

Opposing Roles of Nitric Oxide and Rho-Kinase in Lipid Metabolism in Mice

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Dyslipidemia is a life-style disorder and is one of the important risk factors of cardiovascular diseases. Nitric oxide (NO) exerts beneficial effects on lipid metabolism through activation of hepatic sterol regulatory element-binding protein (SREBP)-2, a transcriptional factor for cholesterol metabolism and expression of LDL receptor, while Rho-kinase, an effector protein of small G protein, RhoA, contributes to the pathogenesis of metabolic syndrome through suppressing the whole body energy consumption. However, the crosstalk between NO and Rho-kinase in regulation of lipid metabolism remains to be elucidated. In the present study, we used male wild-type (WT) mice and mice lacking three isoforms of NO synthase (NOSs^{-/-}). WT mice were fed either normal diet (ND) or high-fat diet (HFD), while NOSs^{-/-} mice were fed ND with or without a selective Rho-kinase inhibitor, fasudil (100 mg/kg/day), for 6 weeks. At 6 weeks, plasma NOx concentration was significantly decreased and Rho-kinase activity and lipid levels were significantly elevated in HFD-fed WT mice and NOSs^{-/-} mice compared with ND-fed WT mice. In the liver, SREBP-2 activity was reduced in NOSs^{-/-} mice. Fasudil ameliorated lipid levels in HFD-fed WT mice and NOSs^{-/-} mice without affecting SREBP-2 activity or LDL receptor expression, whereas it significantly enhanced phosphorylation of AMP-activated kinase (AMPK) in the liver and skeletal muscle. Importantly, the beneficial metabolic effects of fasudil were absent in HFD-fed AMPK^{-/-} mice. These results provide the first evidence that NO and Rho-kinase play opposing roles for the lipid metabolism, suggesting that Rho-kinase inhibitors could be novel therapeutic agents of dyslipidemia.

Keywords: adenosine-5'-monophosphate-activated protein kinase; dyslipidemia; lipid metabolism; nitric oxide; Rho-kinase

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Introduction

Dyslipidemia is a life-style disorder caused by excessive food intake and low physical activity and is one of the important risk factors of cardiovascular diseases (Lim et al. 2011). Although several therapeutic agents are currently available for the treatment of dyslipidemia (e.g. statins, fibrates and ezetimibe), novel therapeutic strategy remains to be developed (Mora et al. 2013).

Impaired production of nitric oxide (NO) by endothelial NO synthase (eNOS), neuronal NO synthase (nNOS) and inducible NO synthase (iNOS) is associated with metabolic disorders (Monti et al. 2003; Yatera et al. 2010), and conversely, metabolic disorders (e.g. hypertension, diabetes and dyslipidemia) further accelerate endothelial dysfunction (Shimokawa 1999). eNOS is thought to be an up-stream molecule of metabolic disorder, because either eNOS^{-/-} mice or n/i/eNOSs^{-/-} mice showed the phenotypes of meta-

bolic syndrome (Duplain et al. 2001; Nakata et al. 2008) and conversely, in endothelium-specific eNOS transgenic mice, diet-induced hypercholesterolemia was prevented (Sansbury et al. 2012). Moreover, activity of sterol regulatory element-binding protein (SREBP)-2, a transcriptional factor for cholesterol metabolism and expression of low-density lipoprotein (LDL) receptor were decreased in high-fat diet (HFD)-fed n/i/eNOSs^{-/-} mice with resultant severe dyslipidemia (Yatera et al. 2010). These findings suggest that eNOS dysfunction is involved in the pathogenesis of dyslipidemia in addition to endothelial dysfunction.

Rho-kinase, an effector of small G protein, RhoA, plays important roles in the pathogenesis of atherosclerotic cardiovascular diseases through vascular smooth muscle proliferation, migration and contraction (Shimokawa and Takeshita 2005). Recently, we have demonstrated that Rho-kinase inhibits AMP-activated kinase (AMPK), one of the key molecules of energy metabolism, with resultant reduc-

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tion in whole body energy consumption and dyslipidemia, suggesting that Rho-kinase is an important therapeutic target of metabolic disorders (Noda et al. 2014).

NO and Rho-kinase are known to negatively interact each other in vasomotor regulation (Takemoto et al. 2002). Rho-kinase suppresses eNOS expression through destabili-

zation of eNOS mRNA, while NO inhibits RhoA expression and Rho-kinase activity through the cGMP and protein kinase G (PKG) pathway (Sauzeau et al. 2003; Kato et al. 2012). Moreover, Rho-kinase inhibits Akt (Wolfrum et al. 2004) and AMPK (Noda et al. 2014), the up-stream molecules of eNOS. However, it remains to be elucidated

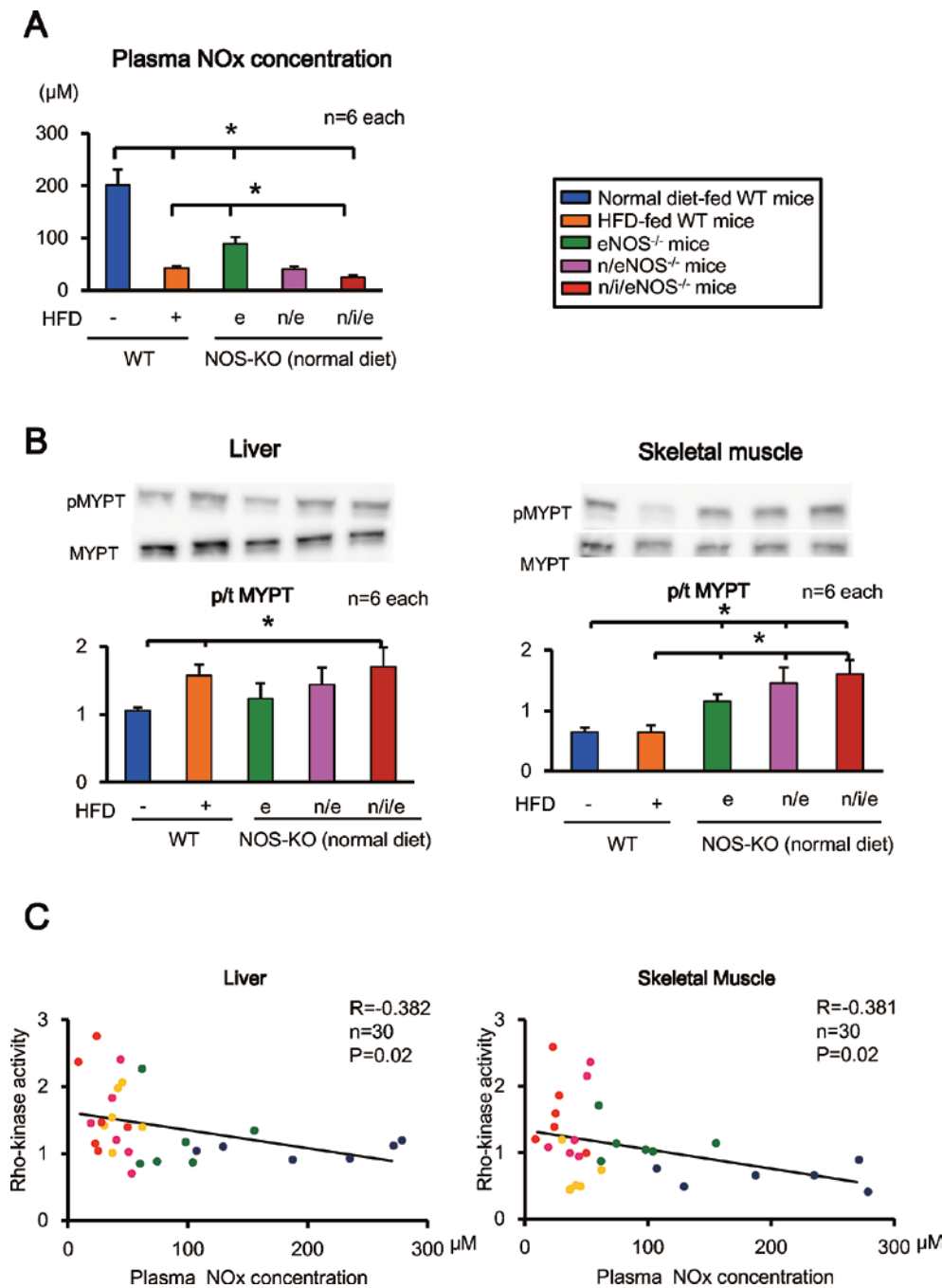


Fig. 1. Plasma NOx concentration and Rho-kinase phosphorylation.

(A) Plasma NOx (NO_2^- and NO_3^-) concentration was measured by Griess method. The NOx concentration was highest in ND-fed WT mice, decreased in ND-fed eNOS^{-/-} mice, and further decreased in HFD-fed WT mice, ND-fed n/eNOS^{-/-} and n/i/eNOS^{-/-} mice. (B) Rho-kinase activity was measured in the liver and the skeletal muscle. Rho-kinase activity was increased in n/i/eNOS^{-/-} mice in both the liver and the skeletal muscle. (C) A significant negative correlation was noted between Rho-kinase activity in the liver and skeletal muscle and plasma NOx concentration. Statistical analysis was performed by one-way ANOVA (A, B) and Spearman correlation coefficients analysis (C). Results are expressed as mean \pm SEM. * $P < 0.05$.

whether NO and Rho-kinase negatively interact in lipid metabolism and if so, what molecular mechanism(s) is involved.

In the present study, we thus addressed this important issue by employing mice lacking singly NOS^{-/-} (eNOS^{-/-}), doubly NOS^{-/-} (n/eNOS^{-/-}), and triply NOS^{-/-} (n/i/eNOS^{-/-}) and AMPK^{-/-} mice.

Methods

Animal preparation

Animal care and the experimental procedures were approved by the Guidelines on Animal Experiments of Tohoku University and the Japanese Government Animal Protection and Management Law (No. 105-2011). C57BL/6N mice were purchased from CREA Japan Inc. (Tokyo, Japan). eNOS^{-/-} mice that were backcrossed to C57BL/6 at least 10 times were originally provided by P. Huang (Harvard Medical

School, Boston, MA). We generated n/i/eNOS^{-/-} mice by crossing doubly NOS^{-/-} mice, as previously reported (Morishita et al. 2005). AMPK α_2 ^{-/-} mice were generated by breeding with CMV-Cre mice and AMPK α_2 floxed mice (Viollet et al. 2003). C57BL/6N mice were fed either a normal diet (ND, CE-2; CREA Japan Inc., Tokyo, Japan) or HFD (60% lard; D12492; Research Diet, NJ, USA) and AMPK α_2 ^{-/-} mice were fed HFD from 6-week-old for 6 weeks. NOS^{-/-} mice were fed a ND. The animals were anesthetized with inhaled isoflurane and intraperitoneal pentobarbital (50 mg/kg), humanely killed by overdose of anesthetic and cervical dislocation, and then the blood was collected from the heart using a plastic microsyringe. Then, the liver and skeletal muscle were excised and weighed, and were prepared for the experiments. We used the soleus as skeletal muscle in all experiments.

Western blot analysis

Western blot analysis was performed using antibodies that spe-

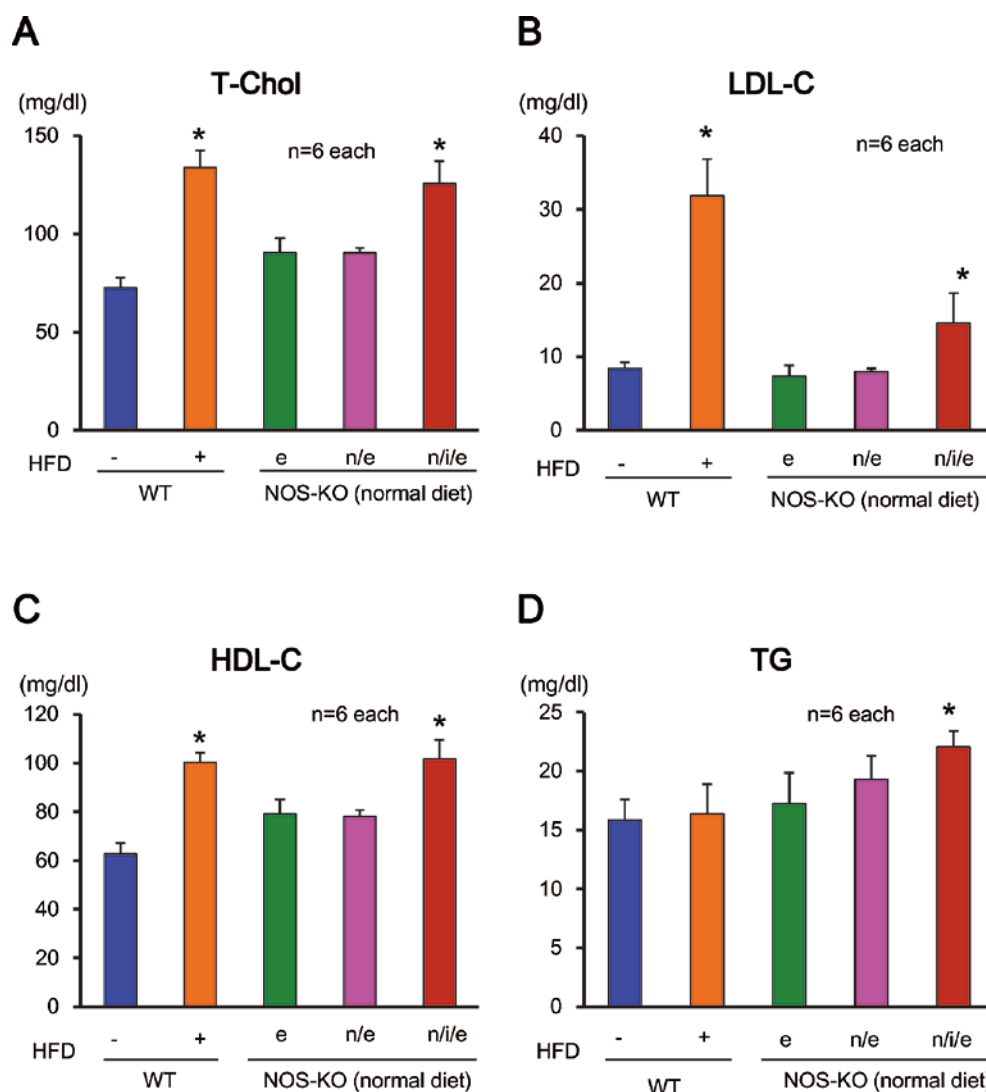


Fig. 2. Abnormal lipid metabolism in n/i/eNOS^{-/-} mice.

(A, C) T-Chol and HDL-C were significantly and similarly increased in HFD-fed WT mice and ND-fed n/i/eNOS^{-/-} mice. (B) LDL-C was significantly increased in HFD-fed WT mice and n/i/eNOS^{-/-} mice. (D) TG was significantly increased in n/i/eNOS^{-/-} mice. Statistical analysis was performed by one-way ANOVA. Results are expressed as mean \pm SEM. **P* < 0.05.

cifically recognize proteins, including AMPK α (Cell Signaling Technology, MA, USA), phospho-AMPK α at Thr172 (p-AMPK, Cell Signaling Technology, MA, USA), SREBP-2 (BD Biosciences, CA, USA), LDL receptor (BD Biosciences, CA, USA), myosin phosphatase targeting subunit-1 (MYPT-1) (BD Biosciences, CA, USA), phospho-MYPT-1 at Thr696 (p-MYPT1, Cell Signaling Technology), eNOS (BD Biosciences, CA, USA) and phospho-eNOS at Ser1177 (BD Biosciences, CA, USA). As a marker of Rho-kinase activity, we

examined the ratio of phospho-MBS to total MBS by Western blot analysis. As a marker of SREBP-2 activity, we examined the expression levels of activated form of SREBP-2 (68 kDa) by Western blot analysis, as reported previously (Yatera et al. 2010). The same amount of extracted protein (10–20 μ g) was loaded for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblot analysis. The regions containing proteins were visualized using ECL prime Western blotting detection system (Amersham

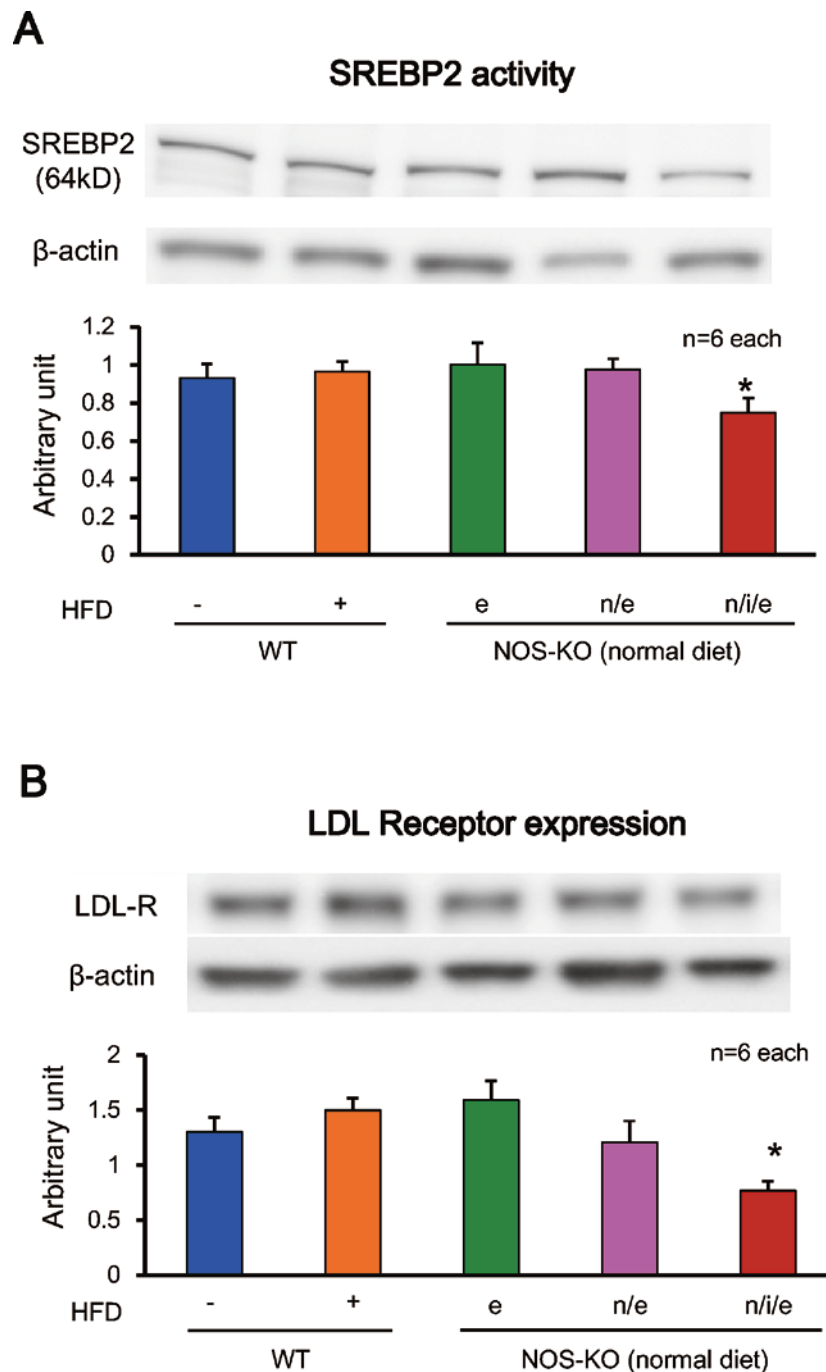


Fig. 3. Hepatic SREBP-2 activity and LDL receptor expression.

(A, B) Both SREBP-2 activity and LDL receptor expression in the liver were significantly decreased only in ND-fed *n/i/eNOS*^{-/-} mice. Results are normalized by the expression of β -actin. Statistical analysis was performed by one-way ANOVA. Results are expressed as mean \pm SEM. * $P < 0.05$.

Biosciences, Buckinghamshire, UK). Each band was normalized by corresponding value of β -actin as an internal control. Densitometric analysis was performed by Image J (NIH) Software (Noda et al. 2012).

Lipid analysis

Serum levels of lipoproteins were analyzed by an on-line dual enzymatic method for simultaneous quantification of total cholesterol, free cholesterol, phospholipids and triglycerides by high-performance liquid chromatography using 2 different gel permeation columns, TSKgel LipopropakXL and Superose 6HR, at Skylight Biotech (Akita, Japan), according to the procedure as previously described (Usui et al. 2002).

Plasma levels of nitrite and nitrate (NOx)

Plasma levels of NOx were analyzed by the Griess method as summation of NO_2^- and NO_3^- , as previously reported (Suda et al. 2002).

Metabolic assessment

Oxygen consumption and carbon dioxide generation was measured by RQ-5000 as previously described (Uno et al. 2006).

Statistical analysis

Comparison of parameters between 2 groups was performed with unpaired Student's *t*-test. Statistical analysis was performed by

one-way ANOVA followed by Bonferroni/Dunn's post-hoc test for multiple comparisons. Relations between variables were determined by Spearman correlation coefficients analysis. Statistical significance was evaluated with IBM SPSS statistics ver. 21 (IBM, NY, USA). A *P* value of < 0.05 was considered to be statistically significant.

Results

Plasma levels of NOx and Rho-kinase activity

We measured plasma levels of NOx and Rho-kinase activity to clarify the relation between the plasma levels of NOx and Rho-kinase activity of each organ in particular metabolic states. Plasma levels of NOx were significantly decreased in HFD-fed wild-type (WT) mice and $\text{eNOS}^{-/-}$, $\text{n/eNOSs}^{-/-}$ and $\text{n/i/eNOSs}^{-/-}$ mice compared with ND-fed WT mice (Fig. 1A). Rho-kinase activity, as evaluated by the extent of phosphorylation of myosin phosphatase targeting subunit 1 (MYPT) at Thr696 (Shimizu et al. 2013), was significantly increased in the liver of HFD-fed WT and $\text{n/i/eNOSs}^{-/-}$ mice and in the skeletal muscle of $\text{NOSs}^{-/-}$ mice (Fig. 1B). Moreover, there was a negative correlation between plasma NOx levels and Rho-kinase activity in the liver and the skeletal muscle among WT and $\text{NOSs}^{-/-}$ mice (Fig. 1C).

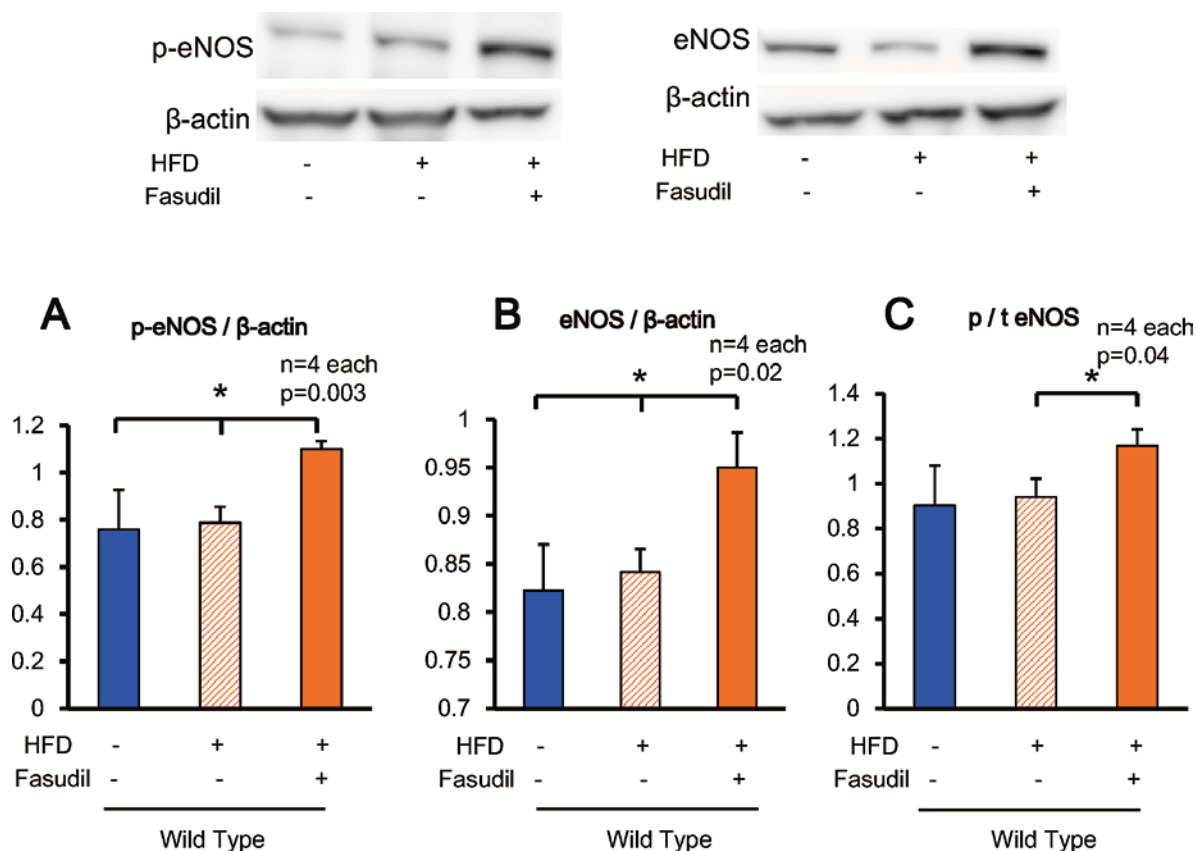


Fig. 4. Effects of fasudil on eNOS in the liver of HFD-fed WT mice.

(A-C) Long-term treatment with fasudil significantly enhanced eNOS phosphorylation, expression and activity (as evaluated by p/t eNOS) in the liver of HFD-fed WT mice. Results are normalized by the expression of β -actin. Statistical analysis was performed by one-way ANOVA. Results are expressed as mean \pm SEM. **P* < 0.05.

Abnormal lipid metabolism in *n/i/eNOS*^{-/-} mice

We measured plasma lipid levels in each genotype of mice to evaluate their lipid metabolism. In WT mice, serum levels of total cholesterol (T-Chol), LDL-C and high-density lipoprotein (HDL)-C were significantly increased in HFD-fed mice compared with ND-fed mice (Fig. 2A-D). Similarly, serum levels of the 3 lipids and triglycerides (TG) were all significantly increased in ND-fed *n/i/eNOS*^{-/-} mice (Fig. 2A-D). The expression of LDL receptor is regulated by hepatic SREBP-2. Activation of SREBP-2 is mediated by a posttranslational cleavage in which the immature protein (120 kDa) is enzymatically truncated into

a smaller mature protein (68 kDa) that enters the nucleus and increases transcription of LDL receptor gene (Li et al. 2011). Thus, the SREBP activity was assessed by the expression levels of the activated form of SREBP-2 (68 kDa), as reported previously. Both SREBP-2 activity and LDL receptor expression in the liver were significantly decreased only in ND-fed *n/i/eNOS*^{-/-} mice (Fig. 3A and B). These results indicate that cholesterol absorption in hepatocytes is decreased and cholesterol metabolism is deteriorated in *n/i/eNOS*^{-/-} mice.

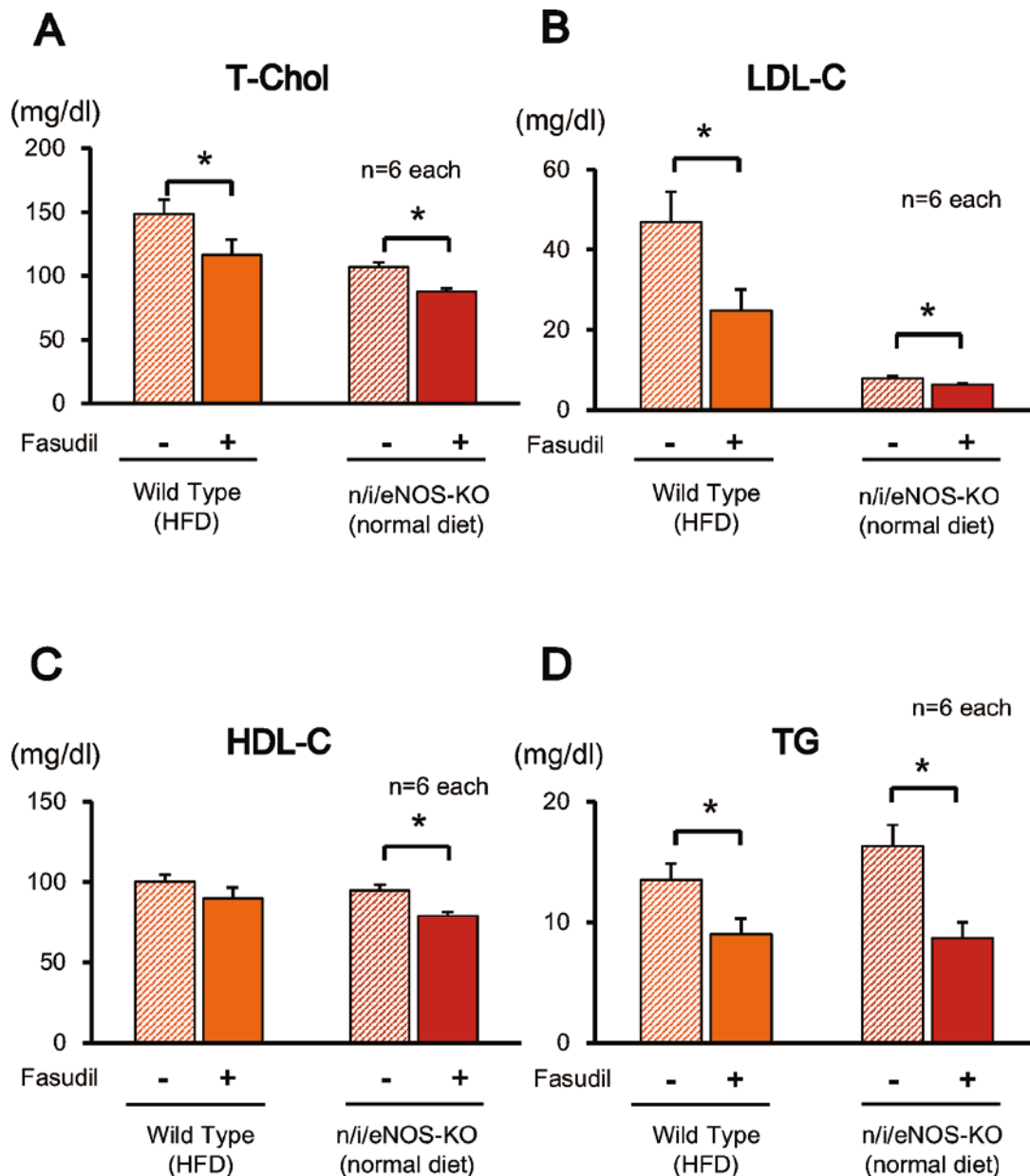


Fig. 5. Effects of fasudil on HFD-fed WT mice and ND-fed *n/i/eNOS*^{-/-} mice.

(A-D) Long-term treatment with fasudil significantly decreased all lipid levels (T-Chol, LDL-C, HDL-C and TG) in both HFD-fed WT mice and ND-fed *n/i/eNOS*^{-/-} mice except for HDL-C in HFD-fed WT mice. Statistical analysis was performed by unpaired Student's *t*-test. Results are expressed as mean \pm SEM. **P* < 0.05.

Fasudil increases eNOS expression and phosphorylation in HFD-fed WT mice

Since Rho-kinase inhibited the expression and activity of eNOS (Takemoto et al. 2002; Sauzeau et al. 2003), we examined the effects of fasudil on eNOS expression and activity in WT mice. As expected, long-term treatment with fasudil significantly enhanced eNOS phosphorylation, expression and activity (as evaluated by phosphorylated/total eNOS) in the liver of HFD-fed WT mice (Fig. 4).

Fasudil improves dyslipidemia in HFD-fed WT mice and *n/i/eNOS*^{-/-} mice

To reveal the role of NO and Rho-kinase in lipid metabolism, we next examined whether fasudil treatment improves lipid profile in HFD-fed WT and *n/i/eNOS*^{-/-} mice. In WT mice, fasudil treatment significantly decreased T-Chol, LDL-C and TG (Fig. 5A-D). Interestingly, T-Chol, LDL-C, HDL-C and TG were all decreased by fasudil treatment even in *n/i/eNOS*^{-/-} mice (Fig. 5A-D), suggesting that the cholesterol-lowering effect of Rho-kinase inhibition was independent of eNOS up-regulation. Fasudil treatment had no effect on hepatic SREBP-2 activity in WT or *n/i/eNOS*^{-/-} mice, whereas it significantly decreased LDL receptor expression in WT but not in *n/i/eNOS*^{-/-} mice (Fig. 6).

Fasudil increases AMPK phosphorylation in HFD-fed WT mice and *n/i/eNOS*^{-/-} mice

To elucidate the mechanisms of the beneficial effects of fasudil on lipid metabolism, we examined AMPK activ-

ity, one of the key molecules of energy metabolism. Fasudil treatment significantly increased phosphorylations of AMPK and acetyl CoA (ACC) in the liver and ACC phosphorylation in the skeletal muscle in WT mice (Fig. 7A and B). Interestingly, these effects of fasudil were unaltered in *n/i/eNOS*^{-/-} mice (Fig. 7C and D). Moreover, fasudil treatment significantly increased O₂ consumption and CO₂ production, but not locomotor activity, in HFD-fed WT mice (Fig. 8).

No noticeable effects of fasudil on dyslipidemia in HFD-fed *AMPK*_{α2}^{-/-} mice

Finally, we examined whether fasudil exerts beneficial effects on lipid metabolism even in *AMPK*_{α2}^{-/-} mice. Serum lipid levels were comparable between HFD-fed *AMPK*_{α2}^{-/-} mice with fasudil and those without it (Fig. 9). Furthermore, the beneficial effects of fasudil on O₂ consumption and CO₂ production were undetectable in *AMPK*_{α2}^{-/-} mice (Fig. 10).

Discussion

The major findings of the present study in mice are that (1) both HFD and genetic deletion of NOSs caused dyslipidemia associated with Rho-kinase activation and reduced SREBP-2 activity and LDL receptor expression in the liver and (2) long-term inhibition of Rho-kinase with fasudil ameliorated HFD-induced dyslipidemia associated with AMPK phosphorylation and increased energy consumption. To the best of our knowledge, this is the first study that demonstrates that NO and Rho-kinase negatively

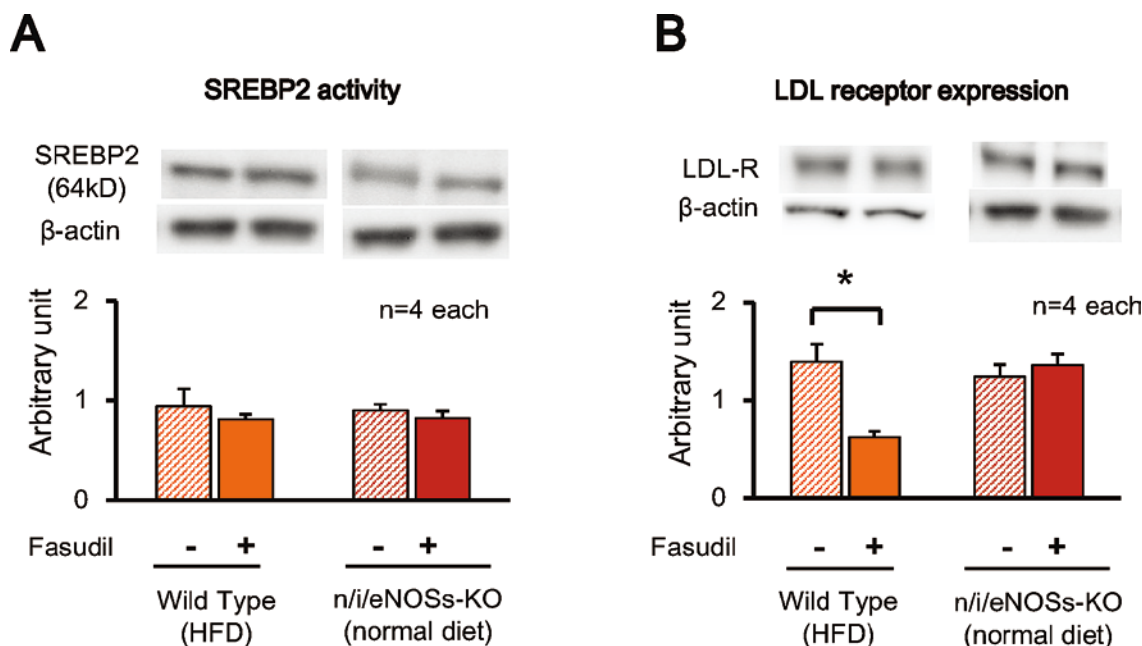


Fig. 6. Effects of fasudil on SREBP activity and LDL receptor expression in the liver.

(A) Long-term treatment with fasudil had no effects on SREBP2 activity in the liver in HFD-fed WT mice and ND-fed *n/i/eNOS*^{-/-} mice. (B) The fasudil treatment significantly decreased LDL receptor expression in the liver in HFD-fed WT mice but not in *n/i/eNOS*^{-/-} mice. Results are normalized by the expression of β -actin. Statistical analysis was performed by unpaired Student's *t*-test. Results are expressed as mean \pm SEM. **P* < 0.05.

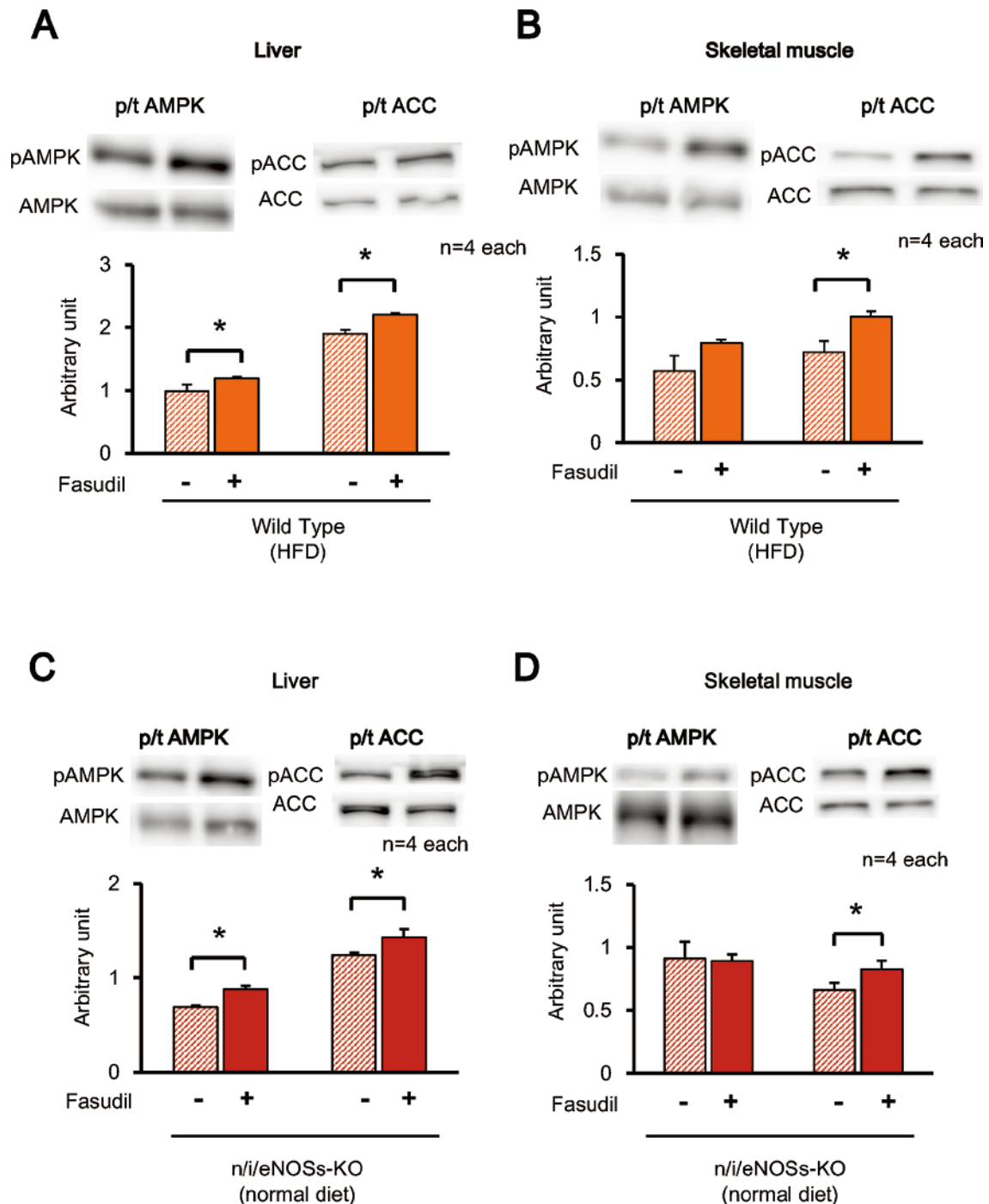


Fig. 7. Effects of fasudil on AMPK and ACC activities in HFD-fed WT mice and *n/i/eNOSs*^{-/-} mice. (A, C) In the liver, the fasudil treatment significantly increased AMPK and ACC activities in both genotypes. (B, D) In the skeletal muscle, the long-term treatment with fasudil significantly increased ACC activity but not AMPK activity in both HFD-fed WT mice and *n/i/eNOSs*^{-/-} mice. Statistical analysis was performed by unpaired Student's *t*-test. Results are expressed as mean \pm SEM. **P* < 0.05.

interact in lipid metabolism, for which SREBP-2/LDL receptor pathway activation by NO and AMPK inhibition by Rho-kinase may be involved.

Mouse models of metabolic disorders

Previous report showed that SREBP-2/LDL receptor

system was decreased in HFD-fed *n/i/eNOSs*^{-/-} mice (Vanhoutte 2009). In the present study, we used HFD-fed WT mice as a model of metabolic disorder. On the other hands, we fed *NOSs*^{-/-} (*eNOS*^{-/-}, *n/eNOSs*^{-/-} and *n/i/eNOSs*^{-/-}) mice with ND since these mice are known as animal models of metabolic disorders (Nakata et al. 2008).

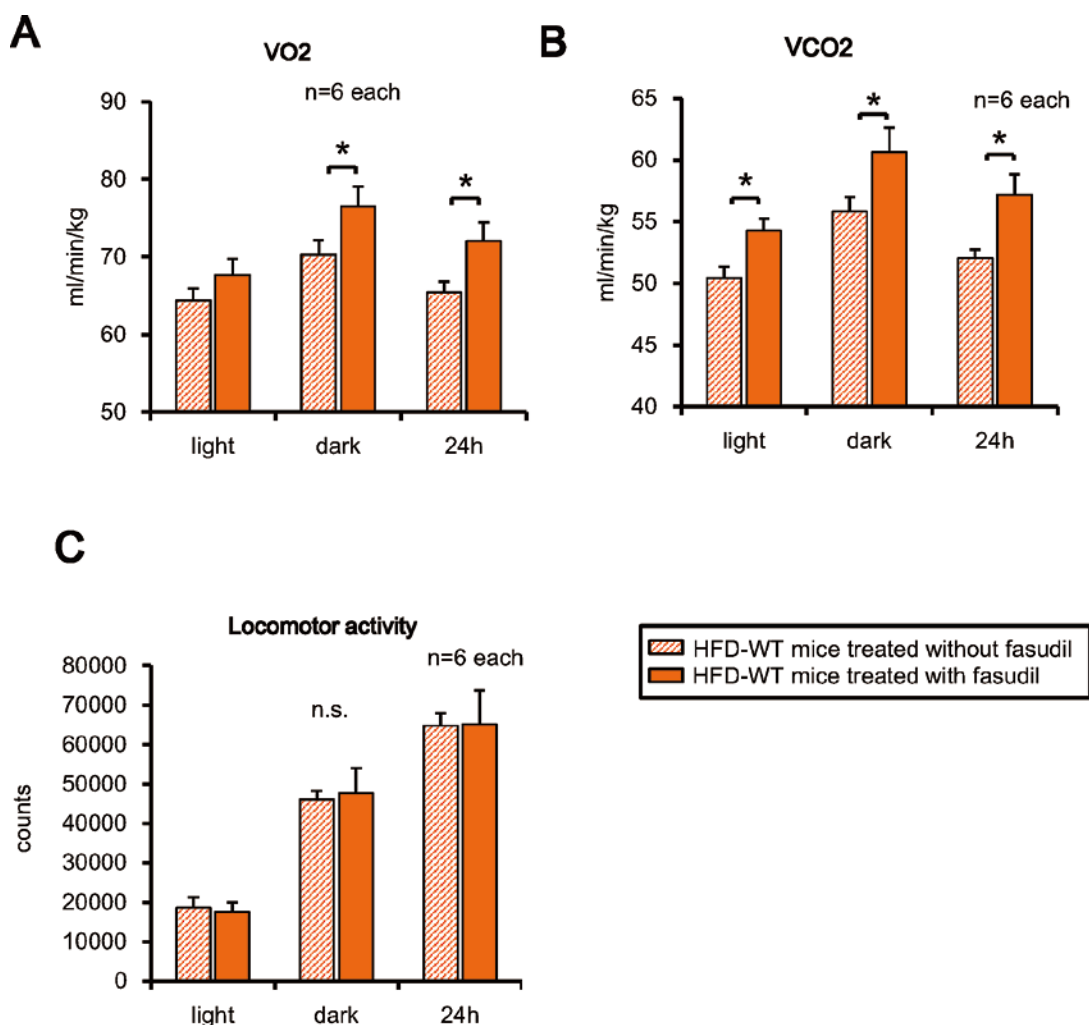


Fig. 8. Energy expenditure in HFD-fed WT mice.

(A) O₂ consumption was increased in HFD-fed WT mice treated with fasudil at dark period and 24 hours but not at light period. (B) CO₂ generation was increased in HFD-fed WT mice treated with fasudil at light period, dark period and 24 hours. (C) Locomotor activity was unaltered by fasudil. Statistical analysis was performed by unpaired Student's *t*-test. Results are expressed as mean ± SEM. **P* < 0.05.

Metabolic disorders, such as hypertension, diabetes and dyslipidemia, cause endothelial dysfunction characterized by eNOS down-regulation and inactivation (Yatera et al. 2010). On the other hand, eNOS inactivation has been thought to be up-stream mechanisms of metabolic disorders (Duplain et al. 2001; Nakata et al. 2008; Sansbury et al. 2012). Both eNOS^{-/-} and n/i/eNOSs^{-/-} mice show the phenotypes of metabolic syndrome (Duplain et al. 2001; Nakata et al. 2008), while in endothelium-specific eNOS transgenic mice, diet-induced hypercholesterolemia was prevented (Sansbury et al. 2012). These findings suggest that eNOS inactivation is up-stream of metabolic disorder, but not the simple result of endothelial dysfunction. It has been reported that SREBP-2 activity and LDL receptor expression were decreased in HFD-fed n/i/eNOSs^{-/-} mice associated with severe dyslipidemia (Yatera et al. 2010). Although it has been reported that serum lipids levels are increased in ND-fed n/i/eNOSs^{-/-} mice (Nakata et al. 2008),

we showed that SREBP-2 activity and LDL receptor expression were decreased even in ND-fed n/i/eNOSs^{-/-} mice in the present study.

Negative interactions between NO and Rho-kinase in lipid metabolism

Rho-kinase plays important roles in the pathogenesis of atherosclerotic cardiovascular diseases through vascular smooth muscle proliferation, migration and contraction (Shimokawa et al. 2001; Shimokawa and Takeshita 2005). Furthermore, the role of Rho-kinase in the pathogenesis of metabolic disorders has also attracted much attention recently, as it was reported that Rho-kinase is activated in metabolic syndrome in animals (Kikuchi et al. 2007; Hara 2011) and humans (Liu et al. 2007).

We have recently demonstrated that Rho-kinase negatively regulates AMPK α_2 activation in vivo and in vitro (Noda et al. 2014). AMPK α_2 is one of the molecules for

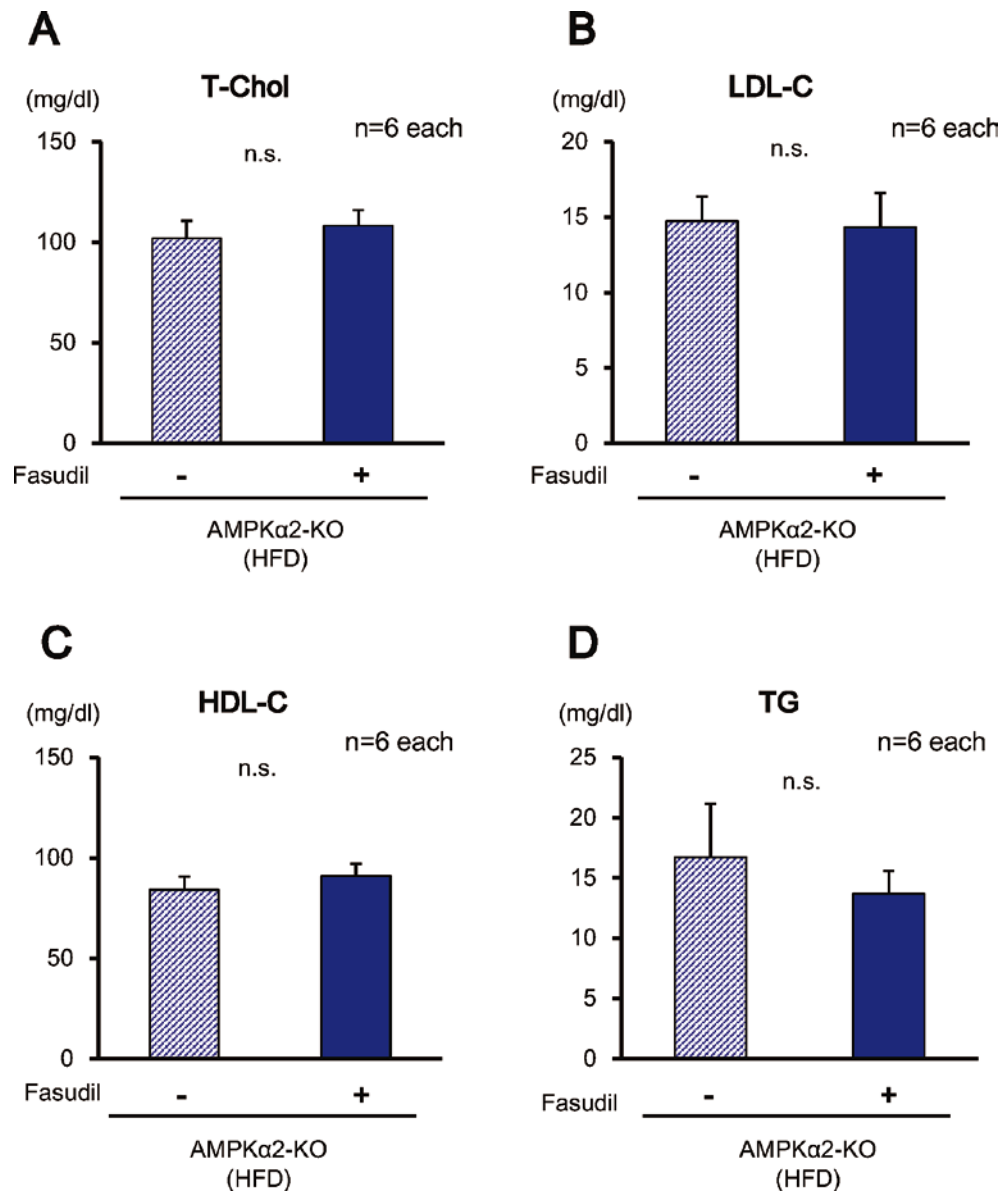


Fig. 9. No Effects of fasudil on dyslipidemia in HFD-fed AMPK $\alpha_2^{-/-}$ mice.

(A-D) The long-term treatment with fasudil had no noticeable effects on dyslipidemia in HFD-fed AMPK $\alpha_2^{-/-}$ mice. Statistical analysis was performed by unpaired Student's *t*-test. Results are expressed as mean \pm SEM.

intracellular fuel gage (Pedersen and Febbraio 2012) and Rho-kinase inhibition increases the whole body energy consumption through AMPK α_2 activation, with consequent reduction in plasma lipids levels (Noda et al. 2014). In the present study, we demonstrated that Rho-kinase inhibition increases AMPK phosphorylation and increases energy consumption, resulting in amelioration of lipid profiles even in *n/i/eNOS* $^{-/-}$ mice. These results indicate that lipid-lowering effects of Rho-kinase inhibition are mediated by AMPK phosphorylation but not by eNOS activation.

Rho-kinase causes instability of eNOS mRNA (Takemoto et al. 2002), whereas NO inhibits RhoA expression and Rho-kinase activity (Sauzeau et al. 2003; Kato et al. 2012). Moreover, Rho-kinase inhibits phosphorylation

of Akt (Wolfrum et al. 2004) and AMPK (Noda et al. 2014), the up-stream molecules of eNOS. Thus, Rho-kinase and eNOS inhibit each other. In the present study, we demonstrated that Rho-kinase was activated in *n/i/eNOS* $^{-/-}$ mice in the liver and skeletal muscle and fasudil up-regulated eNOS activity in the liver. Indeed, there was a negative correlation between Rho-kinase activity in the liver and skeletal muscle and plasma levels of NO_x in mice independent of their genetic backgrounds.

Study limitations

Several limitations should be mentioned for the present study. First, it remains to be elucidated how NO regulates SREBP-2 activity and LDL receptor expression.

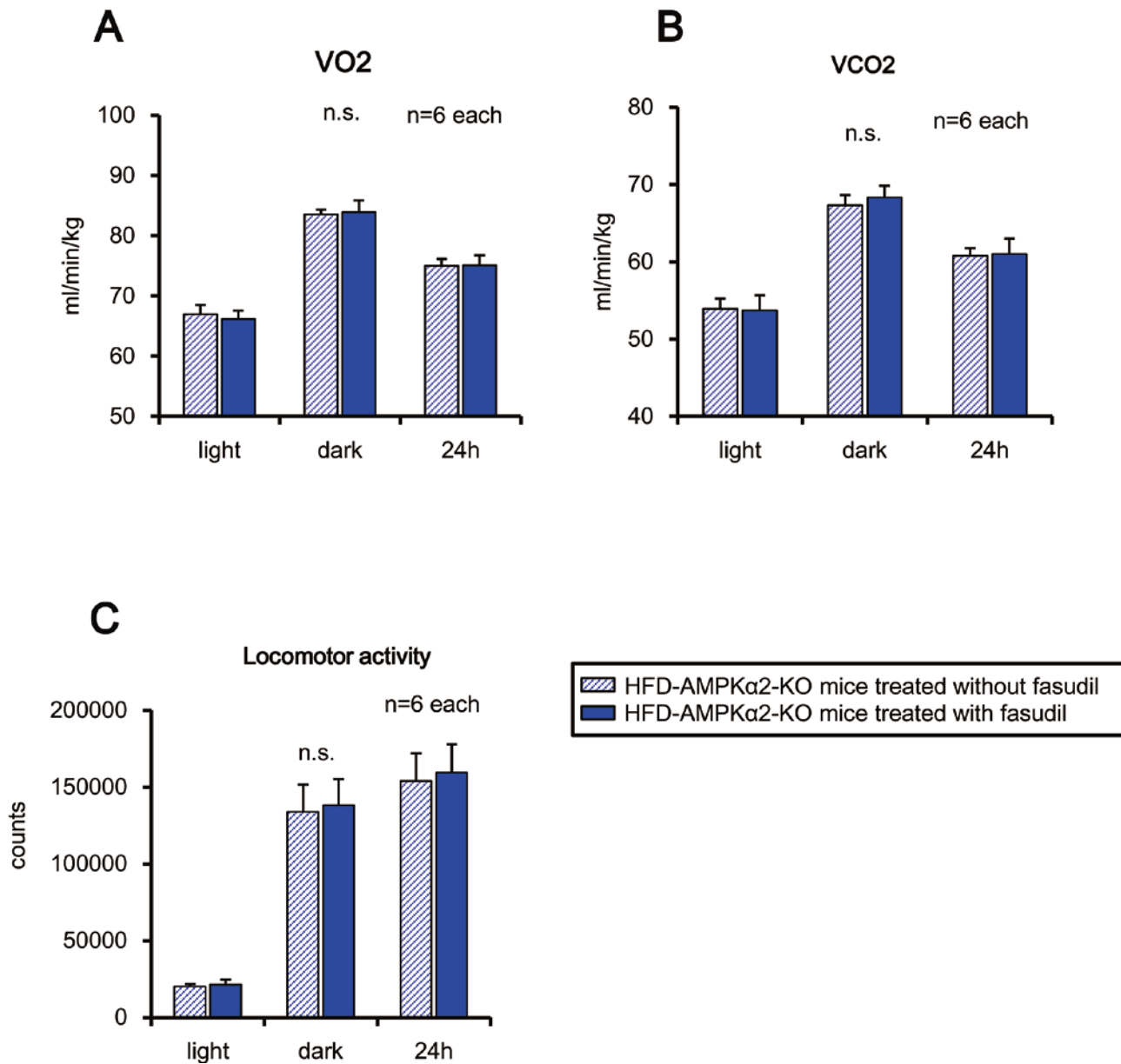


Fig. 10. Energy expenditure in HFD-fed AMPK $\alpha_2^{-/-}$ mice.

(A) O₂ consumption was comparable between HFD-fed AMPK $\alpha_2^{-/-}$ mice with and those without fasudil treatment at light period, dark period and 24 hours in female mice. (B) CO₂ generation was comparable between HFD-fed AMPK $\alpha_2^{-/-}$ mice with and those without fasudil treatment at light period, dark period and 24 hours in female mice. (C) Locomotor activity was unaltered between HFD-fed AMPK $\alpha_2^{-/-}$ mice with and those without fasudil treatment. Statistical analysis was performed by unpaired Student's *t*-test. Results are expressed as mean \pm SEM.

Second, there were some discrepancies in plasma levels of NOx and serum levels of lipids between WT and NOS $^{-/-}$ mice; plasma NOx levels were reduced in all HFD-fed WT and ND-fed NOS $^{-/-}$ mice, while serum lipids levels were increased only in HFD-fed WT and ND-fed n/i/eNOS $^{-/-}$ mice, but not eNOS $^{-/-}$ and n/eNOS $^{-/-}$ mice. These results suggest the involvement of some other factors in the serum lipids levels in addition to NO and Rho-kinase. Third, in the present study, we only examined pharmacological inhibition of Rho-kinase by fasudil but did not examine genetic inhibition using dominant-negative ROCK transgenic mice

(Noda et al. 2014) or ROCK-deficient mice (Shimizu et al. 2013). Finally, since fasudil is known to inhibit some other kinases such as PRK2 that is also one of the Rho effector proteins at similar doses (Kanazawa et al. 2009), all the beneficial effects of fasudil in the present study might not be mediated by Rho-kinase inhibition. This point also remains to be examined in future studies.

Conclusions

In the present study, we were able to demonstrate that NO and Rho-kinase negatively interact in lipid metabolism;

namely, NO activates the SREBP-2/LDL receptor pathway, while Rho-kinase inhibits AMPK. We propose that Rho-kinase may be a novel therapeutic target of metabolic disorders.

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Conflict of Interest

The authors declare no conflict of interest, except for H.S. who is a consultant of Asahi Kasei Pharma Corporation.

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