Author's response to reviews

Title: Deficiency of eNOS exacerbates early-stage NAFLD pathogenesis by changing the fat distribution

Authors:

Yuichi Nozaki (fwix0777@nifty.com)
Koji Fujita (kfujita0819@gmail.com)
Koichiro Wada (kwada@dent.osaka-u.ac.jp)
Masato Yoneda (dryoneda@yahoo.co.jp)
Yoshiyasu Shinohara (shino@hyo-med.ac.jp)
Kento Imao (kento318@yokohama-cu.ac.jp)
Yuji Ogawa (yuji.ogawa01@gmail.com)
Takaomi Kessoku (takaomi-kesso@hotmail.co.jp)
Makoto Nakamura (nakamura@kyumeda.jp)
Satoru Saito (ssai1423@yokohama-cu.ac.jp)
Naohiko Masaki (nmasaki@hospk.ncgm.go.jp)
Yoji Nagashima (ynagas@med.yokohama-cu.ac.jp)
Yasuo Terauchi (terauchi-ty@umin.ac.jp)
Atsushi Nakajima (nakajima-ty@umin.ac.jp)

Version: 2
Date: 18 October 2015

Author's response to reviews: see over
Dear Professors Meng and Afroze,

Thank you very much for your kind E-mail concerning our manuscript entitled, “Deficiency of eNOS exacerbates early-stage NAFLD pathogenesis by changing the fat distribution”

We have now revised the manuscript in line with the suggestions contained in your letter and the reviewers’ comments. The revised portions of the text have been underlined, and our responses to the reviewers’ comments are itemized separately.

We hope that you will find our revised manuscript acceptable for publication in *BMC Gastroenterology* and are looking forward to hearing your decision in the near future.

Sincerely yours,

Atsushi Nakajima, M.D., Ph.D.
Chair Professor
Division of Gastroenterology
Yokohama City University School of Medicine
Response to Reviewer 1
Thank you very much for your useful suggestions. Your suggestions have been addressed in the revised manuscript, which we feel has now been greatly improved as a result.

Comment
Minor Essential Revisions
In the current manuscript submitted by Yuichi Nozaki et al. the authors aimed to investigate the role of eNOS-derived NO in NAFLD pathogenesis using systemic eNOS-knockout mice fed a high-fat diet. Lipid accumulation and inflammation was more extensive in the liver and lipid accumulation was less extensive in the visceral fat tissue in eNOS-knockout mice, compared with wild-type mice, after 12 weeks of being fed a high-fat diet. While systemic insulin resistance was comparable between the eNOS-knockout and wild-type mice fed a high-fat diet, hepatic tissue blood flow was significantly suppressed in the eNOS-knockout mice, compared with the wild-type mice, in mice fed a high-fat diet. The microsomal triglyceride transfer protein activity was down-regulated in eNOS-knockout mice, compared with wild-type mice, in mice fed a high-fat diet. Overall a deficiency of eNOS-derived NO may change the fat distribution of liver and visceral fat, thereby promoting the progression of disease in an HFD-induced, early-stage NASH mouse model by changing the hepatic tissue blood flow. The current study also examined the fat distribution and the pathogenesis of NAFLD/NASH using an imaging procedure in an NAFLD/NASH mouse model with or without the eNOS gene. It is an interesting manuscript with the innovative concept. The overall findings may provide translational potential to address specific clinical issues related to the pathogenesis of NAFLD/NASH. However, some minor issues should be fixed before further consideration.

Comment 1)
The recent data from Sheldon RD et al (Am J Physiol Gastrointest Liver Physiol. 2015 Mar 15;308(6):G540-9) have demonstrated that systemic NOS inhibition in the obese OLETF rats reduced hepatic mitochondrial respiration, increased hepatic triacylglycerol accumulation, and increased hepatic inflammation. The current studies only have very limited data on hepatic tissue blood flow which may too superficial. Therefore some hepatic mitochondrial respiration markers and hepatic inflammation statues should be verified in the current project to further support the central hypothesis.
Response: Thank you for your useful suggestion. According to your suggestion, we have added the following sentences to the Discussion section (page 21, lines 6-13): “Sheldon et al. reported that chronic NOS inhibition via Nω-nitro-L-arginine methyl ester in obese Otsuka Long-Evans Tokushima Fatty rats reduced hepatic mitochondrial respiration, leading to increased hepatic triacylglycerol accumulation, and increased hepatic inflammation, although the specific mechanism remained unclear [8]. They did not examine blood flow in the hepatic tissue; however, the mechanism related to exacerbated early-stage NAFLD pathogenesis under the condition of eNOS deficiency might be associated with the function of hepatic mitochondrial respiration.” Unfortunately, we could not examine the function of hepatic mitochondrial respiration in this study. Consequently, the following sentence mentioning this limitation of our study has been added to the Discussion section (page 22, lines 3-6): “Further studies are needed to verify hepatic mitochondrial respiration markers and hepatic inflammation states, and to examine the relationship between the changes in hepatic blood flow and the degree of liver injury in order to determine the exact underlying mechanism.”

Comment 2)
For hepatic inflammation related studies, neutrophil infiltration detection should be carried out in the liver from eNOS-knockout mice fed a high-fat diet. The presence of neutrophils should be assessed by the measurement of liver tissue myeloperoxidase (MPO), and the degree of neutrophil liver infiltration should be determined by the naphthol AS-D chloroacetate esterase technique.  

Response: Thank you for your useful suggestion. According to your suggestion, we have examined the measurement of liver tissue myeloperoxidase (MPO) and naphthol AS-D chloroacetate esterase. The results showed no significant positive findings in either of the stained samples. We have added the following sentences to the Methods section (page 11, lines 16-20): “Liver samples were excised and embedded in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA, USA) and paraffin for the histological analysis. Formalin-fixed and paraffin-embedded sections were processed routinely using hematoxylin and eosin (H&E), myeloperoxidase (MPO), naphthol AS-D chloroacetate esterase [21] and Sirius-red.” We have also added the following sentences to the Results section (page 16, lines 3-7, page14 lines 11-14): “The liver tissue MPO and Sirius red staining examinations showed no significant positive findings (Figure 2D and 2E), and the additional liver tissue naphthol AS-D chloroacetate esterase staining examination showed negative findings (data not shown).” and “The reason for the negative findings of the liver tissue MPO staining examination
might be related to the model used in this study for early-stage NASH, which showed very mild inflammation, such as very small inflammatory foci detected in the H-E stained samples (Figure 2D).” Your suggestions have been addressed in the revised manuscript, which we feel has now been greatly improved as a result.

**Comment 3)**
Serum ALT and AST values should be included in Table 2.
**Response:** Thank you for your useful suggestion. According to your suggestion, we have included the serum ALT measurements in Table 2. We feel that our manuscript has now been greatly improved as a result.

**Comment 4)**
The fibrosis stage in Table 3 should be verified by Sirius red staining.
**Response:** Thank you for your useful suggestion. According to your suggestion, we have performed a Sirius red staining examination. The results did not show any evidence of significant liver fibrosis. We have added the following sentences to the Methods section (page 11, lines 16-20): “Liver samples were excised and embedded in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA, USA) and paraffin for the histological analysis. Formalin-fixed and paraffin-embedded sections were processed routinely using hematoxylin and eosin (H&E), myeloperoxidase (MPO), naphthol AS-D chloroacetate esterase [21] and Sirius-red.” We have also added the following sentence to the Results section (page 16, lines 3-7): “The liver tissue MPO and Sirius red staining examinations showed no significant positive findings (Figure 2D and 2E), and the additional liver tissue naphthol AS-D chloroacetate esterase staining examination showed negative findings (data not shown).” Your suggestions have been addressed in the revised manuscript, which we feel has now been greatly improved as a result.

**Comment 5)**
The relationship between the changes of hepatic blood flow and the degrees of liver injury should be further established.
**Response:** Thank you for your useful suggestion. As you pointed out, the relationship between the changes in hepatic blood flow and the degree of liver injury should be examined in more detail to clarify the exact mechanism resulting in this phenomenon. Unfortunately, this is one of the limitations of our study; therefore, according to your suggestion, we have added the following sentence mentioning this limitation to the
Discussion section (page 22, lines 3-6): “Further studies are needed to verify hepatic mitochondrial respiration markers and hepatic inflammation states, and to examine the relationship between the changes in hepatic blood flow and the degree of liver injury in order to determine the exact underlying mechanism.”

Comment 6)
To be consistent with Fig. 2 C&D, Fig. 3 also should include four groups instead of two groups.
Response: Thank you for your useful suggestion. According to your suggestion, we have divided the results of the hepatic tissue blood flow measurements shown in Fig. 3 into four groups for each of the right and left lobes. We feel that the figure has now been greatly improved as a result.

Comment 7)
In “List of abbreviations”, the font and size should be consistent.
Response: Thank you for your useful suggestion. According to your suggestion, we have changed the font and size used in the “List of abbreviations” and have changed the format to that used in *BMC Gastroenterology* (page 23, line 1-page 24, line 2).

Response to Reviewer 2
Thank you very much for your useful suggestions. Your suggestions have been addressed in the revised manuscript, which we feel has now been greatly improved as a result.

Comment
In this manuscript entitled “Deficiency of Endothelial Nitric Oxide Synthase Exacerbates Early-stage Non-Alcoholic Fatty Liver Disease Pathogenesis by Changing the Fat Distribution” submitted by Nozaki et al, the authors aimed to demonstrate the role of endothelial nitric oxide synthase (eNOS) derived nitric oxide (NO) in the pathogenesis of NAFLD / NASH. There are several factors or molecules are responsible for the pathogenesis of non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH), but the role of eNOS derived NO in the pathogenesis of NAFLD / NASH is not yet well defined. Therefore to investigate their hypothesis authors performed experiments in systemic eNOS-knockout mice with high-fat diet. Four groups of mice were used in this study. Those mice were (a) wild type eNOS+/+ with basal diet (BD), (b) wild type eNOS/-/ with basal diet (BD), (c)
wild type eNOS+/+ with High fed Diet (HFD), (d) wild type eNOS-/- with High fed Diet (HFD). To explore this study the authors performed measurement of different biochemical markers in plasma and serum, liver triglyceride content, liver/spleen ratio of CT values and visceral fat volume as well. The authors determined the insulin tolerance test (ITT), liver MTP activity assay and hepatic tissue blood flow. CT scan has also been performed. The authors conducted several experiments as well, like as histopathological study on liver and immunohistochemical study as well. The authors also performed qPCR for evaluating at these genes expressions (SREBP-1c, PPAR-#, nNOS, iNOS). It is an interesting study. As a whole the expression of this manuscript is well described. So before going for final submission of this manuscript, it is recommended to take care of this manuscript in terms of the following minor revision. Minor concerns are mentioned below:

**Minor Concerns:**

**Comment 1)**

The manuscript should be carefully checked and need to follow the manuscript format criteria for this journal.

**Response:** Thank you for your useful suggestions. According to your suggestion, we have carefully rechecked the manuscript and have employed a Scientific English editing company (Edanz Group) to revise the manuscript to ensure that it follows the manuscript format criteria for *BMC Gastroenterology*.

**Comment 2)**

It is good to revise by Scientific English editor.

**Response:** Thank you for your useful suggestion. According to your suggestion, we have now had the manuscript revised by a Scientific English editing company (Edanz Group).

**Comment 3)**

The title could be more firm such as “Deficiency of eNOS Exacerbates Early-stage NAFLD Pathogenesis by Changing the Fat Distribution”.

**Response:** Thank you for your useful suggestion. According to your suggestion, we have now changed the title (page 1, lines 1-2). We feel that the manuscript has now been greatly improved as a result.

**Comment 4)**

Materials and Methods section need to be organized in order of experiment, for an
example all the measurements need to be one after another then all the assays and then histological study and then qPCR (gene expression study).

**Response:** Thank you for your useful suggestion. According to your suggestion, we have revised the Methods section (page 9, line 18- page 12, line 7), which we feel has now been greatly improved as a result.

**Comment 5)**
Figure 4-1 is not clear enough, because all the lines for different groups are overlapped. Even though the error bar as well. So it is good to use different color line for each group. So the figure should be clearly visible. Or the figure could be represented by bar graph either way.

**Response:** Thank you for your useful suggestion. According to your suggestion, we have used different colored lines for each group in Fig. 4-1. We feel that Fig. 4-1 has now been greatly improved as a result.