Suppression of iNOS/HIF-1 α /MMP-9/ α -SMA/collagen Axis of Fibrosis and Systemic Hypertension in Thioacetamide-induced Liver Injury by Resveratrol

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Research Article

Keywords: thioacetamide, liver fibrosis, hypertension, rat model, resveratrol, iNOS/ HIF-1 α / α -SMA axi

Posted Date: January 25th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1185091/v1>

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Abstract

Chronic liver injury can lead to hepatic failure and the only available method of treatment would be liver transplantation. The link between nitrosative stress (iNOS), hypoxia-inducible factor-1 α (HIF-1 α), and alpha-smooth muscle actin (α -SMA), in thioacetamide (TAA)-induced liver fibrosis and hypertension in treatment with the anti-inflammatory and antioxidant, resveratrol (RES) was not investigated before. Consequently, we injected rats with either 200 mg/kg TAA for 8 weeks starting at week 2 (model group) or pretreated them before TAA injections with RES (20mg/kg) for two weeks and continued on RES and TAA until being culled at week 10 (protective group). In the model group, we documented the induction of hepatic fibrosis and upregulation of tissue iNOS, HIF-1 α , and the pro-fibrotic biomarkers α -SMA and matrix metalloproteinase-9 (MMP-9) that was significantly ($p \leq 0.0014$) ameliorated by RES. RES also significantly ($p \leq 0.0232$) reduced triglycerides (TG), cholesterol (CHOL), very low-density lipoprotein (vLDL-C), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) induction by TAA. Also, a significant ($p < 0.0001$) positive correlation between iNOS/HIF-1 α / α -SMA/collagen axis and hypertension and liver injury biomarkers was observed. These findings indicate that the hepatotoxic compound, TAA augments iNOS/HIF-1 α /MMP-9/ α -SMA/collagen mediated fibrosis and hypertension, and is inhibited by RES for 10 weeks.

Introduction

Chronic hepatic disease complications represent a major encounter to the health sector including liver transplantation caused by liver failure (Neff et al., 2011). TAA is a severe hepatotoxic agent that causes liver fibrosis (Al-Hashem et al., 2018), cirrhosis and liver cancer (De Minicis et al., 2013), which depends on the length of exposure of the body to this agent. Animals injected with TAA for 6 to 10 weeks caused liver fibrosis and cirrhosis (Wallace et al., 2015). Chronic liver insults such as toxins, chemicals, alcohol abuse, viruses, cholestasis, and autoimmune diseases have been associated with the pathophysiology of hepatic fibrosis that could also lead to liver cirrhosis and ultimately liver failure (Friedman, 2003, Czaja, 2014). Therefore, the prevention of hepatic fibrosis development is a very valuable target aimed by the clinician to prevent the advancement of liver cirrhosis and hepatic failure.

Hepatic stellate cells (HSCs) activation with these insults is the main driver of liver fibrosis since HSCs produce most of the fibrogenic extracellular matrix upon activation (Gressner and Weiskirchen, 2006). Induction of HIF-1 α , and biomarkers of profibrogenesis, α -SMA and collagen type III by carbon tetrachloride (CCl₄) in rats promote liver fibrosis via the activation of HSCs (Zhao et al., 2014). In addition, HIF-1 α induced pulmonary hypertension and fibrosis (Ball et al., 2014). TAA is reported to activate HIF-1 α and α -SMA in HSCs cell lines and animal models of liver disease (Al-Hashem et al., 2018). Furthermore, oxidative stress and inflammation are involved in promoting fibrosis by HSCs and in TAA hepatic intoxication (Robert et al., 2016). Overproduction of reactive nitrogen species (nitrosative stress; iNOS induction) also plays a role in liver disease pathologies such as CCl₄- and cholesterol-induced hepatic fibrosis (Anavi et al., 2015, Yu et al., 2019). Indeed, in animal models of cholesterol-induced liver fibrosis,

iNOS deficient mice developed less liver fibrosis compared with wild type mice that showed a greater fibrotic tissue and upregulation of HIF-1 α and MMP-9 (Anavi et al. 2015).

RES, a plant phytoalexin, is widely used in research for cardiovascular and kidney protection (Chen et al., 2019, Zhao et al., 2018). It also inhibits preeclampsia biomarkers (Cudmore et al., 2012, Al-Ani, 2013), and prevents apoptosis and promotes cell survival (Alayev et al., 2014). Also, numerous types of liver diseases such as sinusoidal obstruction syndrome and hepatic steatosis (Trepiana et al., 2018) were inhibited by RES as well as HSCs (Kawada et al., 1998). Furthermore, in a mouse model of CCl₄-induced liver fibrosis, RES ameliorated fibrosis, which was associated with the inhibition of iNOS (Yu et al., 2019). Therefore, these reports prompted us to speculate the activation of iNOS/HIF-1 α /MMP-9/ α -SMA mediated liver fibrosis and hypertension by TAA which could be inhibited by RES.

Materials And Methods

Animals

24 rat male Albino rats weighing 180-200 gm were included in the experiment. The study follows the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). And approved by the Ethical Committee at King Khalid University, Rats were housed at a controlled temperature ($25 \pm 2^\circ\text{C}$) and relative humidity ($50 \pm 10\%$), with twelve-h light/twelve-h dark cycles, and had free access to food and water.

Experimental Design

One week after adaptation, 24 rats were separated into 4 groups (n= 6 per group). Firstly, the control group (Control) of rats that were non-treated and injected intraperitoneally (i.p.) with the vehicle. Secondly, the resveratrol control group (RES) of rats treated with RES suspension (20 mg/kg, orally) daily for ten weeks. Thirdly, the model group (TAA) of rats that were subjected to i.p. injections with TAA (200 mg/kg, twice per week) for eight weeks (starting at week 3) (Wallace et al., 2015). Fourthly, the protective group (RES+TAA) that includes rats given RES as above from day one till the end of the experiment, at the 10th week, and received TAA as above for eight weeks. After the completion of the experiment, blood samples were collected under anaesthesia using sodium thiopental (40 mg/kg), and animals were killed by cervical dislocation, and liver tissue specimens were harvested.

Histological Examination

Harvested liver specimens were fixed overnight in ten % formalin and then dehydrated with ascending grade of alcohols. Paraffin blocks were prepared by the standard method, and 5 μm thick sections were de-paraffinized and rehydrated. Hepatic sections were then stained with Masson's trichrome to assess the degree of hepatic fibrosis.

Immunohistochemistry of iNOS and α -SMA

Immunohistochemical staining was performed using anti-inducible nitric oxide synthase (iNOS) (Abcam, cat # ab15323) and anti-alpha-smooth muscle actin (α -SMA) (Dako; cat # M0851) as a marker for HSCs activation. Antigen retrieval was conducted, followed by the application of the primary antibody overnight in a humidity chamber and the secondary antibody for 30 minutes. Sections were co-stained with Meyer hematoxylin.

Quantitative real-time polymerase chain reaction (qRT-PCR) of MMP-9

Total RNA was isolated from rats' livers using the RNeasy Mini Kit (Qiagen Pty, Victoria, Australia) and 1 μ g RNA was reverse-transcribed with the complementary DNA (cDNA) synthesis kit (Fermentas, USA). Triplicate cDNA samples and standards were amplified in Master Mix containing SYBR green (Thermo Fisher Scientific Inc, MA, USA) with primers specific for MMP-9 (sense, 5'-CCT GCG TAT TTC CAT TCA TC-3'; antisense, 5'-GCC TTG GGT CAG GTT TAG AG -3') or β -actin. The relative expression was calculated according to the manufacturer's software.

Western Blotting Analysis of HIF-1 α

Proteins from liver tissues were extracted and 20 μ g per sample were subjected to Western blot analysis (Al-Ani et al., 2010). Membranes were probed with anti-HIF-1 α (Thermo Fisher Scientific, MA, USA) at 4⁰C overnight. Protein bands were visualized using the enhanced chemiluminescence (ECL) kit (Amersham-Pharmacia, UK). After normalization by β -actin on the Chemi Doc MP imager, relative expression was obtained using Image analysis software to read the band intensity of the target proteins against the control sample.

Assessment of ALT, AST, triglyceride, cholesterol, vLDL-C, and HDL-C levels

Enzymatic kits (Randox Laboratories, UK) were used to determine the blood levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The blood levels of triglycerides (TG), cholesterol (CHOL), very low-density lipoprotein (vLDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured according to the manufacturer's instructions using the commercial kits supplied by SPINREACT, Spain.

Determination of blood pressure and heart rate

SBP, DBP, and MAP were measured from the conscious rats ails using the tail-cuff technique (BP monitor, LE 5001, LETICIA scientific Instruments, Spain. Rats were warmed for half an hour at 28°C in a thermostatically controlled heating cabinet (Ugo Basile, Italy)) for better detection of the tail artery pulse. The tail was passed through a cuff sensor that was connected to an amplifier (LE 5001, LETICIA scientific Instruments, Spain). The cuff was attached to a sphygmomanometer and BP and heart rate was recorded on a chart, and the averages of three measurements were taken.

Statistical Analysis and Morphometry

Analyses of data were conducted utilizing SPSS with version 10.0 (SPSS, Inc., Chicago, Ill., USA). Statistical comparisons of data were performed using one-way ANOVA followed by Tukey's post hoc test. To detect a probable significance between two different parameters, Pearson correlation was performed. $p \leq 0.05$ was considered statistically significant.

Morphometry of the percentage areas of collagen deposition in Masson's trichrome stained sections and the percentage areas of α -SMA and iNOS positive immunostaining were done using "Leica Qwin 500 C" image analyzer (Cambridge, UK) in 10 non-overlapping fields for each group. Data were also analyzed using analysis of variance (ANOVA) as described above.

Results

Resveratrol (RES) inhibits TAA-induced nitrosative stress (iNOS) and hypoxia (HIF-1 α) in Liver Tissues

Nitrosative stress and hypoxia are known inducible factors of liver fibrosis (Iwakiri, 2015, Roth and Copple, 2015). To test the hypothesis that chronic TAA toxicity can augment tissue levels of nitrosative stress and hypoxia biomarkers and whether RES can protect against the induction of these parameters, we assessed the liver tissue levels of iNOS (Figure 1A-D) and HIF-1 α (Figure 1E) protein in all rats by the end of the experiment. Immunohistochemical analysis of iNOS from liver sections prepared from the control rats showed negative staining (Figure 1A). Whereas, many iNOS positive cells were depicted in liver sections of the TAA group (Figure 1B), which were substantially but not completely inhibited by resveratrol in the RES + TAA group (Figure 1C). The mean area % of iNOS immunostaining in the liver sections of all groups is shown in Figure 1D.

Western blots analysis of hepatic tissue homogenates prepared from the model group (TAA) revealed an increase in HIF-1 α protein expression that was significantly ($p < 0.0001$) ameliorated by RES (Figure 1E). Conversely, the inhibition of HIF-1 α by RES was significantly ($p < 0.0001$) elevated compared with the control rats, which denotes a complete inhibition by RES was not achieved. Also, there is a significant ($p < 0.0001$) correlation ($r = 0.949$) between iNOS score and HIF-1 α (Figure 1F).

Resveratrol (RES) decreases TAA-induced biomarkers of liver fibrosis α -SMA and MMP-9 in injured liver

α -SMA is a well-known pro-fibrogenic marker (Carpino et al., 2005) and MMP-9 is now considered as a pro-fibrotic and not anti-fibrotic marker as previously thought (Murthy et al., 2010). Therefore, we evaluated their tissue levels in the TAA and treated group using immunohistochemistry and qRT-PCR analyses (Figure 2). Using immunohistochemical analysis, hepatic tissue sections of the control group revealed few areas of α -SMA positive cells in the hepatic vascular endothelium (Figure 2A). Many α -SMA positive cells were illustrated in liver sections of the model (TAA) group (Figure 2B), which were completely ($p < 0.0001$) repressed by RES (Figure 2C and D). of rats, MMP-9 mRNA message assessment showed significant augmentation in the model group compared with the control group, which was significantly ($p = 0.0014$), but not completely inhibited by RES in the RES +TAA group (Figure 2E). In

addition, a positive correlation ($r = 0.858$; $p < 0.0001$) was obtained between α -SMA and MMP-9 (Figure 2F).

Resveratrol (RES) protects hepatic tissue against TAA-induced fibrosis and inflammatory cell infiltration

We tested the suggestion that RES can protect liver tissue against damage induced by TAA. Liver tissues obtained from different rat groups were stained with Masson's trichrome and then examined under light microscopy (Figure 3). Unremarkable fine collagen deposition in the portal area and absence of inflammatory cells in the control rats was observed (Figure 3A), whereas, liver sections of the untreated TAA group showed significant course collagen deposition (fibrosis) in the portal area and septum, and inflammatory cells infiltration around the portal tract (Figure 3B). RES treatment significantly ($p < 0.0001$) inhibited collagen deposition (Figure 3C and D) to levels comparable to the control group (Figure 3D).

To support the link between liver fibrosis induced by TAA we determined the correlation between fibrosis score and the tissue levels of iNOS, HIF-1 α , α -SMA, and MMP-9. Figure 3C–H, showed a significant ($p < 0.0001$) positive correlation between collagen deposition and iNOS ($r = 0.921$), α -SMA ($r = 0.958$), HIF-1 α ($r = 0.873$), and MMP-9 ($r = 0.880$).

Resveratrol (RES) decreases dyslipidemia and hypertension induced by TAA

CCl₄ and TAA liver intoxications affect lipid homeostasis and induced dyslipidemia (Boll et al., 2001, Al-Attar and Shawush, 2014). To prove whether RES treatment can protect against TAA-induced dyslipidemia, serum TG, CHOL, vLDL-ch, and HDL-ch levels were measured at week 10 in all rat groups (Figure 4). Compared to the model group (TAA), RES administration significantly ($p \leq 0.023$) reduced TG (Figure 4A), CHOL (Figure 4B), and vLDL-C (Figure 4C), and increased HDL-C (Figure 4D) to levels still significant to the control group. We also measured the levels of RES inhibition to the liver injury biomarkers, ALT (Figure 4E) and AST (Figure 4F) that showed a significant ($p \leq 0.0042$) inhibition.

Dyslipidemia and fibrosis are well-known factors of developing hypertension (Halperin et al., 2006). We measured BP and HR in all rat groups (Figure 5). TAA significantly ($p < 0.0001$) increased SBP (Figure 5A), DBP (Figure 5B), MAP (Figure 5C), and HR (Figure 5D), which were significantly ($p \leq 0.007$) but not completely inhibited by RES. A positive correlation between collagen deposition (fibrosis) and HR ($r = 0.871$; $p < 0.0001$) (Figure 5E) and MAP ($r = 0.804$; $p < 0.0001$) (Figure 5F) was observed.

Discussion

This study investigated the iNOS/HIF-1 α /MMP-9/ α -SMA axis mediated liver fibrosis and hypertension by TAA in the presence and absence of RES in an induced chronic liver injury rat model. We used molecular, biochemical, immunohistochemical, and histological approaches to show that TAA intoxication activated liver tissue iNOS/HIF-1 α / α -SMA axis associated with the induction of liver fibrosis and hypertension, and RES was able to block TAA effects. In addition, RES inhibited TAA-induced matrix metalloproteinase-9 (MMP-9). MMP-9 was reported to participate in liver fibrosis induced by TAA (Lin et al., 2017). Our results

proved that the plant phytoalexin, RES, can ameliorate iNOS/HIF-1 α / α -SMA axis and liver fibrosis and hypertension in a TAA-induced chronic liver injury.

The liver is a known target of TAA intoxication that causes liver fibrosis and cirrhosis (Reif et al., 2004, Al-Hashem et al., 2019), and our data that demonstrated the induction of hepatic fibrosis, iNOS, HIF-1 α , and MMP-9 by TAA (Figures 1-3) are in agreement with the previous report that demonstrated liver fibrosis induced by cholesterol and the enhancement of iNOS, HIF-1 α , MMP-9 in mice that placed iNOS upstream of HIF-1 α and MMP-9 (Anavi et al., 2015). They also demonstrated a profibrotic role for iNOS, both *in vivo* and *in vitro*. However, HIF-1 α gene knockout in macrophages significantly reduced iNOS gene expression (Takeda et al., 2010) suggesting that these two signalling molecules modulate each other's expression. TAA induced iNOS mRNA in a mouse model of liver injury (Tsai et al., 2021) and α -SMA protein in rats (Al-Hashem et al., 2019). This is also in agreement with our study shown in Figures 1 and 2.

Previous studies have shown that RES (i) protected against β -amyloid-induced neurotoxicity via the amelioration of iNOS expression (Huang et al., 2011) ; (ii) inhibited HIF-1 α in pancreatic cancer (Srivani et al., 2020); (iii) deactivated human liver myofibroblasts associated with the inhibition of α -SMA and MMP-2 (Godichaud et al., 2000); (iv) inhibited MMP-9 in chondrosarcoma cells (Gweon and Kim, 2014); (v) decreased liver fibrosis in nonalcoholic steatohepatitis rat model (Kessoku et al., 2016); and (vi) decreased the blood pressure of mice treated with angiotensin II (Prysyazhna et al., 2019). These reports corroborate our data showing substantial inhibition of the iNOS/HIF-1 α / α -SMA/MMP-9/collagen axis and hypertension by RES.

Taken together, we demonstrated in this study that TAA intoxication caused a substantial induction of liver iNOS/HIF-1 α / α -SMA/MMP-9/collagen mRNA and protein expression and hypertension, which was effectively inhibited by RES for 10 weeks.

Declarations

Author contributions

Conceived and designed the experiments: BA-A and AFD. Performed the experiments: HAE, SSK, NSAL, and MAE. Analyzed the data: HAE, MD, MAH, AFD, and BA-A. Wrote the manuscript: BA-A, AFD, and HAE.

The authors declare that all data were generated in-house and that no paper mill was used.

Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to confidential handling of our materials because this manuscript data is part of a big project which is underway.

Statements and Declarations

The authors declare the absence of any conflicts of interest.

Ethical Approval

Institutional review board, PNU, KSA no H-1-R-095

Consent to Participate and Consent to Publish

not applicable

Acknowledgements

We are grateful to Dr Mariam Al-Ani from Face Studio Clinic, 273 Hagley Road, Birmingham, B16 9NB, UK for proofreading the manuscript.

Funding information

This work was funded by the Deanship of Scientific Research at Princess Nourah bint Abdulrahman University, through the Research Funding Program (Grant No# FRP – 1442 - 5).

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Figures

Figure 1

Resveratrol (RES) inhibits TAA-induced liver iNOS and HIF-1α expression. iNOS immunohistochemistry of liver sections (x200) from control (A), TAA (B), and RES+TAA (C) rats' groups are illustrated. Note that arrowheads in (B and C) point to the positive iNOS immunostained cells. A quantitative analysis of iNOS immunostaining area % in liver sections from groups above is exemplified in histograms (D). Liver lysates immunoblotted with antibodies against HIF-1α (E and inset), and b-actin as a loading control (lower bands, inset E). Results represent the mean (±SD). Presented p values are all significant; *p<0.05 versus control, **p<0.05 versus TAA. (F) Correlation between iNOS and HIF-1α. iNOS: inducible nitric oxide synthase; HIF-1α: hypoxia-inducible factor-1α; TAA: thioacetamide.

Figure 2

Resveratrol (RES) inhibits TAA-induced liver α-SMA and MMP-9 expression. α-SMA Immunohistochemistry of liver sections (x200) from control (A), TAA (B), and RES+TAA (C) rats' groups are illustrated. Note that arrowheads point to the positive α-SMA immunostaining in the wall of a blood vessel and septum between hepatic lobules, whereas, arrows in (B) point to the positive α-SMA staining in the perisinusoidal areas. Histograms in (D) exemplify a quantitative analysis of α-SMA immunostaining

area % in liver sections from groups above. Histograms in (E) show a quantitative analysis of MMP-9 relative expression of cDNA messages in liver sections from the above groups and RES control group. Results represent the mean ($\pm$ SD). Presented p values are all significant; * $p < 0.05$ versus control, ** $p < 0.05$ versus TAA. (F) Correlation between α -SMA and MMP-9. α -SMA: alpha smooth muscle actin; MMP-9: matrix metalloproteinase-9; TAA: thioacetamide.

Figure 3

Resveratrol (RES) protects against TAA-induced liver fibrosis. (A-C) Masson's trichrome stained images (x200) for harvested tissues derived from the liver of control (A), TAA (B), and RES+TAA (C) rats' groups are illustrated. Note that arrow in (A and C) points to the fine collagen deposition in the portal area, whereas, arrows in (B) point to the course collagen deposition in the portal area and septum. (D) A quantitative analysis of liver fibrosis determined as % collagen deposition deduced from Masson trichrome stain. Results represent the mean ($\pm$ SD). Presented p values are all significant; * $p < 0.05$ versus control, ** $p < 0.05$ versus TAA. (E-H) Correlation between collagen deposition and iNOS (E), HIF-1 α : (F), α -SMA (G) and MMP-9 (H). TAA: thioacetamide; iNOS: inducible nitric oxide synthase; HIF-1 α : hypoxia-inducible factor-1 α ; α -SMA: alpha smooth muscle actin; MMP-9: matrix metalloproteinase-9.

Figure 4

Resveratrol (RES) inhibits dyslipidemia and liver injury induced by TAA. Blood levels of TG (A), CHOL (B), vLDL-C (C), HDL-C (D), ALT (E), and AST (F) were measured in all rats' groups 8 weeks post TAA intoxication; Control, RES, TAA, and RES+TAA groups. Results represent the mean ($\pm$ SD); $n=6$ for each group. Presented p values are all significant; * $p < 0.05$ versus control, ** $p < 0.05$ versus TAA. TAA:

thioacetamide; TG: triglycerides; CHOL: cholesterol; vLDL-ch: very low density lipoprotein cholesterol; HDL-ch: high-density lipoprotein cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

Figure 5

Resveratrol (RES) inhibits TAA-modulated hypertension and heart rate. Systolic blood pressure (SBP) (A), diastolic blood pressure (DBP) (B), mean arterial pressure (MAP) (C), and heart rate (HR) (D) were measured in all rats' groups 8 weeks post TAA intoxication; Control, RES, TAA, and RES+TAA groups. Results represent the mean ($\pm$ SD); $n=6$ for each group. Presented p values are all significant; * $p<0.05$ versus control, ** $p<0.05$ versus TAA. (E and F) Correlation between collagen deposition and MAP (E), and HR (F). TAA: thioacetamide.

Figure 6

Proposed model for TAA-induced liver fibrosis is inhibited by resveratrol (RES). TAA: thioacetamide; iNOS: inducible nitric oxide synthase; HIF-1 α : hypoxia-inducible factor-1 α ; MMP-9: matrix metalloproteinase-9; α -SMA: alpha smooth muscle actin.

Supplementary Files

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- [HIF1alpha.docx](#)
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