Metformin Protects Endothelial Function in Diet-Induced Obese Mice by Inhibition of Endoplasmic Reticulum Stress Through 5′ Adenosine Monophosphate–Activated Protein Kinase–Peroxisome Proliferator–Activated Receptor δ Pathway

Wai San Cheang, Xiao Yu Tian, Wing Tak Wong, Chi Wai Lau, Susanna Sau-Tuen Lee, Zhen Yu Chen, Xiaqiang Yao, Nanping Wang, Yu Huang

Objective—5′ Adenosine monophosphate–activated protein kinase (AMPK) interacts with peroxisome proliferator–activated receptor δ (PPARδ) to induce gene expression synergistically, whereas the activation of AMPK inhibits endoplasmic reticulum (ER) stress. Whether the vascular benefits of antidiabetic drug metformin (AMPK activator) in diabetes mellitus and obesity is mediated by PPARδ remains unknown. We aim to investigate whether PPARδ is crucial for metformin in ameliorating ER stress and endothelial dysfunction induced by high-fat diet.

Approach and Results—Acetylcholine-induced endothelium-dependent relaxation in aortae was measured on wire myograph. ER stress markers were determined by Western blotting. Superoxide production in mouse aortae and NO generation in mouse aortic endothelial cells were assessed by fluorescence imaging. Endothelium-dependent relaxation was impaired and ER stress markers and superoxide level were elevated in aortae from high-fat diet–induced obese mice compared with lean mice. These effects of high-fat diet were reversed by oral treatment with metformin in diet-induced obese PPARδ wild-type mice but not in diet-induced obese PPARδ knockout littermates. Metformin and PPARδ agonist GW1516 reversed tunicamycin (ER stress inducer)-induced ER stress, oxidative stress, and impairment of endothelium-dependent relaxation in mouse aortae as well as NO production in mouse aortic endothelial cells. Effects of metformin were abolished by cotreatment of GSK0660 (PPARδ antagonist), whereas effects of GW1516 were unaffected by compound C (AMPK inhibitor).

Conclusions—Metformin restores endothelial function through inhibiting ER stress and oxidative stress and increasing NO bioavailability on activation of AMPK/PPARδ pathway in obese diabetic mice. (Arterioscler Thromb Vasc Biol. 2014;34:830-836.)

Key Words: endothelium • nitric oxide • obesity • vasodilation

Obesity is a prevalent chronic disorder clustering with type 2 diabetes mellitus and cardiovascular diseases. The complex mechanisms of its pathogenesis and effective therapeutic approaches are of great interest nowadays. Metformin, a common antidiabetic drug, improves insulin sensitivity and glucose homeostasis and is demonstrated to activate 5′ adenosine monophosphate–activated protein kinase (AMPK) in tissues in humans and rodents. AMPK controls systemic energy balance and metabolism, and its activation exerts vasoprotective effects by inhibiting vascular smooth muscle cell proliferation and increasing NO production. Metformin is found to improve endothelial function and reduce cardiovascular risks in diabetic patients. Peroxisome proliferator–activated receptor δ (PPARδ) is ubiquitously expressed, for example, in adipose and endothelial cells. PPARδ activation promotes fatty acid β-oxidation in adipocytes and skeletal muscle, depletes lipid accumulation, and reduces obesity. PPARδ also regulates glucose homeostasis, as well as protects against atherosclerosis and endothelial dysfunction. Notably, AMPK is shown to form a transcriptional complex with PPARδ and act corporately to regulate transcription of several PPAR target genes, such as uncoupling protein 3 and lipoprotein lipase. AMPK activation in endothelial cells suppresses endoplasmic reticulum (ER) stress, which is associated with atherosclerosis, insulin resistance, obesity, and type 2 diabetes.
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diabetes mellitus.\(^{10}\) On the contrary, AMPK inhibition promotes ER stress and atherosclerosis.\(^ {11}\) To date, no study has examined the possible role of PPAR\(\delta\) in alleviating ER stress and subsequent endothelial dysfunction on AMPK activation. Therefore, we hypothesize that metformin protects against endothelial dysfunction induced by high-fat diet through restoration of AMPK/PPAR\(\delta\) signaling, leading to the inhibition of ER stress and superoxide generation and thus increasing NO bioavailability in the vascular wall.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
PPAR\(\delta\) Contributes to the Beneficial Effect of Metformin on Endothelial Function in High-Fat Diet–Induced Obese Mice and in Mouse Aortae Ex Vivo

Endothelium-dependent relaxation (EDR) in response to acetylcholine was reduced in aortae from both diet-induced obese (DIO) PPAR\(\delta\) wild-type (WT) and knockout (KO) mice compared with control, whereas oral administration of AMPK activator metformin (100 mg/kg per day; 1 week) restored impaired EDR in DIO PPAR\(\delta\) WT (Figure 1A and 1B) but not in KO mice (Figure 1C). In addition, incubation with AMPK inhibitor compound C (5 \(\mu\)mol/L; 30 minutes) inhibited metformin-induced improvement of EDR (Figure 1B). By contrast, phenylephrine-induced contractions in aortae with or without perivascular fats were similar in PPAR\(\delta\) WT and KO mice (Figure I in the online-only Data Supplement). A positive role of ER stress in vascular dysfunction was demonstrated by impaired EDR induced by ex vivo exposure to tunicamycin (ER stress inducer; 2 \(\mu\)g/mL; 16 hours). Coincubation with metformin (100 \(\mu\)mol/L) restored tunicamycin-attenuated EDR, which was inhibited by PPAR\(\delta\) antagonist GSK0660 (500 nmol/L) in aortic rings from C57BL/6J mice (Figure 1D). PPAR\(\delta\) agonist GW1516 (100 nmol/L) also reversed tunicamycin-induced impairment of EDR, which was unaffected by compound C (Figure 1E). One-week treatment with tauroursodeoxycholic acid (ER stress alleviator) at 100 mg/kg per day also markedly improved vasorelaxation in DIO PPAR\(\delta\) WT mice (Figure IIA in the online-only Data Supplement).

Acetylcholine-induced EDR was abolished by NO synthase inhibitor \(\text{L-NAME}\) (Figure III in the online-only Data Supplement), and EDRs to sodium nitroprusside were comparable among groups (Figures IIB and IV in the online-only Data Supplement).

Metformin Activates AMPK Which Acts on PPAR\(\delta\) to Alleviate ER Stress in Aortae of DIO Mice

In aortae from DIO PPAR\(\delta\) WT mice, AMPK\(\alpha\) phosphorylation at Thr\(^{172}\) was downregulated (Figure 2A and 2B), whereas ER stress markers such as cleaved activating transcription factor 6 (ATF6), phosphorylated eukaryotic initiation factor 2\(\alpha\) at Ser\(^{52}\), ATF3, and spliced X-box binding protein 1 were elevated compared with lean control (Figure 2A and 2C–2F).

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AMPK</td>
<td>5′ adenosine monophosphate–activated protein kinase</td>
</tr>
<tr>
<td>ATF6</td>
<td>activating transcription factor 6</td>
</tr>
<tr>
<td>DIO</td>
<td>diet-induced obese</td>
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<tr>
<td>EDR</td>
<td>endothelium-dependent relaxation</td>
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<td>eNOS</td>
<td>endothelial NO synthase</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>KO</td>
<td>knockout</td>
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<td>mouse aortic endothelial cell</td>
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<td>PPAR(\delta)</td>
<td>peroxisome proliferator–activated receptor (\delta)</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>wild-type</td>
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Figure 1. Role of peroxisome proliferator–activated receptor \(\delta\) (PPAR\(\delta\)) in the vascular benefit of metformin. A, Representative traces. B and C, Summarized data. Ach indicates acetylcholine; and Phe, phenylephrine. Endothelium-dependent relaxation in aortae from both diet-induced obese (DIO) PPAR\(\delta\) wild-type (WT) and knockout (KO) mice compared with control, whereas oral administration of AMPK activator metformin (100 mg/kg per day; 1 week) restored impaired EDR in DIO PPAR\(\delta\) WT (Figure 1A and 1B) but not in KO mice (Figure 1C). In addition, incubation with AMPK inhibitor compound C (5 \(\mu\)mol/L; 30 minutes) inhibited metformin-induced improvement of EDR (Figure 1B). By contrast, phenylephrine-induced contractions in aortae with or without perivascular fats were similar in PPAR\(\delta\) WT and KO mice (Figure I in the online-only Data Supplement). A positive role of ER stress in vascular dysfunction was demonstrated by impaired EDR induced by ex vivo exposure to tunicamycin (ER stress inducer; 2 \(\mu\)g/mL; 16 hours). Coincubation with metformin (100 \(\mu\)mol/L) restored tunicamycin-attenuated EDR, which was inhibited by PPAR\(\delta\) antagonist GSK0660 (500 nmol/L) in aortic rings from C57BL/6J mice (Figure 1D). PPAR\(\delta\) agonist GW1516 (100 nmol/L) also reversed tunicamycin-induced impairment of EDR, which was unaffected by compound C (Figure 1E). One-week treatment with tauroursodeoxycholic acid (ER stress alleviator) at 100 mg/kg per day also markedly improved vasorelaxation in DIO PPAR\(\delta\) WT mice (Figure IIA in the online-only Data Supplement). Acetylcholine-induced EDR was abolished by NO synthase inhibitor \(\text{L-NAME}\) (Figure III in the online-only Data Supplement), and EDRs to sodium nitroprusside were comparable among groups (Figures IIB and IV in the online-only Data Supplement).
ER stress markers such as cleaved ATF6, phosphorylated eIF2α (Ser52), and ATF3 were also increased in aortae from DIO PPARδ KO mice. Metformin treatment (100 mg/kg per day; 1 week) enhanced AMPKα phosphorylation in both DIO PPARδ WT and KO mice. However, metformin treatment diminished ER stress in DIO PPARδ WT but not in DIO PPARδ KO mice (Figure 2).

PPARδ Antagonist Blocks the Effect of Metformin in Alleviating ER Stress in Mouse Aortae

Coincubation with metformin (100 μmol/L) enhanced AMPKα phosphorylation at Thr172 (Figure 3A and 3B) and decreased tunicamycin (2 μg/mL; 16 hours)-induced upregulation of ER markers, such as cleaved ATF6, phosphorylated eIF2α (Ser52), and ATF3, in aortae from C57BL/6J mice (Figure 3A and 3C). These inhibitory effects of metformin on ER stress were diminished by GSK0660 (500 nmol/L). GW1516 (500 nmol/L) did not affect AMPK activity (Figure 3D and 3E), whereas it reduced the expression of ER stress markers (Figure 3D and 3F). The effect of GW1516 was unaffected by AMPK inhibitor compound C (5 μmol/L; Figure 4C).

Genetic Knockouts or Antagonists of PPARδ Abolish Metformin-Induced Enhancement of NO Bioavailability in Mouse Aortae and Mouse Aortic Endothelial Cells

In aortae from DIO PPARδ WT mice, phosphorylation of endothelial NO synthase (eNOS) at Ser1177 was reduced, whereas metformin treatment (100 mg/kg per day; 1 week) promoted phosphorylation; nevertheless, metformin failed to enhance eNOS phosphorylation in aortae from DIO PPARδ KO mice (Figure 4D). Tunicamycin (2 μg/mL; 16 hours) decreased phosphorylation of eNOS (Figure 4E and 4F) and inhibited acetylcholine (10 μmol/L)-stimulated nitrite level (Figure 5A) in mouse aortae. Effects of tunicamycin were reversed by cotreatment of metformin (100 μmol/L) and GW1516 (500 nmol/L). Furthermore, tunicamycin treatment reduced the level of phosphorylated eNOS in cultured mouse aortic endothelial cells (MAECs), and this effect was prevented by coinbubation with either metformin or GW1516. Again, PPARδ antagonist showed an upregulated expression of PPARδ in aortae. The ex vivo culture of aortae with tunicamycin (2 μg/mL; 16 hours) did not affect PPARδ expression. Coincubation with metformin (100 μmol/L) increased PPARδ expression, which was inhibited by PPARδ antagonist GSK0660 (500 nmol/L; Figure 4B). GW1516 (500 nmol/L) also increased PPARδ expression, which was unaffected by AMPK inhibitor compound C (5 μmol/L; Figure 4C).

Metformin Increases PPARδ Expression in Mouse Aortae

PPARδ was downregulated in DIO PPARδ WT mice as compared with control lean mice (Figure 4A). DIO PPARδ WT mice treated with metformin (100 mg/kg per day; 1 week) showed an upregulated expression of PPARδ in aortae. The ex vivo culture of aortae with tunicamycin (2 μg/mL; 16 hours) did not affect PPARδ expression. Coincubation with metformin (100 μmol/L) increased PPARδ expression, which was inhibited by PPARδ antagonist GSK0660 (500 nmol/L; Figure 4B). GW1516 (500 nmol/L) also increased PPARδ expression, which was unaffected by AMPK inhibitor compound C (5 μmol/L; Figure 4C).
GSK0660 reversed the effect of metformin (Figure V in the online-only Data Supplement). In MAECs, NO production as represented by the rise of 4-amino-5-methylamino-2',7'-difluorofluorescein fluorescence signal is triggered by the addition of Ca²⁺ ionophore A23187 (1 μmol/L). Tunicamycin (2 μg/mL; 16 hours) lowered NO production, which was restored by cotreatment with metformin (100 μmol/L), whereas GSK0660 (500 nmol/L) blocked the reversal effect of metformin (Figure 5B, 5C, and 5E). GW1516 (100 nmol/L) also augmented NO production in tunicamycin-treated MAECs, but its effect was not inhibited by compound C (5 μmol/L; Figure 5B, 5D, and 5E). Similar results were obtained in lysophosphatidylcholine (30 μmol/L; 16 hours)-treated MAECs (Figure VI in the online-only Data Supplement). Treating MAECs with metformin, GW1516, GSK0660, or compound C alone showed no difference from control (Figure VII in the online-only Data Supplement).

**Metformin Acts Through PPARδ to Inhibit Superoxide Production in Mouse Aortae**

The obese PPARδ WT and KO mice showed a higher level of oxidative stress in aortae compared with lean mice with the same genotype (Figure 6A and 6B). Chronic metformin treatment (100 mg/kg per day; 1 week) decreased the superoxide level in DIO PPARδ WT mice but was not effective in DIO PPARδ KO mice. Exposure to tunicamycin (2 μg/mL; 16 hours) markedly increased superoxide production in aortae from C57BL/6j mice, and this superoxide elevation was reversed by cotreatment with metformin (100 μmol/L; Figure 6C) or GW1516 (100 nmol/L; Figure 6D). GSK0660 (500 nmol/L) blocked the reversal effect of metformin on superoxide generation (Figure 6C). Representative images with larger magnification are shown in Figure VIII in the online-only Data Supplement.

**Discussion**

The present study demonstrates a critical role of PPARδ in inhibiting ER stress and protecting endothelial function by AMPK activation in diabetic obese mice. We observed impaired vasodilatation, upregulated expressions of ER stress markers, and increased superoxide generation in aortae from DIO mice, which were reversed by chronic administration of metformin in vivo. Importantly, these protective effects of metformin were absent in DIO PPARδ KO mice.

Extensive studies have shown that metformin exerts therapeutic effects, promotes eNOS activity, and improves vascular endothelial functions in diabetes mellitus through AMPK activation. The potent and selective AMPK inhibitor, compound C, abolished the vascular protective effect of metformin, supporting that metformin preserves endothelial function most likely via an AMPK-dependent mechanism. l-NAME totally inhibited EDRs in aortae, suggesting that the improved endothelial function by metformin is attributable to increased NO bioavailability.

Perivascular adipose tissue is shown to regulate vascular tone through the release of vasodilators. PPARδ is widely expressed in several tissues, including adipose tissue, and hence may affect vascular function and structural remodeling. However, phenylephrine-induced contractions were similar in aortae from PPARδ WT and KO mice, although they were reduced to the same degree by the presence of perivascular fats, implying that PPARδ is unlikely to affect vascular responses through acting on perivascular adipose tissue.
ER stress is mediated by 3 ER membrane–associated proteins that engage complex downstream signaling pathways, including cleavage of ATF6, activation of eIF2α/ATF3 pathway, and splicing of X-box binding protein 1. All these pathways were exaggerated in obese mice and were suppressed by metformin. Our observations are in line with previous studies reporting that AMPK activation improves vascular endothelial functions and mitigates ER stress and reactive oxygen species (ROS) production associated with diabetes mellitus. Furthermore, PPARδ is known to regulate cardiovascular function: its activation induces angiogenesis and vasculogenesis, inhibits atherosclerosis, and protects endothelial function in diabetic mice. We provide novel findings that PPARδ is downstream of AMPK activation to protect endothelial function, supported by a lack of effect of metformin in obese PPARδ KO mice. This study also demonstrates that PPARδ was downregulated in DIO PPARδ WT mice as compared with control lean mice (Figure 4A). This result agrees with previous studies that PPARδ expression was decreased in hearts and kidneys of diabetic rodents. In addition, metformin increased PPARδ expression in mouse arteries. Although the detailed mechanism of how AMPK/PPARδ regulate ER stress remains incompletely understood, AMPK activation can increase the expression of 150-kDa oxygen-regulated protein (OPR150), which is an ER-associated chaperone by enhancing forkhead box O1 (FOXO1) transcriptional activity and subsequently reduces hepatic ER stress in obese mice. PPARδ was found to act on FOXO signaling pathway as well. PPARδ activation also protects pancreatic β-cells from ER stress through promoting fatty acid oxidation. Further investigation is needed to uncover what PPARδ responsive genes are related to protein degradation and thus regulate ER stress in vasculature.

Metformin treatment did not ameliorate dyslipidemia but improved glucose metabolism (Table I in the online-only Data Supplement), which might partially contribute to the beneficial effect on vascular function. Therefore, we performed ex vivo culture of mouse aortae with tunicamycin that impairs EDR, thus providing direct evidence that ER stress damages endothelial function. Metformin, as well as PPARδ agonist GW1516, reversed impairments in aortae treated with tunicamycin, where ambient glucose and lipid levels were constant. These results strongly suggest that metformin has a direct protective effect on EDR through alleviation of ER stress, independent of any effects on glucose or lipid metabolism in our mouse model of obesity.

AMPK phosphorylation has multiple targets including biosynthetic enzymes, such as acetyl-CoA carboxylase, hydroxymethylglutaryl-CoA reductase, glycogen synthase, and both eNOS and neuronal NO synthase. Whereas, PPARδ agonists GW0742 and L-165041 at higher concentrations (>1 μmol/L) can directly induce vasodilatation, eNOS phosphorylation, and NO production. In addition, our recent study demonstrated that another PPARδ agonist GW1516 protects endothelial function in diabetic db/db mice through an increase of eNOS activity and NO production. The present data show that PPARδ mediates the effect of metformin in increasing eNOS phosphorylation in both mouse aortae and MAECs, as well as NO production, which are consistent with aforementioned observations that PPARδ improves NO bioavailability.
Previous studies also implicated ER stress in oxidative stress. The accumulation of misfolded protein in ER causing ER stress leads to ROS generation through several possible mechanisms.\(^{24}\) First, misfolded proteins bind protein chaperones that consume ATP, stimulating mitochondrial oxidative phosphorylation and, therefore, producing ROS as a byproduct. Second, hydrogen peroxide may be produced during disulfide bond formation in the ER. Alternatively,
glutathione may be depleted during reduction of unstable or improper disulfide bonds in misfolded proteins, followed by a rise in ROS production. Finally, ER stress can lead to Ca\(^{2+}\) leak from the ER and then Ca\(^{2+}\) overload in the mitochondria, None. This work was supported by grants from Hong Kong Research Grant Council (CUHK2/CRF/12G and CUHK464712), National Basic Investment Scheme B.

Although the present study also examined the beneficial effect of metformin against ER stress stimulated by lysophosphatidylcholine, a major component of oxidized low-density lipoprotein-cholesterol in cultured MAECs, we recognize that this in vitro model cannot faithfully mimic the obese condition in mice. In future studies, attempt will be made to culture aortic endothelial cells from DIO mice and to confirm the pathological role of ER stress in vascular dysfunction related to obesity and diabetes mellitus. The present results indicate that the ability of metformin to improve endothelial function was mediated through AMPK and PPAR\(\delta\) with a subsequent alleviation of ER stress and oxidative stress, as well as enhancement of eNOS activity and NO production. These findings highlight the decisive role of PPAR\(\delta\) downstream of AMPK activation to combat against vascular dysfunction in diabetes mellitus and obesity.

Sources of Funding

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Disclosures

None.

References


Significance

5' Adenosine monophosphate–activated protein kinase (AMPK) activation inhibits endoplasmic reticulum (ER) stress, and metformin (AMPK activator) reduces cardiovascular risks in diabetes mellitus. AMPK binds to peroxisome proliferator–activated receptor \(\delta\) (PPAR\(\delta\)). However, whether PPAR\(\delta\) plays a positive role in the beneficial effects of metformin against vascular dysfunction in obesity and diabetes mellitus is unknown. The present study indicates that induction of ER stress leads to elevated oxidative stress and decreased NO bioavailability, subsequently causing endothelial dysfunction. Metformin treatment alleviates ER stress, reduces oxidative stress, increases endothelium-derived NO production, and improves endothelial function through the action of PPAR\(\delta\) in diet-induced obese mice. Overall, these new findings highlight that PPAR\(\delta\) is an essential component downstream of AMPK activation to combat against vascular dysfunction in diabetes mellitus and obesity.
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Metformin Protects Endothelial Function in Diet-Induced Obese Mice by Inhibition of Endoplasmic Reticulum Stress through AMPK-PPARδ Pathway

Wai San Cheang¹; Xiao Yu Tian¹,²; Wing Tak Wong²; Chi Wai Lau¹; Susanna Sau-Tuen Lee³; Zhen Yu Chen³; Xiaoqiang Yao¹; Nanping Wang⁴; and Yu Huang¹

SUPPLEMENTAL MATERIAL

Supplemental Figures

Supplemental Figure I. Phenylephrine (Phe)-induced contractions in aortae were similar in PPARδ wild-type (WT) and PPARδ knockout (KO) mice. Vasoconstrictions in aortae with intact perivascular fats from PPARδ WT and PPARδ KO mice were not significantly different (n.s.) in A the presence of endothelium (+endo) or in B the absence of endothelium (-endo). Data are means ± SEM (n= 4).

Supplemental Figure II. Vasorelaxations in aortae from lean and diet-induced obese (DIO) PPARδ wild-type (WT) mice after tauroursodeoxycholic acid (TUDCA) treatment. Administration of ER stress alleviator TUDCA at 100 mg/kg/day for one week improved A acetylcholine (Ach)-induced endothelium-dependent relaxations in aortae from DIO mice but did not alter B sodium nitroprusside (SNP)-induced endothelium-independent relaxations. Data are means ± SEM (n= 3). *P<0.05 vs Lean; †P<0.05 vs DIO
Supplemental Figure III. Acetylcholine (Ach)-induced endothelium-dependent relaxations in lean PPARδ wild-type (WT) and PPARδ knockout (KO) mice were mainly mediated through nitric oxide pathway. Vasorelaxations in aortae with or without the presence of nitric oxide synthase inhibitor L-NAME. Data are means ± SEM (n= 4).

Supplemental Figure IV. Endothelium-independent relaxations in aortae were not altered in obese mice or by metformin treatment. A and B, Endothelium-independent relaxations were similar in aortae from lean and diet-induced obese (DIO) PPARδ wild-type (WT) and PPARδ knockout (KO) mice after metformin treatment for one week. C and D, Endothelium-independent relaxations were similar in all groups after ex vivo exposure to tunicamycin (tuni, 2 µg/mL), metformin (100 µmol/L), GSK0660 (500 nmol/L), GW1516 (100 nmol/L), or compound C (5 µmol/L) for 16 hours. Data are means ± SEM (n= 5-6).
**Supplemental Figure V.** Metformin increased eNOS activity in mouse aortic endothelial cells (MAECs). A-C. Protein expressions of phosphorylated eNOS at Ser\(^{1177}\) (p-eNOS) to total eNOS (t-eNOS, 140 kDa) in MAECs treated with tunicamycin (2 µg/mL), metformin (100 µmol/L), GW1516 (100 nmol/L), or compound C (5 µmol/L) for 16 hours. *P<0.05 vs Control; †P<0.05 vs Tuni; ‡P<0.05 vs Tuni+metformin. Data are means ± SEM (n= 5).

**Supplemental Figure VI.** Metformin enhanced nitric oxide (NO) bioavailability in mouse aortic endothelial cells (MAECs). A and B (summarized result) fluorescence imaging of A23187 (1 µmol/L)-stimulated NO production in MAECs treated with lysophosphatidylcholine (LPC, 30 µmol/L), metformin (100 µmol/L), GW1516 (100 nmol/L), or compound C (5 µmol/L) for 16 hours. The experiments were carried out at the same time; and thereby shared the same control and LPC-treated groups. *P<0.05 vs Control; †P<0.05 vs LPC; ‡P<0.05 vs LPC+Metformin. Data are means ± SEM (n= 4).
Supplemental Figure VII. Measurement of nitric oxide (NO) production in mouse aortic endothelial cells (MAECs). A and B (summarized result) fluorescence imaging of A23187 (1 µmol/L)-stimulated NO production in MAECs treated with metformin (100 µmol/L), GSK0660 (500 nmol/L), GW1516 (100 nmol/L), or compound C (5 µmol/L) individually for 16 hours. The experiments were carried out at the same time; and thereby shared the same control. Data are means ± SEM (n= 4).

Supplemental Figure VIII. Metformin decreased superoxide production in vascular walls. Representative images of intracellular superoxide production measured in the aortae from lean and diet-induced obese (DIO) PPARδ wild-type (WT) and PPARδ knockout (KO) mice after oral administration of metformin for one week with bigger magnification. Bar: 1000 µm.
Supplemental Table I. Basic parameters of *PPARδ* wild-type (WT) and *PPARδ* knockout (KO) mice on normal (control) or high fat diet (DIO) with or without chronic treatment of metformin.

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<td>52.97±2.61</td>
<td>80.08±5.96*</td>
<td>85.68±4.11*</td>
<td>49.40±1.58</td>
<td>70.25±1.97*</td>
<td>73.60±1.98*</td>
</tr>
<tr>
<td><strong>Non-HDL (mg/mL)</strong></td>
<td>30.26±5.31</td>
<td>86.51±13.66*</td>
<td>88.84±11.36*</td>
<td>8.33±0.93</td>
<td>33.14±3.26*</td>
<td>37.46±6.79*</td>
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<tr>
<td><strong>Non-HDL/HDL</strong></td>
<td>0.59±0.13</td>
<td>1.12±0.22*</td>
<td>1.04±0.13*</td>
<td>0.17±0.02</td>
<td>0.48±0.06*</td>
<td>0.51±0.08*</td>
</tr>
<tr>
<td><strong>Total cholesterol/HDL</strong></td>
<td>1.59±0.13</td>
<td>2.12±0.22*</td>
<td>2.04±0.13*</td>
<td>1.17±0.02</td>
<td>1.49±0.06*</td>
<td>1.51±0.08*</td>
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Data are means ± SEM. *P*<0.05 vs control; †*P*<0.05 vs DIO from each genotype.
Materials and Methods
Animal protocols
The present study was approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong and were consistent with the Guide for the Care and Use of laboratory Animals published by the National Institutes of Health. Male $\text{PPAR}^\delta$ wild-type (WT) and $\text{PPAR}^\delta$ knockout (KO) mice generated from C57BL/6N x Sv/129 background, and C57BL/6J mice were used for this study. $\text{PPAR}^\delta$ WT and KO mice were fed with a rodent diet with 45 kcal% fat (Research Diets Inc., New Brunswick, NJ, USA) for 12 weeks starting from 8 weeks old to generate high-fat diet-induced obese (DIO) mice. They were then treated with metformin (100 mg/kg/day; Sigma-Aldrich, St Louis, MO, USA) or taurine-conjugated ursodeoxycholic acid (TUDCA, 100 mg/kg/day; Sigma-Aldrich) suspended in distilled water or vehicle by oral gavages for 7 days. This concentration of metformin is safe for in vivo animal treatment as referenced to similar studies.2,4

Determination of plasma levels of glucose and cholesterol
After eight hours of fasting, blood was drawn from the mouse tail and blood glucose was measured with a commercial glucometer (Ascensia ELITE®, Bayer, Mishawaka, IN, USA). Plasma levels of total cholesterol, triglyceride, high-density lipoprotein (HDL) and non-HDL were determined by enzyme immunoassay (Mercodia, Sweden).

Functional assay by wire myograph
After mice were sacrificed, thoracic aortae were removed and dissected free adhering connective tissue in oxygenated ice-cold Krebs solution containing (mmol/L): 119 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1 MgCl$_2$, 25 NaHCO$_3$, 1.2 KH$_2$PO$_4$, and 11 D-glucose. Changes in isometric tone of aortic rings (~2 mm in length) were measured by wire myograph (Danish Myo Technology, Aarhus, Denmark). The rings were stretched to an optimal baseline tension of 3 mN and allowed to equilibrate for one hour. Rings were contracted with 60 mmol/L KCl and rinsed in Krebs solution. Endothelium-dependent relaxation (EDR) was determined by cumulative addition of acetylcholine (ACh, 3 nmol/L to 10 µmol/L; Sigma-Aldrich) in phenylephrine (Phe, 3 µmol/L; Sigma-Aldrich) precontracted rings. Some rings were incubated with potent and selective AMPK inhibitor compound C (5 µmol/L, 30 minutes; Sigma-Aldrich), that did not show significant inhibition of several structurally related kinases such as JAK3, PKA, PKCζ, SKY and ZAPK, before EDR measurement. Endothelium-independent relaxation to sodium nitroprusside (SNP, 1 nmol/L to 10 µmol/L; Sigma-Aldrich) was also tested.

Ex vivo culture of mouse aortic rings
Aortic rings were dissected in sterile phosphate buffered saline (PBS) and incubated in Dulbecco’s Modified Eagle’s Media (DMEM; Gibco, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS; Gibco), 100 µg/mL streptomycin and 100 U/mL penicillin. Tunicamycin (ER stress inducer, 2 µg/mL; Sigma-Aldrich), metformin (100 µmol/L), compound C (5 µmol/L), GW1516 ($\text{PPAR}^\delta$ agonist, 100 nmol/L; Alexis Biochemicals, Lausen, Switzerland), or GSK0660 (PPARδ antagonist, 500 nmol/L; Sigma-Aldrich) was added into the culture medium that bathed aortic rings in incubator at 37°C for 16 hours. After the incubation, rings were transferred to fresh Krebs solution for functional studies in myograph and Western blotting. Acetylcholine, phenylephrine, SNP and metformin were dissolved in water and others in DMSO.

Western blot analysis
Protein sample prepared from mouse aorta homogenates were electrophoresed through the 7.5% or 10% SDS-polyacrylamide gels and transferred to an immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). The 1% BSA in 0.05% Tween-20 PBS was used to block non-specific binding sites and the membranes were probed with primary antibodies against phosphorylated AMPKα at Thr$^{172}$, AMPKα (Cell Signaling Technology, Danvers, MA, USA), activating transcription factor 6 (ATF6; Abcam, Cambridge, MA, USA), $\alpha$-smooth muscle actin (Sigma-Aldrich), and GAPDH (Abcam, Cambridge, MA, USA). Protein bands were detected using an enhanced chemiluminescence system (Amersham, Piscataway, NJ, USA) and imaged with a Gel Doc 2000 system (Bio-Rad, Hercules, CA, USA).
UK), phosphorylated eukaryotic initiation factor 2 alpha (eIF2α) at Ser52 (Invitrogen, Carlsbad, CA, USA), eIF2α (Cell Signaling), ATF3, X-box binding protein 1 (XBP1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PPARδ (Cayman Chemical, Ann Arbor, MI, USA), phosphorylated endothelial nitric oxide synthase (eNOS) at Ser1177 (Abcam), eNOS (BD Transduction laboratory, San Diego, CA, USA), and GAPDH (Ambion, Austin, TX, USA), at 4 °C overnight; followed by incubation with horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Carpinteria, CA, USA) at room temperature for 2 hours. The membranes were developed with an enhanced chemiluminescence detection system (ECL reagents, Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed on X-ray films.

Detection of ROS by dihydroethidium (DHE) staining
Aortic rings were frozen and sliced into sections (10 μm) using a Leica CM 1000 cryostat before being incubated in DHE (5 μmol/L; Invitrogen)-containing normal physiological saline solution (NPSS) at 37 °C for 15 min. NPSS contained (mmol/L): 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 5 HEPES (pH 7.4). Fluorescence images were obtained with the Olympus Fluoview FV1000 laser scanning confocal system by measuring the fluorescence intensity at excitation 515 nm and emission 585 nm.

Determination of nitrite level
Aortae were treated with tunicamycin, metformin, compound C, GW1516 or GSK0660 for 16 hours, followed by the addition of 10 μmol/L acetylcholine for 10 min at 37 °C to stimulate nitric oxide generation and incubation with nitrate reductase to reduce nitrate to nitrite. Aortae were homogenized and supernatants were collected for quantification of total nitrite level using a colorimetric assay kit that involved the Griess reaction (Molecular Probes, Eugene, OR, USA). Absorbance was read at 548 nm and compared with a standard nitrite curve. Protein content was measured on tissue homogenate by the Bradford's method and used to normalize the nitrite values.

Primary culture of mouse aortic endothelial cells (MAECs)
Mice were anesthetized with an intra-peritoneal injection of pentobarbital sodium (40 mg/kg). Heparin (100 U/mL in PBS) was infused from the left ventricle into the circulation. Aortae were removed and dissected in sterile PBS. Incubating the aortae with collagenase type IA at 37 °C for 8 minutes detached the endothelial cells that were collected by centrifugation, re-suspended and cultured in endothelial cell growth medium (EGM) supplemented with bovine brain extract (Lonza, Walkersville, MD, USA) till confluency. The cultured MAECs were incubated with different drugs for 16 hours before the measurement of NO using laser confocal fluorescence microscopy.

Measurement of NO production in MAECs
MAECs were loaded with 2 μmol/L 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM DA; Molecular Probes) at room temperature for 10 min. The cells were stimulated with 1 μmol/L calcium ionophore A23187 (Sigma-Aldrich). The fluorescence intensity excited at 495 nm and emitted at 515 nm was determined using Olympus Fluoview FV1000 laser scanning confocal system. Changes in intracellular NO level were displayed as relative fluorescence intensity (F1/F0, where F0 = average fluorescence signals before addition of A23187 and F1 = fluorescence signal at defined time intervals after addition of A23187).

Statistical analysis
Results represent means ± SEM from different groups. The relaxation was presented as percentage reduction of the phenylephrine contraction. Protein expression was quantified by densitometer (FluorChem; Alpha Innotech, San Leandro, CA, USA), normalized to GAPDH and then compared with control. Data were analyzed using Graphpad Prism. Comparisons among groups were made using ANOVA followed by an unpaired Student's t-test. The results were considered statistically significant with P values <0.05.
References


