ANSWERING REVIEWERS

Jan 13, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 7356-review.doc).

Title: Resveratrol inhibits collagen I synthesis by suppressing IGF-1R activation in intestinal fibroblasts

Author: Ping Li, Mei-Lan Liang, Ying Zhu, Yao-Yao Gong, Yun Wang, Ding Heng, Lin Lin

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 7356

The manuscript has been improved according to the suggestions of reviewers:
Many grammatical or typographical errors have been revised.
All the lines and pages indicated above are in the revised manuscript.

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer
Part A (Reviewer 1)
(1)**Comment: In vivo study, authors investigated the expression of IGF-1 and SIRT1 in TNBS-induced rat CD model. They showed upregulated IGF-1 and decreased SIRT1 expression might play important role in this disease model. Then, authors stated that "Previous studies demonstrate that SIRT1 activator, resveratrol decreases inflammatory cytokines and profibrogenic factors in the PG-PS model of Crohn's disease [24]. Consistent with our research, these data clearly indicate that resveratrol may protect against intestinal fibrosis through stimulating SIRT1 expression." I do not think that those previous results from another model could make authors to obtain such a conclusion. To confirm the role of resveratrol in TNBS induced CD models, they should directly use this compound to treat rats with/without TNBS. In addition, how about expression of IGF-1R and p-ERK in vivo?**

Reply: Thank you for these precious comments and suggestions. The protective role of resveratrol in inflammation colitis has been demonstrated in model of colitis induced by dextran sulfate sodium (DSS) (Youn, J., 2009) and trinitrobenzenesulphonic acid (TNBS) (Martin, A.R., 2004). Udai P. Singh's study found that resveratrol may protect against colitis through up-regulation of SIRT1 in immune cells, which functioning as an inverse regulator of NF-κB activation and inflammation in the colon (Udai P. Singh, 2010). According to the above studies, we found that SIRT1 expression in colitis tissues was decreased in TNBS-induced Crohn's disease rats. Therefore, we speculated that SIRT1 may invovled in the process of intestinal fibrosis, and resveratrol may protect against intestinal fibrosis through stimulating SIRT1 expression. In the present manuscript, the focus of our studies was on the effect of resveratrol on collagen I synthesis and its molecular mechanisms in intestinal fibroblasts. In the next stage of our experiment, we

will continue to demonstrate the role of resveratrol and SIRT1 in vivo. Thank you again for pointing this out.

The study of Ref-No.13-14 have shown that the expression IGF-1R was increased in the intestines of ulcerative colitis and Crohn's disease patients by immunohistochemistry (Sipos, F., 2008; El Yafi, F., 2005). Detection of p-ERK1/2 levels in vivo will be affected by many factors, because the colon tissue contains epithelial cells, smooth muscle cells, and many other cell types. Collagen-producing fibroblasts and myofibroblasts are central cell types in intestinal fibrogenesis. Therefore, our studies was aim to explore the effect of resveratrol in intestinal fibroblasts and its possible mechanism. The changes of p-ERK1/2 in colitis tissues could not reflect changes in fibroblasts. As such, there seemed not necessary to detect expression of IGF-1R and p-ERK in vivo.

(2)**Comment: Please show molecular weight of measured proteins in ALL western blot.**

Reply: Thanks for your comments.We have added the molecular weight of measured proteins to all WB graphs.

(3)**Comment: Figure 2A, it looks that expression trend between col1a2 mRNA and collagen I protein is different in MIF cells. Please explain. In addition, how about mRNA expression of col1a1? According to figure legend, both mRNA and protein were collected 24h after IGF-1 incubation. Is it right? The same question is also for Figure 3.**

Reply: Thanks for your comments. We have performed this experiment in duplicate, repeated at least three times and concluded the results. The difference between col1a2 mRNA and collagen I protein may attributed to mRNA or protein stability and posttranslational modification (PTM). Moreover, collagen type I is composed of two alpha 1(I) and one alpha 2(I) chains encoded by two genes (col1a1 and col1a2). And, type I collagen antibody we used in our experiments is not specific for col1a1 or col1a2. So, this phenomenon of different expression trend between mRNA and protein may exist in experiments.

According to the reviewer's suggestion, we have added the expression of col1a1 mRNA in MIFs and CCD-18Co cells, shown in supplymental data figure 1A. Our result shown that IGF-1 also induced col1a1 mRNA expression.

In our preliminary results (see supplymental data figure 1B), we have detected collagen I protein levels in different time points after IGF-1 treatment (24h, 48h and 72h) in CCD-18Co and the maximal stimulation was at 24 hours after IGF-1 incubation. So, we performed our following experiments after 24h IGF-1 treatment.

(4)**Comment: According to the representative WB in Figure 3B, it is difficult not believe these quantificated graph were from WB. WB lanes show that resveratrol did not induce expression of SIRT. If it is true, how could authors stated that ?Resveratrol Treatment Abrogated Collagen I Synthesis Partly through SIRT1"?**

Reply: Thanks for your comments. We have modified SIRT1 bands in figure 3B and quantified again. Our results shown that the expression of SIRT1 mRNA significantly increased after 100μM RSV treatment. In order to prove that resveratrol did induce protein expression of SIRT1, we also treated CCD-18Co cells with different concentration of RSV (50 μM, 100 μM, 150 μM). The results displayed in supplymental data figure 1C.

(5)**Comment: Figure 4B is really difficult understood.**

Reply: We have reorganized the order of figures. Figure 4B was changed to Figure 4C.

We have added the following explanations of the legend of Figure 4C. According to Figure 4A and B, resveratrol inhibited IGF-1R phosphorylation, whereas overexpression SIRT1 WT had no effect on IGF-1R activation. Since binding of IGF-1 to the IGF-1 receptor results in autophosphorylation of the receptor β subunits, and increased receptor tyrosine kinase activity, we further examined whether resveratrol effects the binding of IGF-1 to the IGF-1R. CCD-18Co cells were pretreated with resveratrol (100μM) for 24h to induce SIRT1 expression, then removed and incubated with IGF-1 alone for another 30min (see Figure 4C, Line 4) or both IGF-1 and resveratrol for 30min (see Figure 4C, Line 5), the phosphorylation of IGF-1R and ERK1/2 were tested by immunoblot analysis. The results showed that IGF-1R phosphorylatioin level was down-regulated only after treatment with IGF-1 plus resveratrol for 30min (see Line 5), compared with Line 4. In other words, resveratrol inhibited IGF-1R phosphorylatioin only when resveratrol was incubated together with IGF-1. Therefore, we speculated that resveratrol may inhibited IGF-1 binding to its receptor to decrease phosphorylatioin level of IGF-1R. However, further studies are needed to confirm the findings and elucidate the exact molecular mechanism underlying resveratrol-inhibited activation of IGF-1R.

(6)**Comment: Generally, quality of WB could not support authors' conclusions. It is very critical to confirm whether disruption of SIRT will influence the anti-fibrotic effects of resveratrol in IGF-1-induced collagen I expression.**

Reply: Thanks for your comments. According to this suggestion, we have modified this graph in Figure 3F. Necessary change in the statements has been made in the revised manuscript as well as in the referred figure accordingly. As shown in Figure 3F, depletion of SIRT1 by siRNA (#1 and #3) in CCD-18Co cells blocked the reduction of collagen I expression induced by resveratrol (Figure. 3F). However, disruption of SIRT1 did not influence the anti-fibrotic effects of resveratrol in IGF-1-induced collagen I expression (Figure. 3F). According to this result, we hypothesized resveratrol's inhibition on IGF-1 induced collagen I expression is partly attributed to its negative effect on IGF-1R activity. Resveratrol remarkably inhibited the phosphorylation of IGF-1R and ERK1/2 stimulated by IGF-1. However, overexpression of wild type (WT) or enzyme deficient (HY) SIRT1 had no effect on phosphorylation of IGF1R. In conclusion, resveratrol inhibited collagen I synthesis through two unrelated mechanisms.

(7)**Comment: The orgnization of figures and legends should be improved largely.**

Reply: Thanks for your comments. We have done accordingly.

(8)**Comment: Provide full name of SIRT1 and TNBS in the abstract.**

Reply: Done accordingly.

Part B (Reviewer 2)

(1)**Comment: Please spell out the full name of "TNBS" when it first appeared in the abstract.**

Reply: Thanks for your comments. We have corrected accordingly.

(2)**Comment: The introduction was too long, please try to make it more concise.**

Reply: Done accordingly.

(3)**Comment: Please discuss whether resveratrol now can be used clinically in patients with intestinal fibrosis.**

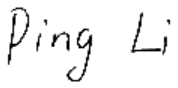
Reply: Thanks for your comments. We have added this part in our discussion. Many

preclinical and clinical studies have shown that resveratrol can prevent or slow the progression of a wide variety of age-associated illnesses, including cancer, diabetes, and coronary, neurodegenerative, and so on. However, most studies of resveratrol on intestinal inflammatory or intestinal fibrosis were performed in experimental colitis. Results obtained by Susana Sánchez-Fidalgo et al showed that dietary supplementation of resveratrol exerted a significant beneficial effect in chronic dextran sulfate sodium (DSS)-induced colitis (Susana Sánchez-Fidalgo, 2010). There are no preclinical and clinical studies have shown that resveratrol can be used clinically in patients with intestinal fibrosis. Moreover, the rapid metabolism of resveratrol diminishes its effectiveness in the colon. Mar Larrosa's study found that resveratrol pro-prodrugs prevented the rapid metabolism of resveratrol and delivered higher quantities of resveratrol to the colon in DSS-induced colitis (Mar Larrosa, 2010). According to previous researches, much more study is needed to explore whether resveratrol can be used in clinic. Long-term epidemiologic studies and controlled clinical trials are also necessary for developing resveratrol to become a standard clinical agent.

3 References and typesetting were corrected

Thank you and all the reviewers for the kind advice.
Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



Ping Li
Department of Gastroenterology,
First Affiliated Hospital of Nanjing Medical University,
Nanjing 210029, Jiangsu Province, China
Tel: +86-25-83718836-6920
Fax: +86-25-83674636
E-mail: liping0607@gmail.com