantly potentiated APAP induced reduction in cells viability. **b** Total RNA isolated using TRIzol reagent, converted to cDNA, and then were quantified using Quantitative real-time polymerase chain reaction (qPCR) analysis and normalized to 18S rRNA. Overexpression of AR augmented APAP-induced reduction on Bcl-x_L mRNA expression. Values are expressed as the mean $\pm$ SE (n = 3/group), ***P < 0.05,

comparison between cells treated with APAP alone versus treated with APAP + pFLAG-AR. **c** Aliquots of 40 μ g of protein extracts were resolved by 12 % SDS-PAGE gel, transferred onto the PVDF membrane, and immunoblotted with the phospho-AMPK, p53, PCNA, and bcl-2 were determined by western blot. Over-expression of AR decreased PCNA, bcl-2, and phospho-AMPK protein expression, and potentiated APAP induced reduction on PCNA and phospho-AMPK. Where as APAP and AR over-expression up-regulated p53, over-expression AR in cells treated with APAP have less p53 indicating to degradation of this protein

inhibition of AMPK promote hydrogen peroxide induced necrosis and cell death [23, 24]. Moreover, the levels of ROS significantly increased in AMPK deficiency mice [25].

The APAP-induced and Nrf2-regulated genes therefore might have very different roles in bioactivation, detoxification and antioxidative defense. The mechanism of overexpression of AR involved in toxicity of APAP remains elusive and surprising, so we mention the possibility that, overexpression of AR may have affects at upstream for MAPK, bcl-2, and GSTA2. To elucidate clearly the effects of AR on MAPK, bcl-2, and GSTA2, further studies appear to be required.

Conclusions

We conclude that APAP induced hepatotoxicity, increased expression of aldose reductase in AML-12, and plasmid overexpressing aldose reductase enhanced APAP-induced oxidative damage and cell death. Overexpression of aldose reductase augmented APAP-induced GSTA2 mRNA induction and significantly decreased AMPK and bcl-2 activity. Therefore, we suggesting the possible mechanism to the cell death caused by overexpression of aldose reductase are probably due to suppressing AMPK and bcl-2 activity, GSTA2 induction, or may due to competition between aldose reductase and glutathione reductase for NADPH.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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