Resveratrol Helps Recovery from Fatty Liver and Protects against Hepatocellular Carcinoma Induced by Hepatitis B Virus X Protein in a Mouse Model

Hsiu-Ching Lin1,5, Yi-Fan Chen2,5, Wen-Hsin Hsu1, Chu-Wen Yang3, Cheng-Heng Kao4*, and Ting-Fen Tsai1,2*

1Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Taipei, Taiwan
2Institute of Molecular and Genomic Medicine, National Health Research Institutes, Zhunan, Miaoli County, Taiwan
3Department of Microbiology, Soochow University, Taipei, Taiwan
4Center of General Education, Chang Gung University, Taoyuan, Taiwan
5These authors contributed equally to this work.

Running title: Resveratrol prevents HBV-associated HCC

Keywords: chemoprevention; fatty liver; HBV X protein; hepatocellular carcinoma; resveratrol

Disclosure of Potential Conflicts of Interest

There are no potential conflicts of interest to declare.

Corresponding authors:

Ting-Fen Tsai, Ph.D., Department of Life Sciences and Institute of Genome Sciences,
National Yang-Ming University, 155 Li-Nong St., Sec. 2, Peitou, Taipei 112, Taiwan. Tel: 886-2-2826-7293; Fax: 886-2-2828-0872; E-mail: tftsai@ym.edu.tw

Cheng-Heng Kao, Ph.D., Center of General Education, Chang Gung University, 259 Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan. Tel: 886-3-2118800 ext. 5484; Email: cheng50@mail.cgu.edu.tw

Word count: 4640 (including title page, abstract, introduction, materials and methods, results, discussion, acknowledgments, and grant support)

Total number of figures: 6
Abstract

Resveratrol (RSV) is a natural polyphenol that has beneficial effects across species and various disease models. Here, we investigate whether RSV is effective against hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) using HBV X protein (HBx) transgenic mice. We found that RSV (30 mg/kg/day) has a therapeutic effect on HBx-induced fatty liver and the early stages of liver damage. RSV decreased intracellular reactive oxygen species and transiently stimulated hepatocyte proliferation. Interestingly, RSV inhibited LXRα and down-regulated the expression of the lipogenic genes, Srebp1-c and Pparγ. The decrease in Srebp1-c seems to further down-regulate the expression of its target genes, Acc and Fas. Additionally, RSV stimulated the activity of Ampk and SirT1. Thus, RSV has a pleiotropic effect on HBx transgenic mice in terms of the down-regulation of lipogenesis, the promotion of transient liver regeneration, and the stimulation of antioxidant activity. Furthermore, at the later pre-cancerous stages, RSV delayed HBx-mediated hepatocarcinogenesis and reduced HCC incidence from 80% to 15%, a 5.3-fold reduction. RSV should be considered as a potential chemopreventive agent for HBV-associated HCC.
**Introduction**

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and has a poor prognosis and low survival rate (1). A large proportion of HCC cases occur in less-developed countries in Asia and Africa, and are typically associated with chronic hepatitis virus infection (mainly HBV and HCV). Interestingly, while the incidence of HCC in less-developed areas is decreasing because of vaccination, the incidence of HCC in well-developed countries, including the United States and Europe, has increased in the last 20 years (2, 3). The etiology of this increase in HCC in developed countries remains to be elucidative, but it is likely to involve HCV and metabolic factors such as obesity and diabetes (4, 5). However, there are currently limited therapeutic regimens available for the effective treatment of HCC. The fact that HCC exhibits a high recurrent rate after resection and is resistant to conventional chemotherapy and radiotherapy renders the disease a very serious health problem at the current time.

In addition, hepatic steatosis (fatty liver), which manifested as an excess accumulation of lipids in hepatocytes, is associated with hepatitis virus infection, various drugs, nutritional factors, and multiple genetic defects in energy metabolism. Fatty liver is a vulnerability factor that can promote liver damage and inflammation, which results in a further progression to cirrhosis and HCC (6). In order to treat fatty liver and protect against the development of more severe forms of end-stage liver disease and cancer, the discovery
and development of chemopreventive agents for HCC is of paramount importance.

Resveratrol (trans-3,5,4’-trihydroxystilbene; RSV) is a polyphenol found in a wide variety of plant species. RSV has been shown to exert beneficial effects across species and various disease models; it can prevent or slow the progression of a wide variety of illnesses, including cancer, cardiovascular disease, diabetes and metabolic disease, as well as enhance stress resistance (7, 8). For liver diseases, previous studies have demonstrated the protective effects of RSV against alcohol-induced fatty liver and liver injury in mice (9, 10). This protective action of RSV in preventing the development of alcoholic fatty liver seems to be associated with an upregulation of the SIRT1 and AMPK signaling pathways in the livers of the ethanol-fed mice (11). The beneficial effect of RSV has also been shown in a rodent model of non-alcoholic fatty liver disease where the disease is induced by a protocol involving a high carbohydrate-fat free modified diet and fasting (12).

The anti-cancer potential of RSV in HCC has been investigated in xenografted nude mice and in rodent models carrying transplanted hepatome cells; in addition, the chemopreventive effect of RSV has also been evaluated in chemical carcinogen-induced HCC in rats (13). Bishayee and Dhir (2009) (14) applied a two-stage protocol of rat hepatocarcinogenesis involving a single intraperitoneal injection of diethylnitrosamine (DEN, 200 mg/kg) followed by promotion with phenobarbital (PB, 0.05%) in the drinking water. Suppression of oxidative stress and the inflammatory response as well as alternations...
in hepatic proinflammatory cytokines have been implicated in the chemopreventive actions of RSV in the DEN-initiated and PB-promoted HCC model (15, 16). However, neither DEN nor PB is epidemiologically associated with HCC in humans. Accordingly, the DEN/PB carcinogen-induced HCC model may not faithfully parallel the normal physiological and pathological situations in terms of the etiological tissue micro-environments of those cells later become cancerous. Thus this model may not recapitulate the spontaneous carcinogenesis progress toward the developed cancerous status.

Therefore, an animal model of hepatocarcinogenesis mimicking the spontaneous progression of HCC development in human patients is required, and this has not yet been explored.

In this study, we investigate the therapeutic effects of RSV on HBV-associated liver damage and fatty liver during the early stages of pathogenesis, and evaluate the potential chemopreventive activity of RSV on HBV-associated HCC at a later pre-cancerous stage. This was done using a transgenic (TG) mouse model that expresses the HBV X protein (HBx) specifically in the hepatocytes. The HBx TG mice spontaneously develop HCC at between 13 months and 16 months of age. The HCC that develops in these HBx TG mice exhibits a well-differentiated morphology of the trabecular pattern, which is similar to that observed in human HCC (17). The HBx TG mice thus provide an animal model for evaluating new chemopreventive and therapeutic agents for HBV-associated HCC under
physiological conditions (18). In addition, we also examined the changes in the hepatic
gene expression profile and signaling pathways before and after RSV treatment at various
time points, and compared these between the HBx TG and wild-type (WT) mice. This
allowed us to explore the potential molecular mechanisms through which the protective
effects of RSV may work.

Materials and Methods

HBx transgenic (TG) mice

We have previously generated four lines of HBx TG mice, namely A105, A106, A110
and A112, in the C57BL/6 background (17). All of the HBx TG lines develop HCC. In this
study, all the animal experiments used male mice of the line A106; this line develops HCC
faster than the other three TG lines (17). All of the mice were housed in a specific pathogen
free facility. All of the animal protocols are consistent with the recommendations outlined
in the “Guide for the Care and Use of Laboratory Animals” (Washington, DC, National
Academy Press). The Institutional Animal Care and Use Committees of the National
Yang-Ming University had specifically approved this study (approval number 981207).

Resveratrol (RSV) administration

To study the therapeutic effect of RSV on the fatty liver and early stage of liver
pathogenesis, 4-week old HBx TG male mice and their WT male littermates were randomly assigned into different groups. RSV (Sigma R5010; 30 mg/kg/day) was dissolved in H₂O and delivered to the mice by oral administration using a feeding needle once a day. Mice were sacrificed at 2, 3, 7 and 14 days after RSV administration. To study the chemopreventive effect of RSV on the pre-cancerous stage of liver carcinogenesis, 12-month old HBx TG male mice and their WT male littermates were used. In this case RSV (Sigma R5010) was mixed with powdered chow at a concentration of 3g/12.5 kg of food to provide a dose of 30 mg/kg/day for a mouse (average body weight 30g, eating 4g of chow daily), and pellets were then reconstituted; this special diet was prepared by Research Diets, Inc. (New Brunswick, NJ 08901, USA). Mice were sacrificed at 16-month old after RSV supplementation to the chow for 4 months. Liver tissues and sera were collected for pathological and biochemical analysis.

Pathological analysis

The number and size of liver nodules were measured at mouse sacrifice. The livers were collected, fixed with formalin and embedded in paraffin. Liver sections were subjected to hematoxylin-eosin (H&E) staining. Fat accumulation was demonstrated by oil red-O staining of cryostat frozen sections (19). Ultrastructural changes in the liver were examined by transmission electron microscopy (TEM) (20).
**Intracellular ROS and GSH levels**

Primary hepatocytes were isolated from mouse livers using the two-step collagenase perfusion method (21). Intracellular ROS levels were determined using an oxidative sensitive fluorescence dye, dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probes) (18). Intracellular GSH levels were determined using a cell-permeable nonfluorescent dye monochlorobimane (MCB; Molecular Probes) that becomes highly fluorescent following reaction with intracellular glutathione (18).

**RNA analysis**

Total RNA was isolated from mouse tissues using TRIzol Reagent (Life Technology). Slot blot hybridization was performed as previously described (22). We execute reverse transcription with 2μg of total RNA using oligo-d(T) as primer and Superscript III reverse transcriptase (Invitrogen Life Technologies). The real-time quantitative PCR was carried out on a Roche LightCycler 480 instrument using a TaqMan probe. All amplifications were carried out in triplicate for each RNA sample and primer set, and all measurements were done using RNA samples from three individual mice. The amount of total input cDNA was normalized using Hprt as an internal control.
Western blotting and immunohistochemistry (IHC) analysis

Western blotting was performed as described previously (23) and detected using VisualizerTM Kit (Upstate 64-201BP). The following antibodies were used: p-Ampk (Cell Signaling 2535, 1:1000); Ampk (Cell Signaling 2532, 1:1000); p-Akt (Upstate 05-736, 1:2000); Akt (Upstate 05-591, 1:2000); β-actin (Sigma A5441, 1:5000); and Gapdh (Millipore MAB374, 1:5000). IHC detection of the Ki67 protein was performed using monoclonal Ki67 antibody (B56; BD PharmingenTM, 1:100) and visualized by the Chemicon IHC SelectTM System (DAB150) according to the manufacturer’s instructions.

Statistical analysis

The results are presented as mean ± SD from at least three independent experiments. Comparisons between two groups were done using a Student’s t test. A p value of <0.05 was considered significant.

Results

RSV promoted recovery from fatty liver and the early stages of liver damage

The animal protocol for RSV treatment is shown in Figure 1A; RSV was used at a concentration of 30 mg/kg/day, which is a feasible daily dose for human (24). Without RSV, at an early stage of the HBx-mediated liver pathogenesis, namely 4 to 6 weeks of age, liver
pathology including fatty changes (microsteatosis), pleomorphic and bizarre nuclei, ballooning of the hepatocytes and abnormal arrangements of the sinusoid, all of which were clearly detectable in the HBx TG mice (Fig. 1B a). Interestingly, oral administration of RSV (30 mg/kg/day) from 4 to 6 weeks of age reduced liver damage and regressed the histopathology of the HBx TG mice in a time-dependent manner (Fig. 1B b-d). The liver pathology of the HBx TG mice recovered significantly after receiving RSV for 7 days (Fig. 1B c) and recovered to normal morphology after receiving RSV for 14 days (Fig. 1B d) compared to the WT control (Fig. 1B e-h). Oil-red O staining of liver cryosections further revealed that fatty liver had completely disappeared in the HBx TG mice after RSV receiving for 14 days (Fig. 1C). There was no obvious difference in the body weight and ratio of liver to body weight in the HBx TG or WT mice with or without RSV (Supplementary Fig. S1). However, the value for serum ALT in the HBx TG mice was significantly reduced at day 14 after receiving RSV. Importantly, no obvious difference in the serum ALT values could be detected in the WT mice with or without RSV treatment, indicating that RSV has no toxic effect on the liver (Supplementary Fig. S2). Thus, our results demonstrated that RSV treatment results in recovery from fatty liver and that RSV exerted therapeutic effects during the early stages of liver pathogenesis in the HBx TG mice.
RSV produced a significant recovery in hepatocyte ultrastructure, increased glutathione (GSH) levels and decreased reactive oxygen species (ROS) levels in the HBx TG livers

To study the efficacy of RSV on hepatocyte ultrastructure, HBx TG livers after receiving RSV were examined by transmission electron microscopy. In the WT mice, no ultrastructural abnormalities were detected with or without RSV treatment (Fig. 2A and B). In the HBx TG mice without RSV, severe ultrastructural alterations were observed in the hepatocytes, including disorganization of rough ER and degeneration of the nuclear envelope and mitochondria (Fig. 2C and D). After receiving RSV, most of the ultrastructural abnormalities were absent at 14 days (Fig. 2E and F) and seem to have completely recovered at 30 days (Fig. 2G and H). Quantification further supported a recovery in mitochondrial volume density among the HBx TG hepatocytes after receiving RSV when compared with WT hepatocytes (Fig. 2I) (25). Previously we have showed that there are persistently increased levels of ROS during liver carcinogenesis of HBx TG mice (18). To study the antioxidant activity of RSV in the liver, the intracellular GSH and ROS levels of the hepatocytes were monitored. Indeed, the intracellular GSH levels were significantly increased (Fig. 2J), whereas the intracellular ROS levels were significantly reduced after receiving RSV for 7 days (Fig. 2K). These results clearly showed that RSV exhibits antioxidant activity in the liver and that it can efficiently reduce the intracellular
ROS levels induced by HBx during HBV-associated carcinogenesis.

**RSV transiently stimulated hepatocyte proliferation, which helps to replace damaged cells in the HBx TG liver**

To study whether hepatocyte proliferation and liver regeneration were affected by RSV, the Ki67 cell proliferation marker was examined by immunohistochemistry staining of liver sections. Our results indicated that Ki67-positive cells were obviously increased after RSV administration (Fig. 3A). In WT mice, proliferation of hepatocyte decreased gradually from 4-week old to 8-week old during maturation (Fig. 3B): 4-week old (2.64±0.16%) → 5-week old (1.2±0.17%; H2O 7d) → 6-week old (0.74±0.13%; H2O 14d) → 8-week old (0.21±0.03%; H2O 30d). In the WT mice treated with RSV, hepatocyte proliferation was not affected after receiving RSV for 3 days; however there was a lower but significant stimulation after receiving RSV for 7 days. Furthermore, the phenomenon of enhanced proliferation disappeared after receiving RSV for 14 days (Fig. 3B). In the HBx TG mice, quantification revealed that after receiving RSV for 3, 7, and 14 days, there was a significant increase in the number of Ki67-positive hepatocytes found in treated TG mice compared to the untreated TG mice (Fig. 3B). Importantly, the proliferation of hepatocytes in the HBx TG mice went back to a basal level after receiving RSV for 30 days; this is the time when the animal has completely recovered from all the liver pathology and
ultrastructure abnormalities following treatment with RSV (Fig. 3B). These results suggested that the enhanced hepatocyte proliferation and liver regeneration induced by RSV helps to replace damaged cells in the HBx TG mice and this may contribute in part to the chemotherapeutic effect of RSV on fatty liver.

**RSV inhibited lipogenic gene expression in the HBx TG livers**

To investigate whether HBx gene expression is affected by RSV and whether this might contribute to the regression of morbid liver pathology after RSV administration, expression of the HBx gene was examined. Our results revealed that the mRNA level of the HBx gene was not inhibited by RSV in the HBx TG mice (Fig. 4A and Supplementary Fig. S3A).

To dissect the molecular mechanisms underlying the beneficial effects of RSV on HBx-mediated fatty liver and histopathology at the early stage of liver damage (4- to 6-week old), we examined the expression of lipogenic genes and the genes related to lipid metabolism in liver. Previous studies have shown that HBx induces lipid accumulation and fatty liver through transcriptional activation of Srebp1-c (sterol regulatory element binding protein 1, isoform c), and peroxisome proliferator-activated receptor gamma (Pparγ) (26, 27). Srebp1-c is a key regulator of lipogenic genes in liver (28). Interestingly, there was an age-dependent decrease of Srebp1-c expression in the liver of WT mice. However, the level of Srebp1-c mRNA was significantly increased in the HBx TG mice at around 3-month old.
and thereafter, compared with age- and sex-matched WT mice (Fig. 4B). Although there was no obvious difference in the expression levels of Srebp1-c between WT and HBx TG mice from 4- to 6-week old, our result revealed that specifically in the HBx TG mice, RSV significantly reduced Srebp1-c mRNA expression as early as 2 days after receiving the RSV (Fig. 4C). Subsequently, expression levels of the downstream target genes of Srebp1-c, namely acetyl-CoA carboxylase (Acc) and fatty acid synthase (Fas), were found to be reduced after the expression of Srebp1-c was lowered (Fig. 4D and E). However, expression of the Srebp1-c target gene stearoyl-CoA desaturase 1 (Scd1) was not affected by RSV (Supplementary Fig. S3B). In addition to Srebp1-c, Pparγ is suggested to be a key regulator for lipid uptake and synthesis in liver (29, 30). Our results revealed that RSV also decreased the expression of Pparγ after RSV had been given for 3, 7, and 14 days in the HBx TG mice (Fig. 4F).

**Inhibition of LXRα seems to be the early event upstream affecting Srebp1-c in the HBx TG liver after receiving RSV**

Previously studies have shown that HBx induces expression of liver X receptor (LXR) and its lipogenic target genes, including Srebp1-c and Ppar, in HBx TG mice (27, 31). Our results revealed that RSV reduced the expression of LXRα mRNA as early as 2 days after receiving RSV (Fig. 4G); however, RSV had no effect on the expression of LXRβ in either
WT or HBx TG mice (Supplementary Fig. S3C). Moreover, we also examined whether RSV affects the activation of Akt by phosphorylation, which has been implicated in the HBx-mediated survival signaling (32) and the activation of Srebp1-c (26). Our results did not show a significant difference in the p-Akt/Akt ratios of HBx TG mice with or without RSV treatment (Supplementary Fig. S4).

Furthermore, because Srebp1-c is also modulated by AMP-activated protein kinase (Ampk) during control of lipid metabolism in the liver (33, 34), we monitored the effect of RSV on Ampk activity by examining the protein levels of phosphorylated Ampk (pAmpk) and total Ampk. We detected a significant increase in the pAmpk/Ampk ratio at day 3, but not at day 2, after the mice had received RSV in both the WT and HBx TG mice (Fig. 4H and I). This activation was one day after the decrease in Srebp1-c in the RSV treated HBx TG mice (Fig. 4C), which suggests that Ampk signaling is unlikely to be the upstream regulator responsible for the RSV-mediated Srebp1-c inhibition in the HBx TG mice.

Moreover, since RSV is an activator of SirT1 (35); we sought to examine the effect of RSV on the hepatic SirT1 activity and gene expression in the RSV treated HBx TG mice. Our results revealed that there was a significant increase in SirT1 enzymatic activity (1.5- to 2-fold) after receiving RSV for 7 and 14 days in both the WT and HBx TG mice (Supplementary Fig. S5A). We further examined the SirT1 protein level in the various livers, and found a similar magnitude of change in enzymatic activity after receiving RSV
(Supplementary Fig. S5B and C); this result indicated that RSV activated SirT1 in liver mainly by upregulating its protein expression.

In summary (Fig. 5), our results reveal that RSV helps the recovery of HBx-induced fatty liver in a coordinating manner by affecting multiple lipid metabolism signaling pathways, which in turn produces reduced lipid synthesis, and prevents the accumulation of hepatic lipids in mice. Specifically, RSV inhibits LXRα and down-regulates the expression of its lipogenic target genes, Srebpl-c and Pparγ; the decrease in Srebpl-c further down-regulates the expression of its target genes, Acc and Fas, both of which are lipogenic-associated enzymes. In addition, our results also show that RSV stimulates the activity of Ampk and SirT1 in the HBx TG liver. The combined effects of these multiple pathway changes seem to be associated directly or indirectly with lipid metabolism. Furthermore, it appears that RSV can transiently induce liver regeneration; this likely helps with the replacement of damaged cells in the HBx TG mice. Moreover, RSV exhibits antioxidant activity, which is accompanied by an increase in the GSH level and a decrease in the ROS level. Taken together, our results indicate that RSV functions as a pleiotrophic chemotherapeutic agent and acts by regulating lipogenesis, promoting transient regeneration, and stimulating antioxidant activity in the liver. These activities together may contribute to the recovery of fatty livers and a reversing of the liver histopathology found in the HBx TG mice.
RSV delayed HBx-mediated hepatocarcinogenesis and significantly reduced HCC incidence at the pre-cancerous stage

Since RSV has a beneficial (therapeutic) effect during the early stages of liver pathogenesis, we further tested the preventive effect of RSV on the later stages of HBx-mediated HCC development. RSV (30 mg/kg/day) was orally administrated to HBx TG and WT mice from 12- to 16-month old (Fig. 6A). In the pre-cancerous mice at 12 months of age and before receiving RSV, hyperplastic nodules measuring between 0.5 to 2.5 mm in diameter could be detected in about 67% of the HBx TG mice (Supplementary Fig. S6A). Furthermore, there was an 80% incidence of HCC in the 16-month old HBx TG mice without any treatment (Fig. 6B and Supplementary Fig. S6B) (17). In WT mice, our results showed that there was no detectable toxicity after receiving RSV for 4 months and there was also no difference in body weight and serum ALT level (a liver damage marker) between the mice treated with RSV and the control group (Fig. 6C and Supplementary Fig. S7). Notably, in the HBx TG mice, there was a significant delay in liver carcinogenesis and a remarkable decrease in HCC incidence after receiving RSV for 4 months. Specifically, no grossly identifiable nodules could be detected in 15% (3/20) of the pre-cancerous HBx TG mice, while 55% (11/20) of the mice contained only small 0.5-2.5 mm hyperplastic nodules and 15% (3/20) of the mice contained 3-6 mm hyperplastic nodules that were later
pathologically confirmed to be benign tumors (Fig. 6D and Supplementary Fig. S8). Out of all the pre-cancerous HBx TG mice, only 15% (3/20) developed HCC. This is a significant reduction after RSV treatment compared to the incidence of HCC in HBx TG mice that have not been treated with RSV, namely 15% compared to 80%, respectively, which is a 5.3-fold reduction of HCC in the RSV treated HBx TG mice compared to the control mice.

Discussion

The central finding in this work is that RSV has therapeutic effects on the early stages of HBx-mediated liver damage, reversing fatty changes and producing a recovery in liver histopathology. Moreover, this study provides evidence for the first time that RSV at 30 mg/kg/day exerts a significant chemopreventive effect on HBx-mediated HCC. Specifically, RSV exhibits anti-carcinogenesis properties by significantly decreasing cancer incidence and delaying the progression of spontaneous HCC in the HBx TG mice.

The molecular mechanism underlying the chemotherapeutic effects of RSV on HBx-induced fatty liver and liver damage seems to be attributable to the pleiotropic actions of RSV (summarized in Figure 5). These actions involve the following. Firstly, RSV inhibits lipogenesis by decreasing LXRα-Srebp1c signaling and, thereby, decreases the expression of its downstream target genes, Acc and Fas; this decrease in the LXRα-Srebp1c signaling is an early event observed 2 days after receiving RSV in the HBx
TG mice. The inhibition of lipogenesis may also be attributable to an increase in the activities of Ampk and SirT1, which can be detected 7 days after receiving RSV in the HBx TG mice. Previously, the RSV-mediated increase Ampk and SirT1 activity has been documented to be associated with the alleviation of alcoholic fatty liver in mice (11).

Secondly, RSV treatment leads to a transient stimulation of liver regeneration that helps to replace damaged hepatocytes in HBx TG mice. Importantly, the proliferation of hepatocytes in the HBx TG mice returned to the basal level of a resting adult liver when the animal had completely recovered from all the pathology and ultrastructure abnormalities; this was after receiving RSV for 30 days. Thirdly, RSV enhances antioxidant activity in the liver by, at least in part, increasing intracellular level of GSH. The increased GSH and decreased lipid content in the hepatocytes of HBx TG mice may both contribute to the reduction in the intracellular ROS after RSV treatment. Previous studies have revealed that RSV has antioxidative properties that can increase hepatic GSH and protect the liver against oxidative stress induced by partial hepatectomy (36) and CCl₄ intoxication (37).

Here, we provided further evidence that RSV also protects the liver from oxidative damage mediated by HBx protein in mice.

It is well established that HCC develops in the presence of chronic liver diseases and is typically associated with fatty liver, fibrosis, cirrhosis from hepatitis virus infection (HBV and HCV), and/or alcoholic liver disease. Additionally, there is increasing evidence to
support the idea that fatty liver is one of the risk factors promoting the development of HCC in association with HBV and HCV (38). Accordingly, eliminating the risk factor of fatty changes and histopathological damage by treating with RSV at an early stage should help to protect the liver against HBx-mediated carcinogenesis, and retard the progression to advanced liver disease and subsequent HCC at the later stage.

The overall safety of RSV has been documented in several in vivo studies. RSV is well tolerated and non-toxic in rodents from low doses (20 mg/kg/day) to high doses (up to 750 mg/kg/day) in a 28-day or 90-day studies (39-41). Only at a very high dose (3000 mg/kg/day) of RSV, which is at least 30 times the routine human dose (the dose as high as 7.5 g per day has been suggested for humans, which is equivalent to a dose of 100 mg/kg/day for a 75 kg person) (7), did rats exhibit clinical signs of toxicity as well as a reduced body weight and food consumption after 4 weeks of receiving RSV. In addition, renal toxicity and nephropathy were also observed in these rats (42). In the present study, our results revealed that oral administration of RSV at 30 mg/kg/day over a period of 4 months seems to have no obvious negative effect that is detrimental to the whole organism. In WT mice, the body weight and serum ALT level did not differ between mice treated with RSV and the control group over the whole treatment period. Moreover, histopathological examination of the organs obtained at autopsy did not reveal any detectable alterations in the treated WT mice. These results provide in vivo evidence that chronic oral consumption...
of RSV at 30 mg/kg/day for up to 4 months does not adversely affect physiological functioning.

Acknowledgments

We thank Yi-Fang Wu and Yao-Kuan Huang for their technical assistance. We thank the Microarray & Gene Expression Analysis Core Facility of the National Yang-Ming University Genome Research Center; the Core Facility is supported by National Science Council.

Grant Support

This work was supported by National Science Council (NSC99-2628-B-010-001-MY3), National Health Research Institutes (NHRI-EX100-9837NI), Center of Excellence for Cancer Research at Taipei Veterans General Hospital (DOH100-TD-C-111-007) and a grant from the Ministry of Education, Aim for the Top University Plan.
References


Protective effect of resveratrol on ethanol-induced lipid peroxidation in rats. Alcohol Alcohol 2006;41:236-239.


2003;278:34268-34276.


**Figure legends**

**Figure 1.** Treatment with RSV reversed fatty liver and early stage liver damage in the HBx TG mice. A, the treatment protocol used with RSV. RSV (30 mg/kg/day) and vehicle (H2O) were orally administrated to the HBx TG and WT mice at 4-week (wk) old. The mice were sacrificed and analyzed after RSV administration for 3, 7 and 14 days. Six to ten mice per group were used. B, (a) H&E staining of a liver section without any treatment from an HBx TG mouse at 6-week old. (b) (c) (d) H&E staining of liver sections from HBx TG mice treated with RSV for 3, 7 and 14 days. (e) H&E staining of a liver section without any treatment from a WT mouse at 6-week old. (f) (g) (h) H&E staining of liver sections from WT mice treated with RSV for 3, 7 and 14 days. C, (a) Oil red-O staining of liver section without any treatment from an HBx TG mouse at 6-week old. (b) (c) (d) Oil red-O staining of liver sections from HBx TG mice treated with RSV for 3, 7 and 14 days. (e) Oil red-O staining of a liver section without any treatment from a WT mouse at 6-week old. (f) (g) (h) Oil red-O staining of liver sections from WT mice treated with RSV for 3, 7 and 14 days. Original magnification: 400X.

**Figure 2.** RSV helped recover hepatocyte ultrastructure, increased GSH and decreased ROS in the HBx TG livers. A and B, TEM of hepatocytes of WT mice.
treated with vehicle (H₂O) for 14 days. N, nucleus. C and D, TEM of hepatocytes of HBx TG mice treated with vehicle (H₂O) for 14 days. RER, rough endoplasmic reticulum; NE, nuclear envelope; Mt, mitochondria. E and F, TEM of hepatocyte of HBx TG mice treated with RSV for 14 days. G and H, TEM of hepatocyte of HBx TG mice treated with RSV for 30 days. Photomicrographs shown in panels B, D, F and H are at the magnification of the boxed area in panels A, C, E and G, respectively. I, comparison of mitochondrial volume density after RSV or vehicle (H₂O) treatment for 30 days. J, intracellular GSH levels of hepatocytes after RSV or vehicle (H₂O) treatment for 3 and 7 days. K, intracellular ROS levels of hepatocytes after RSV or vehicle (H₂O) treatment for 3, 7 and 14 days. *p<0.05. Numbers in bars are mice (I-K); bars represent mean ± SD.

Figure 3. RSV transiently stimulated hepatocyte proliferation in the HBx TG mice. A, (a) (b) IHC staining of the Ki67 cell proliferation marker in liver sections prepared from 4-week old HBx TG and WT mice without RSV treatment. (c) (d) IHC staining of Ki67 protein in liver sections prepared from HBx TG and WT mice treated with RSV for 3 days. (e) (f) IHC staining of Ki67 protein in liver sections prepared from HBx TG and WT mice treated with RSV for 7 days. (g) (h) IHC staining of Ki67 protein in liver sections prepared from HBx TG and WT mice treated with RSV for 14
days. (i) (j) IHC staining of Ki67 protein in liver sections prepared from HBx TG and

WT mice treated with RSV for 30 days. Original magnification, 400X. B,

quantification of hepatocyte proliferation as monitored by Ki67 positive staining.

Between 600-1000 hepatocytes from each mouse were examined for the presence of

Ki67 positive staining. The mean for each group is expressed as a percentage of total

hepatocytes counted. *p<0.05; **p<0.005. Numbers in bars are mice (B); bars

represent mean ± SD.

**Figure 4.** Expression of HBx and the genes associated with lipogenesis in the HBx TG livers.

RSV inhibited LXRα expression while enhanced the level of p-Ampk in the HBx TG livers.

A, HBx mRNA levels in the livers of 6-week old HBx TG mice with or without RSV

treatment for 14 days were detected by slot blot hybridization. HBx gene expression was not

inhibited by RSV treatment. The 28S rRNA was used as an internal control for RNA loading.

B, expression of the Srebp1-c mRNA was activated in the HBx TG livers in an

age-dependent manner without any treatment. C, a significant decrease in Srebp1-c mRNA

level was detected as early as 2 days after RSV treatment in the HBx TG mice, and this

preceded the inhibition of the Acc and Fas mRNA expression. D, a significant decrease in

Acc mRNA level was detected in the HBx TG mice after RSV treatment for 3, 7 and 14 days.

E, a significant decrease in Fas mRNA level was detected in the HBx TG mice after RSV
treatment for 7 and 14 days. F, a significant decrease in Pparγ mRNA level was detected in
the HBx TG mice after RSV treatment for 3, 7 and 14 days. The relative mRNA levels of the
Srebp1-c, Acc, Fas and Pparγ were measured by real-time quantitative RT-PCR; the amount
of total input cDNA was normalized using Hprt as an internal control. G, RSV reduced the
expression of LXRα mRNA as early as 2 days after the RSV treatment. H, a representative
Western blot of the p-Ampk and total Ampk protein. I, the p-Ampk/Ampk ratio increased
significantly at day 3 after RSV treatment, but not at day 2. *p<0.05; **p<0.005. Numbers in
bars are mice (B-G and I); bars represent mean ± SD.

**Figure 5.** Summary of lipogenesis, liver regeneration, and antioxidant activity in HBx TG
mice after RSV treatment. The levels of gene expression related to lipogenesis, the results
related to hepatocyte proliferation and the level of antioxidant activity are summarized as a
function of time after RSV treatment from 4- to 6-week old.

**Figure 6.** RSV delayed hepatocarcinogenesis and reduced HCC incidence in the
pre-cancerous stage of HBx TG mice. A, summary of the incidence of hyperplasia nodules
and HCC in the HBx TG mice with or without RSV treatment; nd, not determined. B, a
representative liver of an HBx TG mouse at 16-month old without any treatment; mo, months.
C, a representative liver of a WT mouse at 16-month old with RSV treatment for 4 months. D,
the 16-month old HBx TG mice after RSV treatment for 4 months can be divided into four groups: group #1, no grossly identifiable nodules detected; group #2, livers contain small 0.5-2.5 mm hyperplastic nodules; group #3, livers contain 3-6 mm hyperplastic nodules; group #4, livers with HCC. All of the hyperplastic nodules and HCC were pathologically confirmed. Arrows indicate hyperplastic nodules; arrow head indicates HCC.
Figure 1

A

Genotyping  Treatment  Analysis

HBx TG  Age (wk)  3  4  5  6

RSV treatment (30 mg/kg/day)

3 days  7 days  14 days

B

<table>
<thead>
<tr>
<th></th>
<th>HBx</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (6-wk old)</td>
<td>a</td>
<td>e</td>
</tr>
<tr>
<td>3 d</td>
<td>b</td>
<td>f</td>
</tr>
<tr>
<td>7 d RSV (30 mg/kg/day)</td>
<td>c</td>
<td>g</td>
</tr>
<tr>
<td>14 d</td>
<td>d</td>
<td>h</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>HBx</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (6-wk old)</td>
<td>a</td>
<td>e</td>
</tr>
<tr>
<td>3 d</td>
<td>b</td>
<td>f</td>
</tr>
<tr>
<td>7 d RSV (30 mg/kg/day)</td>
<td>c</td>
<td>g</td>
</tr>
<tr>
<td>14 d</td>
<td>d</td>
<td>h</td>
</tr>
</tbody>
</table>
Figure 2

A-C: WT and HBx liver sections showing normal liver architecture.
D-F: HBx-RSV 14d liver sections with increased mitochondria (Mt) and rough endoplasmic reticulum (RER).
G-I: HBx-RSV 30d liver sections with further mitochondrial changes.

J: Graph showing GSH levels with a significant increase in HBx-RSV treated mice compared to controls.
K: Graph showing intracellular ROS levels, with a significant decrease in HBx-RSV treated mice compared to controls.

* indicates statistically significant differences.
**Figure 3**

**A**

<table>
<thead>
<tr>
<th>untreated (4 wks)</th>
<th>RSV (30 mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 d</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
</tr>
<tr>
<td></td>
<td>14 d</td>
</tr>
<tr>
<td></td>
<td>30 d</td>
</tr>
</tbody>
</table>

- HBx
- WT

**B**

- **Ki67 positive hepatocyte (%)**
- **WT**
- **HBx**

**Legend:**
- **WT**
- **HBx**
- **H2O2**
- **RSV**

**Significance:**
- *P < 0.05
- **P < 0.01
**Figure 4 continued**

**G**

![Graph showing mRNA levels of LXRα for different conditions and time points](image)

**H**

![Western blots showing p-Ampk and Gapdh for different conditions and time points](image)

**I**

![Graph showing the ratio of p-Ampk/Ampk for different conditions and time points](image)
Figure 5

RSV treatment (days)

Inhibit Lipogenesis

↓ LXRα expression
↓ Srebp1-c expression
↓ Pparγ expression
↓ Acc expression
↑ p-Ampk level

↓ Fas expression
↑ SirT1 activity

Promote Regeneration

↑ Hepatocyte proliferation
↑ Ki67 positive hepatocytes

Stimulate Antioxidant Activity

↑ GSH level
↓ ROS level
Figure 6

A

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>Hyperplasia</td>
<td>67%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>HCC</td>
<td>0%</td>
<td>50%</td>
<td>60%</td>
<td>70%</td>
</tr>
<tr>
<td>RSV treatment</td>
<td>Hyperplasia</td>
<td>67%</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>HCC</td>
<td>0%</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

B

HBx (16 mo)

C

WT (16 mo, RSV)

D

<table>
<thead>
<tr>
<th>No nodule</th>
<th>Nodule size 0.5 – 2.5 mm</th>
<th>Nodule size 3 - 6 mm</th>
<th>HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% (3/20)</td>
<td>55% (11/20)</td>
<td>15% (3/20)</td>
<td>15% (3/20)</td>
</tr>
</tbody>
</table>
Resveratrol Helps Recovery from Fatty Liver and Protects against Hepatocellular Carcinoma Induced by Hepatitis B Virus X Protein in a Mouse Model

Hsiu-Ching Lin, Yi-Fan Chen, Wen-Hsin Hsu, et al.

Cancer Prev Res  Published OnlineFirst June 1, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-12-0001

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.