Role of Peroxisome Proliferator-Activated Receptors in Diabetic Vascular Dysfunction

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DECLARATION

The experiments described in this dissertation were carried out in the Department of Physiology, and later the Vascular and Metabolic Biology Laboratory of the School of Biomedical Sciences, the Chinese University of Hong Kong, between January 2008 and December 2010. This work is solely that of the author. No part of this dissertation is being concurrently submitted for any other degree, diploma or other qualification at this or any other institutions.

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I am grateful to my husband Jack Wong, who is also the person introducing me to this lab at the very beginning. Over the years, we worked together on many research projects and exchange opinions on science and life.

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Finally, I owe my deepest gratitude to my parents for their endless support, for allowing me to live a life far away from them, although they missed me a lot.

I always remember the poetry by Walt Whitman:"The untold want, by life and land ne'er granted, Now, Voyager, sail thou forth, to seek and find." I shall continue with the hard work and seek the truth of science.

ABSTRACT

Type 2 diabetes mellitus and obesity represent a global health problem worldwide. Most diabetics die of cardiovascular and renal causes, thus increasing the urgency in developing effective strategies for improving cardiovascular outcomes, particularly in obesity-related diabetes. Recent evidence highlights the therapeutic potential of peroxisome proliferators activated receptor (PPAR) agonists in improving insulin sensitivity in diabetes.

Firstly, I demonstrated that adipocyte-derived adiponectin serves as a key link in PPARγ-mediated amelioration of endothelial dysfunction in diabetes. Results from *ex vivo* fat explant culture with isolated arteries showed that PPARγ expression and adiponectin synthesis in adipose tissues correlate with the degree of improvement of endothelium-dependent relaxation in aortas from diabetic *db/db* mice. PPARγ agonist rosiglitazone elevates the adiponectin release and restores the impaired endothelium-dependent relaxation *ex vivo* and *in vivo*, in arteries from both genetic and diet-induced diabetic mice. The effect of PPARγ activation on endothelial function that is mediated through the adiponectin- AMP-activated protein kinase (AMPK) cascade is confirmed with the use of selective pharmacological inhibitors and *adiponectin*$^{-/-}$ or *PPARγ*$^{+/-}$ mice. In addition, the benefit of PPARγ activation *in vivo* can be transferred by transplanting subcutaneous adipose tissue from rosiglitazone-treated diabetic mouse to control diabetic mouse. I also revealed a direct effect of adiponectin to rescue endothelium-dependent relaxation in diabetic mouse aortas, which involves both AMPK and cyclic AMP-dependent protein kinase signaling pathways to enhance nitric oxide formation accompanied with inhibition of oxidative stress. These novel findings clearly demonstrate that adipocyte-derived adiponectin is prerequisite for PPARγ-mediated improvement of endothelial function in diabetes, and thus highlight the prospective of subcutaneous adipose tissue as a potentially important intervention target for newly developed PPARγ agonists in the alleviation of diabetic vasculopathy.

Aside from an indirect effect of PPARγ activation to reduce insulin resistance and to facilitate adiponectin release, PPARγ agonist could also exert direct effects on blood vessels. I provided a first line of experimental evidence demonstrating that PPARγ agonist rosiglitazone up-regulates the

endothelin B receptor (ET_BR) expression in mouse aortas and attenuates endothelin-1-induced vasoconstriction through an endothelial ET_BR-dependent NO-related mechanism. ET_BR up-regulation inhibits endothelin-1-induced endothelin A receptor (ET_AR)-mediated constriction in aortas and mesenteric resistance arteries, while selective ET_BR agonist produces endothelium-dependent relaxations in mesenteric resistance arteries. Chronic treatment with rosiglitazone *in vivo* or acute exposure to rosiglitazone *in vitro* up-regulate the ET_BR expression without affecting ET_AR expression. These results support a significant role of ET_BR in contributing to the increased nitric oxide generation upon stimulation with PPARγ agonist. This study provides additional explanation for how PPARγ activation improves endothelial function.

While agonists of PPARα and PPARγ are clinically used, PPARδ is the remaining subtype that is yet to be a target for current therapeutic drugs. Little is available in literature about the role of PPARδ in the regulation of cardiovascular function. The third part of my thesis focused on elucidating cellular mechanisms underlying the beneficial effect of PPARδ activation in the modulation of endothelial function in diabetes. PPARδ agonists restore the impaired endothelium-dependent relaxation in high glucose-treated aortas and in aortas from diabetic *db/db* mice through activation of a cascade involving PPARδ, phosphatidylinositol 3-kinase, and Akt. PPARδ activation increases Akt and endothelial nitric oxide synthase and nitric oxide production in endothelial cells. The crucial role of Akt is confirmed by selective pharmacological inhibitors and transient transfection of dominant negative Akt plasmid in these cells. Treatment with PPARδ agonist GW501516 *in vivo* augments endothelial function in diabetic *db/db* and diet-induced obese mice. The specificity of GW501516 for PPARδ is proven with the loss of its effect against high glucose-induced impairment of endothelium-dependent relaxation in aortas from *PPARδ* knockout mice. In addition, oral administration of GW501516 *in vivo* fails to improve endothelial function in diet-induced obese *PPARδ* deficient mice.

To summarize, the present investigation has provided a few lines of novel mechanistic evidence in support for the positive roles of PPARγ and PPARδ activation as potentially therapeutic targets to combat against diabetic vasculopathy.

論文摘要

糖尿病與肥胖症多發心血管並發症，開發治療心血管疾病的藥物成了當務之急。最近的研究顯示過氧化酶體增殖物激活受體PPARs可以改善糖尿病相關胰島素抵抗且有降血脂作用，因此本研究主要探討了PPAR受體激活對糖尿病血管病變的保護作用。

第一部分的實驗主要探討了脂肪細胞分泌的脂聯素(adiponectin)在胰島素增敏劑噻唑烷二酮(thiazolidinedione)即PPARγ激動劑rosiglitazone引起的血管保護作用中的功能。首先建立體外培養脂肪組織與離體血管的模型用來研究脂肪細胞分泌的脂肪因子與血管功能之間的直接作用。結果顯示PPARγ的表達，adiponectin的生成，與rosiglitazone刺激的脂肪組織產生adiponectin從而引起瘦素受體敲除*db/db*小鼠主動脈內皮依賴性舒張功能的改善正相關；Rosiglitazone在體外與體內實驗中均能改善*db/db*小鼠和高脂飲食小鼠主動脈的舒張功能。進一步的實驗利用各種抑製劑與adiponectin敲除和PPARγ雜合基因型小鼠證明，PPARγ激活引起的內皮細胞功能改善是通過激活脂肪細胞的PPARγ受體，生成adiponectin，並作用在內皮細胞的腺苷一磷酸激活蛋白激酶AMPK/內皮一氧化氮合成酶eNOS通路，增加AMPK與eNOS磷酸化，增加一氧化氮NO產生。Rosiglitazone慢性治療對*db/db*小鼠內皮的保護作用可以通過移植皮下脂肪組織轉移到對照組小鼠，顯示了皮下脂肪產生的adiponectin的重要性。這些結果顯示了脂肪產生的adiponectin在PPARγ激活改善內皮功能中的關鍵作用，提示了PPARγ在糖尿病血管病變治療中的重要性。

第二部分的實驗研究了PPARγ除了改善胰島素抵抗，增加adiponectin產生以外，在內皮細胞上的直接作用是通過上調內皮細胞上內皮素B型受體(ET_BR)的表達，增加NO產生，從而抑制的內皮素ET-1引起的收縮作用。Rosiglitazone作用於離體血管24小時後，ET_AR介導的ET-1引起的收縮降低，ET_BR表達增加。Rosiglitazone慢性治療C57BL/6J小鼠後，在主動脈與腸系膜阻力血管上，ET-1引起的收縮減少，ET_BR表達增加，ET_BR激動劑Sarafotoxin S6c可引起腸系膜阻力血管的NO依賴性的舒張。這些結果提示了ETBR是PPARγ的在內皮細胞上的一個重要靶點，進一步解釋了PPARγ的血管保護作用。

第三部分的實驗研究了PPARδ在糖尿病內皮功能失調中的作用與機理。PPARδ激動劑GW501516與GW0742可以恢復高糖處理C57小鼠主動脈後降低的內皮依賴性舒張功能，並改善*db/db*小鼠主動脈的舒張功能，恢復高糖處理後減少的原代小鼠主動脈內皮細胞中的NO釋放。使用PPARδ拮抗劑與PPARδ敲除小鼠證明了PPARδ激動劑的特異性。PPARδ激動劑對內皮依賴性舒張功能的改善和內皮細胞NO釋放可以被PI3K或Akt抑製劑阻斷，同時PPARδ激動劑也可增加Akt與eNOS的磷酸化。在db/db小鼠與高脂飲食小鼠中，GW501516的慢性治療可以改善內皮依賴性舒張功能，增加Akt與eNOS的磷酸化，在高脂飲食處理的PPARδ敲除小鼠中，GW501516的作用消失，證實了PPARδ的特異性。這些結果顯示，PPARδ通過激活PI3K/Akt/eNOS通路，增加NO釋放，從而改善糖尿病小鼠的內皮功能。

綜上所述，此論文的三項相關實驗研究提示了PPARγ與PPARδ在糖尿病血管功能障礙中的保護作用，這些信號傳遞為糖尿病血管病變的治療途徑提供了新的作用機理與新的藥物靶點。

ABBREVIATIONS

ACh:	acetylcholine
AMPK:	AMP-activated protein kinase
CA-Akt:	constitutively active Akt1 plasmid
DAF-FM:	4-Amino-5-methylamino-2',7'-difluorofluorescein
DIO:	diet-induced obese
DHE:	dihydroethidium
DKO:	double knockout
DMEM:	Dulbecco's Modified Eagle's Media
DMSO:	Dimethyl sulfoxide
DN-Akt:	dominant negative Akt1 construct
EDHF:	endothelium-derived hyperpolarizing factors
EDR:	endothelium-dependent relaxation
EDRF:	endothelium-derived relaxing factors
eNOS:	endothelial nitric oxide synthase
ET-1:	endothelin-1
ET_AR:	endothelin A receptor
ET_BR:	endothelin B receptor
HUVEC:	human umbilical vein endothelial cells
KO:	knockout
L-NAME:	N^G-nitro-L-arginine methyl ester
MAEC:	mouse aortic endothelial cells
MRA:	mesenteric resistance arteries
NO:	nitric oxide
PKA:	protein kinase A
PI3K:	phosphatidylinositol-3-kinase
PGI_2:	prostacyclin
PPAR:	peroxisome proliferator-activated receptor
PPARδ:	peroxisome proliferator-activated receptor delta
PPARγ:	peroxisome proliferator-activated receptors gamma
ROS:	reactive oxygen species
RXR:	retinoid receptor
TZD:	thiazolidinedione
WT:	wild-type

PUBLICATIONS and AWARDS

Publications

Original research article

1. Yuen, CY, Wong, WT, **Tian, XY**, Wong, SL, Lau, CW, Yu, J, Tomlinson, B, Yao, X, Huang, Y (2011) Telmisartan inhibits vasoconstriction via PPARγ-dependent expression and activation of endothelial nitric oxide synthase. ***Cardiovascular Research*** (In press)
2. Yang, Q, Xue, HM, Wong, WT, **Tian, XY**, Huang, Y, Tsui, KW, Ng, KS, Wohlfart, P, Li, H, Xia, N, Tobias, S, Underwood, MJ, He,GW (2011) Endothelial nitric oxide synthase enhancer AVE3085 restores endothelial function and reduces blood pressure in spontaneously hypertensive rats. ***British Journal of Pharmacology*** (Accepted) (**Co-first author**)
3. Yung, LM, Wong, WT, **Tian, XY**, Leung, FP, Chen, ZY, Lau, CW, Yao, X, Huang, Y (2011) Inhibition of renin-angiotensin system reverses endothelial dysfunction and oxidative stress during estrogen deficiency in ovariectomized rats. ***PLoS One*** (Accepted)
4. Wong, SL, Wong, WT, **Tian, XY**, Lau, CW, Huang, Y (2010) Prostaglandins in action: indispensible roles of cyclooxygenase-1 and -2 in endothelium-dependent contractions. In ***Advances in Pharmacology***, Ed: Paul M Vanhoutte (Invited book chapter) vol 60C:pp61-83.
5. Wong, WT, **Tian, XY**, Chen, Y, Leung, FP, Liu, L, Lee, HK, Ng, CF, Xu, A, Yao, X, Vanhoutte, PM, Tipoe, GL, Huang, Y (2010). Bone morphogenic protein-4 impairs endothelial function through oxidative stress-dependent cyclooxygenase-2 upregulation: implications on hypertension. ***Circulation Research*** 107(8): 984-991. (**Co-first author**)
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9. Han, WQ, Wong, WT, **Tian, XY**, Huang, Y, Wu, LY, Zhu, DL, Gao, PJ (2010). Contributory role of endothelium and voltage-gated potassium channels in apocynin-induced vasorelaxations. ***Journal of Hypertension*** 28(10): 2102-2110.
10. Wong, WT, Wong, SL, **Tian, XY**, Huang, Y (2010). Endothelial dysfunction: the common consequence in diabetes and hypertension. ***Journal of Cardiovascular Pharmacology*** 55(4): 300-307.

11. Cheang, WS, Wong, WT, Shen, B, Lau, CW, **Tian, XY,** Tsang, SY, Yao, X, Chen, ZY, Huang, Y (2010). 4-aminopyridine-sensitive K^+ channel contributes to NaHS-induced membrane hyperpolarization and relaxation in the rat coronary artery. ***Vascular Pharmacology*** 53(3-4): 94-98.
12. Liu, CQ, Wong, SL, Leung, FP, **Tian, XY**, Lau, CW, Lu, LM, Yao, X, Chen, ZY, T Yao, Huang, Y (2010) Phosphodiesterase inhibition ameliorates prostanoid TP receptor-mediated impairment of vasorelaxation induced by cyclic AMP-elevating dilator. ***European Journal of Pharmacology*** 632(1):45-51.
13. Yung, LM, Leung, FP, Wong, WT, **Tian, XY**, Yung, LH, Chen, ZY, Yao, XQ, Huang, Y (2008) Tea polyphenols benefit vascular function. ***Inflammopharmacology*** 16(5): 230-234.

Manuscripts under revision or resubmitted

1. **Tian, XY**, Wong, WT, Lu Y, Xu, A, Chen, ZY, Liu, WS, Lee, VW, Lau, CW, Yao, X, Huang, Y. (2011) Rosuvastatin improves endothelial function of *db/db* mice: role of angiotensin II type 1 receptor and oxidative stress. ***British Journal of Pharmacology***. (Resubmitted after revision)
2. Yung, LH, **Tian, XY**, Wong, WT, Leung, FP, Chen, Y, Kong, SK, Ng, SM, Lai, PS, Yung, LM, Yao, X, Vanhoutte, PM, Huang, Y (2010) Bone morphogenic protein-4 induces endothelial cell apoptosis through oxidative stress-dependent p38MAPK/JNK1 pathway. ***Journal of Molecular and Cellular Cardiology***. (Resubmitted after revision)
3. Wong, WT, **Tian, XY**, Xu, A, Lau, CW, Yun, J, Lee, V, Wang, Y, Lam, KSL, Vanhoutte, PM, Huang, Y (2010) The obligatory role of adipocyte-derived adiponectin in restoring endothelial function in rosiglitazone-treated diabetic mice. ***Cell Metabolism*** (Under revision) (**Co-first author**)
4. Chan, YC, **Tian, XY**, Leung, FP, Yung, LM, Lau, CW, Chen, ZY, Yao, X, Laher, I, Huang, Y (2010) Raloxifene improves vascular reactivity in pressurized septal coronary arteries of ovariectomized hamsters fed cholesterol diet ***Pharmacological Research*** (Revision)
5. Wong WT, **Tian XY**, Leung FP, Ng CF, Lee HK, Yao X, Au CL, Lau CW, Vanhoutte PM, Huang Y (2011) ROS-stimulated Production of Cyclooxygenase-2-derived Prostaglandin $F_{2\alpha}$ Cause Endothelial Dysfunction in Renal Arteries of Renovascular Hypertensive Rats ***Antioxidants & Redox Signaling*** (Under revision) **(co-first author)**

Manuscripts recently submitted

1. **Tian, XY**, Wong, WT, Lau, CW, Luo, J, Tsang, SY, Leung, FP, Bian, ZX, Yao, X, Chen, ZY, Huang, Y (2010) NaHS relaxes rat and mouse cerebral artery through inhibition of L-type Ca^{2+} channels (Submitted)
2. Liu, L, Wong, WT, **Tian, XY**, Liu, J, Lau, CW, Wang, YX, Xu, G, Xu, A, Lam, KSL, Chen, ZY, Yao, X, Huang Y (2010) Dipeptidyl-peptidase 4 inhibitor improves endothelial function of spontaneously hypertensive rats through activation of GLP-1/GLP-1 receptor/AMPK/NO cascade. (submitted)
3. Cheang, WS, Wong, WT, **Tian, XY**, Yang, Q, Lee, HK, He, GW, Yao, X, Huang, Y (2011) Endothelial nitric oxide synthase enhancer reduces

oxidative stress and restores endothelial function in *db/db* mice (Submitted) **(co-corresponding author)**

Academic awards and travel grants

1. 1st Prize of Chaired Poster Presentation for Young Investigator Awards, at the 14th Annual Scientific Meeting of the Institute of Cardiovascular Science and Medicine, Hong Kong (18 December 2010)
2. 2nd prize for Young Investigator Award competition (Oral presentation) 4th Scientific Meeting of the Asian Society for Vascular Biology in Hong Kong. (November 2010)
3. 2nd prize for Young Investigator Award competition (Oral presentation) at 12th Hong Kong Diabetes and Cardiovascular Risk Factors, East meet West Symposium. (October 2010)
4. 2nd Prize of Young Investigator Awards Competition at The International Forum of Cardiovascular Committee of Integrative Medicine, China (Jiangmen), August 6-9, 2010
5. 1st Prize of Young Investigator Award Competition (Oral Presentation) Scientific Conference on Cardiovascular Sciences Across the Strait, Kunming, Yunnan, China. (August 2009)
6. 1st Prize of Chaired Poster Presentation for Young Investigator Awards, at the 11th Annual Scientific Meeting of the Institute of Cardiovascular Science and Medicine, Hong Kong (December 2008)
7. Outstanding Postgraduate Student Oral Presentation Award in the 10th Scientific Meeting of Hong Kong Pharmacology Society (December 2008)
8. Postgraduate Oral Presentation Award, Faculty Research Day 2008, Faculty of Medicine, Chinese University of Hong Kong (July 2008)
9. Outstanding Abstract Prize (for the oral category) at the Third International Symposium on Healthy Aging, Hong Kong (1-2 March 2008)
10. CUHK International Conference Travel Grant (Japan, June 2009)
11. A travel grant from Hong Kong Pharmacology Society (August 2008)

Scientific meetings attended

1. 4th Scientific Meeting of the Asian Society for Vascular Biology in Hong Kong. (November 2010)
2. 12th Hong Kong Diabetes and Cardiovascular Risk Factors, East meet West Symposium. (October 2010)
3. The International Forum of Cardiovascular Committee of Integrative Medicine, China (Jiangmen), August 6-9, 2010
4. Joint Scientific meeting of Hong Kong Society of Neurosciences & the Biophysical Society of Hong Kong. University of Hong Kong. (June 2010) Oral Presentation for Young Investigator Award Competition.
5. Scientific Conference on Cardiovascular Sciences Across the Strait, Kunming, Yunnan, China. (August 2009)
6. East meet West Symposium in Hong Kong (October 2009) Oral presentation for Young Investigator Award Competition
7. 10th International Symposium on Mechanisms of Vasodilatation. Japan, (June 2009) Oral Presentation in "Endothelial Cells" Session.

8. Annual Scientific Meeting of Hong Kong Society of Endocrinology, Metabolism and Reproduction. Hong Kong, November 2008. Oral Presentation Competition.
9. 3rd Scientific Meeting of the Asian Society for Vascular Biology. Singapore, August 2008. Oral Presentation Competition.

Conference abstracts

1. **XY Tian**, WT Wong, J Tian, P Zhang, N Wang, Y Huang (2010) Rosiglitazone upregulates endothelial expression of endothelin B receptor and attenuates endothelium-i-induced vasoconstriction. ***J HK Coll Cardiol***, Vol 18:90 (P8).
2. J Liu, WT Wong, **XY Tian**, LM Liu, SL Wong, CW Lau, J Yu, X Yao, Y Huang (2010) Hemin restores the impaired endothelium-dependent vasodilatation in diabetic *db/db* mice through PI3K/Akt pathway. ***J HK Coll Cardiol***, Vol 18:76 (P19).
3. Limei Liu, Wing Tak Wong, **Xiao Yu Tian**, Jian Liu, Chi Wai Lau, Yi-Xiang Wang, Gang Xu, Aimin Xu, Karen SL Lam, Zhen Yu Chen, Xiaoqiang Yao, Yu Huang (2010) DIPEPTIDYL-PEPTIDASE 4 INHIBITOR IMPROVES ENDOTHELIAL FUNCTION OF SPONTANEOUSLY HYPERTENSIVE RATS THROUGH ACTIVATION OF GLP-1/GLP-1 RECEPTOR/AMPK/NO CASCADE. ***J HK Coll Cardiol***, Vol 18:64 (O3).
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5. WS Cheang, **XY Tian**, WT Wong, SY Tsang, CW Lau, X Yao, Y Huang (2010) NaHS relaxes rat cerebral arteries through inhibiting L-type calcium channel. ***J HK Coll Cardiol***, Vol 18:69 (P5).
6. Y Lu, **XY Tian**, WT Wong, LM Liu, J Liu, CW Lau, X Yao, Y Huang (2010) Advanced glycation End Product induces Endothelial dysfunction In Mouse Aortas through mitochondrial Reactive oxygen species. ***J HK Coll Cardiol***, Vol 18:79 (P32).
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8. LM Liu, WT Wong, **XY Tian**, J Liu, CW Lau, G Xu, X Yao, AM Xu, KS Lam, **Yu Huang** (2010) Dipeptidyl-peptidase 4 inhibitor sitagliptin restores the relaxations to exendin 4 in spontaneously hypertensive rats. 4th Scientific Meeting of Asian Society of Vascular Biology and PMV Research Symposium, 21-22 November 2010, Hong Kong
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11. WS Cheang, WT Wong, **XY Tian**, Q Yang, CW Lau, GW He, Y Huang (2010) eNOS enhancer AVE3085 restores endothelial function in *db/db* mice through increasing NO bioavailability. 12th Hong Kong Diabetes & Cardiovascular Risk Factors-East Meets West Symposium (1-2 Oct 2010). Page 23, Abstract Ab15.

12. **X.Y. Tian**, W.T. Wong, L.M. Liu, G. Xu, S.T. Lee, N.P. Wang and Y. Huang (2010) PPARdelta Activation Protects Endothelial Function in Diabetic Mice Through PI3K/Akt Pathway. 12th Hong Kong Diabetes & Cardiovascular Risk Factors-East Meets West Symposium (1-2 Oct 2010). Page 23, Abstract Ab20.
13. **XY Tian**, WT Wong, CW Law, Wang YX, Chen ZY, Wong YL, Mak CS, Y Huang (2010) Chronic intake of melamine impairs renal blood flow and renovascular function in rats. Health Research Symposium 2010, Hong Kong 11 Sept 2010. Page 54, Ab54.
14. **XY Tian**, WT Wong, FP Leung, AM Xu, ZH Jiang, RNS Wong, Y Huang (2010) Ginsenosides protect endothelial cell function in diabetic mice: Role of AMP-activated protein kinase. The International Forum of Cardiovascular Committee of Integrative medicine, China (Jiangmen), August 6-9, 2010. Proceedings, page 262-263.
15. **XY Tian**, WT Wong, Y Huang (2010) Hydrogen Sulfide Relaxes Rat Cerebral Arteries in Vitro: The Role of L-type Calcium Channel Inhibition and Myosin Light Chain Phosphatase Stimulation. 28th Scientific Meeting of the Hong Kong Society of Neuroscience, 7-8 June 2010. page 30-31, Abstract Y2
16. WS Cheang, WT Wong, **XY Tian**, Q Yang, CW Lau, GW He, **Y Huang** (2010) Pharmacological enhancement of eNOS restores endothelial function in type 2 diabetic db/db mice. The Fifth International Symposium on Healthy Aging, Hong Kong, page:44;Abstract:OP3
17. Y Lu, **XY Tian**, WT Wong, Y Huang (2009) Cyclooxygenase-derived prostanoids mediate endothelial dysfunction induced by advanced glycation end products. *J HK Coll Cardial* 17(2):63 (P20)
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19. CY Yuen, WT Wong, **XY Tian**, J Yu, B Tomlinson, X Yao, Y Huang (2009) Telmisartan increases endothelium-dependent relaxations through increasing nitric oxide bioavailability in mouse resistance arteries. *J HK Coll Cardial* 17(2):55 (P4)
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21. LM Liu, WT Wong, **XT Tian**, A Xu, J Liu, K Lam, Y Huang (2009) GLP-1 receptor activation attenuates endothelium-dependent contractions in rat renal arteries. *J HK Coll Cardial* 17(2):56 (P5)
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CHAPTER I

Introduction

1.1 Endothelial cell function

The inner surface of the vascular wall is covered by a thin lining of cells known as endothelial cells. Furchgott and Zawadzki discovered chemical substances present in endothelial cells that can relax blood vessels in response to acetylcholine, a neurotransmitter of parasympathetic nerves and then named them as endothelium-derived relaxing factors (EDRFs) (Furchgott *et al.*, 1980). Later it was worked out the chemical identity of EDRF and the likely cellular mechanism of its action, and concluded that EDRF was in fact nitric oxide (NO), which was immediately after recognized as an important gaseous molecule in cardiovascular physiology and pathology. Endothelium-derived NO is a powerful regulator of vascular homeostasis. By virtue of its ability to activate soluble guanylyl cyclase and increase intracellular cyclic GMP, NO relaxes the underlying vascular smooth muscle to improve vascular compliance and to reduce vascular resistance. In addition, endothelium-derived NO inhibits platelet adhesion and aggregation; suppresses leukocyte adhesion and vascular inflammation; and limits the proliferation of the underlying vascular smooth muscle cells. Furthermore, NO is mitogenic for endothelial cells, and increases the regeneration of the endothelial monolayer. In large conduit vessels such as the coronary artery, NO plays a critical role in defending against vascular inflammation and lesion formation.

1.2. Regulation of the NOS pathway

Endothelial nitric oxide synthase (eNOS) metabolizes L-arginine to NO and L-citrulline. Endothelial shear stress, as well as a variety of humoral or paracrine factors such as acetylcholine, adenosine diphosphate, thrombin and vasopressin, is known to induce vasodilatation, secondary to phosphorylation and activation of eNOS (Cooke *et al.*, 1991a; Cooke *et al.*, 1991b; Nishida *et al.*, 1992). The ability of the endothelium to respond to shear stress or other stimuli, and to induce relaxation of the underlying vascular smooth muscle, is impaired in older individuals (Egashira *et al.*, 1993; Gerhard *et al.*, 1996; Taddei *et al.*, 2001) and those with diabetes, hypertension, hypercholesterolemia, or tobacco exposure (Cooke, 2004; Creager *et al.*, 1990). An impairment of eNOS not only reduces the ability of a blood vessel to relax, but also broadly disrupts vascular homeostasis. In addition to relaxing vascular smooth muscle, NO is a potent inhibitor of platelet adhesion and aggregation (Cooke *et al.*, 1990; Stamler *et al.*, 1989). In addition, NO suppresses vascular inflammation by reducing the expression of leukocyte adhesion molecules and inflammatory cytokines (Tsao *et al.*, 1996; Tsao *et al.*, 1995; Tsao *et al.*, 1994; Tsao *et al.*, 1997). Consistent with these observations, in animal models, the enhancement of NO synthesis (as with L-arginine administration or over-expression of eNOS protein) reduces the progression of atherosclerosis and myointimal hyperplasia (Candipan *et al.*, 1996; Cooke *et al.*, 1992; von der Leyen *et al.*, 1995). The importance of NO in vascular homeostasis is supported by a large number of studies revealing that an impairment of endothelial vasodilator function is an independent risk factor for cardiovascular morbidity and mortality (Gokce *et al.*, 2003; Schachinger *et al.*, 2000; Suwaidi *et al.*, 2000).

A number of conditions associated with cardiovascular diseases are also known to impair the NOS pathway. For example, diabetes mellitus is associated with mitochondrial dysfunction and oxidative stress (Brownlee, 2005) that can accelerate the degradation of NO (Hink *et al.*, 2001). Furthermore, diabetes mellitus favours the production of advanced glycation end products (AGEs) which can also disrupt eNOS activation (Musicki *et al.*, 2005b; Wells *et al.*, 2001). Aging alters the phosphorylation and activation of eNOS in experimental animals (Musicki *et al.*, 2005a). Dyslipidemia is another major cause for the impaired endothelial vasodilator function. Hypercholesterolemia enhances the inhibitory interaction of caveolin-1 with eNOS, an effect that can be reversed by diet control and exercise (Musicki *et al.*, 2008).

1.3 The AMP-activated protein kinase (AMPK)

AMPK is a metabolic sensor with high sensitivity for the cellular energy status. It is a heterotrimeric protein consisting of catalytic and regulatory subunits (Gao *et al.*, 1996; Woods *et al.*, 1996). The protein kinase complex is activated in response to an increase in the ratio of AMP to ATP within the cell. Binding of AMP activates AMPK allosterically and induces phosphorylation of a threonine residue (Thr^{172}) within the activation domain of the subunit by an upstream kinase, the tumor suppressor LKB1 (Shaw *et al.*, 2004; Shaw *et al.*, 2005). Furthermore, binding of AMP inhibits the dephosphorylation of Thr^{172} by protein phosphatases, whereas a high concentration of ATP inhibits AMPK activation (Davies *et al.*, 1995; Suter *et al.*, 2006). AMPK is activated by a wide array of metabolic stresses, including hypoxia (Mu *et al.*, 2001), ischemia (Altarejos *et al.*, 2005; Mount *et al.*, 2005), oxidative and hyperosmotic

stresses (Barnes *et al.*, 2002; Qin *et al.*, 2008; Toyoda *et al.*, 2004), and rise in intracellular calcium ions (Leclerc *et al.*, 2004; Yamauchi *et al.*, 2008). Furthermore, exercise and glucose deprivation also activate AMPK, which suggests a role in exercise adaptations and cell function. In general, activation of AMPK triggers catabolic pathways that produce ATP, and turns off anabolic pathways that consume ATP, to maintain cellular energy stores (Canto *et al.*, 2009; Hardie, 2003; Osler *et al.*, 2008). Metformin and TZDs, two widely prescribed drugs for the treatment of type 2 diabetes mellitus (T2DM), are also reported to increase AMPK activity (Mauvais-Jarvis *et al.*, 2001), underlining the potential role of the AMPK pathway in the treatment of T2DM. Pharmacological activation of AMPK can be achieved by treatment of cells with an artificial activator, 5-aminoimidazole-4-carboxamide1–beta–D -ribofuranoside (AICAR). AICAR is taken up by the cells and phosphorylated to form 5-aminoimidazole-4-carboxamide ribonucleoside (ZMP), an AMP mimetic, and confers the activating effects of AMP on the AMPK pathway (Corton *et al.*, 1995).

1.4 The Phosphatidylinositol-3-kinase (PI3K) and Akt

Phosphatidylinositol-3-kinase (PI3K) is the upstream regulator of Akt, a serine/threonine protein kinase which activates eNOS (Dimmeler *et al.*, 1999). It was reported that vascular endothelial growth factor (VEGF) activates eNOS through Akt (Feliers *et al.*, 2005; Youn *et al.*, 2009). Akt can also be activated by shear stress (Boo *et al.*, 2002; Fisslthaler *et al.*, 2000). Akt-induced eNOS activation is also responsible for endothelium-dependent relaxation induced by adrenomedullin (Hamid *et al.*, 2006) and insulin (Montagnani *et al.*, 2001). Akt-dependent eNOS phosphorylation is also regulated by interaction of

Hsp90 with Akt (Chen *et al.*, 2004). Hyperglycemia can induce glycosylation of the eNOS phosphorylation site at Ser^{1177}, which is mainly regulated by Akt, and causes inactivation of eNOS (Salt *et al.*, 2003).

In diabetes, Akt/eNOS pathway in the endothelial cell is inhibited, which is related to endothelial dysfunction (Chen *et al.*, 2007; Du *et al.*, 2001; Kobayashi *et al.*, 2004; Molnar *et al.*, 2005). Akt activity is regulated by adipokines, fatty acid, insulin, *etc.* and has become a useful target in protecting endothelial cells and ameliorating endothelial dysfunction in diabetes (Chen *et al.*, 2008; Davis *et al.*, 2006; Jesmin *et al.*, 2007; Ota *et al.*, 2008; Shah *et al.*, 2007; Zhang *et al.*, 2007; Zhong *et al.*, 2007b). Of note, PI3K/Akt can also be regulated by the peroxisome proliferator-activated receptors (PPARs). In endothelial cells and mouse aortas, PPARγ protects endothelial function and enhances angiogenesis through increasing Akt and eNOS phosphorylation, which is dependent on the upregulation of VEGF and its receptor (Cho *et al.*, 2004; Huang *et al.*, 2008). PPARα activators bezafibrate and WY-14643 activate eNOS through PI3K and p38 MAPK (Bulhak *et al.*, 2009; Wang *et al.*, 2006b). PPARδ also modulates Akt in myocardium, skeletal muscle, endothelial cells, and vascular smooth muscle cells (Coll *et al.*; Li *et al.*, 2009; Wang *et al.*, 2006a; Zhang *et al.*, 2002).

1.5 Endothelial dysfunction in diabetes

Endothelial dysfunction, characterized by a diminished release of endothelium-derived NO and/or an augmented release of contracting prostanoids and ROS, is an important early event in the initiation and development of hypertension, diabetes and atherosclerosis. Micro- and macro-vascular dysfunctions are currently the major causes of morbidity and

mortality in patients with diabetes mellitus. Endothelial dysfunction plays a critical role in the development of diabetic vasculopathy, which is associated with the reduced bioavailability of NO resulting from overproduction of ROS, lipid peroxidation, and increased production of adhesion molecules (Khan et al., 1996). Impaired endothelium-dependent vasodilatations have been observed in type I and II diabetes from both clinical settings and animal studies (Choudhary et al., 2007; Lin et al., 2002; Prior et al., 2005). The forearm vasodilator response to the muscarinic acetylcholine receptor agonist, methacholine is impaired in patients with insulin-dependent diabetes (type I diabetes) (Johnstone et al., 1993) and non-insulin-dependent diabetes (type II diabetes) (Tan *et al.*, 2002; Williams *et al.*, 1996). Besides, the impaired endothelium-dependent vasodilatations could be also demonstrated in animal models of type I diabetes (Chang *et al.*, 1993; Dai *et al.*, 1993; Nassar *et al.*, 2002) and type II diabetes (Elmi *et al.*, 2008; Gao *et al.*, 2007; Pannirselvam *et al.*, 2002). The complex mechanisms by which hyperglycemia modifies the endothelial function include increased oxidative stress (Laight *et al.*, 2000), glycation of proteins and lipids (Vlassara, 1992) and activation of protein kinase C (Hink *et al.*, 2001).

1.5.1 Oxidative stress in diabetic endothelial dysfunction

Oxidative stress is caused by a disturbed balance between oxidant enzymes and antioxidant enzymes tilting towards an increase in ROS overproduction. Oxidative stress is a critical factor in diabetic endothelial dysfunction. Under a hyperglycemic condition the antioxidant enzymes like SOD and catalase are down-regulated, resulting in an increased generation of oxygen-derived free radicals (Giugliano *et al.*, 1996). Increases in ROS lead to impairment of

endothelium-dependent vasodilatations through the reduction of NO bioavailability. It had been recently demonstrated that superoxide anion scavengers like superoxide dismutase improve the impaired endothelium-dependent vasodilatations in *db/db* diabetic mice (Elmi *et al.*, 2008; Moien-Afshari *et al.*, 2008). Chronic treatments with antioxidants including vitamin E and vitamin C were reported to prevent the development of endothelial dysfunction in diabetic patients and animals (Keegan *et al.*, 1995; Ting *et al.*, 1996). However, it remains controversial as to the beneficial effects of the use of antioxidants in the treatment of vascular dysfunction in diabetes because in clinical settings antioxidant treatment does not always yield a protective effect in reversing endothelial dysfunction in type II diabetic patients (Gazis *et al.*, 1999).

1.5.2 Reduced NO bioavailability in diabetes

Bioavailability of endothelial-derived NO, as determined by the relative ratio of NO over ROS levels, is an important index for determining endothelial function. Any situation in which there is a reduced eNOS activity or elevated ROS production can normally lead to a reduced NO bioavailability and thus impairs endothelium-dependent vasodilatations. The phosphorylation of eNOS was found to be impaired in diabetic mouse aortas (Zhang *et al.*, 2009; Zhong *et al.*, 2007a), renal arteries (Zhong *et al.*, 2007b), and mesenteric resistance arteries (Su *et al.*, 2008). Under hyperglycemic and enhanced oxidative stress states, the phosphorylation of eNOS at Ser1177 site was also found to be diminished in human umbilical vein endothelial cells (Vasquez *et al.*, 2007; Wang *et al.*, 2009). As afore-mentioned, ROS production is increased in diabetic vascular tissues due to the imbalance between the oxidant and

antioxidant enzymes. These highly reactive oxygen free radicals act quickly to remove NO, and thus further reduce the NO bioavailability. Furthermore, the interaction between NO and superoxide radicals leads to the formation of peroxynitrite, another highly reactive free radical species that causes lipid peroxidation, DNA damage and protein nitration and collectively damages the vascular function (Bloodsworth *et al.*, 2000).

1.6 Peroxisome proliferator-activated receptors (PPARs)

PPARγ plays a role in various physiological and pathophysiological events, including adipocyte differentiation (Tontonoz *et al.*, 1994) and the response to insulin. PPARs belong to the nuclear receptors superfamily that functions as transcription factors regulating gene expressions. Three types of PPARs have been identified and named as PPARα (NR1C1), PPARβ/δ (NR1C2), PPARγ (NR1C3). PPARs play important roles in the regulation of cellular differentiation, development and metabolism (carbohydrate, lipid and protein). PPARα is predominantly expressed in cells with high rates of fatty acid catabolism such as those found in liver, heart, kidney and skeletal muscle (Braissant *et al.*, 1996). PPARγ is mainly associated with adipose tissue (Escher *et al.*, 2000). PPARδ is abundantly and ubiquitously expressed at much higher levels than PPARγ and PPARα (Kliewer *et al.*, 1992). The expression of PPARδ has been also detected in vascular cells including endothelial cells (Piqueras *et al.*, 2007), smooth muscle cells, and macrophages (Welch *et al.*, 2003).

1.6.1 Physiological functions of PPARs

All PPARs heterodimerize with the retinoid receptor (RXR) and bind to specific

regions of DNA of the target genes (Gearing *et al.*, 1993). These DNA sequences are called peroxisome proliferator hormone responsive elements (PPREs). The consensus sequence of the DNA is AGGTCAXAGGTCA, with X being a random nucleotide. This sequence usually occurs in the promoter region of the gene, and when the ligands bind with the PPARs, transcription of the target genes is activated. The RXR also forms heterodimers with other nuclear receptors including vitamin D receptors and thyroid hormone receptors. PPARs can be activated by a wide range of structurally diverse endogenous and synthetic ligands (Michalik *et al.*, 2006). Endogenous ligands for the PPARs including free fatty acids and eicosanoids, are identified, and synthetic ligands for the PPARs developed are also developed for the treatment of diabetes and dyslipidemia (Varnecq *et al.*, 1999).

1.6.2 PPAR ligands

Ligands	***PPARs***
Natural and endogenous ligands	
Mono-unsaturated fatty acids	α and β/δ
Poly-unsaturated fatty acids	α, β/δ and γ
Saturated fatty acids	α and β/δ
Eicosanoids (prostaglandins, prostacyclin, thromboxane and leukotrienes)	α
15-Deoxy-Δ12, 14-PGJ2	γ
Leukotriene B4	γ
Prostacyclin	β/δ
Retinoic acid	β/δ
Synthetic ligands	
Wy14643	α
Fatty acyl-CoA dehydrogenase inhibitors	α
GW409554	α

GW2433	α
GW2331	α
GW7647	α
Bezafibrate	α
Gemfibrozil	α
Fenofibrate	α
Ciprofibrate	α
Clofibrate	α
Pioglitazone	γ
Rosiglitazone	γ
Troglitazone	γ
Rivoglitazone	γ
Ciglitazone	γ
MCC-555	γ
GW1929	γ
S26948	γ
Leukotriene B4 analogs	α and γ
Muraglitazar	α and γ
Aleglitazar	α and γ
Tesaglitazar	α and γ
GW501516	β/δ
GW0742	β/δ
L-165041	β/δ

1.7 PPARγ

1.7.1 Physiological function of PPARγ

PPARγ activation enhances the lipid storage capacity of the adipose mass, and also increases the number of small, insulin-sensitive adipocytes so as to improve insulin sensitivity (Yamauchi *et al.*, 2001b). PPARγ is implicated in the regulation of lipid metabolism (Rosen *et al.*, 2001), as well as the maturation of

monocyte / macrophages and the control of inflammatory reactions (Moore *et al.*, 2001). In human, loss-of-function mutation of PPARγ is associated with insulin resistance and diabetes (Barroso *et al.*, 1999). Although PPARγ deficient ($PPAR\gamma^{-/-}$) mice show embryonic lethality due to placental dysfunction (Barak *et al.*, 1999), partial reduction of PPARγ activity by heterozygous PPARγ deficiency leads to mild insulin resistance (Barak *et al.*, 1999; Matsui *et al.*, 2004). In addition, targeted deletion of PPARγ in skeletal muscle, macrophage, adipose tissue, or endothelium suggests that PPARγ regulates glucose homeostasis (He *et al.*, 2003; Kanda *et al.*, 2009; Norris *et al.*, 2003; Odegaard *et al.*, 2007).

1.7.2 PPARγ ligands

There are endogenous ligands for PPARγ, including unsaturated fatty acids, leukotriene B_4, 15-deoxy-delta12,14-PGJ_2, and nitrolinoleic acid (Paruchuri *et al.*, 2008; Schopfer *et al.*, 2005; Vamecq *et al.*, 1999). Of note, 15-Deoxy-delta12,14-PGJ_2 is an eicosanoid formed by cyclooxygenase (Bell-Parikh *et al.*, 2003). The synthetic ligands for PPARγ that were in clinical use as anti-diabetic agents are rosiglitazone and pioglitazone (Woodcock *et al.* 2010). There are convincing results demonstrating that TZDs improve insulin sensitivity in a PPARγ-dependent manner (Yamauchi *et al.*, 2001a). Human intervention studies with TZDs report reduced fasting insulin concentrations, glucose concentrations, and improved whole body insulin sensitivity (Fonseca *et al.*, 2000; Kahn *et al.*, 2006). Although the use of rosiglitazone was restricted due to the risk of myocardial infarction,(Nissen *et al.*, 2007), the insulin-sensitizing action of PPARγ is still important, and a recent report described that selective activation of PPARγ in adipocytes improves

whole-body insulin sensitivity (Sugii *et al.*, 2009), suggesting that PPARγ is still an important therapeutic target for type 2 diabetes.

1.7.3 PPARγ in vasculature

As PPARγ is also expressed in the endothelial cells, the anti-inflammatory actions of PPARγ were mostly examined by using glitazones or PGJ_2 or gain-of-function, which inhibits endothelial cell activation by a PPARγ-dependent mechanism (Verrier *et al.*, 2004). PPARγ activation inhibits NF-κB activated transcription of chemokines (Marx *et al.*, 2000), and the expression of adhesion molecules *in vitro* and *in vivo* (Jackson *et al.*, 1999; Pasceri *et al.*, 2000; Wang *et al.*, 2002). Importantly, PPARγ also reduces ROS production by inhibiting the expression of NADPH oxidases (Hwang *et al.*, 2005; Hwang *et al.*, 2007), and protein kinase-C activation (Verrier *et al.*, 2004). Apart from being anti-inflammatory, PPARγ also increases NO bioavailability. TZDs or PGJ_2 stimulate eNOS phosphorylation and interaction of eNOS with heat shock protein 90 to increase NO release (Polikandriotis *et al.*, 2005) through a p38 MAPK-mediated pathway (Ptasinska *et al.*, 2007).

1.8 PPAR β/δ

While the agonists of PPARα and PPARγ are clinically used, PPARδ is the remaining subtype that is not yet a target for current drugs despite the fact that synthetic ligands for PPARδ were developed (Berger *et al.*, 1999) and found to exert beneficial effects on lipid and glucose metabolism (Narkar *et al.*, 2008).

1.8.1 Function of PPARδ in diabetes and obesity

The function of PPARδ was studied using newly synthesized PPARδ ligands. A

high-affinity synthetic PPARδ ligand, GW501516 can reduce weight gain and decrease the circulating triglyceride level in diet-induced obese mice and in *ob/ob* mice, which is attributed to the increased peripheral fatty acid catabolism (Tanaka *et al.*, 2003; Wang *et al.*, 2003). GW501516 and another PPARδ agonist, L165041 elevate HDL-cholesterol in *db/db* mice (Lee *et al.*, 2006b; Leibowitz *et al.*, 2006). Whether or not PPARδ activation could reduce adiposity in humans remains to be revealed. PPARδ also regulates glucose homeostasis. In a mouse model of diet-induced obesity, administration of GW501516 lowers the plasma insulin level and improves glucose tolerance and insulin sensitivity (Tanaka *et al.*, 2003). These benefits of GW501516 are lost in *PPARδ*$^{-/-}$ mice. Collectively, the limited results obtained in mice suggest that PPARδ could be a potential therapeutic target for combating against obesity and insulin resistance.

1.8.2 PPARδ in the cardiovascular system

It had been reported that prostacyclin protects endothelial cells against H_2O_2 through PPARδ-mediated expression of its target gene 14-3-3α which prevents Bad-dependent apoptosis (Liou *et al.*, 2006). On the other hand, PPAR-δ agonists possess angiogenic properties. For example, GW501516 stimulates human endothelial cell proliferation with increased mRNA expression of vascular endothelial growth factor α and its receptor flt-1 (Stephen *et al.*, 2004). In human endothelial cells, both GW0742 and GW501516 inhibit the TNFα- or interleukin 1β-induced expression of adhesion molecules and monocyte adhesion to the endothelial cells (Fan *et al.*, 2008). The PPARδ agonists decrease the production of ROS in endothelial cells, probably in relation to an increase in gene expression of anti-oxidant enzymes,

including superoxide dismutase-1, catalase, and thioredoxin (Fan *et al.*, 2008).

Recent limited studies suggest that PPARδ activation may retard the development of atherosclerosis as PPARδ activation is found to reduce the expression of ICAM-1, MCP-1 and other inflammatory cytokines, and to attenuate development and progression of atherosclerosis in mice (Graham *et al.*, 2005; Li *et al.*, 2004). The anti- atherogenic effect of PPARδ activation may also be associated with the decreased circulating levels of pro-inflammatory cytokines and TNFα expression in macrophage and increased cholesterol efflux and the reversed cholesterol transport and fatty acid catabolism (Graham *et al.*, 2005; Lee *et al.*, 2006a; Oliver *et al.*, 2001). PPARδ expressed in the myocardium (Cheng *et al.*, 2004; Schiffrin *et al.*, 2003) is involved in the transcriptional regulation of lipid metabolism (Barger *et al.*, 2000).

Cardiomyocyte-specific *PPARδ*$^{-/-}$ mice exhibit myocardial lipid accumulation, hypertrophy and heart failure with reduced lifespan (Cheng *et al.*, 2004) and *in vitro* activation of PPARδ inhibits hypertrophy of neonatal rat cardiomyocytes (Planavila *et al.*, 2005; Smeets *et al.*, 2008), hence supporting its physiological role in maintaining normal cardiac function. However, the role of PPARδ in the regulation of vascular tone and the development of hypertension remains largely unknown. Obviously, PPARs play an important role in cardiovascular physiology and pathophysiology. Detailed understanding of PPARs-mediated regulation of the cardiovascular function will help to delineate the precise mechanisms by which PPARs modify cellular activities associated with cardiovascular diseases and to identify more effective therapeutic targets. The proposed study will thus focus on the contribution of altered PPARδ activity to the induction and maintenance of endothelial dysfunction in diabetes.

1.9 Adiponectin

Adipose tissue, once considered simply as a lipid storage depot, is now known to be a dynamic endocrine organ that secretes various adipokines. Obese and type 2 diabetic patients exhibit altered profiles of adipokines. Adiponectin is one of the most abundant plasma proteins (~1–17 mg/mL) accounting for approximately 0.01% of the total protein content of human plasma (Arita *et al.*, 1999; Fang *et al.*, 2006). Circulating levels of adiponectin decrease in obesity as well as in patients with cardiovascular diseases, hypertension and metabolic syndrome (Choi *et al.*, 2004; Esposito *et al.*, 2003; Hara *et al.*, 2007; Ouchi *et al.*, 1999).

1.9.1 Physiological function of adiponectin

Adiponectin structurally belongs to the complement 1q family and is known to form a characteristic homomultimer (Scherer *et al.*, 1995). Circulating adiponectin exists predominantly as three distinct oligomeric complexes (Wang *et al.*, 2008; Xu *et al.*, 2005). The basic building block of oligomeric adiponectin is a tightly-associated homotrimer, which is formed via hydrophobic interactions within its globular domains. Two trimers self-assemble into a disulfide-linked hexamer, which further associates into a high molecular weight (HMW) multimeric complex (Tsao *et al.*, 2003). Studies have suggested that high molecular weight adiponectin may be the major bioactive isoform responsible for its insulin-sensitizing activity (Qiao *et al.*, 2008). Adipose tissue is considered as the major site of endogenous adiponectin production. Insulin sensitizer PPARγ agonists increase adiponectin levels in mice and humans, as well as in 3T3-L1 adipocytes in vitro (Kadowaki *et al.*, 2008; Yamauchi *et al.*, 2007). Indeed, epidemiological

studies on different ethnic groups have identified low level of circulating adiponectin, especially its HMW oligomeric complex, as an independent risk for type 2 diabetes, hypertension, atherosclerosis and myocardial infarction (Zhu *et al.*, 2008).

1.9.2 Adiponectin receptors

Two subtypes of adiponectin receptors (adipoR1 and adipoR2) have been identified (Yamauchi *et al.*, 2003). AdipoR1 and AdipoR2 are integral membrane proteins containing seven transmembrane domains, but they are structurally and functionally distinct from classical G protein coupled receptors (GPCRs) (Kadowaki and Yamauchi, 2005). The binding of adiponectin to adipoR1 and adipoR2 mediates increased AMPK and PPARγ activation, fatty-acid oxidation and glucose uptake (Kadowaki *et al.*, 2005; Yamauchi *et al.*, 2002). Recent works identified the AdipoR1/R2 interacting protein APPL1 as a direct interacting partner of adipoR1 and adipoR2 (Cheng *et al.*, 2007; Mao *et al.*, 2006). APPL1, a 70 amino acid-adaptor protein, APPL1 appears to play a key role in coupling the adiponectin receptors to their downstream signalling cascades (Mao *et al.*, 2006; Xin *et al.*, 2010; Zhou *et al.*, 2009).

1.9.3 Role of adiponectin in the cardiovascular disease

Unlike most other adipokines with pro-inflammatory actions, adiponectin possesses anti-inflammatory and anti-diabetic properties. Adiponectin protects against insulin resistance (Berg *et al.*, 2001; Combs *et al.*, 2001), atherosclerosis (Okamoto *et al.*, 2002), hypertension (Ohashi *et al.*, 2006), heart failure (Shibata *et al.*, 2005), and other obesity-related cardiovascular diseases. It is noted that adiponectin knockout mice are more susceptible to

diet-induced insulin resistance (Berg *et al.*, 2001; Kubota *et al.*, 2002), endothelial dysfunction (Kumada *et al.*, 2003; Ouchi *et al.*, 2003), hypertension (Ohashi *et al.*, 2006), atherosclerosis (Kubota *et al.*, 2002) and heart failure (Shibata *et al.*, 2005). Adiponectin serves as a vasodilator that induces eNOS phosphorylation and NO production (Hattori *et al.*, 2003; Ouchi *et al.*, 2004; Xi *et al.*, 2005). It had been demonstrated that circulating levels of adiponectin are positively associated with flow-induced vasodilatation of the brachial artery in patients (Tan *et al.*, 2004). Aortic rings isolated from adiponectin knock-out mice display lowered eNOS phosphorylation and NO production, and impaired relaxation (Cao *et al.*, 2009). Administration of recombinant adiponectin in rats with high fat diet-induced obesity restored eNOS activity, NO production and endothelium-dependent relaxation (Deng *et al.*, 2010). Adiponectin enhances eNOS activity and NO production in endothelial cells via AMP-activated protein kinase (AMPK)-mediated phosphorylation of eNOS at Ser^{1177} (Chen *et al.*, 2003).

Besides, adiponectin inhibits oxidized low density lipoprotein (LDL)-induced ROS generation through inhibition of NADPH oxidase in bovine endothelial cells (Motoshima *et al.*, 2004). Adiponectin also reverses high glucose-induced ROS production in HUVECs through a cAMP/PKA dependent mechanism (Ouedraogo *et al.*, 2006). Importantly, it is also noted that aortas from adiponectin knockout mice show high levels of superoxide anion and peroxynitrite which were reversed by recombinant adiponectin (Cao *et al.*, 2009). Recombinant adiponectin also suppressed superoxide anion and peroxynitrite production in aortic rings isolated from rats fed with a high-fat diet (Li *et al.*, 2007), which was associated with an increase in eNOS activity. Furthermore, adiponectin inhibits proliferation and migration of vascular

smooth muscle cells (Arita *et al.*, 2002; Okamoto *et al.*, 2002; Wang *et al.*, 2005). It had been demonstrated that adiponectin knockout mice exhibited an enhanced proliferation of vascular smooth muscle cells and increased neointimal thickening after mechanical injury (Kubota *et al.*, 2002), which could be reversed by adenovirus-mediated expression of adiponectin (Okamoto *et al.*, 2002).

1.10 Endothelin-1 and vascular function

Endothelin-1 (ET-1), a 21-amino acid peptide, is a potent vasoconstrictor and pro-inflammatory substance that is primarily produced by the endothelial cells. Increased production and activity of ET-1 is associated with arterial hypertension, pulmonary hypertension, and cerebral vasospasm. ET-1 is formed from pre-pro-ET-1 via big ET-1 by ET converting enzymes (Yoshimura *et al.*, 1997). ET-1 acts in an autocrine or paracrine pattern upon stimuli such as angiotensin II in various cell types such as cardiomyocytes (Ito *et al.*, 1993), leukocytes (Sessa *et al.*, 1991), and endothelial cells (Schiffrin *et al.*, 1998). ET-1 is a potent vasoconstrictor with pro-longed action, which involves voltage-dependent Ca^{2+} channels, protein kinase C activation, and also the release of thromboxane A_2 in different arteries (Kasuya *et al.*, 1989; Miyauchi *et al.*, 1996; Rizzoni *et al.*, 1997; Taddei *et al.*, 1993; Yoshida *et al.*, 1994; Yousif, 2006).

1.10.1 Function of ET-1 receptors

Two types of endothelin receptors, ET_AR and ET_BR, were G protein-coupled receptors (Elshourbagy *et al.*, 1993). ET_AR is mainly expressed in vascular smooth muscle layer and responsible for vasoconstriction, while ET_BR is

expressed mainly in endothelial cells, and to less extent in the smooth muscle cells (Hosoda *et al.*, 1991; Ogawa *et al.*, 1991).

Activation of ET_BR leads to NO production as it is functionally coupled to eNOS signaling including interaction with caveolin and Akt (Hirata *et al.*, 1993; Kwok *et al.*, 2009; Liu *et al.*, 2003; Murohara *et al.*, 1996; Noiri *et al.*, 1997; Tsukahara *et al.*, 1994). ET_BR also mediates the clearance of ET-1 (Bohm *et al.*, 2003; Burkhardt *et al.*, 2000; Honore *et al.*, 2005; Ozaki *et al.*, 1995) and antagonizes the effect of ET_AR, thus modulates the vascular tone.

1.10.2 Regulation of ET-1 by PPARγ

The production and function of ET-1 can be regulated by PPARγ. PPARγ activation inhibits endothelin-1 production induced by thrombin through inhibition of transcriptional factor activator protein-1 (Delerive *et al.*, 1999), thus reduces cardiac hypertrophy (Sakai *et al.*, 2002). PPARα and PPARγ ligands inhibit oxidized LDL-induced protein kianse C activation and ET-1 production, improve endothelial function, reduce vascular remodeling, and lower blood pressure in hypertension (Iglarz *et al.*, 2003; Martin-Nizard *et al.*, 2002). In addition, PPARγ activation can also inhibit the downstream pathways of ET-1 such as ET-1-induced vascular inflammation (Montezano *et al.*, 2007), and ET-1-induced calcineurin/NFAT-dependent cardiac hypertrophy (Bao *et al.*, 2008).

1.11 Justification, long-term significance and objectives of the present project

Endothelial cell function is important for modulating local vascular tone and maintaining normal vascular function. Endothelial dysfunction is

characterized by a diminished NO bioavailability as a result of reduced NO production and/or increased production of ROS. The degree of endothelial dysfunction predicts the severity of cardiovascular risks. Impaired endothelium-dependent vasodilatation is observed in diabetic patients and animal models of diabetes.

PPARs such as rosiglitazone and pioglitazone are current anti-diabetic targets to correct insulin resistance and dyslipidemia (Fonseca *et al.,* 2000; Kahn *et al.,* 2006). PPAR ligands also possess pleotropic actions apart from the metabolic effects. An emerging role of PPARs in the development of cardiovascular disease is being increasingly recognized and activation of PPARs inhibits vascular inflammation, atherosclerotic progression, and oxidative stress as well as promotes angiogenesis. In view of the importance of PPARs in endothelial function, the present study aimed at investigating the positive involvement of endothelial PPARγ and PPARδ activation in ameliorating endothelial dysfunction in type 2 diabetes by using diabetic *db/db* mice, and diet-induced obese mice, *PPARγ heterozygous* and *PPARδ* knockout mice. The modulation of PPARγ and PPARδ on the expression and activity of eNOS and its upstream regulators are of particular interest. The results of the present study should provide novel experimental evidence in support of the clinical effects of PPARγ and PPARδ activators in alleviating endothelial dysfunction in diabetes.

In addition, the effect of adiponectin, which is a PPARγ-dependent adipokine, was also examined, plasma adiponectin level correlates with endothelial dysfunction in patients with type 2 diabetes, hypertension, and coronary heart disease. However, the role of adipose tissue and its product, adiponectin in endothelial dysfunction in diabetes is still unclear. Of

importance, adipose tissue is not only an energy storage but also a major endocrine organ, which regulates glucose homeostasis. The present study also examined the role of adipose-tissue derived adiponectin in ameliorating endothelial dysfunction by using multiple approaches including *in vitro*, *ex vivo*, and *in vivo* models. The results from the present study shall provide novel evidence favoring the beneficial impact of adiponectin in improving endothelial cell function in diabetes.

The objectives of the present study were therefore to investigate:

1. whether PPARγ activation in adipose tissue could enhance the adiponectin production which improves endothelial function through an AMPK-dependent pathway;
2. whether endothelial ET_BR could be a target of PPARγ to enhance the NO production and thus favorably modulates vascular tone;
3. whether PPARδ activation could improve endothelial function in diabetes though a PI3K/Akt-dependent pathway.

In order to achieve the afore-mentioned objectives, a combination of experimental approaches was employed in the present study and they included vasoreactivity study, biochemical assays, cell culture, imaging, knockout animals, and other molecular biology techniques.

CHAPTER II

Methods and Materials

2.1 Animals

The use of animals for my experiments was approved by the Ethical Committee for Animal Research, Chinese University of Hong Kong (CUHK). Animals that were supplied by the CUHK Laboratory Animal Service Center including: male leptin receptor deficient *db/db* (homozygous) and age-matched *db/m*$^{+}$ heterozygous mice generated from the C57BL/KsJ; male C57BL/6J mice. *PPARβ/δ*$^{-/-}$, and age-matched *PPARβ/δ* wild type littermates (Peters *et al.*, 2000); PPARγ heterozygous-deficient mice (*PPARγ*$^{+/-}$) mice (Yu *et al.*, 2008) and PPARγ wild-type (*PPARγ*$^{+/+}$) controls. *PPARγ*$^{+/-}$ mice were used because all homozygous PPARγ knockout animals were embryonically lethal due to placental dysfunction (Yu *et al.*, 2008). Animals supported by Dr. Xu Aimin from the Department of Medicine and Department of Pharmacology and Pharmacy, the University of Hong Kong, are: adiponectin knockout (*Adn*$^{-/-}$) generated from C57BL background, *Adn*$^{-/-}$ and *db/db* double knockout (*DKO*) (Ma *et al.*, 2002; Zhou *et al.*, 2008). All animals were housed at room temperature (25 °C) with alternating 12-hr light / 12-hr dark cycle and fed on standard rat chow and water *ad libitum*.

2.1.1 Animal model: diet-induced obese mouse

Diet-induced obese (DIO) mice were generated by C57BL/6J, *PPARβ/δ*$^{-/-}$ and age-matched *PPARβ/δ* wild type (*WT*) littermates at the age of 6-7 weeks which were fed with high fat diet for 10 weeks (Rodent diet with 45% kcal% fat, D12451, Research Diets Inc. New Brunswick, NJ, USA). Body weight and fasting blood glucose were monitored biweekly. Plasma glucose levels were determined using a commercial blood glucose meter (Ascenia Elite XL, Bayer, IN, USA).

2.1.2 Drug treatments in animal studies

1. Male *db/db* and *DKO* mice aged at 12 weeks with fasting blood glucose over 20 mmol/L were randomly divided into several groups and administered orally with rosiglitazone (10 mg/kg/day; PPARγ agonist, GSK No: BRL-49653-C) or vehicle for 4 weeks.
2. Male C57BL/6J mice (10-weeks old) were subjected to receive daily oral administration of rosiglitazone (10 mg/kg/day; PPARγ agonist, GSK No: BRL-49653-C) or vehicle for 2 weeks.
3. Male *db/db* mice aged 12 weeks with fasting blood glucose over 20 mmol/L; DIO mice (C57BL/6J, *PPARβ/δ KO* and *PPARβ/δ WT*) at the age of 16 weeks; and age-matched C57BL/6J were divided into several groups, and they received oral administration of GW501516 (PPARδ agonist, 5mg/kg/day) or vehicle for one week.

2.2 Measurement of basic parameters

2.2.1 Oral glucose tolerance test

In *db/db* and *db/m*$^{+}$ mice, after 6 hrs of fasting, glucose was loaded 1.2 g/kg with a 10% glucose solution via oral gavage, and the plasma glucose level was measured subsequently at 15, 30, 60 and 120 min with a commercial glucometer (Ascenia Elite XL, Bayer, IN, USA)

2.2.2 Lipid profile

After animals were sacrificed, blood was drawn from the inferior vena cava and collected in heparin coated test tube. Plasma was separated by centrifugation and stored at -80 °C until further assay. Plasma levels of total cholesterol and triglyceride were determined using enzymatic methods (Stanbio, Boerne, TX, USA). A blank was prepared by substituting 0.01 mL of distilled water from the cholesterol sample. Samples were mixed and incubated for 15 min at 37 °C. Absorbance was read at 500 nm using a spectrophotometer. Briefly, triglycerides are converted to glycerol and fatty acids, and then into NADH. Finally, the

formation of colored formazan took place in response to the addition of 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium. Absorbance at 500 nm was recorded and the reading was directly proportional to the concentration of triglycerides in the sample. For the measurement of the level of high-density lipoprotein (HDL), the low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) portions were removed by the addition of HDL cholesterol assay reagent (Sigma, kit number 352-4). The remaining level of cholesterol, that is HDL, was obtained.

2.4 Isometric force measurement

After animals were sacrificed by CO_2 inhalation, the thoracic aorta, or small intestine with mesentery was rapidly removed and placed in oxygenated ice-cold Krebs-Henseleit solution. Segments of blood vessels including aortas, or mesenteric resistant arteries were carefully dissected free from adjacent connective tissues. Changes in isometric tension of mouse aortas or mesenteric resistance arteries were recorded in a Multi Myograph System (Danish Myo Technology A/S, Denmark) as previously described (Wong *et al.*, 2010a). Mouse aortas of 3 mN and mesenteric resistance arteries of 1 mN were then allowed to equilibrate for 60 min before the start of the experiment. Each experiment was performed on rings prepared from different animals.

2.4.1 Organ culture of mouse aorta

Mouse thoracic aortic rings (2 mm in length) were incubated in a Dulbecco's Modified Eagle's Media (DMEM, Gibco, Gaithersberg, MD, USA) culture media supplemented with 10% fetal bovine serum (FBS, Gibco), plus 100 IU/mL penicillin and 100 µg/mL streptomycin (Wong *et al.*, 2010a). Recombinant mouse full-length adiponectin (5 µg/mL) (Wang *et al.*, 2006) and drugs including rosiglitazone (1 µmol/L, PPARγ agonist, GSK No: BRL-49653-C), compound C (5 µmol/L, AMP-activated protein kinase (AMPK) inhibitor, Sigma-Aldrich, St. Louis, MO, USA), H89 (1 µmol/L, protein kinase A (PKA) inhibitor, Millipore, Temucula, CA, USA), Rp-cAMP (10 µmol/L, PKA inhibitor, RBI, Natick, MA,

USA), SQ22536 (100 µmol/L, adenylyl cyclase inhibitor, Tocris Bioscience, Bristol, UK), rabbit polyclonal antibodies against mouse adiponectin (5 µg/mL) (Zhou *et al.*, 2008); GW501516 (0.1 µmol/L, PPARδ agonist, Alexis Biochemicals, Lausen, Switzerland), GW0742 (0.1 µmol/L, PPARδ agonist, Tocris Bioscience), wortmannin (0.1 µmol/L, PI3K inhibitor, Tocris Bioscience), LY294002 (10 µmol/L, PI3K inhibitor, Tocris Bioscience), GSK0660 (1 µmol/L, PPARδ antagonist Sigma-Aldrich) were added individually. After the incubation period, ring segments were transferred to fresh Krebs solution, mounted in a myograph, and changes in isometric force were recorded.

2.4.2 *Ex vivo* fat tissue explant culture

The method was modified from an established adipose tissue culture technique (Delporte *et al.*, 2002). After the mice were sacrificed, adipose tissues (subcutaneous, visceral, perivascular) were weighted to an equal amount, rinsed in phosphate-buffered saline (PBS), and incubated in Dulbecco's modified Eagle's medium/Ham's F12 medium (HyClone, Ogden, UT, USA). The samples were centrifuged briefly to separate the fat explants from precipitated cells, and re-suspended in serum-free medium. Drugs including rosiglitazone malate (PPARγ agonist, 1 µmol/L, GSK No: BRL-49653-C), GW9662 (PPARγ antagonist, 5 µmol/L), and rabbit polyclonal antibodies against mouse adiponectin (5 µg/mL) were added individually. After twelve hours of incubation, aliquots of the medium were collected for either assaying adiponectin or incubating aortic rings from *db/db* mouse following the same protocol of organ culture as mentioned above.

2.4.3 Experimental protocols

Each ring was first contracted by 60 mmol/L KCl and rinsed several times in Krebs solution. To examine endothelium-dependent relaxation, after washout, phenylephrine (Phe, 1 µmol/L, α_1-adrenoceptor agonist) was used to produce a steady contraction and subsequently relaxed by cumulative addition of acetylcholine (ACh), the muscarinic acetylcholine receptor agonist.

To test the responsiveness and sensitivity of blood vessels in response to stimulation of endothelin-1 (ET-1), the concentration-dependent contractions to ET-1 (1-50 nmol/L) were compared in control, rosiglitazone-treated rings in the absence and presence of 100 μmol/L N^G-nitro-L-arginine methyl ester (L-NAME). The effects of endothelin receptor antagonists including ABT627 (ET_AR antagonist) and A192621 (ET_BR antagonist) were tested on ET-1-induced contractions.

Endothelium-independent relaxations to sodium nitroprusside (SNP) (1 nmol/L - 1 μmol/L) were studied in rings without endothelium.

2.5 Tissue Culture

2.5.1 Primary culture of mouse aortic endothelial cells

The method for primary culture of mouse aortic endothelial cells (MAECs) was modified from Kobayashi et al. (Kobayashi *et al.*, 2005). Briefly, mice were anaethesized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Heparin (100 U/mL in PBS) was infused into the circulation from the left ventricle. The aortas were dissected in DMEM, and incubated with collagenase type II for 15 minutes at 37 °C. Detached endothelial cells were collected by centrifugation, re-suspended in 25 cm^2 flasks supplemented with 20% FBS-DMEM, then cultured in endothelial cell growth medium (EGM, Clonetics, Lonza, Walkersville, MD, USA) supplemented with bovine brain extract (BBE, Clonetics) till confluent. The cultured endothelial cells were then incubated with normal medium, high glucose (30 mmol/L) medium or high glucose medium plus individual drugs for 36 hours before collecting cells for Western blotting or measuring NO by laser confocal fluorescence microscopy.

2.5.2 Culture of human umbilical cord vein endothelial cells

Human umbilical cord vein endothelial cells (HUVECs) obtained from Lonza (CC-3317) were grown in EGM supplemented with BBE and 1% penicillin and streptomycin (GIBCO). Cells were grown in 75 cm^2 flasks and maintained at 37 °C in a 95% humidified air / 5% CO_2 atmosphere. Medium was changed every

two days. Confluent cells were passaged by trypsinization (0.25% trypsin with 2.5 mmol/L EDTA in PBS). Experiments were performed on cells at passage 4-8 when 80-90% confluency was achieved.

2.6 Western Blotting

Aortas were snap frozen in liquid nitrogen and subsequently homogenized in ice-cold RIPA lysis buffer that contained 1 μmol/L leupetin, 5 μmol/L aprotonin, 100 μmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L sodium fluoride, and 2 mg/mL β-glycerolphosphate. HUVECs or MAECs were harvested by trypsinization and homogenized with RIPA. The lysates were incubated for 30 min on ice and then centrifuged for 20 min at 20,000 g. The supernatant was collected and analyzed for protein concentration using the Lowry method (Bio-Rad, Hercules, CA, USA). Sample buffer containing 5% β-mercaptoethanol was added to the sample, and then denatured by boiling for 10 min. For each sample, 50 μg of protein was separated with 7.5% - 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), together with the prestained and biotinylated size marker. The resolved proteins were electrophorectically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) using wet transfer (Bio-Rad) at 100 V for 60 min at 4 °C. The membranes were blocked with 5% non-fat milk or 1% bovine serum albumin (BSA) dissolved in phosphate buffer saline with 0.1% Tween-20 (PBST) for 1 hour at room temperature. Primary antibodies against target proteins (information summarized in Section 2.13.4) were incubated at 4 °C overnight, while the corresponding secondary antibodies conjugated to horseradish peroxidase (HRP) (DakoCytomation, Carpinteria, CA) were used at a dilution of 1:3000 and incubated for 1 hour at room temperature. The membranes were developed with enhanced chemiluminescence detection solutions (ECL reagents; Amersham Pharmacia, Pittsburgh, PA, USA) and exposed on X-ray films. Densitometry was performed using a documentation program (Flurochem, Alpha Innotech Corp. San Leandro, CA, USA). GAPDH or β-actin was selected as housekeeping protein for checking

equal loading of each sample. Summarized data represented the mean of 4-5 separate experiments.

2.7 Immunohistochemistry

Aortic rings were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated, processed and embedded in paraffin. Cross sections at 5 μm were cut on microtome (Leica Microsystems, Germany). After rehydrated to water, sections were microwave boiled in 0.01 mol/L citrate buffer (pH 6.0) for 10 min for antigen retrieval, then incubated for 15 min with 3% H_2O_2 at room temperature to block endogenous peroxidase activity. After washed with phosphate buffer saline (PBS), sections were blocked in 5% normal goat or donkey serum according to the host species (Jackson Immunoresearch, West Grove, PA) for 1 hour at room temperature. Primary antibodies (anti-ET_BR, 1:100, Abcam, Cambridge, UK) diluted in normal serum were incubated overnight at 4°C. The slides were washed with PBS three times (5 min each). Biotin-SP conjugated goat anti-rabbit secondary antibodies (1:500, Jackson Immunoresearch) diluted in PBS were added and incubated for 1 hr at room temperature. Slides were washed with PBS three times (5 min each) and incubated for 30 min with streptavidin-HRP conjugate (1:500, Zymed laboratory, San Francisco, CA) at room temperature, and washed. Positive staining was developed as brown precipitate by 3,3'-diamonobenzidine tetrachloride (DAB) chromogen substrate (Vector laboratory, Burlingame, CA). Slides were rinsed with water and counterstained with hematoxylin. Pictures were taken under Leica DMRBE microscope with a SPOT-RT digital camera and SPOT Advanced software (Diagnostic Instruments, Sertling Heights, MI) and intensities of signals were analyzed by ImageJ (National Institute of Health, USA).

2.8 Detection of ROS by dihydroethidium fluorescence

The amount of intracellular ROS production was determined using dihydroethidium (DHE) (Molecular Probes, Eugene, OR), which binds to DNA when oxidized to emit fluorescence (Robinson *et al.*, 2006). Aortic rings from

db/m^{+} and *db/db* mice were obtained as described above in functional study. To verify the endothelial origin of the ROS production, the endothelial layer was removed by rolling the luminal surface with fine forceps tips before loading DHE dye. Frozen sections of the aortic ring were prepared in 10-μm thickness using a cryostat (Shandon, Pittsburgh, PA, USA) and incubated in Krebs solution containing DHE (5 μmol/L) for 10 min at 37 °C. Fluorescent intensity was measured by confocal microscope (FV1000, Olympus, Tokyo, Japan) at excitation/emission of 488/605 nm to visualize the fluorescence signal.

2.9 Measurement of NO by confocal fluorescence microscopy

Fluorimetric measurements were performed on MAECs using the Olympus Fluoview FV1000 laser scanning confocal system mounted on an inverted IX81 Olympus microscope, equipped with a 10X objective (NA 0.5). 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate, Molecular Probes, Eugene, OR, USA) was used as NO indicator. The cells were incubated with 1 μmol/L DAF-FM diacetate in the dark for 15 minutes and then washed for 20 minutes. The amount of NO in response to 1 μmol/L A23187 was evaluated by measuring the fluorescence intensity excited at 495 nm and emitted at 515 nm. The cells were stimulated with the calcium ionophore A23187 (1 μmol/L, Tocris) because there was little calcium or NO signal in response to acetylcholine in the cultured endothelial cells. Changes in intracellular NO production were displayed as relative fluorescence intensity (F_1/F_0, where F_0 = control before A23187, and F_1 = administration of A23187).

2.10 Cyclic AMP measurement

After organ culture, mouse aortic segments were frozen rapidly in liquid nitrogen and stored at -80 °C until homogenization in ice-cold 0.1 mol/L HCl using a glass homogenizer. The homogenate was centrifuged at 2000 g for ten minutes at 4 °C. The supernatant was extracted and the protein content was determined using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. The tissue content of cyclic AMP was determined by direct measurement using an

EIA kit (Assay Design). The tissue content of cyclic AMP is presented as pmol/mg protein. Forskolin (100 nmol/L, 1 hour) was used as a positive control.

2.11 Transfection Condition

MAECs and HUVECs were transfected with either a constitutively active Akt1 plasmid (CA-Akt) or a dominant negative Akt1 construct (DN-Akt) from Dr. Wu Zhenguo from the Department of Biochemistry, Hong Kong University of Science and Technology by electroporation using Nucleofector II machine (Amaxa/Lonza, Walkersville, MD, USA) following the procedure in manufacturer's instruction. About 70% of endothelial cells were successfully transfected by respective protocols as indicated by control transfection using a GFP-expressing pCAGGS vector.

2.12 Drugs, chemicals and other reagents

2.12.1 Chemicals

Chemicals	Description	Solvent	Source
A192621	ET_BR antagonist	DMSO	Abbott laboratories, Abbott Park, IL, USA
A23187	Calcium ionophore	DMSO	Tocris Bioscience, Bristol, UK
ABT627	ET_AR antagonist	DMSO	Abbott laboratories
Acetylcholine hydrochloride (ACh)	Muscarinic acetylcholine receptor agonist	H_2O	Sigma, St. Louis, MO. USA
Akt inhibitor V/API-2/ triciribine/TCN	Akt inhibitor	DMSO	Sigma
Compound C	AMPK inhibitor	DMSO	Sigma
Endothelin-1	Endothelin-1 receptor agonist	H_2O	Tocris Bioscience
GSK0660	PPARδ antagonist	DMSO	Sigma
GW0742	PPARδ agonist	DMSO	Tocris Bioscience
GW501516	PPARδ agonist	DMSO	Alexis Biochemicals, Lausen, Switzerland
GW9662	PPARγ antagonist	DMSO	Sigma
H89	PKA inhibitor	DMSO	Millipore, Temucula, CA, USA
LY294002	PI3K inhibitor	DMSO	Tocris Bioscience
N^G-nitro-L-arginine methyl ester (L-NAME)	Nitric oxide synthase (NOS) inhibitor	H_2O	Sigma
Phenylephrine	α-adrenergic receptor	H_2O	Sigma/RBI
Rp-cAMP	PKA inhibitor	DMSO	RBI, Natick, MA, USA
Rosiglitazone	PPARγ agonist	DMSO	GlaxoSmithKline, NC, USA
Sarafotoxin S6c	ET_BR agonist		Tocris Bioscience
Sodium nitroprusside	Exogenous NO donor	H_2O	Sigma
SQ22536	Adenylyl cyclase inhibitor	DMSO	Tocris Bioscience
U46619	TP receptor agonist	DMSO	Sigma
Wortmannin	PI3K inhibitor	DMSO	Tocris Bioscience

2.12.2 Composition of Krebs solution

The solution was freshly prepared before the experiments. The pH value was maintained at 7.4 by continuously bubbling with 95% O_2 plus 5% CO_2 at 37 °C.

Chemicals	Final concentration (mmol/L)
NaCl	119
$HaHCO_3$	25
$MgCl_2$·6H2O	1
KCl	4.7
KH_2PO_4	1.2
$CaCl_2$	2.5
D-glucose	11.1

2.12.3 Reagents for Western blot analysis

Reagents for sample preparation		
RIPA buffer		
	NaCl	8 g
	KCl	0.2 mmol/L
	Na_2PO_4	1.44 mmol/L
	KH_2PO_4	0.24 mmol/L
	NP-40	1%
	Sodium dodecyl sulfate (SDS)	0.1%
	Sodium deoxycholate	0.5%
Protease inhibitors		
	5 mg/mL aprotonin	5 µg/mL
	200 mM EDTA	1 mmol/L
	200 mM EGTA	1 mmol/L
	259 mg/mL β-glycerolphosphate	2 mg/mL
	10 mg/mL leupetin	1 µg/mL
	100 mM phenylmethylsulfonyl fluoride (PMSF)	1 mM
	125 mM sodium fluoride	1 mmol/L
	100 mM sodium orthovanadate	1 mmol/L
Reagents for gel preparation (stacking and separating)		
30% acrylamide		made up to 100 mL
	Acrylamide	29.2 g
	Methylene bis-acrylamide	0.8 g
1.5 M Lower Tris-base buffer (pH 8.8)		made up to 100 mL
	Tris base	18.17 g
	10% SDS	4 mL

0.5 M Upper Tris-base buffer (pH 6.8)		made up to 100 mL
	Tris base	6.047 g
	10% SDS	4 mL
Others		
	Tetramethylethylene diamide (TEMED)	2% in final solution
	Ammonium persulphate (freshly prepared)	0.1% in final solution
Buffers for electrophoresis, transfer, and washing		
SDS gel loading buffer (2X)		
	Tris (from 1M Tris-HCl, pH 6.8)	125 mmol/L
	SDS	4%
	Glycerol	20%
	Bromophenol blue	0.06%
	β-mecaptoethanol	10% freshly add
Electrophoresis running buffer		Adjust pH to 8.3
	Tris	25 mmol/L
	Glycine	250 mmol/L
	SDS	0.1%
Transfer buffer		
	Tris base	48 mmol/L
	Glycine	39 mmol/L
	SDS	0.037%
	Methanol	20%
Phosphate buffered saline with Tween-20 (PBST)		Adjust pH to 7.4
	NaCl	135 mmol/L
	$NaHPO_4$	3.2 mmol/L
	KH_2PO_4	0.5 mmol/L
	KCl	1.3 mmol/L
	Tween 20	0.05%

2.12.4 Primary antibodies

Primary antibodies for target proteins were diluted in 1% bovine serum albumin (dissolved in PBST) or 5 % non-fat milk for western blotting or in 5 % host serum for immunostaining and incubated overnight at 4 °C.

Antigen	Host species	Type	Application	Company
AdipoR1	rabbit	polyclonal	WB (1:200)	Alpha Diagnostic, San Antonio, TX. USA

AdipoR2	rabbit	polyclonal	WB (1:200)	Alpha Diagnostic
Adiponectin	rabbit	polyclonal	WB (1:2000)	Dr. Xu Aimin Department of Medicine, HKU
Akt1	rabbit	polyclonal	WB (1:1000)	Cell Signaling Technology
AMPKα	rabbit	polyclonal	WB (1:1000)	Cell Signaling Technology, Beverly, MA, USA
Endothelial nitric oxide synthase (eNOS)	rabbit	monoclonal	WB (1:500)	BD Transduction Laboratories, San Jose, CA. USA
GAPDH (6C5)	mouse	monoclonal	WB (1:5000)	Ambion Inc. Austin, TX. USA
Phosphor-eNOS (Ser^{1177})	rabbit	polyclonal	WB (1:1000)	Upstate Biotechnology, Lake Placid, NY. USA
Phosphor-AMPKα (Thr^{172})	rabbit	polyclonal	WB (1:1000)	Cell Signaling Technology
Phospho-Akt (Ser^{473})	rabbit	polyclonal	WB (1:1000)	Cell Signaling Technology
Phospho-Akt (Thr^{308})	rabbit	polyclonal	WB (1:1000)	Cell Signaling Technology
PPARγ	rabbit	polyclonal	WB (1:1000)	Cell Signaling Technology

2.13 Statistical analysis

Results were means ± sem on n blood vessels from separate animals. The cumulative concentration-response relationship was analyzed with a non-linear curve fitting (GraphPad Prism, Version 4.0). The pD_2 was calculated as the negative logarithm of the dilator concentration that induced 50% of the maximal relaxation (E_{max}). Protein expression analysis was normalized to GAPDH and then expressed relative to control. Student's *t*-test (unpaired two-tailed) was used and concentration-response curves were analyzed by two-way ANOVA followed by Bonferroni post-tests. Levels of probabilities of less than 0.05 were regarded as significant.

CHAPTER III

The obligatory role of adiponectin in restoring endothelial function in PPARγ agonist-treated diabetic mice

3.1 Introduction

Obesity and diabetes are common risk factors for the initiation of vascular dysfunction. Adipose tissue is now recognized as an important metabolic and endocrine organ in the regulation of glucose metabolism. Dysregulation of adipose tissue participates in the development of insulin resistance and vascular complications of diabetes (Hajer *et al.*, 2008).

The gene expression pattern of adiponectin in subcutaneous and visceral adipose tissue and the levels of circulating adipokines predict insulin resistance and diabetic risk (Samaras *et al.*, 2010). Although obesity is a common contributor to insulin resistance, the molecular link between the increased adiposity and impaired vascular function in human is not fully elucidated. Adipose tissue also contributes to the regulation of vascular tone (Fesus *et al.*, 2007; Galvez-Prieto *et al.*, 2008; Verlohren *et al.*, 2004). Chronic inflammation of adipose tissue leads to vascular dysfunction, due to a diminished production of vasoprotective cytokines and increased release of inflammatory cytokines by adipocytes. However, the role, if any, of adipose tissue in vascular benefit of anti-diabetic drugs is unclear.

Adiponectin is an adipose-secreted protein that exerts both anti-atherogenic and insulin-sensitizing effects, and a reduced production of adiponectin is closely coupled to insulin resistance (Kadowaki *et al.*, 2006;

Whitehead *et al.*, 2006; Zhu *et al.*, 2008). The plasma concentration of adiponectin in obese subjects is lower than that in non-obese subjects and correlates inversely with body mass index (Arita *et al.*, 1999). Moreover, hypoadiponectinemia is associated with the attenuated endothelium-dependent dilatation in both diabetic and non-diabetic human subjects (Ouchi *et al.*, 2003; Shimabukuro *et al.*, 2003; Tan *et al.*, 2004; Torigoe *et al.*, 2007). The nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) is a major regulator of adipocyte function and controls the secretion of adipokines, in particular promoting the production of adiponectin (Crossno *et al.*, 2006; Maeda *et al.*, 2001), while limiting the generation of pro-inflammatory TNFα, IL-6 and IL-1β (Jiang *et al.*, 1998). The insulin sensitizing drugs thiazolidinediones (TZDs) including rosiglitazone and pioglitazone, are high-affinity ligands which act on PPARγ in liver, skeletal muscle, and adipose tissue. TZDs also increase plasma adiponectin levels in insulin-resistant humans (Yang *et al.*, 2002; Zhu *et al.*, 2008). PPARγ ligands improve endothelial function through multiple mechanisms including stimulating eNOS (Calnek *et al.*, 2003; Cho *et al.*, 2004; Liang *et al.*, 2009; Yasuda *et al.*, 2009), inhibiting inflammatory target genes (Kanda *et al.*, 2009; Lee *et al.*, 2009; Orasanu *et al.*, 2008), and down-regulating NAD(P)H oxidases (Ceolotto *et al.*, 2007).

Although TZDs are widely used to restore insulin sensitivity in patients with type 2 diabetes (Yki-Jarvinen, 2004) the molecular mechanisms that confer its vascular protection and vasodilatory function are poorly understood. The present experiments were designed to test the hypothesis that adipocyte-derived adiponectin plays an indispensable role in the amelioration of endothelial dysfunction in diabetes following chronic treatment with PPARγ

agonists. The present results demonstrate that subcutaneous adipose tissue is an important target for PPARγ agonists to improve diabetic endothelial function. The adipocytes-derived adiponectin by two independent cellular mechanisms preserves the bioavailability of nitric oxide (NO).

3.2 Experimental procedures

3.2.1 Chemicals

Acetylcholine, N^G-nitro-L-arginine methyl ester (L-NAME), phenylephrine and Rp-cAMP were dissolved in water, while others in DMSO. All drugs and chemicals were purchased from Sigma-Aldrich, unless specified.

3.2.2 Animals

Male leptin receptor$^{-/-}$ (*db/db*) mice and leptin receptor$^{-/-}$/adiponectin$^{-/-}$ double knockout (*db/Adn* DKO) mice (Zhou *et al.*, 2008) and their lean littermates; adiponectin knockout (*Adn*$^{-/-}$) mice (Ma *et al.*, 2002) with a C57BL/6J background and their wild type controls; and PPARγ heterozygous-deficient mice (*PPARγ*$^{+/-}$) mice (Yu *et al.*, 2008) and PPARγ wild-type (*PPARγ*$^{+/+}$) controls were used for this study. *PPARγ*$^{+/-}$ mice were used because all homozygous PPARγ knockout animals were embryonically lethal due to placental dysfunction (Yu *et al.*, 2008). The mice were housed in a temperature-controlled holding room (22–23°C) with a 12-hour light/dark cycle, and fed a standard chow and water. All of the experiments were conducted under the institutional guidelines for the humane treatment of laboratory animals.

3.2.3 Oral glucose tolerance test (OGTT)

After eight hours of fasting, mice were loaded with glucose solution (1.2 g/kg) by oral gavage. Blood was drawn from the mouse tail and plasma glucose was measured at times 0, 15, 30, 60 and 120 min with a commercial glucometer.

3.2.4 Functional assay

After mice were sacrificed, thoracic aortas were removed rapidly and placed in oxygenated ice-cold Krebs solution that contained (mmol/L): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. Changes in isometric tone of the rings were recorded in myograph (Danish Myo Technology, Aarhus, Denmark) (Wong *et al.* 2010). The rings were stretched to an optimal baseline tension of 3 mN and then allowed to equilibrate for 60 minutes before the experiment commenced. Rings were first contracted with 60 mmol/L KCl and rinsed in Krebs solution, and after several washouts, phenylephrine (1 μmol/L) was used to produce a steady contraction. Then acetylcholine (ACh) (10 nmol/L – 10 μmol/L) was added cumulatively.

3.2.5 Organ culture of mouse arterial rings

Mouse thoracic aortic rings (2 mm in length) were incubated in a Dulbecco's Modified Eagle's Media (DMEM, Gibco, Gaithersberg, MD, USA) culture media supplemented with 10% fetal bovine serum (FBS, Gibco), plus 100 IU/mL penicillin and 100 μg/mL streptomycin (Wong et al. 2010a). Mouse full-length adiponectin (5 μg/mL) and drugs including compound C (5 μmol/L, AMP-activated protein kinase (AMPK) inhibitor, SigmaAldrich, MO, USA), H89 (1 μmol/L, protein kinase A (PKA) inhibitor, Millipore, Temucula, CA, USA), Rp-cAMP (10 μmol/L, PKA inhibitor, RBI, Natick, MA, USA), SQ22536 (100 μmol/L, adenylyl cyclase inhibitor, Tocris, Bristol, UK), rabbit polyclonal antibodies against mouse adiponectin (5 μg/mL) (Zhou *et al.*, 2008) were added individually. After the incubation period, ring segments were transferred to fresh Krebs solution, mounted in a myograph, and changes in isometric force were recorded.

3.2.6 *Ex vivo* fat tissue explant culture

The method was modified from an established adipose tissue culture technique (Delporte *et al.*, 2002). After the mice were sacrificed, adipose tissues (subcutaneous, visceral, perivascular) were weighted to an equal amount, rinsed in phosphate-buffered saline (PBS), and incubated in Dulbecco's modified Eagle's medium/Ham's F12 medium (HyClone, Ogden, UT, USA). The samples were centrifuged briefly to separate the fat explants from precipitated cells, and re-suspended in serum-free medium. Drugs including rosiglitazone malate (PPARγ agonist, 1 μmol/L, GSK No: BRL-49653-C), GW9662 (PPARγ antagonist, 5 μmol/L), and rabbit polyclonal antibodies against mouse adiponectin (5 μg/mL) (Zhou *et al.*, 2008) were added individually. After twelve hours of incubation, aliquots of the medium were collected for either assaying adiponectin or incubating aortic rings from db/db mouse following the same protocol of organ culture as mentioned above (Figure 1A). In order to avoid rejection, transplantation was performed between littermates from the same mother.

3.2.7 Fat Transplantation

The surgical procedures were approved by the Animal Experimental Ethics Committee, CUHK. Methods were modified from several groups *(Gabriely et al.*, 2002; Gavrilova *et al.*, 2000; Tran *et al.*, 2008). To avoid rejection, donor fat grafts were taken from *db/db* littermates. Mice were anaesthetized with a mixture of 35 mg/kg ketamine and 7 mg/kg xylazine. Fat transplantation was performed using fat pads removed from either the subcutaneous dorsal area. Fat pads were removed, cut into approximately 200 mg pieces, and kept in saline warmed at 37 °C until transplantation. Recipient mice were

anesthetized. For each recipient mouse, a total of 1.0 g subcutaneous fat were removed from the dorsal area, similar amount of donor slices of fat were transplanted into the dorsal area. All mice received subcutaneous injection of antibiotics penicillin and streptomycin after surgery and housed in individual cages for 2 weeks before sacrifice. Fat grafts were examined visually to see whether it was necrotic after sacrifice, which was excluded if this occurred.

3.2.8 Detection of ROS by dihydroethidium (DHE) fluorescence

The amount of intracellular ROS production was determined using DHE (Molecular Probes, Eugene, OR, USA). Aortic rings from *db/m*$^{+}$ and *db/db* mice were incubated in culture medium. Frozen sections (10 μm thick) of the ring were cut using a cryostat and incubated for ten minutes at 37 °C in Krebs solution containing 5 μmol/L DHE. The fluorescence intensity was measured with a confocal microscope (FV1000, Olympus, Tokyo, Japan) at an excitation/emission of 488/605 nm to visualize the signal, and analyzed using Olympus Fluoview Version 1.5.

3.2.9 Measurement of intracellular cyclic AMP

After organ culture, mouse aortic segments were frozen rapidly in liquid nitrogen and stored at -80 °C until homogenization in ice-cold 0.1 mol/L HCl using a glass homogenizer. The homogenate was centrifuged at 2000 g for ten minutes at 4 °C. The supernatant was extracted and the protein content determined using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. The tissue content of cyclic AMP was determined by direct measurement using an EIA kit (Assay Design). The tissue content of cyclic

AMP is presented as pmol/mg protein. Forskolin (100 nmol/L, 1 hour) was used as a positive control.

3.2.10 Western blotting

Protein samples prepared from aorta homogenates or fat tissue explants were electrophoresed through a 10% SDS-poly-acrylamide gel and transferred onto an immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). Nonspecific binding sites were blocked with 5% non-fat milk or 1% BSA in 0.05% Tween-20 TBS. The blots were incubated overnight at 4°C with the primary antibodies: polyclonal anti-phosphor-eNOS at Ser^{1177} (1:1000, Upstate Biotechnology, Lake Placid, NY, USA); anti-phosphor-AMPKα at Thr^{172}, polyclonal anti-eNOS, anti-AMPK (1:1000, Cell Signaling, USA), monoclonal anti-PPARγ (1:1000, Cell Signaling, USA), rabbit polyclonal antibodies against mouse adiponectin (1:1000) (Zhou *et al.*, 2008); followed by HRP-conjugated secondary antibody (DakoCytomation, Carpinteria, CA, USA). Monoclonal anti-GAPDH (1:5000, Ambion, Cambridge, UK) was used as a housekeeping protein. For detection of adiponectin in culture medium of fat explants, equal amount of fat explants and equal volume of medium were subjected to Western blots.

3.2.11 Primary culture of mouse aortic endothelial cells

The method was modified from Kobayashi et al. (Kobayashi *et al.*, 2005). Briefly, mice were anaethesized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Heparin (100 U/mL in PBS) was infused into the circulation from the left ventricle. The aortas were dissected in DMEM, and incubated with collagenase type II for 15 minutes at 37 °C. Detached

endothelial cells were collected by centrifugation, resuspended with 20% FBS-DMEM, then cultured in endothelial cell growth medium (EGM) supplemented with bovine brain extract (Lonza, Walkersville, MD, USA) till confluent. The cultured endothelial cells were then incubated with normal medium, high glucose (30 mmol/L) medium or high glucose medium plus 5 μmol/L mouse recombinant full-length adiponectin for 36 hours before measuring NO by laser confocal fluorescence microscopy.

3.2.12 Measurement of NO by laser confocal fluorescence microscopy

Fluorimetric measurements were performed on primary mouse aortic endothelial cells using the Olympus Fluoview FV1000 laser scanning confocal system mounted on an inverted IX81 Olympus microscope, equipped with a 10X objective (NA 0.5). 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate, Molecular Probes, Eugene, OR, USA) was used as NO indicator. The cells were incubated with 1 μmol/L DAF-FM DA in the dark for 10 minutes and then washed for 20 minutes. The amount of NO in response to 1 μmol/L A23187 was evaluated by measuring the fluorescence intensity excited at 495 nm and emitted at 515 nm. The cells were stimulated with the Ca^{2+} ionophore A23187 because there was no calcium or NO signal in response to acetylcholine in the cultured endothelial cells. Changes in intracellular NO production was displayed as relative fluorescence intensity (F_1/F_0, where F_0 = control and F_1 = administration of A23187).

3.2.13 Statistics

Results represent means ± SEM from different mice. Concentration-response curves were analyzed by non-linear regression curve fitting using GraphPad Prism software (Version 4.0) to calculate E_{max} as the maximum response and pD_2 as the negative logarithm of the drug concentration that produced half of E_{max}. The protein expression was quantified by densitometer (FluorChem, Alpha Innotech, San Leandro, CA), normalized to GAPDH and then compared with control. Comparisons among groups were made using ANOVA followed by an unpaired Student's t test. The *p* values less than 0.05 were accepted to indicate statistically significant differences.

3.3 Results

3.3.1 Adipose tissue is required for PPARγ activation-induced restoration of the impaired endothelium-dependent relaxation in *db/db* mouse aorta

In order to investigate the role of adipose tissue in PPARγ induced endothelial protective effect *in vivo*, an *ex vivo* fat explant organ culture method is used to examine the effect of adipokines released from different fat depots on vascular function (Figure 3.1A). Rosiglitazone (1 μmol/L, 12 hours)-activated adipose tissue (pool of subcutanesous and visceral fat depots) from either non-diabetic *db/m*$^{+}$ or diabetic *db/db* mice significantly improved, to a similar extent, endothelium-dependent relaxations (EDR) in response to acetylcholine (ACh) in *db/db* mouse aortas precontracted with phenylephrine (1 μmol/L) (Figure 3.1B).

However, 12-hour exposure of isolated *db/db* mouse aortas to rosiglitazone alone without fat explant did not improve EDR (Figure 3.2A). The EDR was increased markedly only by medium from rosiglitazone-activated subcutaneous adipose tissue (Figure 3.2B), while medium from visceral adipose tissue had no effect (Figure 3.2C), and that from perivascular adipose tissue caused a moderate potentiation of EDR (Figure 3.2D).

Adipose tissue from *db/db* mouse expressed significantly less PPARγ as shown by Western blotting. The expression level of PPARγ corresponded to the effect on EDR from different fat depots of *db/db* and *db/m*$^{+}$ mice. Subcutaneous adipose expressed the highest level of PPARγ followed by perivascular adipose while visceral adipose contained the least amount of PPARγ (Figure 3.3A). The adiponectin release in response to rosiglitazone in

subcutaneous adipose tissue was significantly higher than that in perivascular and visceral adipose tissue from *db/db* mouse (Figure 3.3B). The following experiments were performed using only subcutaneous adipose tissue from *db/db* mouse in organ culture.

3.3.2 PPARγ activation increases adiponectin release and improves endothelium-dependent relaxation

I next tested the effects of PPARγ antagonist GW9662 and anti-adiponectin neutralizing antibody to establish an essential role of adipose tissue-derived adiponectin in mediating PPARγ-dependent improvement of EDR in *db/db* mice. Both anti-adiponectin antibody (5 μg/mL) and GW9662 (5 μmol/L) prevented the effect of PPARγ-treated fat explant from *db/m*$^{+}$ (Figure 3.4A) and *db/db* mice (Figure 3.4B).

The pivotal role of adipocyte-derived adiponectin was further confirmed as EDR in *db/db* mice was not restored by rosiglitazone-treated fat explant from *Adn*$^{-/-}$ mice (Figure 3.5A). Similarly, improvement of EDR in *db/db* mice with rosiglitazone-treated fat explant from *PPARγ*$^{+/-}$ mice was attenuated (Figure 3.5B). EIA and Western blotting showed that rosiglitazone elevated the amount of adiponectin released by fat explants from *db/db* and *db/m*$^{+}$ mice but not from *Adn*$^{-/-}$ mice, and that this increase was inhibited by GW9662 (Figure 3.6A). Rosiglitazone-stimulated adiponectin release was much less in fat explants from *PPARγ*$^{+/-}$ mice which might account for the small improvement of EDR in *db/db* mouse aortas, correlating with less PPARγ expression (Figure 3.6B).

3.3.3 *In vivo* rosiglitazone treatment improves endothelial function in diabetic mice through an adiponectin-dependent

mechanism

3.3.3.1 Rosiglitazone treatment improves endothelial function in *db/db* and DKO mice

Administration of *db/db* mice with rosiglitazone (10 mg/kg body weight/day) for four weeks improved oral glucose tolerance (Figure 3.7A). Rosiglitazone treatment in DKO mice also improved glucose tolerance to a similar extent, as showed in Figure 3.7A and summarized data by area under curve in Figure 3.7B. Rosiglitazone treatment increased plasma adiponectin in *db/db* mice (adiponectin level in μg/mL: 2.86 ± 0.22 in vehicle-treated mice vs 8.08 ± 1.45 in rosiglitazone-treated mice, $p<0.05$), which was very low in DKO mice and DKO mice treated with rosiglitazone (Figure 3.7C).

In vivo rosiglitazone treatment also improved lipid profile in *db/db* mice. Fat composition as showed by fat weight comparing to body weight is similar in *db/db* mice and *db/db* or *DKO* mice treated with rosiglitazone (Figure 3.8A). However, plasma concentrations of total cholesterol and triglyceride were significantly reduced in *db/db* mice treated with rosiglitazone comparing with control (Figure 3.8B&C).

Endothelium-dependent relaxation to acetylcholine in aortas was significantly reduced in *db/db* compared with *db/m*$^{+}$ mice (Figure 3.9A&B). Administration of *db/db* mice with rosiglitazone (10 mg/kg body weight/day) for four weeks potentiated endothelium-dependent relaxations to acetylcholine in aortas from *db/db* mice, but not in those from *DKO* mice (Figure 3.9A&B).

Aortas from *db/db* mice exhibited a significantly less phosphorylation of AMPK at Thr^{172} and eNOS at Ser^{1177} compared with those from *db/m*$^{+}$ mice.

In vivo treatment with rosiglitazone restored the reduced phosphorylation of AMPK and eNOS (Figure 3.10A-C).

Aortas from *db/db* mice showed slightly less expressions of AdipoR1 and AdipoR2 as compared with those from db/m^+ (Figure 3.10B&E&F). Rosiglitazone treatment increased AdipoR2 expression in aortas from *db/db* mice, however, the difference was not significant (Figure 3.10B&F). AdipoR1 expression was unchanged after rosiglitazone treatment (Figure 3.10B&E).

3.3.3.2 Rosiglitazone treatment improves endothelial function in DIO mice

In order to verify the effect of rosiglitazone in type 2 diabetes apart from genetic model of diabetes such as *db/db* mice, I also used DIO induced mice. Aortas from DIO mice have the reduced EDRs compared with age-matched C57BL/6J littermates. Rosiglitazone treatment in DIO mice also restored EDRs in aortas (Figure 3.11A). In addition, improved relaxations after *in vivo* rosiglitazone treatment were inhibited by overnight incubation with compound C or anti-adiponectin antibody, but unaffected by GW9662 (Figure 3.11B). Reduced phosphorylations of AMPK and eNOS in aortas from DIO mice were restored after rosiglitazone treatment (Figure 3.11C&D).

3.3.4 In vivo fat transplantation improves endothelial function in diabetic mice

In order to further confirm the importance of subcutaneous adipose tissue in endothelial protection in response to PPARγ activation in diabetic mice, I established fat transplantation model in diabetic mice. Fat transplantation

were performed using subcutaneous flank fat of donor *db/db* mice, removing an similar amount of subcutaneous fat of recipient *db/db* mice, and placing the fat grafts into the same dorsal subcutaneous area from the donor to the recipient as showed in the schematic Figure 3.12A. Some donors or recipients were treated with rosiglitazone (10 mg/kg/day for 4 weeks) prior to fat transplantation. EDRs were studied in control recipient mice receiving fat graft from rosiglitazone treated mice (C+RF) (Figure 3.12B). Rosiglitazone-treated recipient mice receiving fat graft from either rosiglitazone-treated donors (R+RF) or from control donors (R+CF) have similar EDRs as those C+RF littermates, which were also significantly improved compared with impaired EDRs from control recipients receiving fat grafts from control donor mice (C+CF) (Figure 3.12B). In addition, AMPK and eNOS phosphorylations also increased in aortas from control mice receiving fat transplant from rosiglitazone-treated mice; or those from rosiglitazone-treated mice receiving fat transplants either from control or rosiglitazone-treated mice (Figure 3.12 C&D).

3.3.5 Adiponectin increases NO bioavailability through AMPK and PKA

Adiponectin (5 μg/mL) augmented EDR in *db/db* mouse aortas (Figure 3.13A). The effect of adiponectin was abolished by incubation with anti-adiponectin antibody (5 μg/mL), while unaffected by PPARγ antagonist GW9662 (Figure 3.13A). Improved EDR in response to adiponectin was inhibited by the AMPK inhibitor compound C (5 μmol/L) (Figure 3.13B). Treatment with the cyclic AMP-dependent protein kinase (PKA) inhibitors, H89 (1 μmol/L) or Rp-cAMP (10 μmol/L), or the adenylyl cyclase inhibitor SQ22536 (10 μmol/L)

significantly attenuated the effect of adiponectin (Figure 3.13C). Combined treatment with compound C and H89 did not cause further inhibition (Figure 3.13B). Adiponectin improved EDRs in aortas from DIO mice, which was inhibited by anti-adiponectin antibody or compound C, but unaffected by GW9662 (Figure 3.13D).

Western blots from *db/db* mouse aortas showed that adiponectin increased the phosphorylation of AMPK at Thr^{172}, and eNOS at Ser^{1177}. Compound C but not H89 inhibited the adiponectin-stimulated AMPK phosphorylation (Figure 3.14A). Compound C, H89, and SQ22536 also attenuated the adiponectin-stimulated eNOS phosphorylation (Figure 3.14B).

The aortas of *db/db* mouse contained higher levels of ROS compared with those of db/m^+ mouse as revealed by DHE fluorescence intensity. Treatment with 5 μg/mL adiponectin reduced ROS and this effect was abolished by H89 or SQ22536, but not by compound C (Figure 3.15A&B). Forskolin, a cyclic AMP-elevating agent at 100 nmol/L produced a similar effect as adiponectin in reducing ROS (Figure 3.15A&B).

Biochemical assays confirmed that both adiponectin and forskolin raised the tissue content of cyclic AMP in db/m^+ and *db/db* mouse aortas. Only SQ22536 but not the other inhibitors prevented the adiponectin-induced increase in cyclic AMP level (Figure 3.16).

The NO production in response to A23187 (1 μmol/L) was significantly blunted in primary cultured mouse aortic endothelial cells (MAECs) incubated in high glucose (30 mmol/L, HG) when compared with its mannitol osmotic control (NG) (Figure 3.17A&B). Incubation of 5 μg/mL adiponectin restored the reduced NO production in endothelial cells under high glucose condition (Figure 3.17A&B).

3.4 Discussion

The present study defines an obligatory role of adipose tissue, particularly subcutaneous fat depots, in an improvement of endothelial function in diabetic mice following PPARγ activation. It demonstrates that adipocyte-derived adiponectin is the primary mediator that improves endothelial function and does so by both AMPK- and PKA-mediated mechanisms. The present findings suggest that adipose tissue can be an important therapeutic target in the protection of vascular dysfunction in diabetes through the production and release of anti-inflammatory vaso-active hormones of which adiponectin plays an indispensable role in protecting vascular function.

The present study employs multiple approaches aided by the use of relevant genetically modified mice to demonstrate a crucial role of adipocyte-derived adiponectin in PPARγ agonist-induced endothelial cell protection in diabetic mice. Differential expression levels of PPARγ in the three studied fat depots were observed with the PPARγ expression being most abundant in subcutaneous, intermediate in perivascular and least in visceral adipose tissues. The PPARγ level positively correlated with the amount of adiponectin released in different fat depots upon PPARγ activation by rosiglitazone and also corresponded to the extent of adiponectin-mediated improvement in endothelium-dependent relaxations in aortas from *db/db* mice in response to ex vivo PPARγ ligands on fat explant. The present results indicate that PPARγ agonists do not act on the endothelium directly since exposure to rosiglitazone did not augment the relaxation without the presence of adipose tissue. Although adipose tissues from *db/db* mice expressed less PPARγ and secreted less adiponectin than those from non-diabetic *db/m*$^{+}$ mice, PPARγ

activation in subcutaneous fat explants from *db/db* mice shows similar effectiveness in augmenting the acetylcholine-induced relaxation of *db/db* mouse aortas. The specificity of PPARγ was further verified by using a PPARγ antagonist GW9662 and studying *PPARγ*$^{+/-}$ mice. The latter expectedly exhibited a reduced PPARγ expression. The obligatory role of adipocyte-derived adiponectin in the vascular benefits of PPARγ agonist was also supported by the observation that the administration of a neutralizing anti-adiponectin antibody *in vitro* could eliminate the beneficial vascular effect of PPARγ activated fat explants, and that PPARγ activation in fat explants from *adiponectin*$^{-/-}$ mice failed to improve the relaxation of *db/db* mouse aortas. These evidences from fat explant experiment suggest that adiponectin production in response to PPARγ activation from adipose tissue improved endothelial function of diabetic mice.

The beneficial effect of PPARγ activation is further confirmed by the chronic oral administration of rosiglitazone to diabetic mice. The chronic TZD treatment markedly augmented endothelium-dependent relaxations in aortas from *db/db* mice, improved glucose tolerance, and increased serum adiponectin level. The observed vascular benefit could be a consequence of systemic improvement in insulin sensitivity in diabetic mice after treatment with rosiglitazone. However, the experiments with *db/Adn DKO* mice performed to verify the adiponectin-dependent endothelial protection of the *in vivo* treatment, demonstrated that the potentiating effect of rosiglitazone on endothelial function was largely blunted in aortas of these animals, indicating an indispensable role of adiponectin in preventing diabetic vascular dysfunction.

To further the importance of subcutaneous adipose tissue in diabetic

mice in response to PPARγ activation, I continued to examine whether the benefit from subcutaneous adipose tissue can be transferred from rosiglitazone-treated mice to untreated mice *in vivo*, in order to confirm the role of adipose tissue by a more definitive method. Visceral fat removal or subcutaneous fat transplantation is effective to reverse or prevent insulin resistance and glucose intolerance in diabetic mice (Gabriely *et al.*, 2002; Gavrilova *et al.*, 2000; Tran *et al.*, 2008). Therefore, in the present study, fat graft transplantation was applied to see whether the benefits of PPARγ activation in adipose tissue on endothelial function can also be transferred. Data showed that fat graft from rosiglitazone-treated *db/db* mice was able to result in improvement of endothelial function after implanting into control *db/db* mice, suggesting that subcutaneous adipose tissue was the major source to release vaso-protective adipokines. Interestingly, this benefit could last for a period which is in my experiment for two weeks in the recipient mice after rosiglitazone treatment stopped, because the recipient did not receive continuously rosiglitazone treatment. Moreover, in rosiglitazone-treated recipients receiving fat grafts from control donors, the protective effect on endothelial function by rosiglitazone treatment could also be prolonged even if the amount of subcutaneous adipose tissue was reduced which was partially replaced with fat from control donors. This approach strengthened my hypothesis that subcutaneous adipose tissue is the major source to release vaso-protective adiponectin in response to PPARγ activation in diabetic mice.

TZDs are reported to stimulate adiponectin transcription through PPAR-responsive element in its promoter in adipocytes and in adipose tissues of obese mice and promote adiponectin secretion from adipocytes (Combs *et al.*, 2002; Iwaki *et al.*, 2003; Maeda *et al.*, 2001). This PPARγ-dependent

adiponectin production is responsible for regulation of glucose homeostasis and improvement of insulin sensitivity in diabetic animal models and type 2 diabetic patients (Anghel *et al.*, 2007; Combs *et al.*, 2002; He *et al.*, 2003; Kim *et al.*, 2007; Nawrocki *et al.*, 2006; Yang *et al.*, 2004; Yang *et al.*, 2002). The present study also demonstrates an increased adiponectin release from fat explants upon PPARγ activation. Although the PPARγ expression was less in adipose tissue from *db/db* mice, PPARγ activation in fat explants from these mice showed similar effectiveness to release adiponectin as fat from non-diabetic mice. A possible explanation is that TZDs improve insulin sensitivity and reverse the proinflammatory changes in adipocytes to facilitate the release of vaso-protective adipokines (Chatterjee *et al.*, 2009; Chui *et al.*, 2005; He *et al.*, 2003; Marchesi *et al.*, 2009).

Treatment with TZDs improves cardiovascular outcomes such as hypertension and atherosclerosis (Calkin *et al.*, 2005; Collins *et al.*, 2001; Duan *et al.*, 2008; Joner *et al.*, 2007; Ryan *et al.*, 2004; Wang *et al.*, 2004; Yue TI *et al.*, 2001). *In vivo* TZD treatment, through adiponectin-dependent mechanisms, reduces pathological revascularizations in the ischemic retina (Higuchi *et al.*, 2010), and inhibits plasminogen activator inhibitor-1 production (Hoo *et al.*, 2007). The present study suggests that the vascular benefit of TZD treatment is largely dependent on adiponectin instead of a systemic improvement of insulin sensitivity since glucose tolerance of *db/db* and *db/Adn DKO* mice improved to a similar extent upon rosiglitazone treatment, but endothelium-dependent relaxations were improved by the treatment only in the former. However, the direct effect of long-term beneficial effects of TZDs on endothelial cells and vascular smooth muscle cells can not be excluded. There were several reports suggesting that TZDs also acts directly

on the vasculature to exert anti-inflammatory and anti-oxidative effects by inhibition of several cytokines and chemokines such as tumor necrosis factor (TNF)-α, matrix metalloproteinase 9 (MMP9), and IκBα expression (Bishop-Bailey *et al.*, 2002; Chang *et al.*, 2009; de Dios *et al.*, 2003; Giannini *et al.*, 2004; Goetze *et al.*, 2002; Law *et al.*, 2000; Marx *et al.*, 1998; Orasanu *et al.*, 2008). In type 2 diabetic patients, TZDs also have anti-inflammatory effects. TZDs could reduce monocyte chemoattractant protein-1 (MCP-1), C-reactive protein (CRP), and soluble vascular cell adhesion molecules (sVCAM)-1, etc. (Ghanim *et al.*, 2006; Hanefeld *et al.*, 2007; Kahn *et al.*, 2010; Kanda *et al.*, 2009; Lombardi *et al.*, 2008; Marx *et al.*, 2003; Mohanty *et al.*, 2004; Orasanu *et al.*, 2008).

To further investigate the direct effect of adiponectin on vascular function, the effect of full-length mouse recombinant adiponectin was studied. The observations that the adiponectin augmented endothelium-dependent relaxations, increased AMPK and eNOS phosphorylation, and reduced ROS production in aortas from *db/db* mice, suggest that both the AMPK and cyclic AMP/PKA signaling cascade contribute to the effect of adiponectin in increasing NO bioavailability. Indeed, the AMPK inhibitor compound C markedly attenuated the vascular effect of adiponectin and abolished the adiponectin-stimulated increases in phosphorylation of AMPK at Thr^{172} and reduced eNOS phosphorylation at Ser^{1177}. The present findings are in line with the observation that AMPK activation is involved in adiponectin-stimulated production of NO in cultured endothelial cells (Chandrasekar *et al.*, 2008; Chen *et al.*, 2003; Cheng *et al.*, 2007; Deng *et al.*, 2010; Gonon *et al.*, 2008; Ouchi *et al.*, 2004). The present results demonstrate the functional importance of AMPK activity in adiponectin-induced vascular benefit in intact

arteries of diabetic mice. The adiponectin induced improvement of endothelium-dependent relaxations can also be mediated by the cyclic AMP/PKA cascade. This conclusion is based on the observation that the responses of *db/db* mouse aortas to adiponectin were inhibited by inhibitors of adenylyl cyclase and PKA. These agents also reduced eNOS phosphorylation. Further experiments demonstrated that adiponectin increased the cyclic AMP content in *db/db* mouse aortas through activation of adenylyl cyclase, independently of AMPK. Importantly, PKA was also involved in the adiponectin-induced ROS reduction in aortas from *db/db* mice. These findings suggest that the vascular effect of adiponectin is also partially mediated through PKA signaling pathways. Previous studies in human umbilical vein endothelial cells showed that cyclic AMP/PKA signaling mechanisms mediate the inhibitory effect of adiponectin on high glucose-induced H_2O_2 generation (Ouedraogo *et al.*, 2006). In patients, hypoadiponectinema is associated with increased oxidative stress (Lautamaki *et al.*, 2007). Likewise, in this study, adiponectin inhibited the ROS production in aortas from *db/db* mice. This effect was abolished by H89 and SQ22536, but not by compound C, suggesting the major involvement of the cyclic AMP/PKA pathway in lowering ROS. A reduced production of ROS by adiponectin should further enhance NO bioavailability. However, the possibility can not be discounted that *in vivo* TZD treatment results in direct inhibition of oxidative stress as activation of endothelial PPARγ also exert anti-inflammatory and antioxidant effects (Beyer *et al.*, 2008; Ceolotto *et al.*, 2007). Moreover, the anti-oxidative effect of adiponectin may also due to the inhibition of NADPH oxidase activity, suppression of IκBα expression and antagonism of TNF-α as reported previously (Devaraj *et al.*, 2008; Li *et al.*,

2007; Ohashi *et al.*, 2007; Tao *et al.*, 2007; Wang *et al.*, 2009; Zhang *et al.*, 2010).

Several previous reports suggest that adiponectin receptors both AdipoR1 and AdipoR2 are expressed in endothelial cells (Cheng *et al.*, 2007; Goldstein *et al.*, 2004; Tan *et al.*, 2004). In bovine aortic endothelial cells, AdipoR1 has higher affinity to globular adiponectin, while AdipoR2 has similar affinity for both globular and full-length adiponectin, which we used in our experiment (Motoshima *et al.*, 2004). In human endothelial cells, both AdipoR1 and AdipoR2 mediate the effect of adiponectin to stimulate eNOS activity (Cheng *et al.*, 2007). The expressions of both receptors have also been shown in aortas and coronary arterioles of *db/db* mouse (Zhang *et al.*, 2010). The present study showed the expression of adiponectin receptor AdipoR1 and AdipoR2 in the aortas by Western blots. AdipoR2 expression reduced in aortas from *db/db* mice compared with *db/m*$^{+}$, and increased after rosiglitazone treatment, while AdipoR1 was not altered, which is similar to the previous report (Zhang *et al.*, 2010), suggesting that the sensitivity to adiponectin was reduced in diabetic and non-diabetic mouse arteries, which is also in line with previous study (Cheng *et al.*, 2007).

In summary, the present study demonstrates that PPARγ activated adipocyte-derived adiponectin plays an obligatory role in TZD induced improvement of endothelial function in diabetes. Adiponectin increases the NO bioavailability by activating AMPK and cyclic AMP/PKA signaling. The present results also support a differential role of various fat depots, which is directly related to the amount of adiponectin released upon PPARγ activation. Subcutaneous adipose tissue could be an important intervention target for newly developed PPARγ agonists in the alleviation of diabetic vasculopathy.

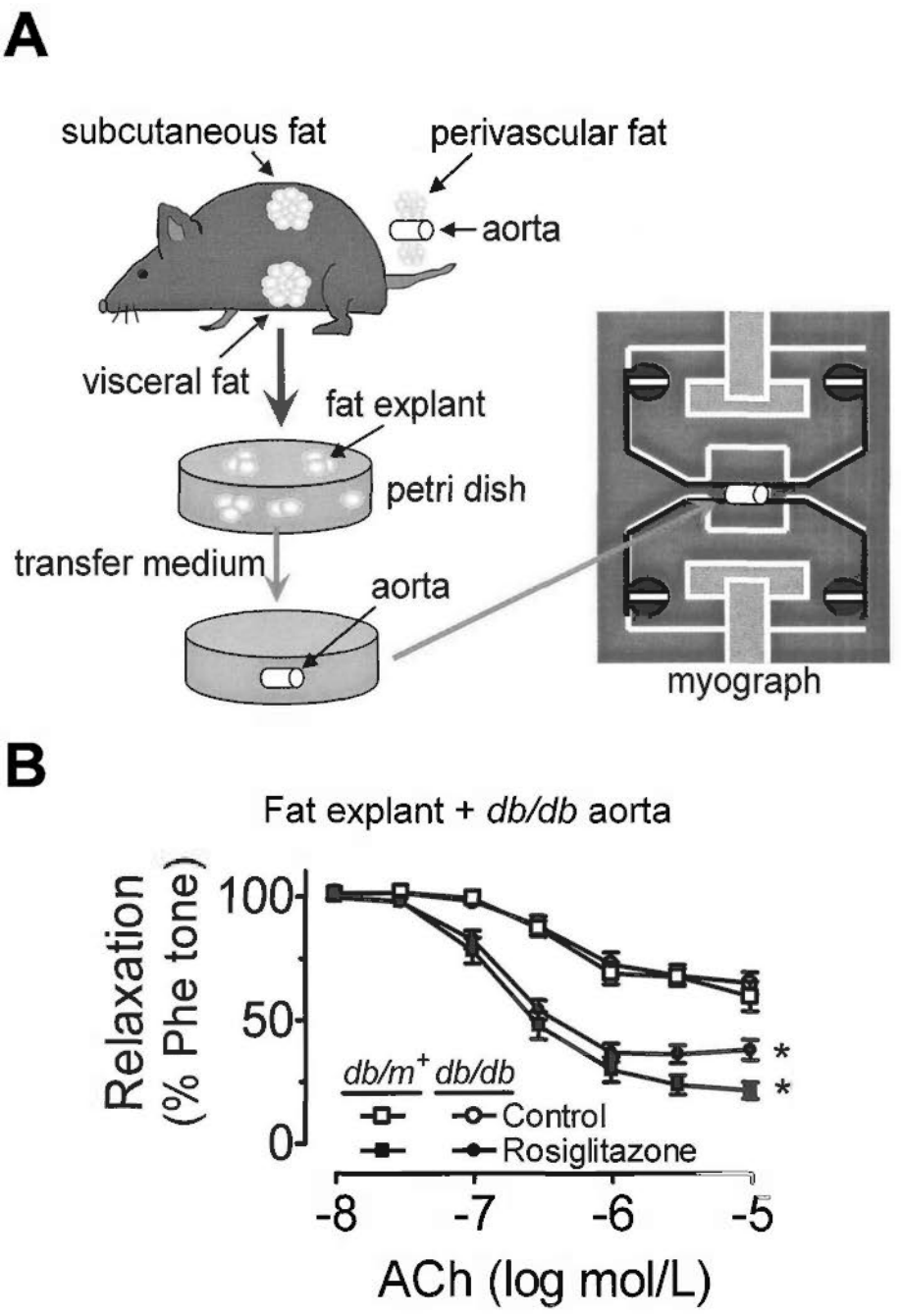

Figure 3.1. Adipose tissue is required for PPARγ activation-induced amelioration of the impaired endothelium-dependent relaxation in *db/db* mouse aortas. (A) Schematic of fat explant experiments. (B) Acetylcholine-induced endothelium-dependent relaxations in *db/db* mouse aortas after incubation in culture medium from rosiglitazone-treated fat explants (pool of subcutaneous, visceral and perivascular fats) from *db/db* and *db/m*$^+$ mice. Results are means ± SEM of 6 – 8 experiments from different mice. *p<0.05 vs control within each group.

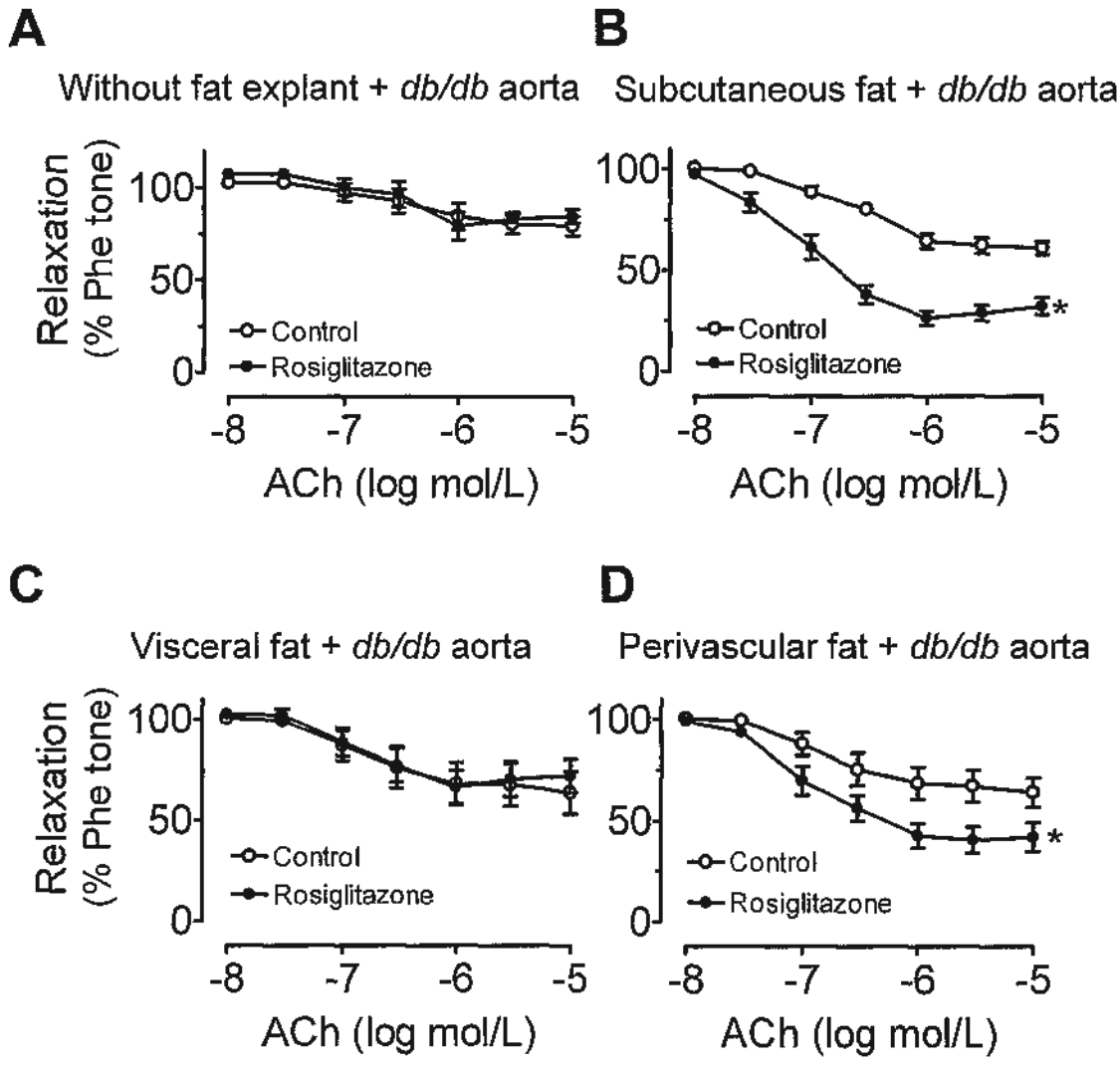

Figure 3.2. The differential effects of fat depots on EDRs in response to rosiglitazone. (A) Effect of 12-hour exposure of isolated *db/db* mouse aortas to 1 μmol/L rosiglitazone alone. (B) Effect of 12-hour incubation of fat explant from subcutaneous adipose tissue with 1 μmol/L rosiglitazone. (C) Effect of 12-hour incubation of fat explant from visceral adipose tissue with 1 μmol/L rosiglitazone. Results are means ± SEM of 6 – 8 experiments from different mice. (D) Effect of 12-hour incubation of fat explant from perivascular adipose tissue with 1 μmol/L rosiglitazone. Results are means ± SEM of 6–8 experiments from different mice. *$p<0.05$ vs control within each group.

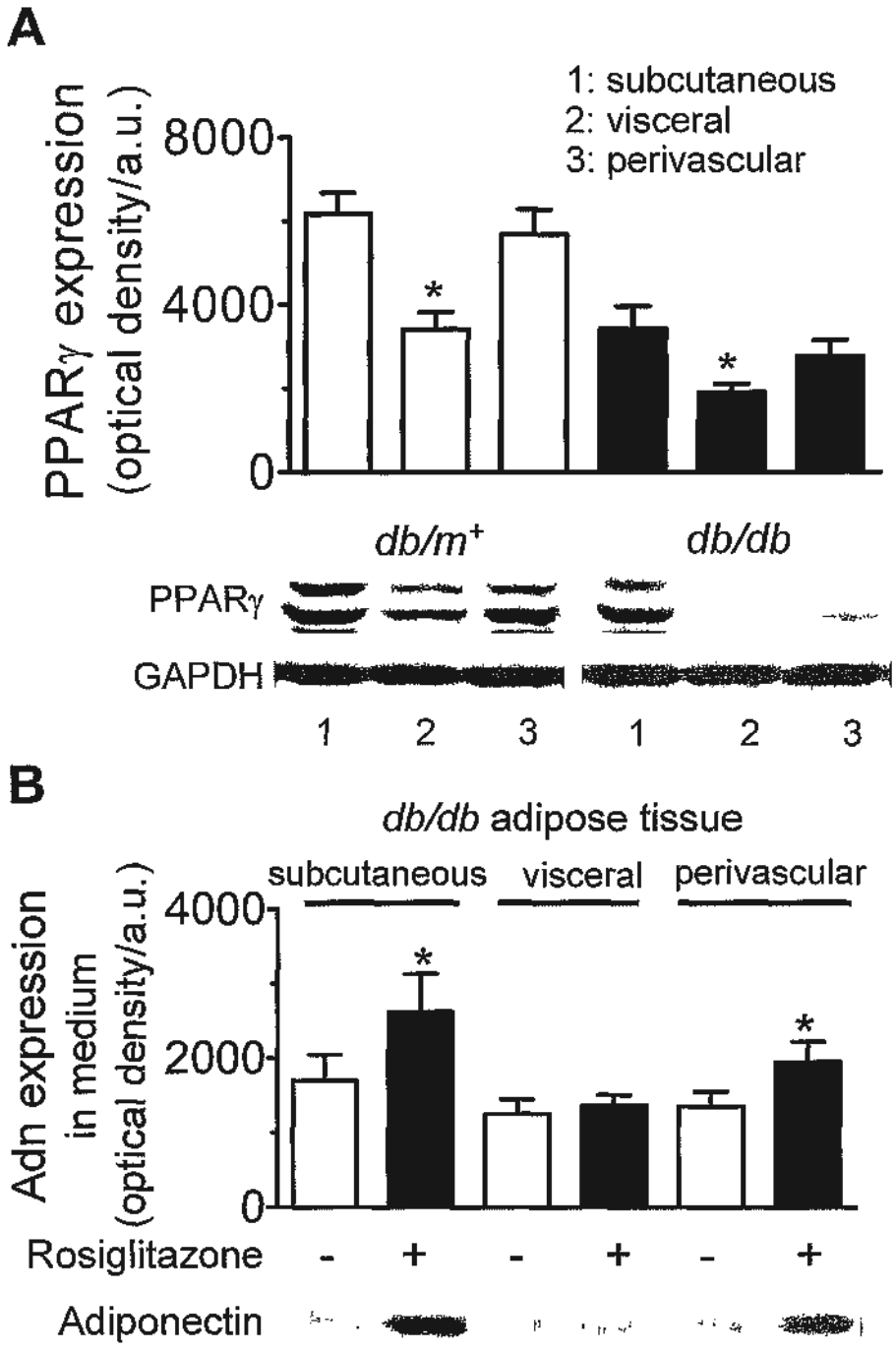

Figure 3.3. PPARγ expression and adiponectin production in different fat depots in response to rosiglitazone. (A) The protein expression of PPARγ in subcutaneous, visceral and perivascular adipose tissues from *db/m*$^+$ and *db/db* mice. (B) The levels of adiponectin present in culture medium after incubation of subcutaneous, visceral, and perivascular fat explants in control or in response to rosiglitazone (1 μmol/L, 12 hr). Results are means ± SEM of 4 experiments. *p<0.05 vs control within each group.

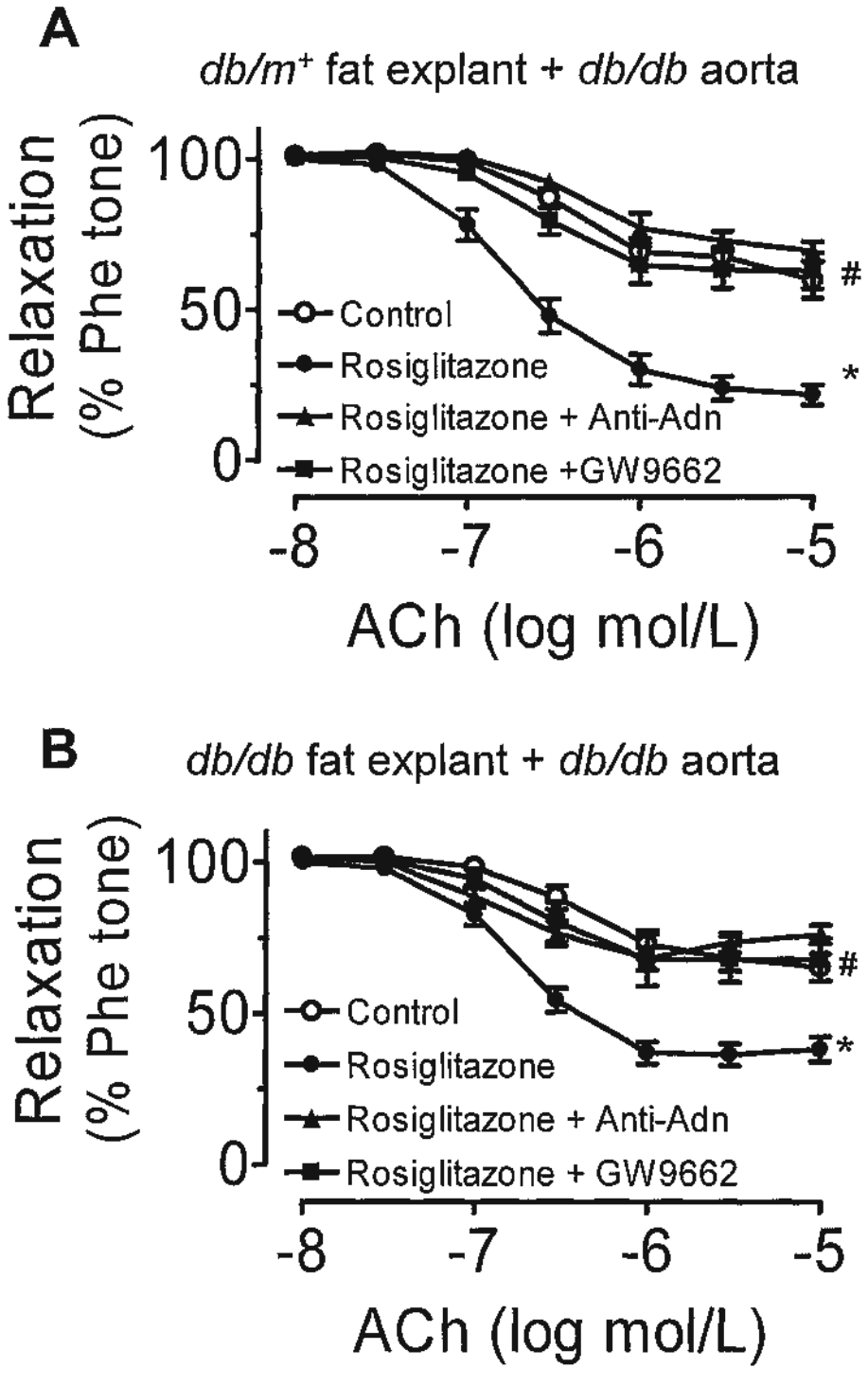

Figure 3.4. Role of adiponectin in PPARγ agonist induced vascular benefit. (A, B) Anti-adiponectin antibody (anti-Adn, 5 µg/mL) and GW9662 (5 µmol/L, PPARγ antagonist) abolished the effect of PPARγ-activated subcutaneous fat explants by rosiglitazone (1 µmol/L, 12 hr) (*db/m*$^+$: A; *db/db*: B) to improve the EDR in aortas from db/db mice. Results are means ± SEM of 4-6 experiments. *p<0.05 vs control, #p<0.05 vs rosiglitazone.

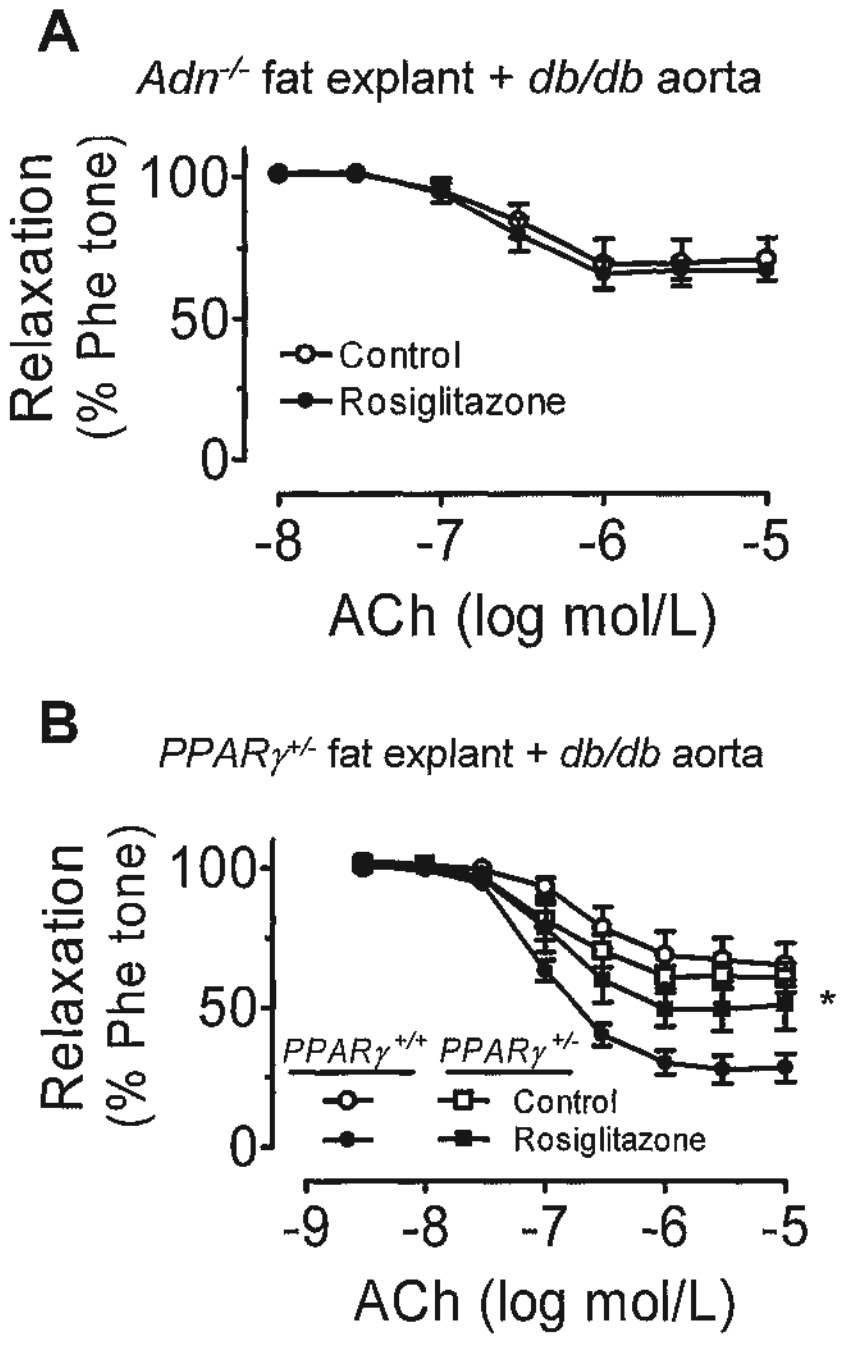

Figure 3.5. Effect of rosiglitazone on fat explants from *adiponectin*$^{-/-}$ and *PPARγ*$^{+/-}$ mice. (A) EDR of aortas from db/db mice did not improve after incubation in medium of fat explants from *adiponectin*$^{-/-}$ (*Adn*$^{-/-}$) mice treated with rosiglitazone. (B) Effect of rosiglitazone-treated subcutaneous fat explants from *PPARγ*$^{+/-}$ mice was less effective to improve EDR in aortas from *db/db* mice. Results are means ± SEM of 4-6 experiments. *p<0.05 vs control.

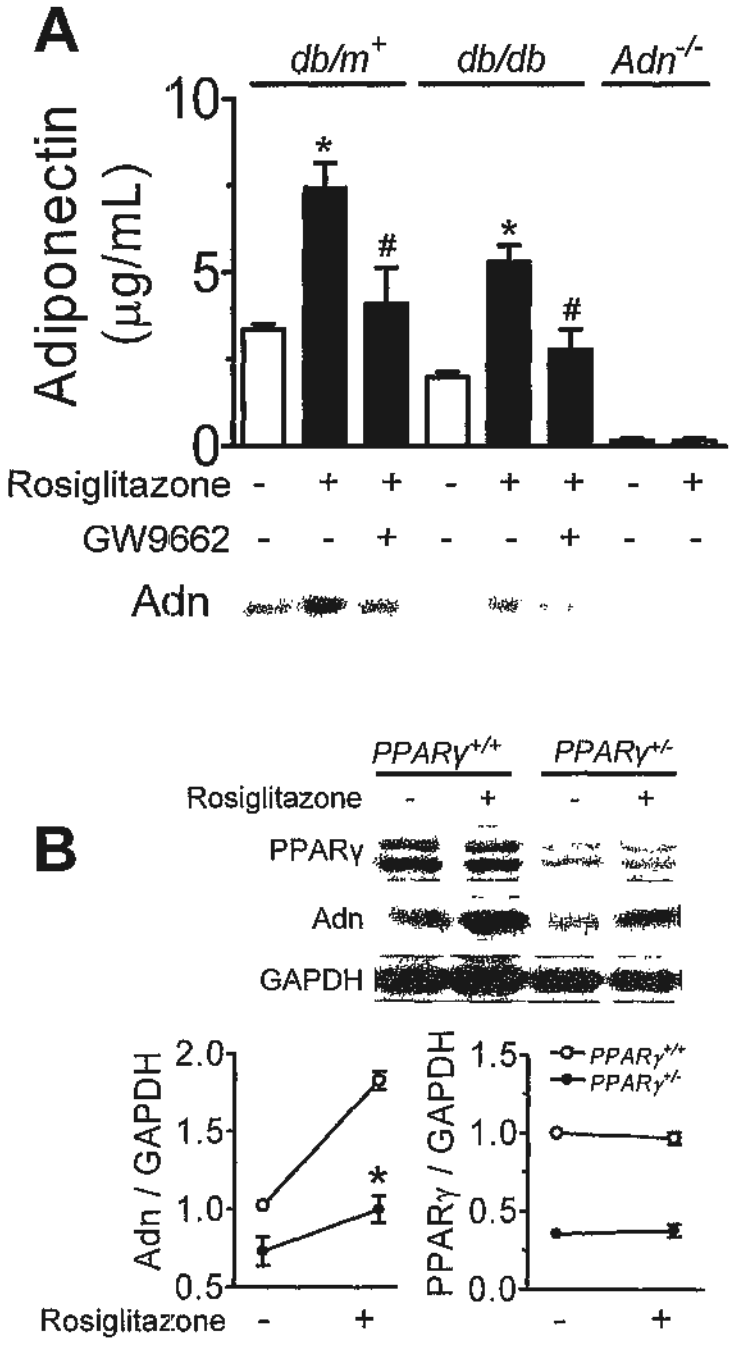

Figure 3.6. The levels of adiponectin present in culture medium from fat explants. (A) Adiponectin release in control or in response to rosiglitazone with or without GW9662 in fat explants from *db/m*$^{+}$, *db/db*, and *Adn*$^{-/-}$ mice. (B) The expression of PPARγ and adiponectin release from fat explants of *PPARγ*$^{+/-}$ mice or those from their *PPARγ WT* littermates. Adiponectin release in the culture medium was measured by Western blots using equal amount of medium from each group. Results are means ± SEM of 4-6 experiments. *$p<0.05$ vs control, #$p<0.05$ vs rosiglitazone.

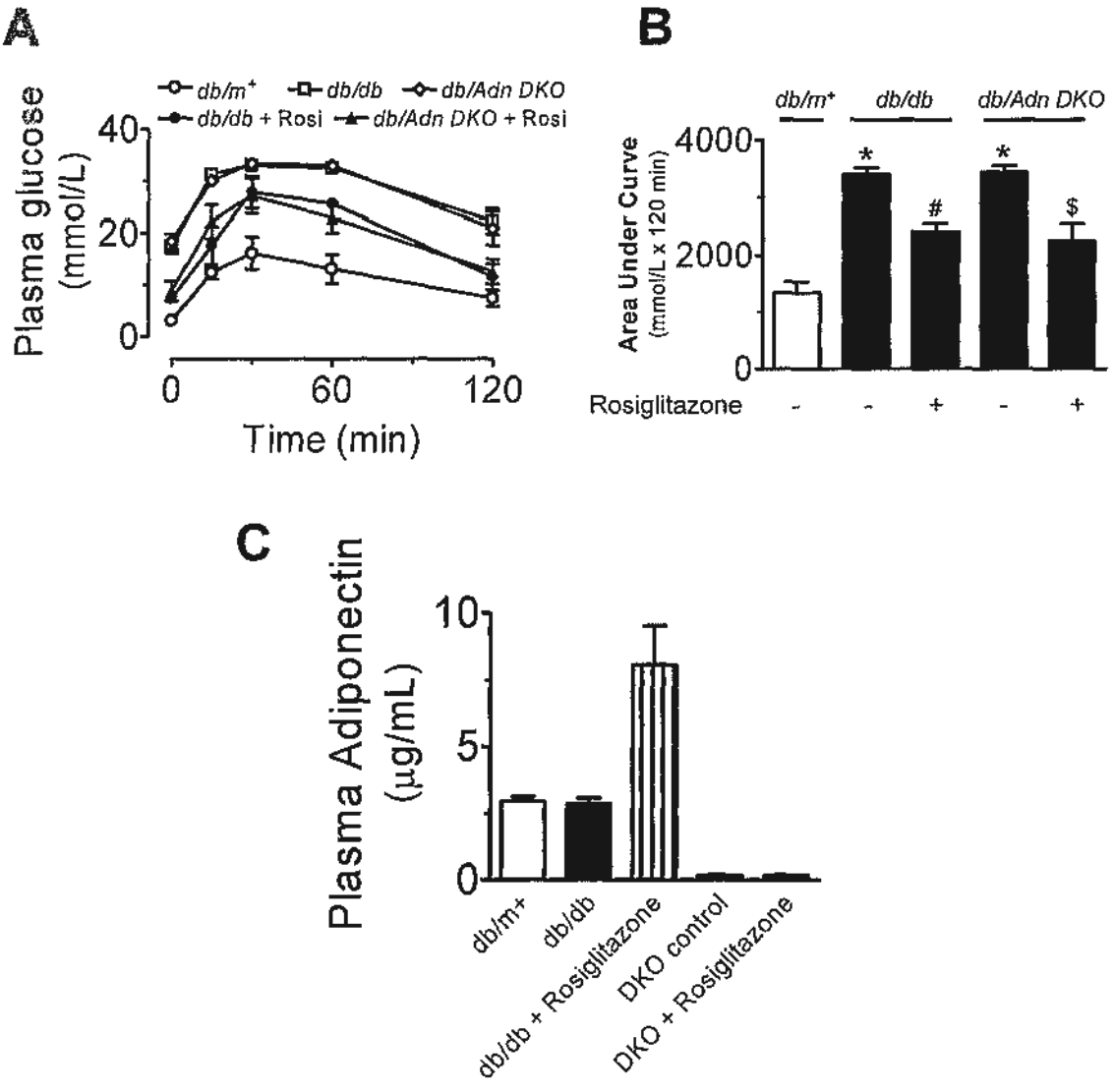

Figure 3.7. Metabolic parameters. (A) Oral glucose tolerance test showed that rosiglitazone treatment improved glucose tolerance in both *db/db* and *db/Adn DKO* mice, compared with *db/db* and *DKO* control mice. (B) Area under curve of oral glucose tolerance test of all the groups. (C) Plasma concentration of adiponectin (µg/mL) of all the groups. **p*<0.05 vs *db/m*$^{+}$, #*p*<0.05 vs *db/db*, †*p*<0.05 vs *db/db*+Rosiglitazone and $p<0.05 vs *db/Adn DKO*.

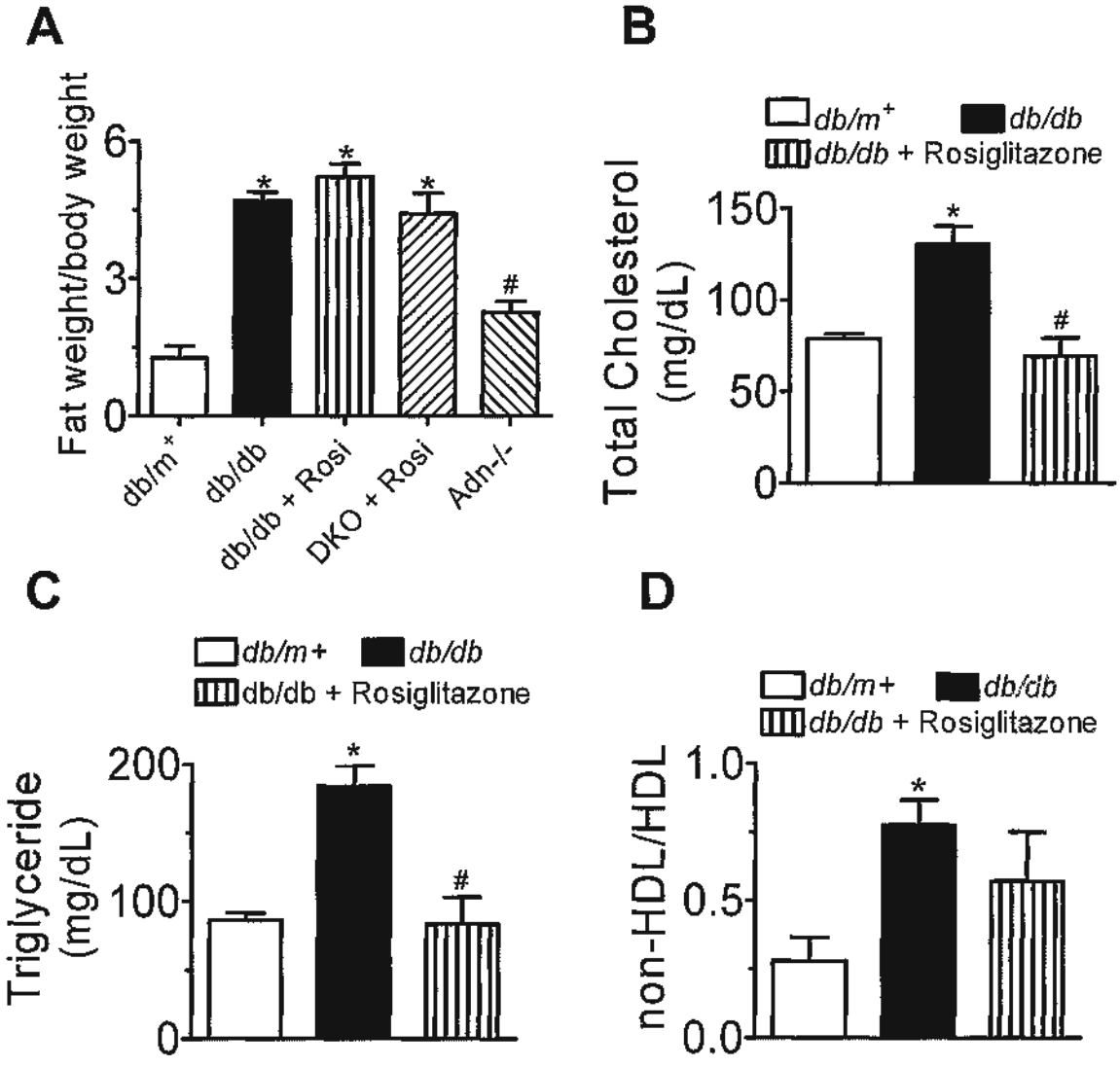

Figure 3.8. *Lipid profile.* (A) Fat weight / body weight % value of all the groups. (B) Plasma total cholesterol level in *db/m*$^{+}$, *db/db*, and *db/db*+Rosiglitazone. (C) Plasma triglyceride level in *db/m*$^{+}$, *db/db*, and *db/db*+Rosiglitazone. (D) Plasma non-HDL/HDL ratio in *db/m*$^{+}$, *db/db*, and *db/db*+Rosiglitazone. Results are means ± SEM of 6 mice. *p<0.05 vs *db/m*$^{+}$, #p<0.05 vs *db/db*.

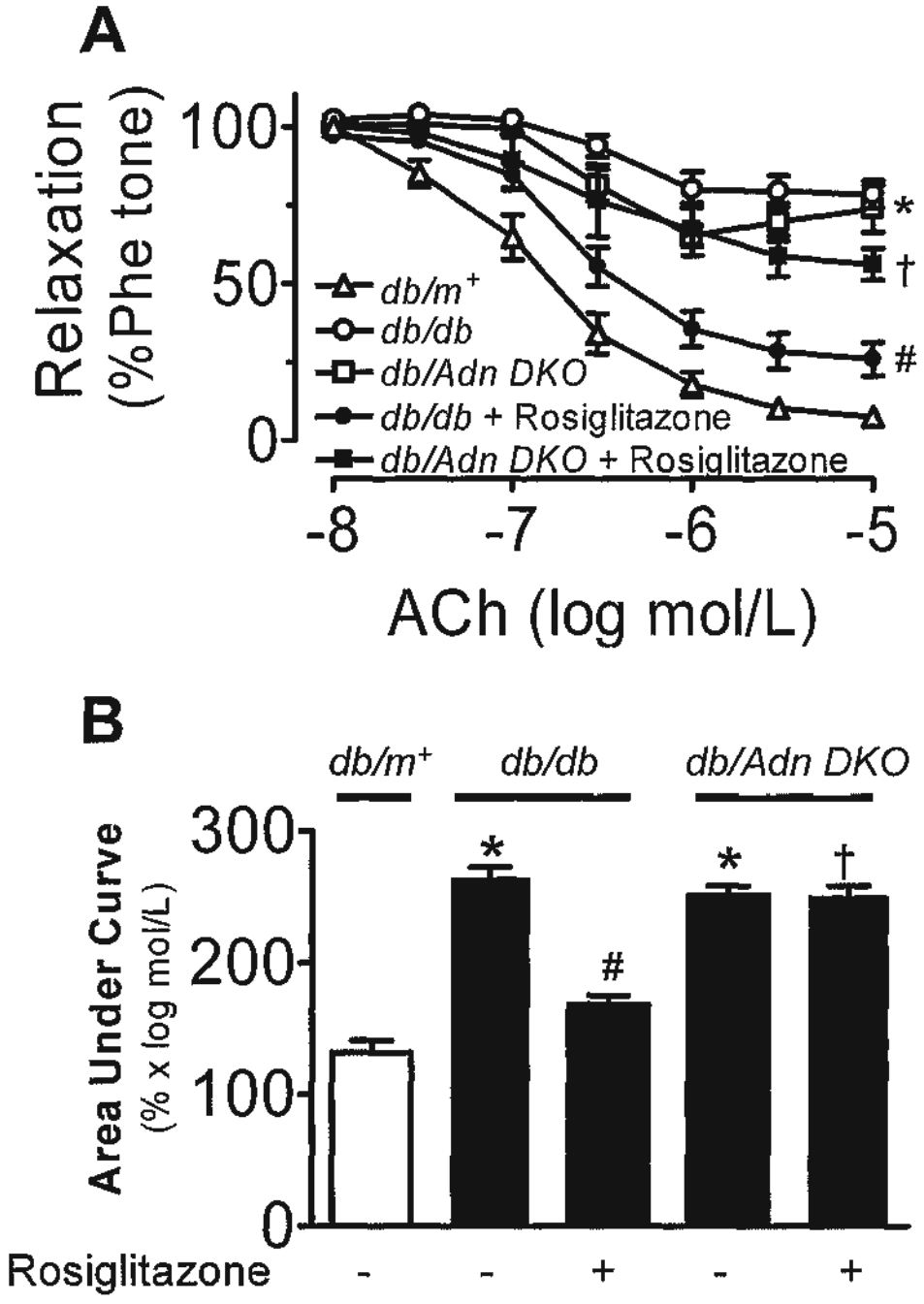

Figure 3.9. *In vivo* rosiglitazone treatment improved endothelial function in diabetic mice through adiponectin-dependent mechanism. (A, B) Chronic treatment with rosiglitazone improved EDR in aortas from *db/db* mice. Potentiation of EDR was abolished in aortas from *db/Adn DKO* mice. (B) Area under curve of relaxation curve in response to ACh. Results are means ± SEM of 6 mice. *$p<0.05$ vs *db/m*$^+$, #$p<0.05$ vs *db/db*, †$p<0.05$ vs *db/db*+Rosiglitazone and \$p<0.05 vs *db/Adn DKO*.

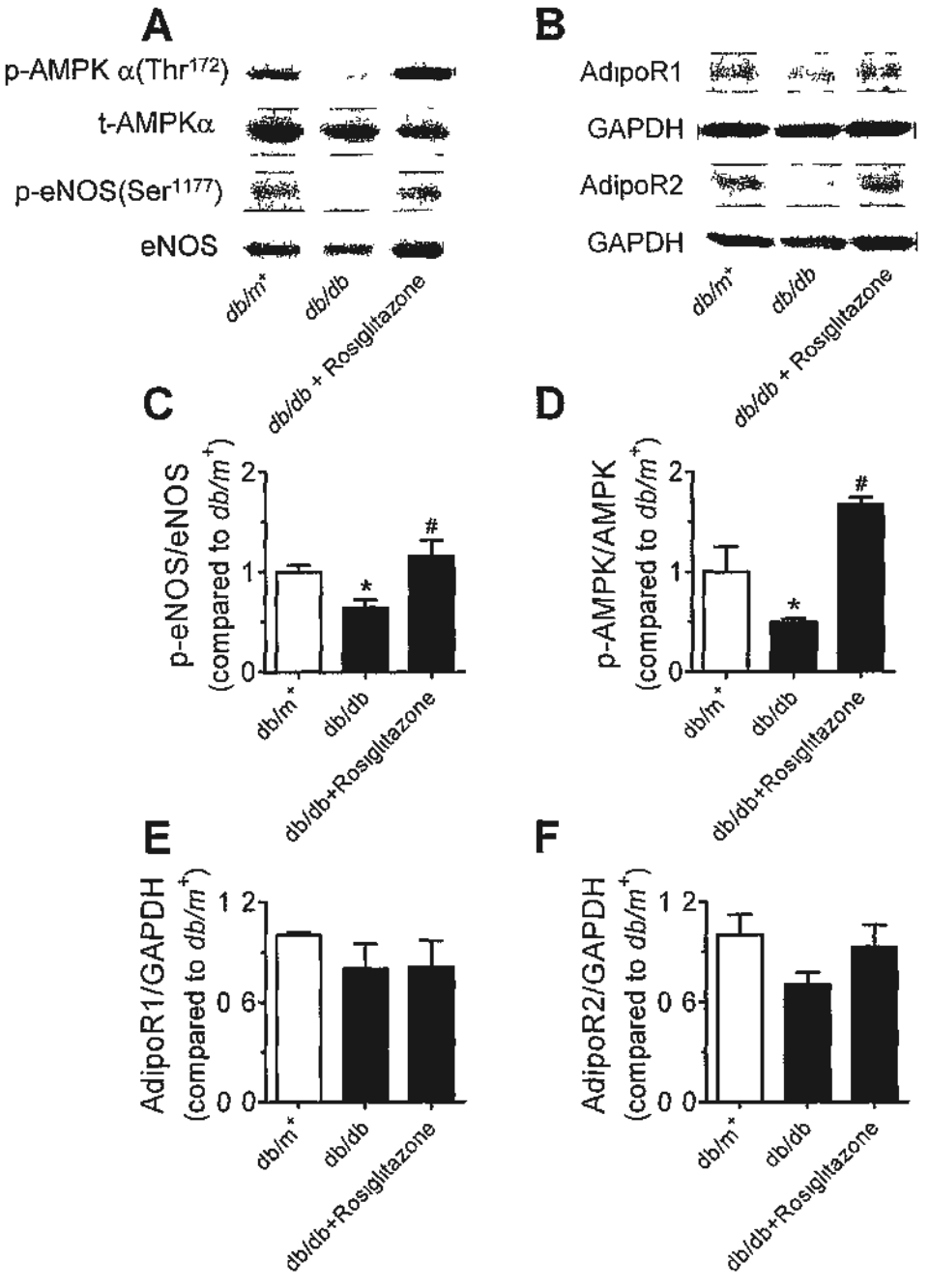

Figure 3.10. Rosiglitazone treatment increasd eNOS and AMPK activity in aortas from diabetic mice. (A) Western blots showed the increased AMPKα and eNOS phosphorylation with total AMPKα and eNOS levels unchanged in aortas from *db/db* mice after rosiglitazone treatment. (C&D) Summarized data of p-AMPK and p-eNOS levels compared with total AMPK or eNOS in aortas from *db/m*$^{+}$, *db/db*, and *db/db*+Rosiglitazone. (B) Western blots showed the expressions of AdipoR1 and AdipoR2 in aortas. (E&F) Summarized data of AdipoR1 and AdipoR2 expressions compared with GAPDH in aortas from *db/m*$^{+}$, *db/db*, and *db/db*+Rosiglitazone. Results are means ± SEM of 6 mice. **p*<0.05 vs *db/m*$^{+}$, #*p*<0.05 vs *db/db*. (D) Expression of AdipoR2 in aortas from *db/m*$^{+}$, *db/db*, *db/db*+Rosiglitazone, and *Adn*$^{-/-}$ mice. Data are representative from 3 blots.

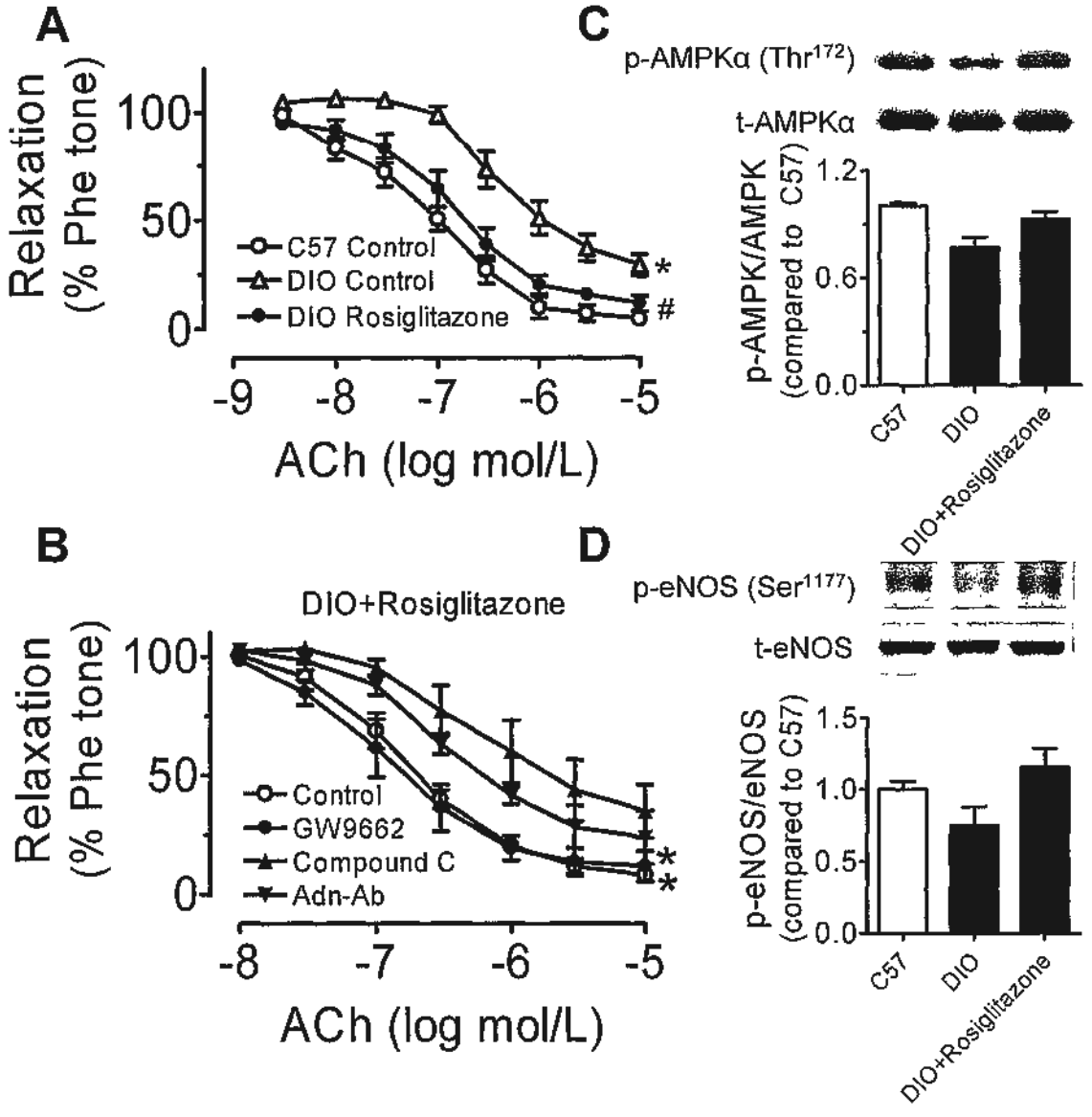

Figure 3.11. Rosiglitazone treatment improved endothelial function in DIO mice. (A) Chronic treatment with rosiglitazone improved EDR in aortas from DIO mice, compared with aortas from DIO control. Results are means ± SEM of 6 mice. *p<0.05 vs C57, #p<0.05 vs DIO. (B) Improved EDRs in aortas from rosiglitazone treated DIO mice were reduced in the presence of compound C (5 μmol/L) or anti-adiponectin antibody (Adn-Ab, 5 μg/mL), but unaffected by GW9662 (5 μmol/L). Results are means ± SEM of 6 mice. *p<0.05 vs control. (C&D) Western blots showed the increased AMPKα and eNOS phosphorylation with total AMPKα and eNOS levels unchanged in aortas from DIO mice after rosiglitazone treatment. Results are means ± SEM of 6 mice. *p<0.05 vs C57, #p<0.05 vs DIO.

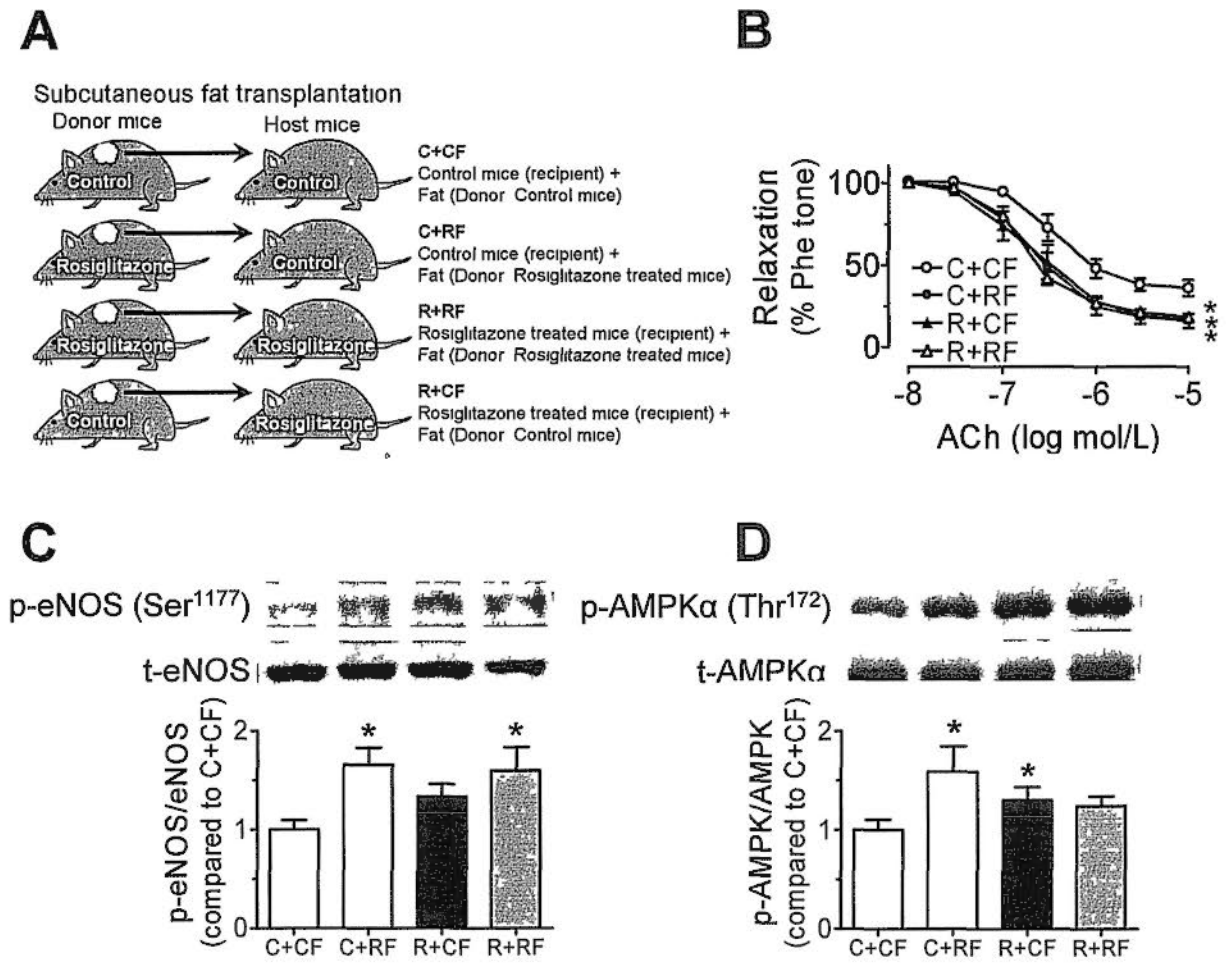

Figure 3.12. Fat transplantation from rosiglitazone-treated *db/db* mice improved endothelial function in control *db/db* mice. (A) Schematic of fat transplantation procedure. (B) Improved EDRs observed in aortas from rosiglitazone-treated *db/db* mice receiving fat grafts from either control (R+CF) or rosiglitazone-treated *db/db* mice (R+RF), and also from control *db/db* mice receiving fat grafts from rosiglitazone-treated *db/db* mice (C+RF), compared with impaired EDRs from control db/db mice (C+CF). Results are means ± SEM of 6 mice. *p<0 05 vs C+CF. (C&D) Western blots showed the increased AMPKα and eNOS phosphorylation with total AMPKα and eNOS levels unchanged in aortas from C+RF mice compared with those from C+CF mice Results are means ± SEM of 3-4 mice. *p<0 05 vs C+CF.

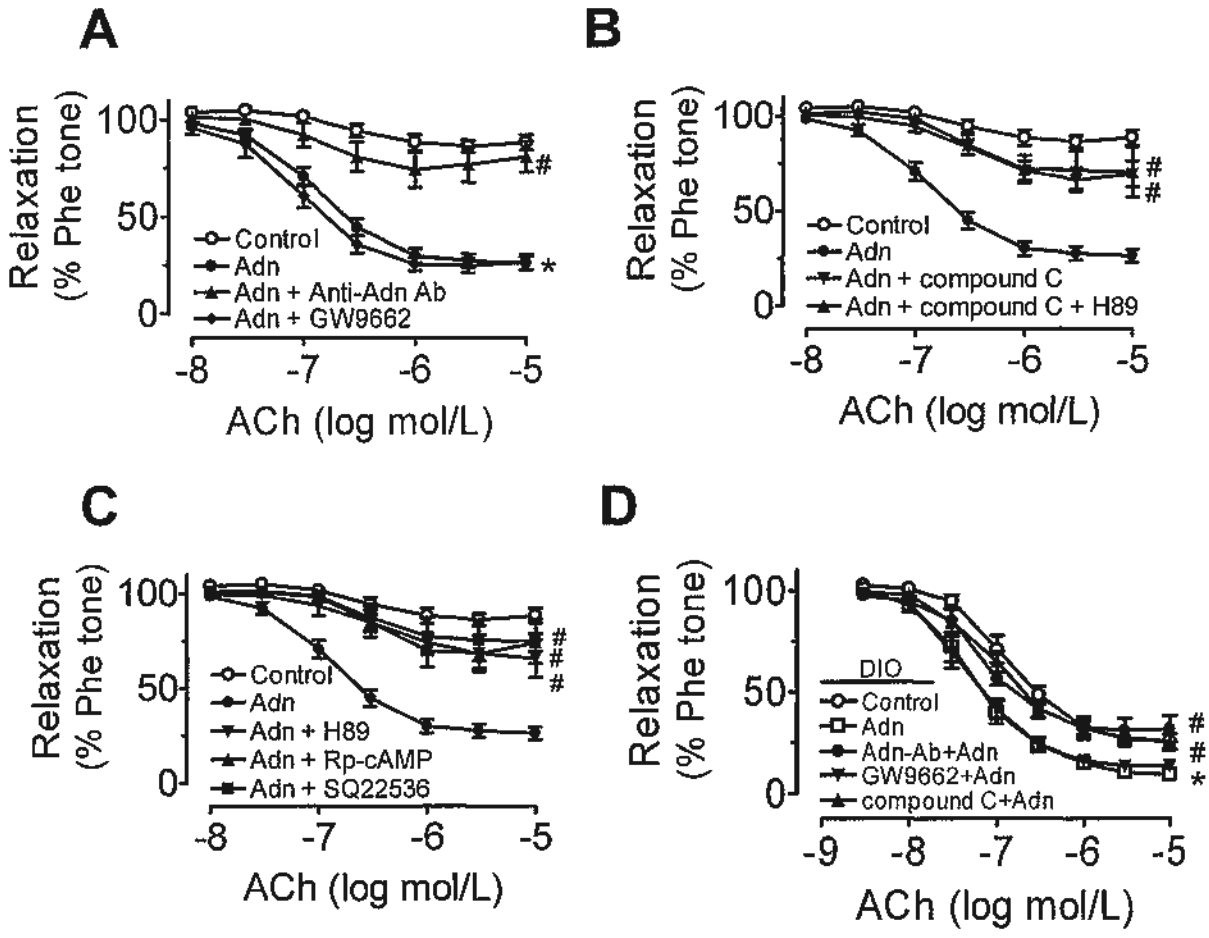

Figure 3.13. Adiponectin improved endothelial function through AMPK and PKA signaling in *db/db* and DIO mice. (A) Adiponectin (Adn, 5 μg/ml) alleviated the impaired EDRs in *db/db* mouse aortas and this effect was reversed by anti-adiponectin antibody (Anti-Adn Ab, 5 μg/ml), but unaffected by GW9662 (5 μmol/L, PPARγ antagonist). (B) Effects of compound C (5 μmol/L, AMPK inhibitor) and compound C plus H89 (1 μmol/L, PKA inhibitor). (C) Effects of H89 (1 μmol/L), Rp-cAMP (10 μmol/L, PKA inhibitor) or SQ22536 (100 μmol/L, sAC inhibitor). (D) Adiponectin (Adn, 5 μg/ml) alleviated the impaired EDRs in DIO mouse aortas and this effect was reversed by anti-adiponectin antibody (Adn-Ab, 5 μg/ml), compound C (5 μmol/L), but unaffected by GW9662 (5 μmol/L). Results are means ± SEM of 6 mice. *p<0.05 vs control; #p<0.05 vs adiponectin.

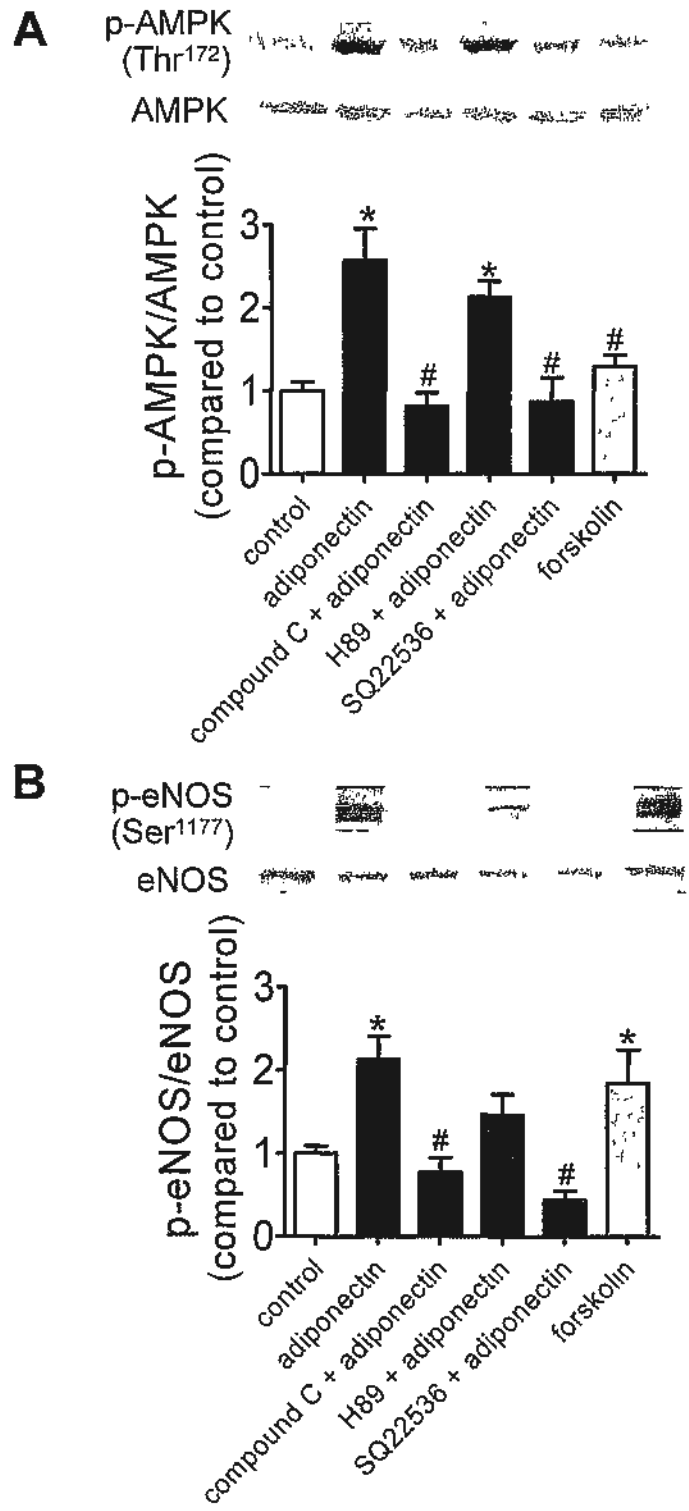

Figure 3.14. Adiponectin increased AMPK and eNOS phosphorylation in *db/db* mouse aortas. (A) Adiponectin (5 µg/mL) increased the phosphorylation of AMPKα at Thr^{172}, and inhibited by compound C (5 µmol/L) and SQ22536 (100 µmol/L) but unaffected by H89 or foskolin (PKA activator, 0.1 µmol/L). (B) Adiponectin (5 µg/mL) increased the phosphorylation of eNOS at Ser^{1177}, inhibited by compound C and SQ22536. Results are means ± SEM of 4-6 experiments. *p<0.05 vs Control, #p<0.05 vs adiponectin. Control group and *Adn* group in

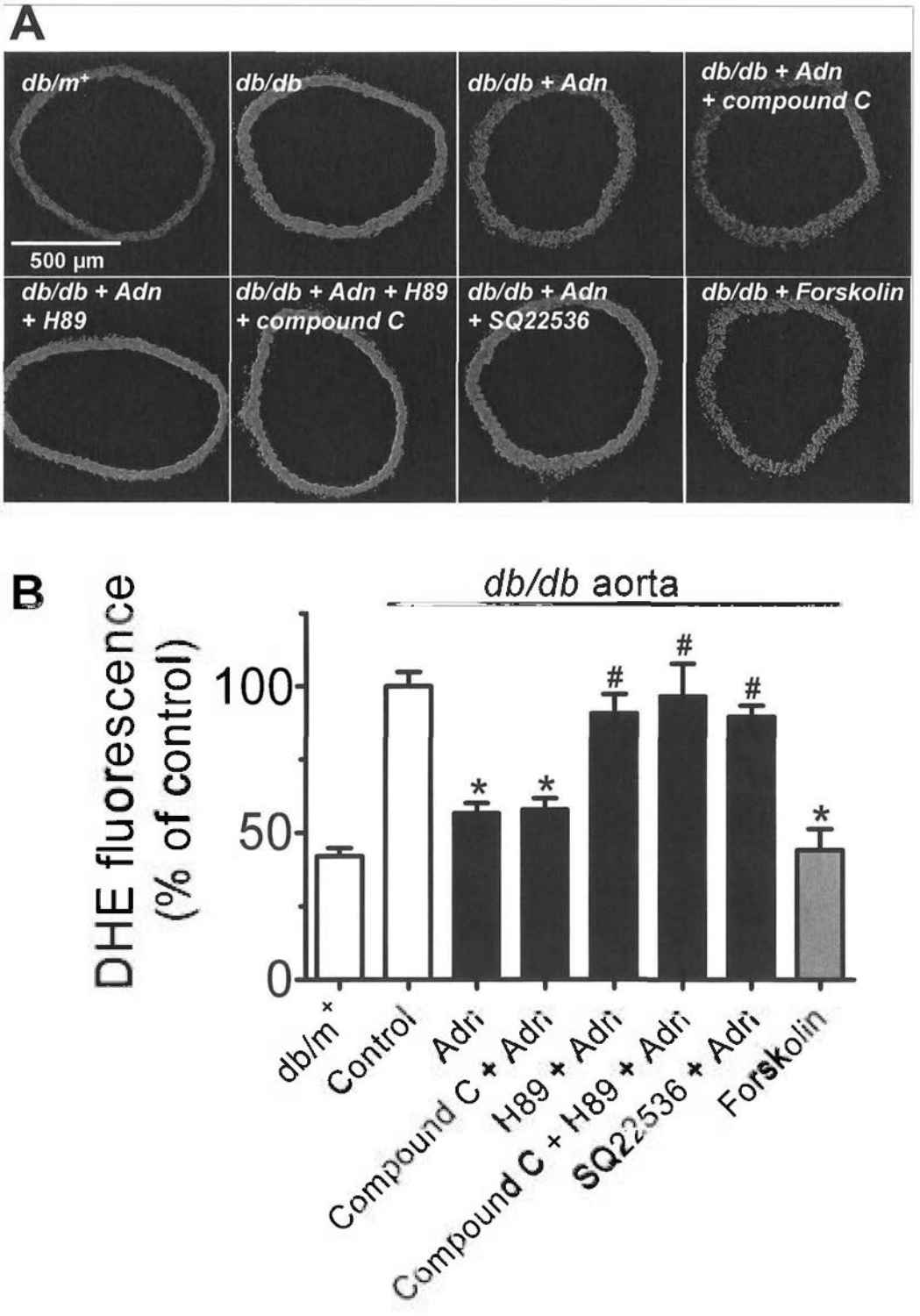

Figure 3.15. Adiponectin reduced ROS generation and increases NO bioavailability. Representative images (A) and summarized data (B) showing adiponectin (5 µg/ml) reduced ROS accumulation as determined by DHE fluorescence intensity in the vascular wall of aortas from *db/db* mice and this effect was reversed by H89 or SQ22536, but not by compound C with forskolin serving as positive control. Results are means ± SEM of 6-8 mice. **p*<0.05 vs db/db control and #*p*<0.05 vs db/db+adiponectin.

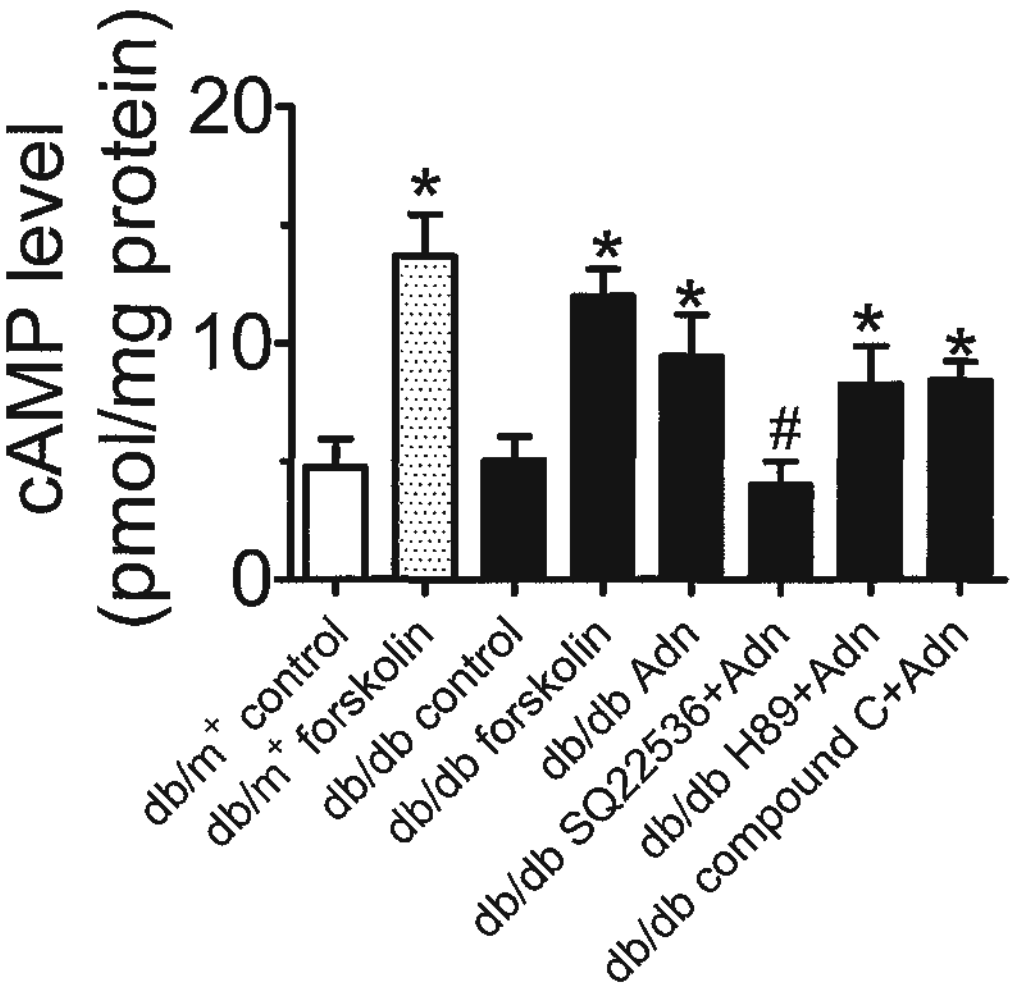

Figure 3.16. Adiponectin increased intracellular cyclic AMP concentration. (A) Adiponectin (5 µg/ml) increased production of cyclic AMP (cAMP) in aortas from *db/db* mice and this effect in *db/db* mouse aortas was inhibited by SQ22536 but not by H89 or compound C. Results are means ± SEM of 4 mice. *$p<0.05$ vs control and #$p<0.05$ vs adiponectin.

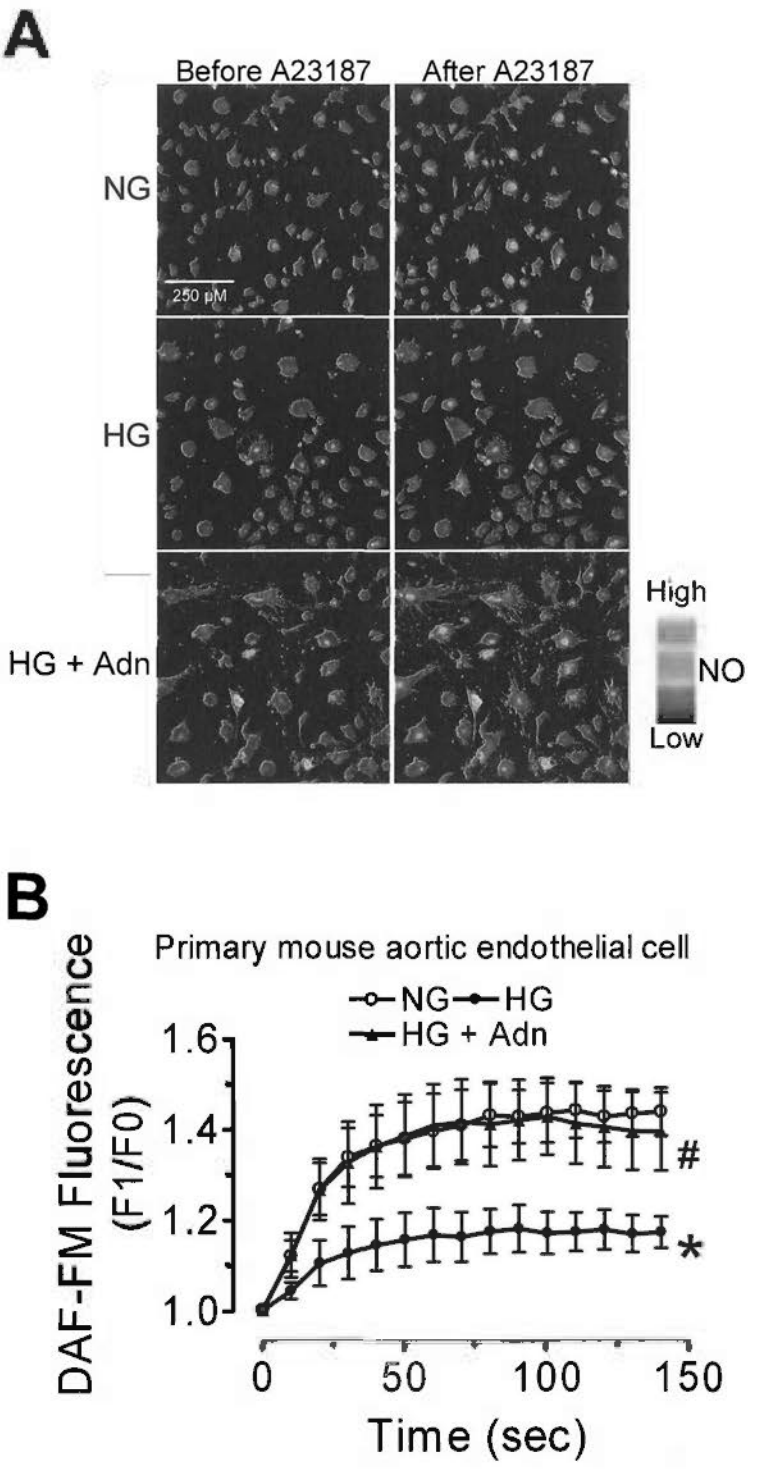

Figure 3.17. Adiponectin increased NO production in response to A23187 in MAECs. Representative images (A) and summarized data (B) showing adiponectin (5 μg/ml) enhanced the nitric oxide production in responses to 1 μmol/L A23187 under high glucose (30 mmol/L, HG) condition. Results are means ± SEM of 6 experiments. *p<0.05 vs control within each group. #p<0.05 vs adiponectin (Adn). NG: normal glucose (5 mmol/L glucose + 25 mmol/L mannitol as osmotic control of HG)

CHAPTER IV

Up-regulation of endothelial expression of ETB Receptor by PPARγ activation attenuates endothelin-1 induced vasoconstriction

4.1 Introduction

Thiazolidinediones (TZDs), such as peroxisome proliferator-activated receptor (PPAR)-γ ligands rosiglitazone and pioglitazone are widely used insulin-sensitizing drugs for type 2 diabetic patients. TZDs target at organs such as liver, skeletal muscle, and adipose tissue to improve glucose homeostasis, reverse insulin resistance, and improve lipid profile for treatment of type 2 diabetes (Etgen *et al.*, 2002; Yang *et al.*, 2002). Besides, TZDs also exert cardiovascular benefits in diabetic or non-diabetic patients with other diseases (Campia *et al.*, 2006; Hsieh *et al.*, 2009; Staels *et al.*, 2008; Villacorta *et al.*, 2009). In addition, TZDs have direct protective effects on endothelial function independent of their insulin-sensitizing action (Chetty *et al.*, 2006; Duan *et al.*, 2008; Ghanim *et al.*, 2006; Hanefeld *et al.*, 2007; Lehrke *et al.*, 2005; Martens *et al.*, 2006; Moreno *et al.*, 2004).

The endothelium maintains vascular tone and homeostasis by liberating vasoactive factors such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factors (EDHFs) (Vanhoutte *et al.*, 2009; Wong *et al..,* 2010a). Apart from vasodilatory action, endothelium-derived NO also exerts a vaso-protective effect by inhibiting the production of inflammatory cytokines that are responsible for vascular smooth muscle cell migration, leukocyte adhesion, and platelet aggregation (Laroux *et al.*, 2000; Taylor,

2001; Wang *et al.*, 2002). In addition to the suppression of inflammatory gene expression in endothelial cells and vascular smooth muscles by PPARγ agonists, such as vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1), E-selectin expression, and NF-κB activation (Duan *et al.*, 2008; Pasceri *et al.*, 2000), to my particular interest, PPARγ agonists also inhibit endothelin-1 (ET-1) production by interfering with activator protein-1 signaling pathway in human vascular endothelial cells (Sakai *et al.*, 2002). In *in vivo* studies, rosiglitazone was found to decrease blood pressure (Ling *et al.*, 2005; Ryan *et al.*, 2004) and to improve the endothelium-dependent relaxation of carotid arteries without affecting the expressions of endothelial nitric oxide synthase (eNOS), angiotensin II type 1 receptors and preproendothelin-1 (Ryan *et al.*, 2004), which are major contributors in controlling blood pressure. Moreover, the modulation of blood pressure and improvement of renal or vascular function in response to PPARγ ligands have been attributed to the role of PPARγ in inhibiting the production and secretion of ET-1 (Bao *et al.*, 2008; Iglarz *et al.*, 2003b; Martin-Nizard *et al.*, 2002; Montezano *et al.*, 2007). PPARγ ligands improve endothelial function in diabetic rats partially through reducing the effect of ET-1 (Matsumoto *et al.*, 2007). However, the mechanisms underlying the vaso-protective effects of TZDs remain to be fully elucidated.

In the present study, I observed that PPARγ rosiglitazone attenuated ET-1-induced contraction. This effect was dependent on the endothelium, mediated by endothelin B receptor (ET_BR)-dependent nitric oxide pathway. ET_BR agonist caused endothelium-dependent relaxation in mouse arteries. In addition, rosiglitazone increased the ET_BR expression in a PPARγ-dependent manner.

4.2 Materials and Methods

4.2.1 Reagents and chemicals

Polyclonal rabbit anti-ET_AR and anti-PPARγ were obtained from Santa Cruz, CA, Polyclonal rabbit anti-ET_BR antibody from Abcam, Cambridge, UK. Fetal bovine serum (FBS) and Dulbeco's Modified Eagle's Media (DMEM) were purchased from Invitrogen (Carlsbad, CA, USA). GW9662, phenylephrine, acetylcholine, endothelin-1, U46619, N^G-nitro-L-arginine methyl ester (L-NAME), BSA, leupeptin, Triton X-100 and PMSF were purchased from Sigma Chem. Co. (St. Louis, MO); rosiglitazone were obtained from GlaxoSmithKline (Research Triangle, NC, USA, GSK No: BRL-49653-C). ET_AR antagonist ABT627 and ET_BR antagonist A192621 were bought from Abbott laboratories (Abbott Park, IL, USA). Rosiglitazone, U46619, ABT627, and A192621 were dissolved in DMSO.

4.2.2 Drug treatment

Adult male C57BL/6J mice (10-weeks old) were supplied by the Animal Service Center of Chinese University of Hong Kong and housed under a 12-h light / 12-h dark cycle and fed *ad libitum*. Mice received daily oral administration of 10 mg kg^{-1} rosiglitazone or vehicle via gastric gavage for 2 weeks. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

4.2.3 Blood vessel preparation

The mice were sacrificed by cervical dislocation. Thoracic aortas and mesenteric resistance arteries were dissected out, cleaned of adhering connective tissues, and cut into several ring segments of ~2 mm in length

each. Isolated mouse aortic rings were incubated in Dulbeco's DMEM supplemented with 10% FBS, plus 100 IU/ml penicillin and 100 µg/ml streptomycin with rosiglitazone (1 or 10 µmol/L) or vehicle control for 24 hr, then transferred into Krebs solution, and mounted in a myograph for real-time measurement of changes in arterial tone (Wong *et al.*, 2010b).

4.2.4 Isometric tension measurement

Each ring was suspended between two small tungsten wires in an organ chamber (Multi Myograph System, Aarhus, Denmark) filled by 5 ml of Krebs-Henseleit solution of the following composition (in mmol/L): 119 NaCl, 4.7 KCl, 25 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 1.2 KH_2PO_4, and 11 D-glucose. The bathing solution was constantly oxygenated by 95% O_2 plus 5% CO_2 and maintained at 37°C (pH of 7.4). Rings were placed under a previously determined optimal resting tension of 3 mN for aortas and 1 mN for mesenteric resistance arteries (MRA) and left for 90-min equilibration. The majority of experiments were carried out on endothelium-intact rings in which acetylcholine at 1 µmol/L produced over 90% of relaxation in phenylephrine-preconstricted vessels, supporting a functional integrity of the endothelium. The concentration-dependent contractions to ET-1 (1-50 nmol/L) were compared in control, rosiglitazone-treated rings in the absence and presence of 100 µmol/L N^G-nitro-L-arginine methyl ester (L-NAME). The effects of antagonists of both endothelin receptor A (ET_AR) and ET_BR were tested on ET-1-induced contractions. In some rings, the endothelium was mechanically disrupted, which was confirmed by a complete loss of relaxation to acetylcholine; the effect of rosiglitazone was tested in these rings. Finally, it

was examined whether rosiglitazone treatment could non-specifically reduce contractions to other constrictors such as elevated KCl and U46619.

4.2.5 Protein extraction and Western blotting

Aortas were isolated and frozen in liquid nitrogen following rosiglitazone treatment and homogenized in RIPA lysis buffer. Protein samples prepared from aorta homogenates were electrophoresed through a 10% SDS-poly-acrylamide gel, transferred onto an immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Nonspecific binding sites were blocked with 1% BSA in 0.05 % Tween-20 phosphate-buffered saline. The blots were incubated overnight at 4°C with primary antibodies: polyclonal rabbit anti-ET_AR (Santa Cruz, CA) or anti-ET_BR antibody (Abcam, Cambridge, UK) overnight at 4°C. The protein expression was quantitated with densitometer (FluorChem, Alpha Innotech, San Leandro, CA), normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

4.2.6 Immunohistochemistry

Cross sections in 5 μm thickness were cut in paraffin-embedded aortic rings, treated with citrate buffer for antigen retrieval, incubated with 3% H_2O_2 to block endogenous peroxidase, and blocked in 5% normal goat serum. Anti-ET_BR antibody (1:100, Abcam, Cambridge, UK) was added and incubated overnight at 4 °C, followed by Biotin-SP conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA), then incubated with streptavidin-HRP conjugate (Zymed, San Francisco, CA), and visualized by DAB (Vector, Burlingame, CA).

4.2.7 Statistical Analysis

Quantitative data are means ± SEM. Arterial contractions were expressed as active tension [tone developed/ (2x ring length in mm)]. Statistical analyses were performed with 1- or 2-way ANOVA or Student's *t*-test, Bonferroni *post-hoc* tests were performed when more than 2 treatments were compared (GraphPad Prism software, Version 4.0, San Diego, CA), with statistical significance set at $P<0.05$. Non-quantitative results were representative of at least 3 independent experiments.

4.3 Results

4.3.1 ET-1 induced vasoconstriction is attenuated by rosiglitazone

Rosiglitazone treatment (1 or 10 μmol/L, 24 h) significantly reduced the constrictive responses to ET-1 in aortic rings from C57BL/6J mice (Figure 4.1A). Figure 4.1B presented original recordings in isolated mouse aortas with endothelium and showed that ET-1 produced concentration-dependent contractions which were significantly reduced by 24-h treatment with 1-10 μmol/L rosiglitazone (Figure 4.1B). By contrast, the acute (30-min) exposure to 10 μmol/L rosiglitazone did not modulate ET-1-induced contractions (data not shown). Rosiglitazone (24 h) treatment did not affect U46619, a thromboxane receptor agonist-induced contractions (Figure 4.1C). Likewise, the 60 mmol/L K^+ containing Kreb's solution-induced contraction was comparable in control (2.48 ± 0.10 mN/mm) and rosiglitazone-treated (2.41 ± 0.13 mN/mm) rings ($P>0.05$). In mesenteric resistance arteries (MRAs), ET-1 induced vasoconstriction was also inhibited by rosiglitazone (10 μmol/L, 24 h) (Figure 4.1D).

4.3.2 ET_BR and NO contributes to attenuated ET-1 contraction

To examine which ET receptor subtype was affected by rosiglitazone and the role of endothelium, ET-1-induced vasoconstriction were examined in both endothelium-intact and endothelium-denuded aortic rings, in the presence or absence of L-NAME, an inhibitor of nitric oxide synthase (NOS), or in the presence of ET_AR antagonist ABT627 and ET_BR antagonist A192621. The contraction of isolated mouse aortas in response to ET-1 was likely to be mediated through ET_AR activation since the selective ET_AR antagonist ABT627 (10 nmol/L) abolished the contraction in control and

rosiglitazone-treated rings (Figure 4.2A). The attenuated ET-1-induced contraction of rosiglitazone-treated rings was restored by the presence of a selective ET_BR antagonist, A192621 (10 nmol/L) (Figure 4.2B), while this antagonist did not modulate the evoked contractions in control rings. The difference in the amplitude of contractions between control and rosiglitazone-treated rings was lost in endothelium-intact rings that had been previously exposed to 100 μmol/L L-NAME for 30 min (Figure 4.2C) or in rings without endothelium (Figure 4.2D).

4.3.3 Rosiglitazone increase ET_BR in mouse aortas

To investigate how rosiglitazone affected the expression of ET_BR, male C57BL/6J mice thoracic aortas were treated with 1 and 10 μmol/L rosiglitazone for 24 h and examined for the ET_BR protein level. Western blotting results showed an up-regulation of ET_BR with rosiglitazone treatment in endothelium-intact but greatly less in endothelium-denude mouse aortas (Figure 4.3A), while the ET_AR expression was unchanged (Figure 4.3B). Immunohistochemical staining data also showed that ET_BR was expressed at low levels in both endothelial cells and vascular smooth muscle cells of normal mouse aortas (Figure 4.3C), and rosiglitazone treatment increased the ET_BR expression which was primarily confined to the endothelial cells (Figure 4.3D).

4.3.4 *In Vivo* rosiglitazone treatment attenuates ET-1-induced vasoconstrictions

To further support the *in vitro* effects, C57BL/6J mice were treated with rosiglitazone at 10 mg kg^{-1} for 2 weeks and vascular reactivity was examined on myograph. ET-1-induced contractions were attenuated after rosiglitazone treatment in both the aortas (Figure 4.4A) and MRAs (Figure 4.5A). In the

presence of NOS inhibitor, L-NAME (100 μmol/L), the inhibitory effect of rosiglitazone treatment on ET-1-induced vasoconstrictions was abolished in aortas (Figure 4.4B) and MRAs (Figure 4.5B). The ET_BR antagonist, A192621 also abolished rosiglitazone-attenuated vasoconstrictions in response to ET-1 in aortas (Figure 4.4C) and MRAs (Figure 4.5C).

4.3.5 Rosiglitazone treatment increases ET_BR expression in mouse aortas

Rosiglitazone treatment *in vivo* up-regulated the ET_BR expression (Figure 4.6A) while leaving the ET_AR expression unaltered (Figure 4.6B) in mouse aortas. In addition, the eNOS expression also increased while phosphor-eNOS (Ser^{1177}) to eNOS ratio were unaltered in mouse aortas after rosiglitazone treatment (Figure 4.6C&D).

4.3.6 Rosiglitazone treatment enhances ET_BR agonist-induced relaxation

Sarafotoxin 6c (S6c), a selective ET_BR agonist did not induce vasodilatation in MRAs in vehicle-treated mice (Figure 4.7A). Rosiglitazone treatment enhanced S6c-induced relaxation in MRAs (Figure 4.7A) which was abolished in the presence of L-NAME (Figure 4.7B) or A192621 (Figure 4.7C).

4.4 Discussion

In the present study, I demonstrate for the first time that rosiglitazone up-regulates ET_BR expression in mouse aortas and attenuates ET-1-induced vasoconstriction through an endothelial ET_BR-dependent NO-related mechanism. I also showed that selective ET_BR agonist can produce induce endothelium-dependent relaxations in mouse mesenteric resistance arteries.

ET-1 is a potent vasoconstrictor that can be synthesized in the vascular smooth muscle cells of the vascular wall as well as the endothelial cells from preproET-1 and endothelin-converting enzyme-1 which correlated with atherosclerosis or hypertension in patients (Rossi *et al.*, 1999; Schiffrin, 2001). ET-1 also plays an important role in cardiac hypertrophy, heart failture, and pulmonary hypertension (Galie *et al.*, 2004; Munter *et al.*, 2001; Zolk *et al.*, 1999). The function of ET-1 was mediated through two types of the ET-1 receptor: ET_AR and ET_BR. ET_AR is mainly expressed in vascular smooth muscle layer and responsible for vasoconstriction, while ET_BR are expressed mainly in endothelial cells, and to less extent smooth muscle cells (Hosoda *et al.*, 1991; Ogawa *et al.*, 1991). To my particular interest, activation of ET_BR leads to NO production as it is functionally coupled to eNOS signaling (Kwok *et al.*, 2009; Liu *et al.*, 2003; Murohara *et al.*, 1996; Noiri *et al.*, 1997; Tsukahara *et al.*, 1994). ET_BR also mediates the clearance of ET-1 (Bohm *et al.*, 2003; Burkhardt *et al.*, 2000; Honore *et al.*, 2005; Ozaki *et al.*, 1995) and antagonize the effect of ET_AR, thus modulating the vascular tone.

Interestingly, PPARγ activators inhibit the production and function of ET-1. For example, in endothelial cells, PPARγ and PPARα agonists can suppress ET-1 secretion by down-regulation of thrombin-activated transcription of

human ET-1 promoter (Delerive *et al.*, 1999), and inhibit oxidized low-density lipoprotein-induced ET-1 production in the endothelial cells (Martin-Nizard *et al.*, 2002). PPARγ ligands also inhibit cardiac hypertrophy in rats via the suppression of activator protein-1 (AP-1) (Irukayama-Tomobe *et al.*, 2004; Sakai *et al.*, 2002). Besides, PPARs also exert anti-hypertensive effects in ET-1 related hypertension (Bae *et al.*; Iglarz *et al.*, 2003a; Iglarz *et al.*, 2003b).

Although the effect of TZDs on ET-1 expression have been assessed in both *in vitro* and *in vivo* models, up to date, it is still unknown whether PPARγ ligands can suppress ET-1-induced vasoconstriction in blood vessels, which would be a direct evidence that can explain the reported anti-hypertensive effect of PPARγ in ET-1 related hypertension. The functional importance of ET_BR is also not very clear in hypertension. In my experiments, I showed that 24-h incubation with rosiglitazone attenuated the ET-1-induced vasoconstriction. In mouse aortas and resistance arteries, the contractile response to ET-1 is dependent on ET_AR because selective ET_AR antagonist ABT627 abolished the ET-1-induced contraction. On the other hand, ET_BR antagonist A192621 reversed the suppressed ET-1-induced contraction after rosiglitazone treatment, without affecting that from un-treated control mice, suggesting that the attenuated contraction is most likely caused by the enhanced expression and function of ET_BR. In addition, removal of endothelium or inhibition of NO production by L-NAME also eliminated the effect of rosiglitazone, indicating an endothelial origin of functional ET_BR involved in suppressing the ET-1-induced contraction in an NO-dependent manner in response to PPARγ activation. This was confirmed by upregulation of ET_BR but not ET_AR in rosiglitazone-treated mouse aortas with or without endothelium. Immunostaining data also showed an increase of the ET_BR

expression that was mainly confined to the endothelium despite a similar expression of ET_BR in the smooth muscle layers with or without rosiglitazone treatment. Further experiments revealed that *in vivo* rosiglitazone treatment have a similar effect in attenuating the ET-1-induced vasoconstriction and in elevating the ET_BR expression in both conduit and resistance arteries in mice. Since there have been many reports showing the anti-hypertensive effect of PPARγ agonists in clinical and experimental settings (Benkirane *et al.*, 2006; Ledingham *et al.*, 2005; Potenza *et al.*, 2009; Ryan *et al.*, 2004), the present findings suggest additional benefit of rosiglitazone in protecting endothelial function through enhancing the NO production. This mechanism may account for the anti-hypertensive action of rosiglitazone.

As showed by our colleagues in Peking University, PPARγ agonists, rosiglitazone and troglitazone increased the ET_BR expression both at mRNA and protein levels in a concentration-dependent manner in HUVECs. The upregulation of ET_BR was prevented by co-incubation with PPARγ antagonists GW9662 and BADGE. Moreover, constitutively active PPARγ by adenoviral overexpression in HUVECs also leads to an ET_BR upregulation. This finding was further confirmed by the reporter gene luciferase activity assay, suggesting that rosiglitazone can increase the ET_BR gene promoter activity in PPARγ-dependent manner (Tian *et al.*, 2010). The results with chromatin immunoprecipitation assays confirm that PPARγ directly bound to the PPAR-responsive elements (PPRE) site of human ET_BR gene. These observations suggest that the ET_BR gene is a direct target of transcriptional factor PPARγ to activate of the human ET_BR gene transcription by PPARγ binding to the PPAR-responsive element in the ET_BR promoter.

The prevalence of hypertension in type 2 diabetic patients is higher then non-diabetics (Gress *et al.*, 2000; Sowers *et al.*, 2001). TZDs are used as insulin-sensitizing drugs in diabetic patients and they are found to lower blood and protect endothelial function (Kelly *et al.*, 2007). Endothelial cell PPARγ have been reported to be the target for TZDs to exert anti-inflammatory effects against the development of atherosclerosis (Chang *et al.*; Wang *et al.*, 2002), suggesting that PPARγ may be associated with a direct protection of endothelial function. The present study showed that *in vivo* rosiglitazone treatment, as well as *ex vivo* organ culture with rosiglitazone inhibited ET-1-induced vasoconstriction in an ET_BR-NO-dependent mechanism, suggesting that ET_BR is a direct target of PPARγ activation in mediating endothelial cell protection. In addition, my results demonstrated for the first time that activation of ET_BR by sarafotoxin 6c (S6c) leads to endothelium-dependent vasodilatation in resistance arteries, which is a direct evidence for the vasodilatory action of ET_BR, and helps to explain how TZDs improve endothelial function and modulate vascular tone in animal models of hypertension and diabetes (Potenza *et al.*, 2009; Walker *et al.*, 1999).

In summary, the present study shows that PPARγ activation inhibits ET-1-induced vasoconstriction through upregulation of ET_BR and enhancement of NO production in the endothelium, which contributes to the protection of endothelial function induced by PPARγ ligands.

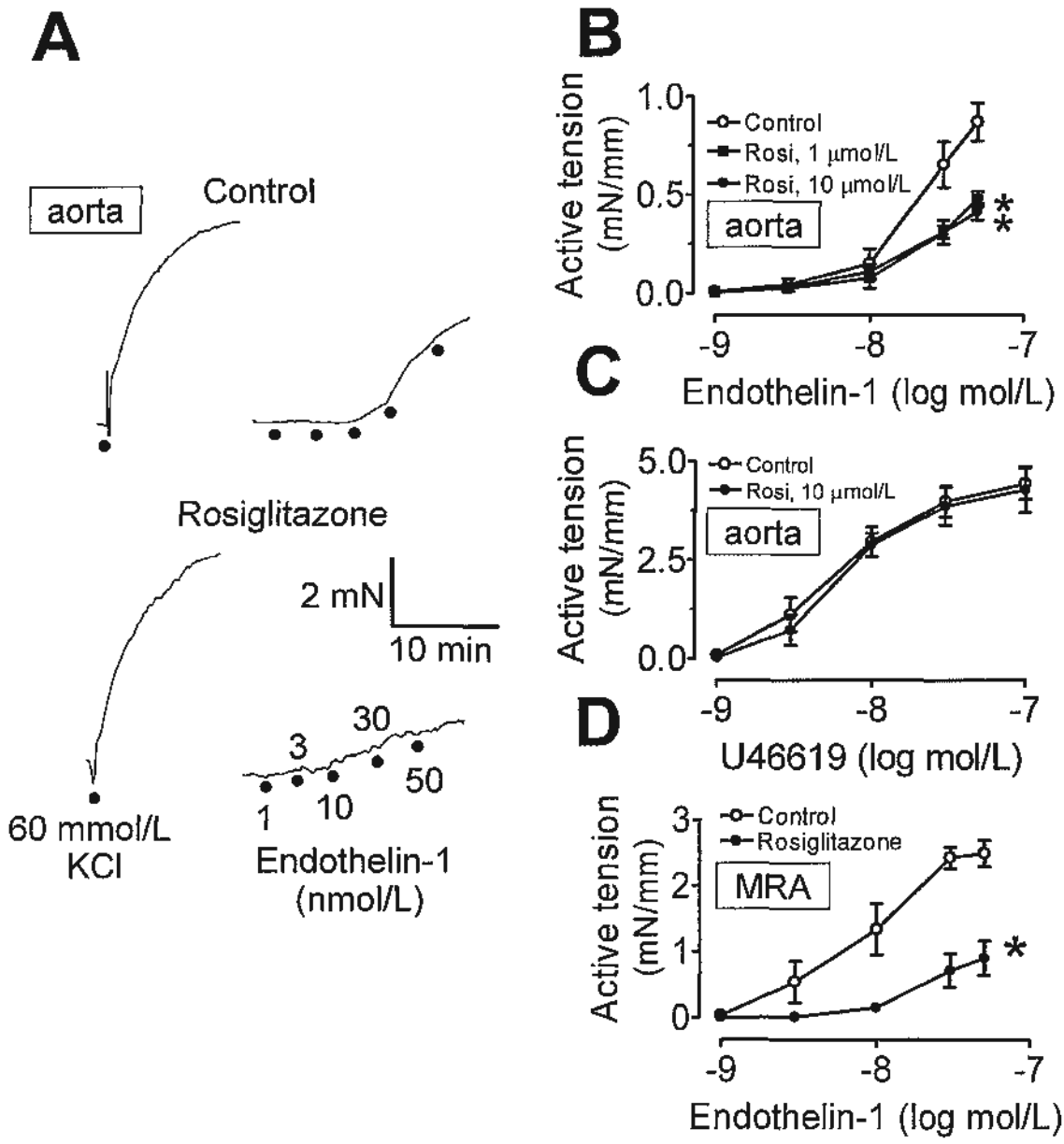

Figure 4.1. (A) Original records showing endothelin-1-induced vasoconstrictions in endothelium-intact mouse aortic rings that had been treated with 10 µmol/L rosiglitazone for 24 hours. (B) Concentration-dependent vasoconstrictions to endothelin-1 in rosiglitazone (1-10 µmol/L)-treated aortic rings (n=4-7). (C) Concentration-dependent contractions to U46619 in rosiglitazone (10 µmol/L)-treated aortic rings (n=5). (D) Concentration-dependent vasoconstrictions in mesenteric resistance arteries (MRAs) (n=6). Results are means ± SEM of n mice. *p<0.01 vs control.

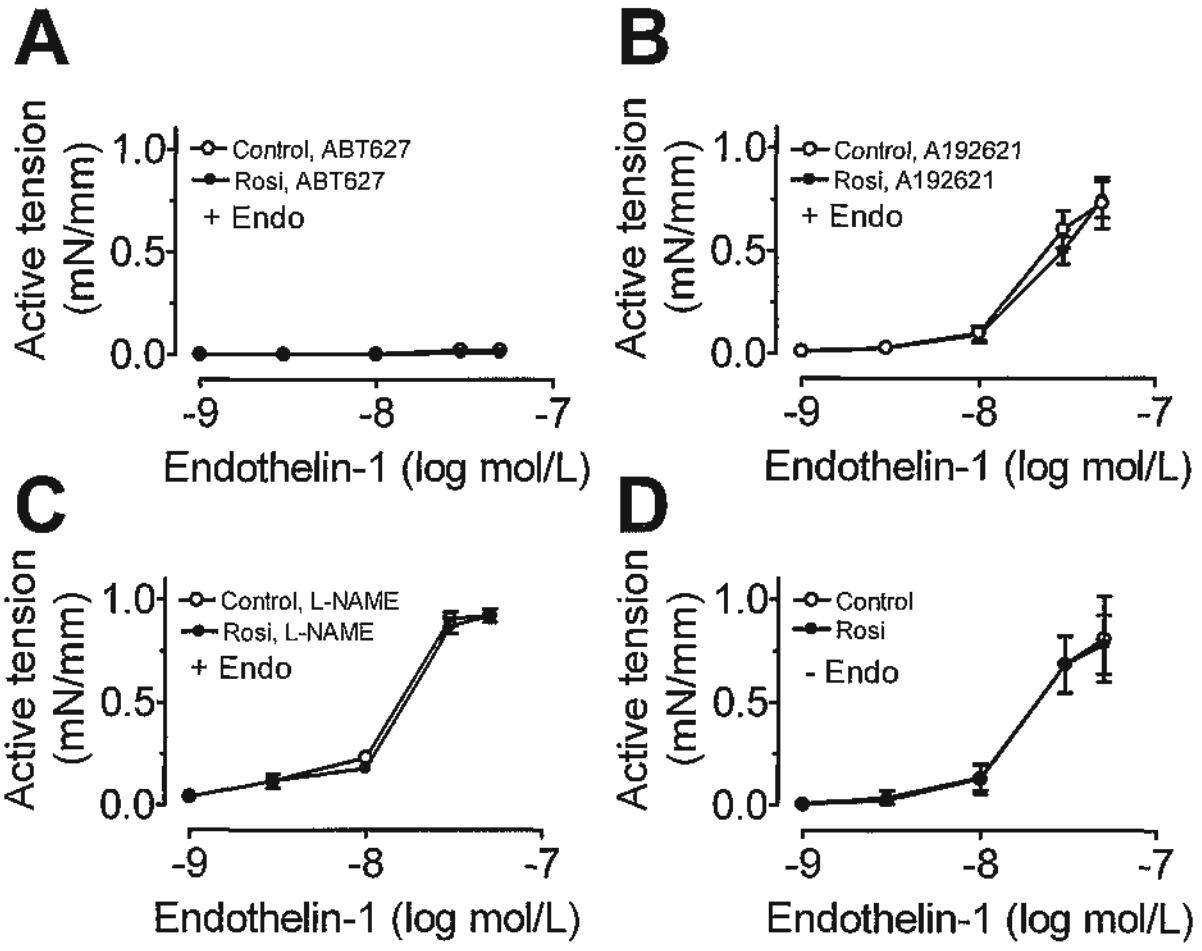

Figure 4.2. Concentration-dependent contractions to endothelin-1 in rosiglitazone (10 µmol/L, 24 h)-treated mouse aortic rings in the presence of 10 nmol/L ABT627 (A), of 10 nmol/L A192621 (B), of 100 µmol/L L-NAME (C) and in aortic rings without endothelium (D). Results are means ± SEM of 4-6 experiments.

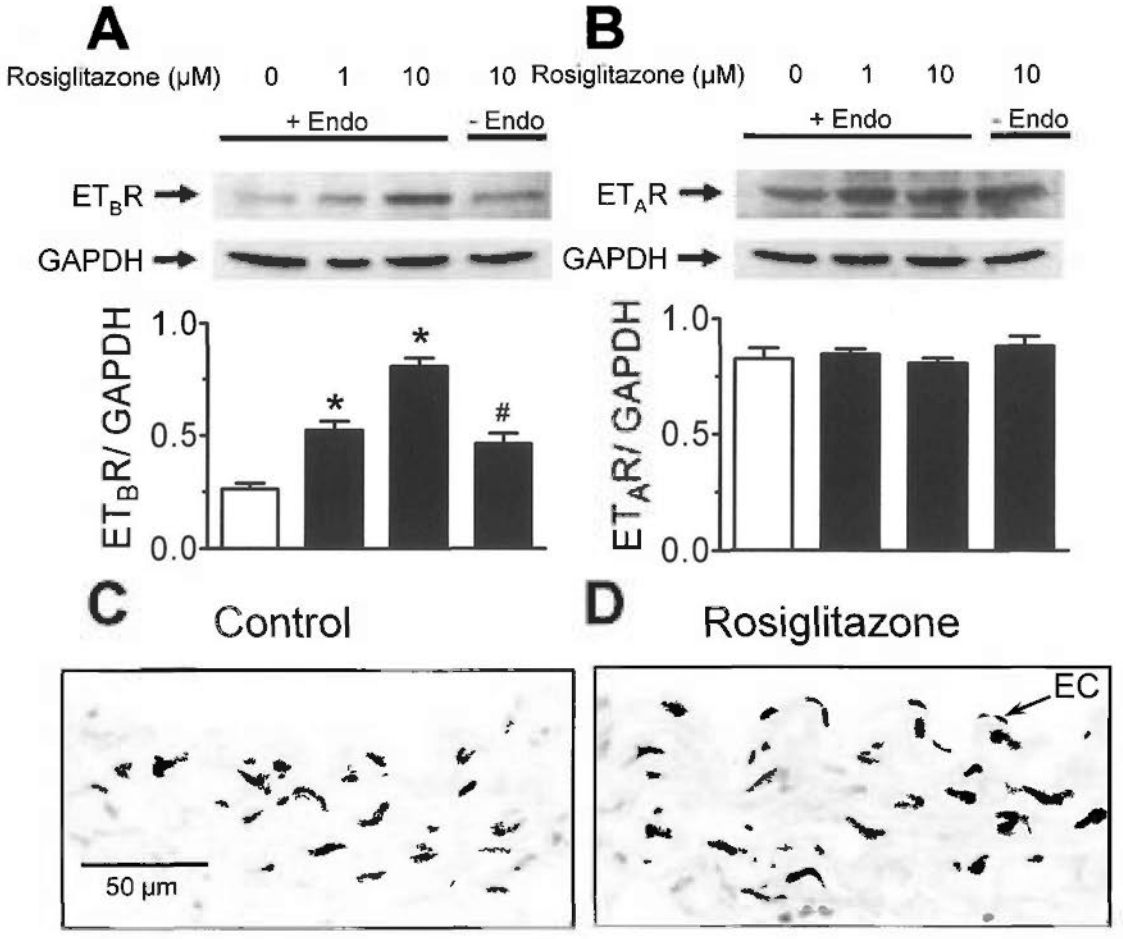

Figure 4.3. Western blotting for ET_B receptor (A) and ET_A receptor (B) in control and rosiglitazone (10 μmol/L, 24 h)-treated mouse aortas. Results are means ± SEM of 3 experiments. *p<0.05 vs control; # p<0.05 vs Rosiglitazone (10 μmol/L). Immunohistochemical staining of ET_B receptor in mouse aortas (C, D) and arrow indicates endothelial cells (EC). Data are representative for 3 times from different mice.

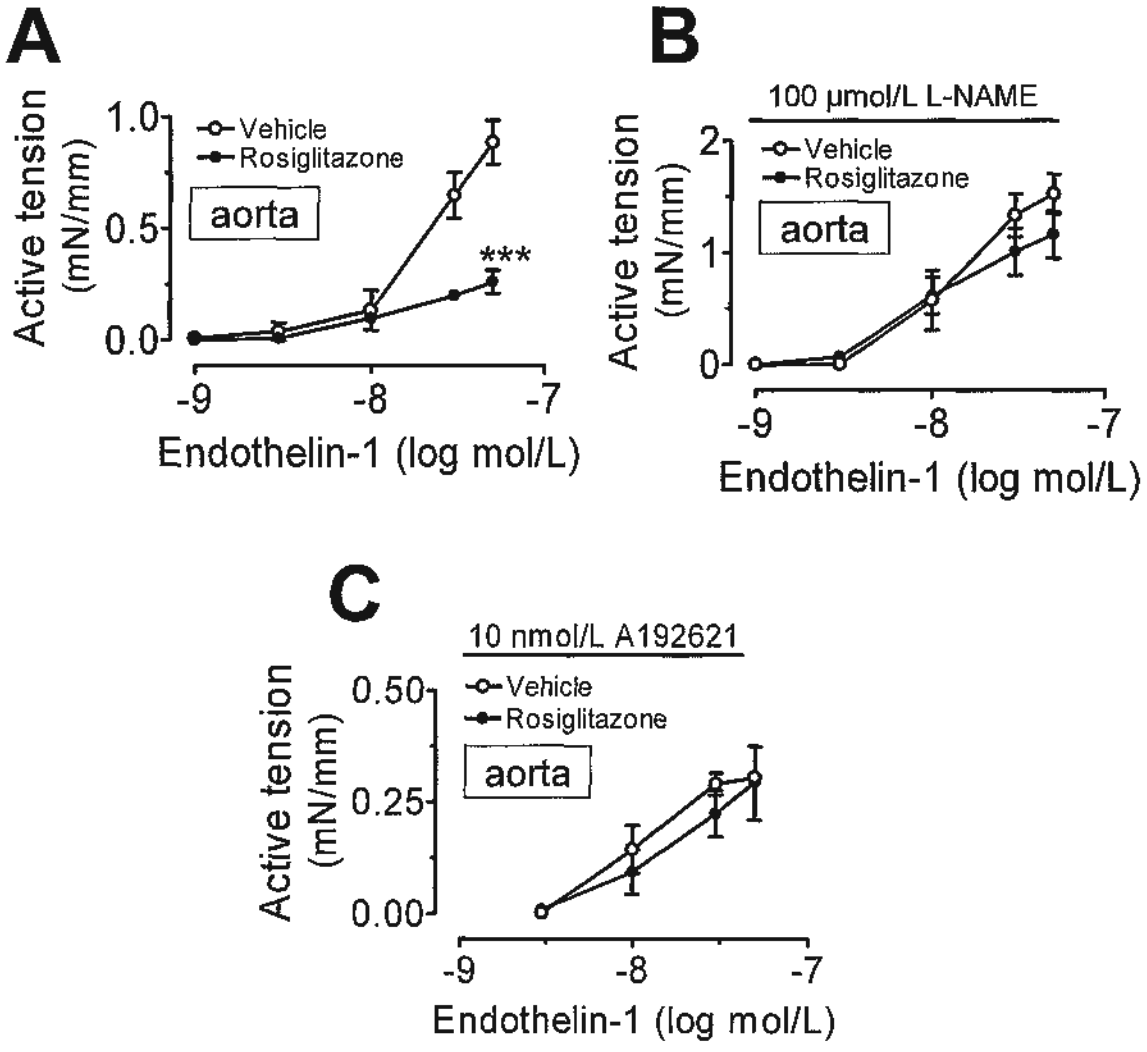

Figure 4.4. Chronic treatment with rosiglitazone by oral gavage reduced endothelin-1-induced vasoconstrictions in mouse aortas (A) and were abolished by the presence of 100 µmol/L L-NAME (B) or 10 nmol/L A192621 (C). Data are means ± SEM of 5 experiments from different mice. *** $p<0.001$ vs vehicle.

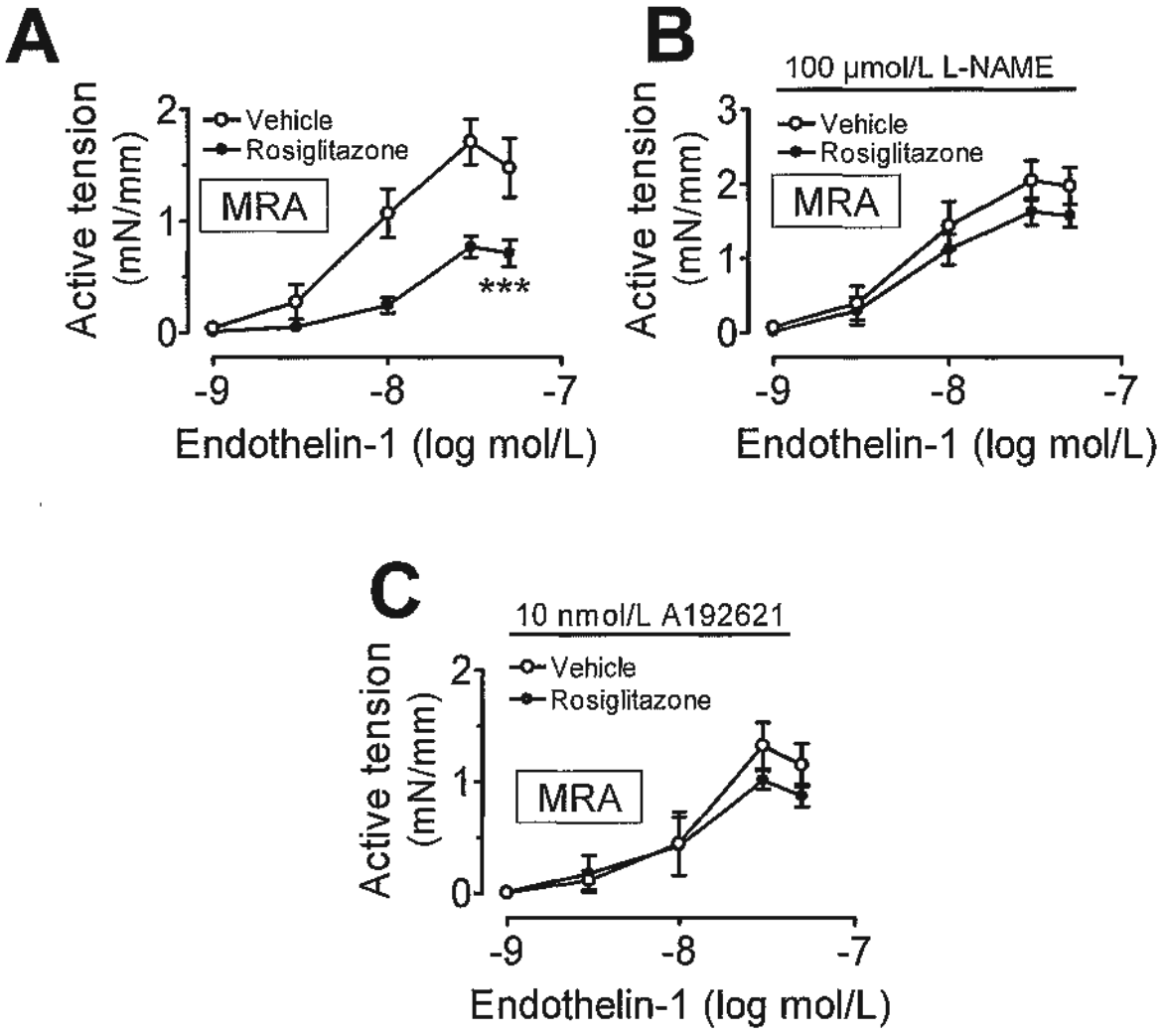

Figure 4.5. Chronic treatment with rosiglitazone by oral gavage reduced endothelin-1-induced vasoconstrictions in MRAs (A) and were abolished in the presence of 100 µmol/L L-NAME (B) or 10 nmol/L A192621 (C). Results are means ± SEM of 5 experiments from different mice. *** $p<0.001$ vs vehicle.

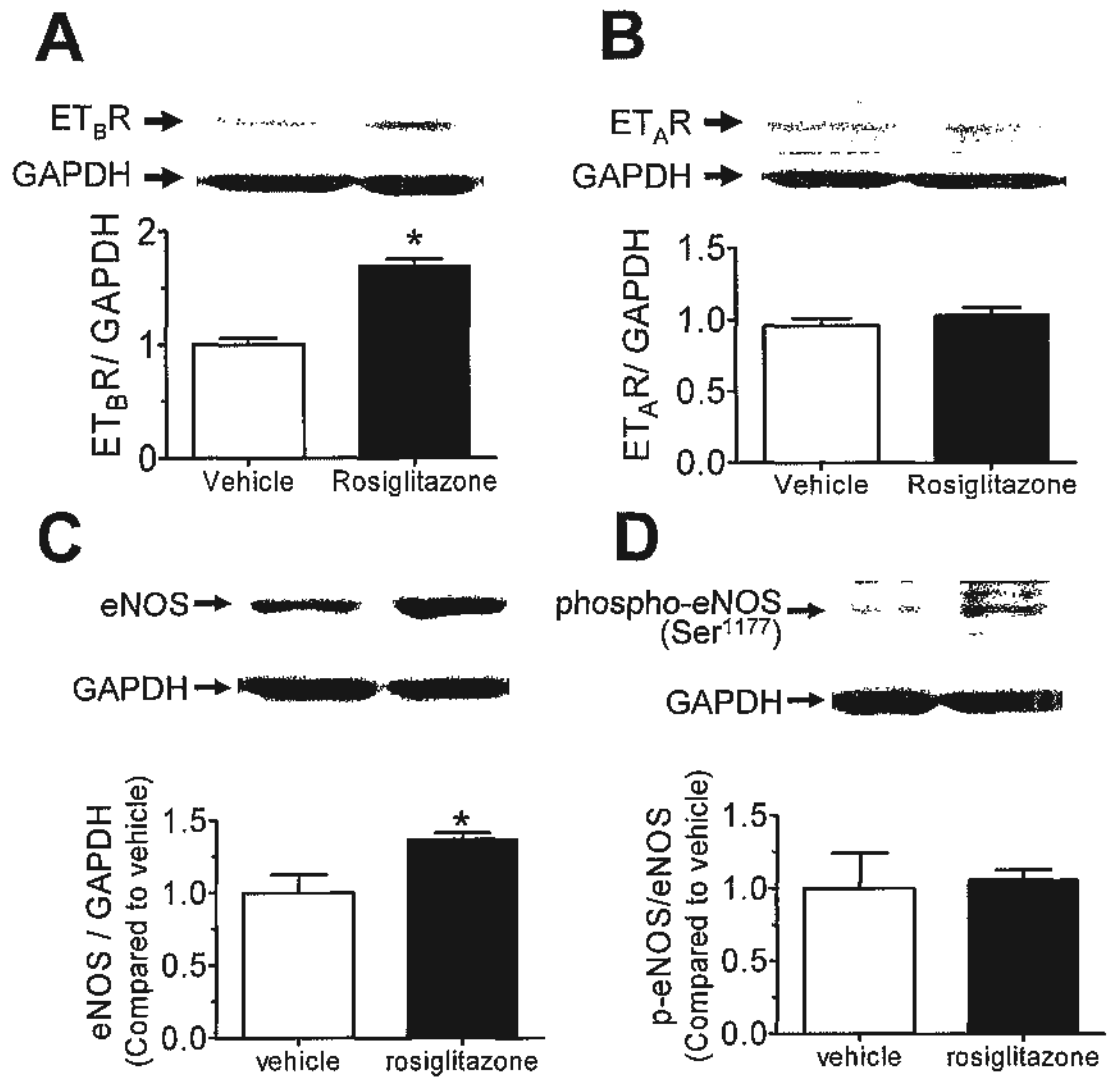

Figure 4.6. Western blotting revealed that *in vivo* rosiglitazone treatment upregulated the ET_BR expression (A) without affecting the ET_AR expression (B) in mouse aortas. Rosiglitazone treatment also increases the eNOS expression (C) without affecting the eNOS activity as indicated by unchanged phosphor-eNOS/eNOS ratio (D). Results are means ± SEM of 5 experiments from different mice. *p<0.05 vs vehicle.

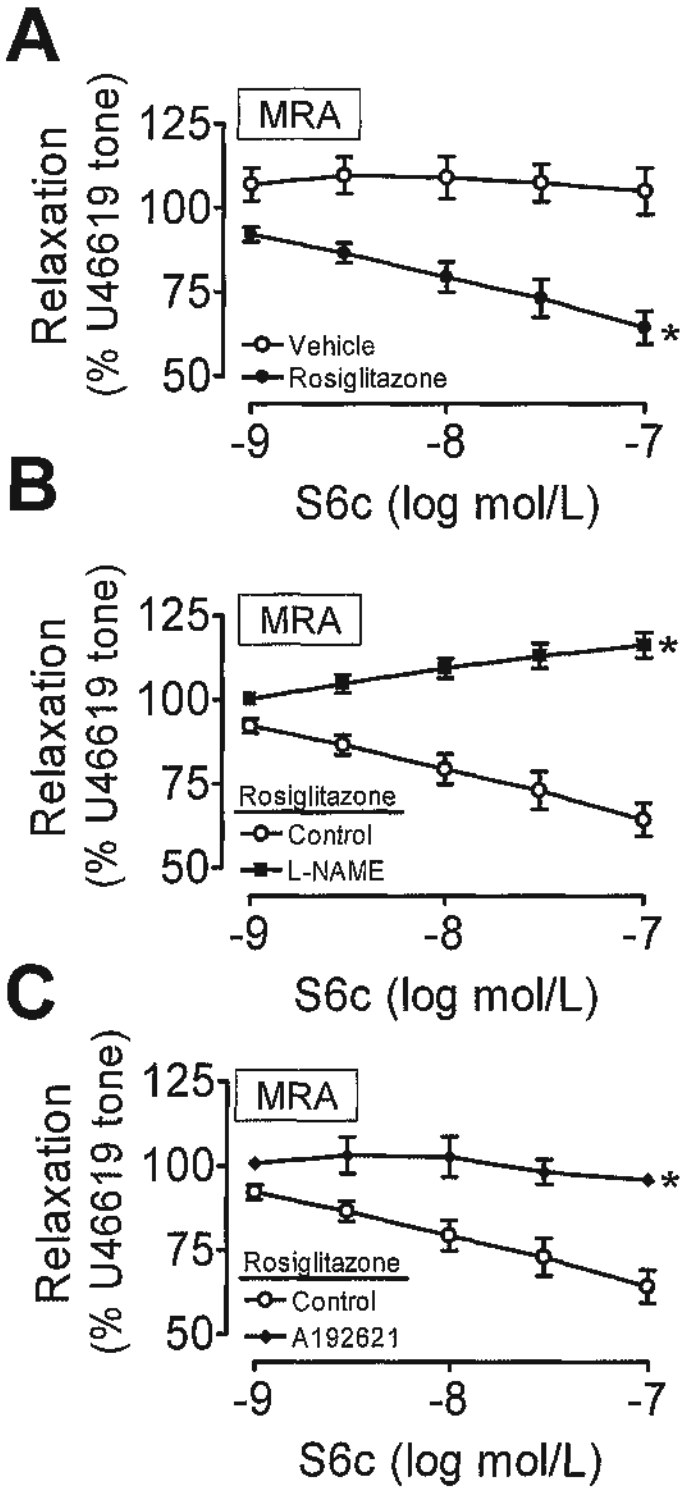

Figure 4.7. the selective ET_BR agonist, sarafotoxin 6c (S6c)-induced vasodilatations in MRAs only observed in rosiglitazone treated mice (A). *p<0.05 vs vehicle. Vasodilatations induced by S6c were abolished by 100 µmol/L L-NAME (B) or 10 nmol/L A192621 (C). Results are means ± SEM of 5 experiments from different mice. *p<0.05 vs Control.

CHAPTER V

PPARδ activation protects endothelial function in diabetic mice through PI3K/Akt pathway

5.1 Introduction

PPARδ is the least studied isoform of peroxisome proliferators-activated receptors (PPARs) and it is ubiquitously expressed in tissues such as liver, brain, skin, and adipose tissue (Peters *et al.*, 2000; Qin *et al.*, 2008). Recently, the role of PPARδ in obesity and diabetes has been examined by using loss-of-function study or synthetic PPARδ ligands. PPARδ deficiency may lead to a reduced adipogenesis (Barak *et al.*, 2002). On the contrary, PPARδ knockout mouse is more prone to weight gain on high-fat diet, which can be ameliorated by the synthetic PPARδ agonist GW501516 (Tanaka *et al.*, 2003; Wang *et al.*, 2003). PPARδ agonists GW501516, GW0742, and L-165041 can improve the lipid profile in obese animal models through increasing HDL and decreasing LDL cholesterol and triglyceride (Leibowitz *et al.*, 2000; Oliver *et al.*, 2001; van der Veen *et al.*, 2005).

PPARδ also regulates glucose homeostasis in type 2 diabetes. PPARδ activation in *db/db* mice improves hepatic and peripheral insulin sensitivity by increasing glucose consumption and also promotes fatty acid synthesis in the liver (Lee *et al.*, 2006). GW501516 enhances the HDL level and facilitates triglyceride clearance in healthy human subjects by up-regulation of fatty acid oxidation in skeletal muscles (Sprecher *et al.*, 2007). GW501516 can also lower plasma levels of triglyceride, LDL cholesterol and insulin in obese men (Riserus *et al.*, 2008). In *db/db* mice, either GW0742 treatment or hepatic

ove-rexpression of PPARδ attenuates hepatic steatosis by regulating lipogenesis (Qin *et al.*, 2008). In general, PPARδ is beneficial against obesity insulin resistance and metabolic disease.

The metabolic effect of PPARδ activation is likely to be associated with cardiovascular benefits in diabetes. However, the direct effects of PPARδ activation on the vascular wall such as angiogenesis and endothelial function are less studied. PPARδ is expressed in endothelial cells (Piqueras *et al.*, 2007). Importantly, one of the endogenous agonistic ligands for PPARδ is prostacyclin, which can be released by the endothelium. Prostacyclin promotes pro-angiogenic function of endothelial progenitor cells in a PPARδ-dependent manner (Gupta *et al.*, 2000; He *et al.*, 2008). Prostacyclin and PPARδ agonist L-165041 can prevent apoptosis induced by H_2O_2 through PPARδ-dependent up-regulation of 14-3-3α expression which prevents Bad-triggered apoptosis (Liou *et al.*, 2006). These experimental observations suggest that PPARδ may play a positive role in vascular activities such as angiogenesis, apoptosis and endothelial activation. GW0742 can reduce the expression of pro-inflammatory adhesion molecules and reduces atherosclerotic lesion which is partially related to the beneficial effect of PPARδ agonists on lipid profile. In addition, a direct anti-inflammatory effect of PPARδ activation has been verified in both *in vivo* and *in vitro* experimental models (Fan *et al.*, 2008; Graham *et al.*, 2005; Li *et al.*, 2004).

To my interest, a recent report suggests that PPARδ activation by GW501516 enhances vasculogenesis in a mouse model of hind limb ischemia through the stimulation of phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signaling pathway (Han *et al.*, 2008). PI3K/Akt participates in the regulation of the activity of endothelial nitric oxide synthase (eNOS) in

endothelial cells (Oudit *et al.*, 2004; Shiojima *et al.*, 2002). Up to date, no study has examined the possible role of PPARδ in endothelial dysfunction of diabetes. Therefore, in the present study I investigated the effect of PPARδ activation on endothelial dysfunction in diabetic mice and determined whether or not PI3K/Akt could contribute to the vascular benefit of PPARδ activation. To achieve this, a combination of experimental approaches (functional, molecular and biochemical studies) was employed. *db/db* diabetic mice, high-fat diet-induced obese mice and PPARδ knockout mice with and without chronic oral treatment with the PPARδ agonist were used. Functional assay was performed on microvessel myograph.

5.2 Experimental procedures

5.2.1 Chemicals

Acetylcholine, N^G-nitro-L-arginine methyl ester (L-NAME), phenylephrine and sodium nitroprusside (SNP) were dissolved in water, while others in DMSO. PPARδ agonist GW501516 was from Alexis Biochemicals, Lausen, Switzerland. Ca^{2+} ionophore A23187, PPARδ agonist GW0742, PI3K inhibitor LY294002 were from Tocris Bioscience, Bristol, UK. PPARδ antagonist GSK0660, PI3K inhibitor wortmannin, Akt inhibitor V (API-2/triciribine/TCN) were purchased from Sigma-Aldrich, St Louis, MO, USA.

5.2.2 Animals

Male leptin receptor$^{-/-}$ (*db/db*) mice, with their lean *db/m*$^{+}$ littermates; Wild type *PPARβ/δ*$^{+/+}$ and *PPARβ/δ*$^{-/-}$ mice generated from C57BL/6NXSv/129 background were used for this study. *PPARβ/δ*$^{+/+}$ and *PPARβ/δ*$^{-/-}$ mice were generated as described previously (Peters *et al.*, 2000). This mouse line has

been verified in brain, liver, skin, adipose tissue (Kim *et al.*, 2004; Kim *et al.*, 2005; Lee *et al.*, 2006; Marin *et al.*, 2006; Muller-Brusselbach *et al.*, 2007; Peters *et al.*, 2000; Shan *et al.*, 2008). The mice were housed in a temperature-controlled holding room (22–23°C) with a 12-hour light/dark cycle, and fed a standard chow and water. All of the experiments were conducted under the institutional guidelines for the humane treatment of laboratory animals.

Diet-induced obese (DIO) mice were generated on C57BL/6J mice, *PPARβ/δ*$^{-/-}$ and age-matched *PPARβ/δ*$^{+/+}$ wild type (*WT*) littermates at the age of 6 weeks which were fed for 8-10 weeks with high fat diet (Rodent diet with 45 % kcal% fat, D12451, Research Diets Inc. New Brunswick, NJ, USA). Mice were treated with GW1516 or vehicle by oral gavage at the dosage of 5 mg/kg/day for 7-10 days 8-9 weeks after high-fat feeding in DIO (C57/BL/6J fed on high-fat diet), age-matched C57BL/6J, *db/db*, *PPARβ/δ*$^{+/+}$ and *PPARβ/δ*$^{-/-}$ DIO mice, respectively.

5.2.3 Functional assay

After mice were sacrificed, thoracic aortas were removed rapidly and placed in oxygenated ice-cold Krebs solution that contained (mmol/L): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. Changes in isometric tone of the aortic rings were recorded in myograph (Danish Myo Technology, Aarhus, Denmark) (Wong *et al.* 2010a). The rings were stretched to an optimal baseline tension of 3 mN and then allowed to equilibrate for 60 minutes before the experiment commenced. Rings were first contracted with 60 mmol/L KCl and rinsed in Krebs solution. After several washouts, phenylephrine (1 μmol/L) was used to produce a steady contraction and

acetylcholine (ACh) (10 nmol/L – 10 μmol/L) was added cumulatively to induce endothelium-dependent relaxation.

5.2.4 Organ culture of mouse aortic rings

Mouse thoracic aortic rings (2 mm in length) were incubated in a Dulbecco's Modified Eagle's Media (DMEM, Gibco, Gaithersberg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), plus 100 IU/mL penicillin and 100 μg/mL streptomycin. Drugs including GW501516 (PPARδ agonist, 0.1 μmol/L, Alexis Biochemicals, Lausen, Switzerland), GW0742 (PPARδ agonist, 0.1 μmol/L, Tocris Bioscience, Bristol, UK), GSK0660 (PPARδ antagonist, 1 μmol/L, St Louis, MO, USA), LY294002 (PI3K inhibitor, 5 μmol/L, Tocris), wortmannin (PI3K inhibitor, 0.1 μmol/L, Sigma), Akt inhibitor V (API-2/triciribine/TCN, Akt inhibitor, 5 μmol/L, Sigma) were individually added into the culture medium that bathed the aortic rings. High glucose condition was achieved by the addition of 25 mmol/L glucose, while 25 mmol/L of mannitol was used as the osmotic control. After the incubation period, the rings were transferred to a chamber filled with fresh Krebs solution and mounted in a myograph for measurement of changes in isometric force.

5.2.5 Western blotting

Protein samples prepared from mouse aorta homogenates were electrophoresed through a 10% SDS-poly-acrylamide gel and transferred onto an immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). Nonspecific binding sites were blocked with 1% BSA in 0.05% Tween-20 TBS. The blots were incubated overnight at 4°C with the primary antibodies: polyclonal anti-phosphor-eNOS at Ser^{1177} (1:1000, Upstate Biotechnology, Lake Placid, NY, USA); anti-phospho-Akt at Ser^{473}

and Thr^{308}, monoclonal anti-Akt1 (1:1000, Cell Signaling technology, Danvers, MA, USA), monoclonal anti-eNOS (1:1000, BD Transduction Laboratory, San Diego, CA, USA); followed by HRP-conjugated secondary antibody (DakoCytomation, Carpinteria, CA, USA). Monoclonal anti-GAPDH (1:5000, Ambion, Cambridge, UK) was used as a housekeeping protein.

5.2.6 Primary culture of mouse aortic endothelial cells

The method was modified based on the early reported procedure (Kobayashi *et al.*, 2005; Magid *et al.*, 2003). Briefly, mice were anaethesized with an intra-peritoneal injection of pentobarbital sodium (40 mg/kg). Heparin (100 U/mL in PBS) was infused into the circulation from the left ventricle. The aortas were dissected in DMEM, and incubated with collagenase type II for 15 minutes at 37 °C. Detached endothelial cells were collected by centrifugation, re-suspended in 20% FBS-DMEM, then cultured in endothelial cell growth medium (EGM) supplemented with bovine brain extract (Lonza, Walkersville, MD, USA) till confluency. The cultured endothelial cells were then incubated in normal medium (with 25 mmol/L mannitol), high glucose (30 mmol/L) medium with or without the presence of different drugs for 36 hours before the measurement of NO using laser confocal fluorescence microscopy.

5.2.7 Culture of human umbilical cord vein endothelial cells

Human umbilical cord vein endothelial cells (HUVECs) obtained from Lonza (CC-3317) were grown in EGM supplemented with BBE and 1% penicillin plus streptomycin (GIBCO). The cells were grown in 75 cm^2 flasks and maintained at 37 °C in a 95% humidified air / 5% CO_2 atmosphere. Medium was changed every two days. Confluent cells were passaged by trypsinization

(0.25% trypsin with 2.5 mmol/L EDTA in PBS). Experiments were performed on cells at passage 6-8 at the time 80-90% confluency was obtained.

5.2.8 Transient transfection

MAECs and HUVECs were transfected with either a constitutively active Akt plasmid (CA-Akt), or a dominant negative Akt construct (DN-Akt), or control plasmid by electroporation using Nucleofector II machine (Amaxa/Lonza, Walkersville, MD, USA) following the procedure provided by the manufacturer. DNA plasmids were generously provided by Dr Wu Zhenguo from the Department of Biochemistry, Hong Kong University of Science and Technology (Xu *et al.*, 2000). About 70% of endothelial cells were successfully transfected using these protocols as indicated by control transfection using a GFP-expressing pCAGGS vector.

5.2.9 Measurement of NO by laser confocal fluorescence microscopy

Fluorimetric measurements were performed on primary mouse aortic endothelial cells using the Olympus Fluoview FV1000 laser scanning confocal system mounted on an inverted IX81 Olympus microscope, equipped with a 10X objective (NA 0.5). 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA, Molecular Probes, Eugene, OR, USA) was used as the NO indicator. The cells were incubated with 1 μmol/L DAF-FM DA in the dark for 10 minutes and then washed for 20 minutes. The amount of NO in response to 1 μmol/L A23187 was evaluated by measuring the fluorescence intensity excited at 495 nm and emitted at 515 nm. The cells were stimulated with the calcium ionophore A23187 because there was no calcium or NO signal in response to acetylcholine in the cultured endothelial cells. Changes

in intracellular NO production was displayed as relative fluorescence intensity (F_1/F_0, where F_0 = control and F_1 = administration of A23187).

5.2.10 Statistics

Results represent means ± SEM from different mice. Concentration-response curves were analyzed by non-linear regression curve fitting using GraphPad Prism software (Version 4.0) to calculate E_{max} as the maximum response and pD_2 as the negative logarithm of the drug concentration that produced half of E_{max}. The protein expression was quantified by densitometer (FluorChem, Alpha Innotech, San Leandro, CA), normalized to GAPDH and then compared with control. Comparisons among groups were made using ANOVA followed by an unpaired Student's t test. The *p* values less than 0.05 were accepted to indicate statistically significant differences.

5.3 Results

5.3.1 PPARδ ligands improve endothelium-dependent relaxations impaired by high glucose

In order to investigate the effect of PPARδ activation on endothelial function, organ culture of isolated aortic rings in culture medium containing different pharmacological agents was performed. Exposure to 30 mmol/L glucose-containing DMEM (HG) for 36 hrs significantly reduced endothelium-dependent relaxations (EDRs) to ACh in aortas from C57BL/6J mice as compared with those incubated in 5 mmol/L glucose-containing DMEM (NG) (Figure 5.1A&B). Co-incubation with PPARδ agonistic ligands GW0742 (0.1 μmol/L) (Figure 5.1A) GW1516 (0.1 μmol/L) (Figure 5.1B) restored the impaired EDRs in high glucose-containing medium.

In order to verify the specificity of GW1516 on PPARδ, *PPARδ WT* and *PPARδ KO* mice were used. The genotype of *PPARδ WT* and *PPARδ KO* mice was confirmed by PCR-genotyping using designed primers provided by Dr. ST Lee (Department of Biochemistry, Chinese University of Hong Kong) (data not shown). Exposure to high glucose reduced EDRs in aortas from both *PPARδ WT* and *PPARδ KO* mice to a similar extent (Figure 5.2A&B). Co-incubation with GW1516 improved EDRs in aortas from *PPARδ WT* mice (Figure 5.2A), but not in those from *PPARδ KO* mice (Figure 5.2B). Endothelium-independent relaxations to SNP were similar among all groups (Figure 5.2C&D).

5.3.2 PI3K/Akt contributes to the beneficial effect of PPARδ agonist GW1516

PPARδ antagonist GSK0660 (1 μmol/L) abolished the improved EDRs in

aortic rings treated with GW1516 (0.1 μmol/L) in exposure to high glucose (HG, 30 mmol/L, 36 hrs) (Figure 5.3A). Co-incubation with LY294002 (PI3K inhibitor, 5 μmol/L) (Figure 5.3C), wortmannin (PI3K inhibitor, 0.1 μmol/L) (Figure 5.3B), or Akt inhibitor V (Akt inhibitor, 5 μmol/L) (Figure 5.3D), also inhibited the improved EDRs in GW1516-treated rings bathed in high glucose-containing culture medium.

5.3.3 PPARδ ligands improve endothelium-dependent relaxations in aortas from *db/db* mice

Treatment with PPARδ agonists, GW1516 (0.1 μmol/L) or GW0742 (0.1 μmol/L) markedly improved EDRs which were impaired in aortas from *db/db* mice (Figure 5.4A). GSK0660 (1 μmol/L) antagonized the effect of GW1516 (0.1 μmol/L) on EDRs in aortas from *db/db* mice (Figure 5.4B). Co-incubation with LY294002 (5 μmol/L) (Figure 5.4C), wortmannin (0.1 μmol/L) (Figure 5.4C), or Akt inhibitor V (5 μmol/L) (Figure 5.4D), also inhibited the improved EDRs in GW1516-treated *db/db* mouse aortas.

5.3.4 PPARδ agonists enhance the nitric oxide production in cultured endothelial cells

In cultured mouse aortic endothelial cells (MAECs), addition of the Ca^{2+} ionophore A23187 induced a rise of the DAF-FM diacetate fluorescence which reflects the level of NO production in normal glucose (NG)-containing medium, which was similar in cells treated with GW1516 in NG group (Figure 5.5 and Figure 5.6A&D). In cells treated with high glucose (30 mmol/L, 36 hrs), the NO production diminished, which was restored by co-treatment with 0.1 μmol/L GW1516 (Figure 5.5 and Figure 5.6A&D). GW0742 at 0.1 μmol/L

produced a similar effect as GW1516 in high glucose-treated cells (Figure 5.5 and Figure 5.6B&D). The PPARδ antagonist, GSK0660 (1 μmol/L) eliminated the effect of GW1516 (0.1 μmol/L) to increase the A23187-stimulated NO production (Figure 5.5 and Figure 5.6B&D). Co-incubation with LY294002 (5 μmol/L), wortmannin (0.1 μmol/L), or Akt inhibitor V (5 μmol/L) also inhibited the effect of GW1516 (Figure 5.5 and Figure 5.6C&D).

5.3.5 GW1516 increases the phosphorylation of eNOS and Akt in mouse aortic endothelial cells

In high glucose-treated MAECs (30 mmol/L, 36 hrs), eNOS phosphorylation at Ser^{1177} and Akt phosphorylation at Ser^{473} and Thr^{308} reduced, which was reversed by GW1516 (0.1 μmol/L). Co-incubation with GSK0660 (1 μmol/L), LY294002 (5 μmol/L), or wortmannin (0.1 μmol/L) inhibited the effect of GW1516 (Figure 5.7 and Figure 5.8).

5.3.6 Regulation of Akt activity affects the NO production in endothelial cells

To further confirm the role of Akt in the effect of GW1516 on NO production, I over-expressed the constitutively active Akt (CA-Akt) by transient transfection. Increasing Akt activity by CA-Akt slightly increased NO production in high glucose-treated MAECs (Figure 5.9A&C). GW1516 also restored NO production in high glucose-treated MAECs that were transfected with CA-Akt (Figure 5.9A&C). I also used dominant negative Akt construct (DN-Akt) to suppress the Akt activity. Suppression of the Akt activity by DN-Akt inhibited the restoration by GW1516 of NO production in high glucose-treated MAECs (Figure 5.9B&C).

Akt over-expression by CA-Akt increased Akt phosphorylation at Ser^{473} and Thr^{308}, and slightly increased eNOS phosphorylation at Ser^{1177}. Phosphorylation of Akt was significantly inhibited by DN-Akt, without affecting the level of eNOS phosphorylation (Figure 5.10A). GW1516 (0.1 μmol/L) did not further increase Akt phosphorylation in CA-Akt transfected cells, but slightly increased eNOS phosphorylation. However, the effect of GW1516 was abolished in DN-Akt transfected cells (Figure 5.10A&B).

5.3.7 GW1516 treatment *in vivo* improves endothelial function in *db/db* mice.

GW1516 was administered by oral gavage (5 mg/kg/day. 7-10 days) to *db/db* mice. EDRs in aortas from *db/db* mice were significantly reduced compared with those from *db/m*$^+$ mice (Figure 5.11A) while EDRs in aortas from *db/db* mice were markedly augmented following GW1516 treatment (Figure 5.11A). By contrast, endothelium-independent relaxations to SNP were similar in *db/m*$^+$, *db/db*, or *db/db* treated with GW1516 (Figure 5.11B). Phosphorylations of eNOS at Ser^{1177} and Thr^{308} were restored in aortas from GW1516-treated *db/db* mice (Figure 5.12).

5.3.8 GW1516 treatment *in vivo* improves endothelial function in diet-induced obese mice in a PPARδ-specific manner.

To produce diet-induce obese mice (DIO), normal C57BL/6J mice were fed on high-fat diet for about 10 weeks. GW1516 was administered by oral gavage (5 mg/kg/day. 7-10 days) to DIO, age-matched C57BL/6J control, *PPARδ KO*, and age-matched *PPARδ WT* mice on high-fat diet. EDRs were significantly impaired in aortas from DIO *PPARδ KO*, *PPARδ WT* mice, or C57BL/6J mice

(Figure 5.13A and Figure 5.14A&B). GW1516 treatment *in vivo* restored EDRs in aortas from DIO mice (Figure 5.13A) and in those from DIO *PPARδ WT* mice (Figure 5.14A), but not in those from DIO *PPARδ KO* mice (Figure 5.14B). Again, endothelium-independent relaxations to SNP were similar among all groups (Figure 5.13B and Figure 5.14C&D). Reduced eNOS and Akt phosphorylation upon high-fat feeding in DIO mice was restored after GW1516 treatment only in aortas from *PPARδ WT* mice, but not in those from *PPARδ KO* mice (Figure 5.15 and Figure 5.16).

5.4 Discussion

In the present study, I have demonstrated that PPARδ activation improved endothelial function in diabetic mouse through the activation of PI3K/Akt. Firstly, I showed that PPARδ agonists GW501516 (GW1516) and GW0742 can cause direct improvement of endothelium-dependent relaxation (EDRs) in aortas which was reduced by 36-h exposure to high glucose. The effects of PPARδ ligands were PPARδ-specific, which was verified by three following approaches: (1) two selective PPARδ agonists GW1516 and GW0742 exhibited similar effects in improving EDRs in aortas and in augmenting the NO production in cultured mouse aortic endothelial cells; (2) the PPARδ antagonist GSK0660 antagonized the beneficial effect of GW1516; and (3) in *PPARδ KO* mice, the effect of GW1516 was absent. In addition, stimulation with PPARδ agonists also improved ACh-induced vasodilator response in aortas from *db/db* mice. Secondly, the effect of PPARδ activation in mouse aortas and in cultured mouse aortic endothelial cells appears to be mediated through PI3K/Akt signaling cascade based on the following observations. (1) The effect of PPARδ agonists on arteries or endothelial cells was inhibited by pharmacological inhibitors of both PI3K and Akt. (2) PPARδ agonist stimulated a rise in Akt and eNOS phosphorylation and this effect was sensitive to PI3K/Akt inhibition and PPARδ antagonism. (3) The suppression of the Akt activity by transient transfection inhibited the effect of GW1516 to restore the diminished NO production or to increase the reduced eNOS and Akt phosphorylation caused by high glucose exposure in cultured endothelial cells. (4) Increasing the Akt activity by over-expression restored the decreased NO production induced by high glucose. Lastly, the beneficial

effect of PPARδ ligand was verified *in vivo* using two types of diabetic mouse models, *e.g.*, the genetic obese *db/db* mice, and high-fat diet-induced obese mice, which were also related to the activation of PI3K/Akt.

Although PPARδ is expressed in the vascular cells (Piqueras *et al.*, 2007; Tanaka *et al.*, 2003), the role of PPARδ in the regulation of cardiovascular function is not thoroughly understood. The effect of PPARδ activation in the vasculature is mainly focused on angiogenesis and inflammatory response. PPARδ activation induces angiogenesis and vasculogenesis (Han *et al.*, 2008; He *et al.*, 2008). The anti-inflammatory and anti-atherosclerotic effects of PPARδ agonistic ligands have also been examined in different mouse models of atherosclerosis and PPARδ activation decreases the size of atherosclerotic lesions and suppresses the expression of adhesion molecules to activate endothelial cells (Fan *et al.*, 2008; Graham *et al.*, 2005; Li *et al.*, 2004; Liou *et al.*, 2006; Takata *et al.*, 2008). However, there is no study examining the possible beneficial impact of PPARδ agonists against diabetic vascular dysfunction. The present study is probably the first of its kind showing that PPARδ agonists can cause a direct effect to restore endothelium-dependent vasodilatory function in isolated aortas from *db/db* mouse and in aortas subjected to high glucose challenge. This notion is supported by the beneficial effect of GW1516 treatment *in vivo* in two types of diabetic and obese mice. Given that PPARδ agonists can ameliorate dyslipidemia, the beneficial effect of GW1516 might be partially due to its favorable modulation of lipid metabolism *in vivo*. To verify this possibility, *ex vivo* organ culture of isolated mouse aortas treated with PPARδ agonists was performed. The results from these experiments strongly suggest that such a direct effect may exist as reflected by the effect of GW1516 to augment the

NO production in cultured mouse aortic endothelial cells *in vitro*.

Existing evidence indicates that PPARs benefit endothelial function. Although the function of PPARδ is less studied, PPARγ agonists, pioglitazone and rosiglitazone can improve endothelium-dependent dilatation in resistance arteries in angiotensin II-induced hypertension and reduce hypertension (Diep *et al.*, 2002). PPARα and PPARγ agonists inhibit the thrombin-activated endothelin-1 synthesis (Delerive *et al.*, 1999). PPARγ also elevates the NO bioavailability through increasing NO biosynthesis (Kleinhenz *et al.*, 2009) through activation of p38 (Ptasinska *et al.*, 2007), and/or decreasing endothelial production of superoxide anions (Hwang *et al.*, 2005). PPAR activators are also effective to improve vascular function in diabetic patients (Campia *et al.*, 2006; McMahon *et al.*, 2005; Werner *et al.*, 2007). Both animal and clinical studies suggest that PPARs can be potential targets for pharmaceutical intervention in the protection of endothelial function in diabetes. Given the recently publicized adverse effect of rosiglitazone on cardiovascular outcomes in diabetic patients (Home *et al.*, 2007; Nissen *et al.*, 2007), PPARδ could be an alternative target for the treatment of atherosclerosis, hypertension and other cardiovascular events in diabetic patients.

The present study shows that the activation of PI3K/Akt pathway is essential for the beneficial effect of PPARδ agonists on endothelial function. In cancer cells, PPARδ agonist up-regulates VEGF, which promotes the cell survival through PI3K/Akt-dependent mechanisms (Wang *et al.*, 2006). In keratinocytes, Akt mediates the anti-apoptotic effect of PPARδ (Di-Poi *et al.*, 2002). There is also an interaction between PPARδ and PI3K/Akt in other cell types (Han *et al.*, 2005; Zhang *et al.*, 2002). A recent study described that

GW501516 promotes vasculo-genesis through genomic transcription and non-genomic activation of PI3K/Akt via interaction with p85α, a regulatory subunit of PI3K (Han *et al.*, 2008). In addition, GW0742 and L-165041 at higher concentration (> 1 μmol/L) can directly induce endothelium-dependent relaxation, NO generation and eNOS phosphorylation which are partially related to activation of PI3K/Akt pathway in the rat aorta (Jimenez *et al.* 2010). The results in the present study support the aforementioned observations suggesting that PI3K/Akt is one of the most likely downstream targets for PPARδ activation. Nevertheless, I have provided the first line of evidence demonstrating that benefit of PPARδ activation on the vasodilatory function is associated with the stimulation of PI3K/Akt.

The specificity of GW1516 on the PPARδ receptor was substantiated in *PPARδ KO* mouse by demonstrating (1) high glucose exposure reduced endothelium-dependent relaxations in aortas from *PPARδ KO* and *WT* mice and this impairment is not reversed by co-treatment of GW1516 only in *PPARδ KO* mice; (2) chronic GW1516 treatment *in vivo* did not rescue the impaired relaxations in aortas from diet-induced obese *PPARδ KO* mice; and (3) GW1516 treatment *in vivo* did not normalize the reduced eNOS and Akt phosphorylation in the aortas from diet-induced obese *PPARδ KO* mice. The *PPARδ KO* mouse line used in this study is generated by Peters *et al.* (Peters *et al.*, 2000), which has been used by several different groups (Ghosh *et al.*, 2007; Han *et al.*, 2008; Lee *et al.*, 2006; Shan *et al.*, 2008). There is another line of *PPARδ KO* mouse generated by Barak *et al.* (Barak *et al.*, 2002). Both lines exhibit a reduced adipose store, suggesting the contribution of PPARδ in lipogenesis. *PPARδ KO* mice generated by Barak *et al.* exhibited glucose intolerance and metabolic inactivity on the normal chow (Lee *et al.*, 2006),

which is different from the line used in the present study. However, similar insulin resistance was observed in both *PPARδ KO* and *PPARδ WT* mice placed on a high-fat diet (Lee *et al.*, 2006). In the present study, the effect of high-fat feeding on the endothelium-dependent relaxation was comparable in *PPARδ KO* and *PPARδ WT* mice. The response to high glucose exposure was also similar between *WT* and *KO* mice. It is worthwhile noting that the present study used mice at the age of around 16 weeks, while Lee *et al.* used mice at much older age (> 6 months). The mouse line used in the present study also developed mild glucose intolerance in both *WT* and *KO* mice at older age (> 6 months) (data not shown).

More recently, some clinical trials examined the effect of GW501516 on dyslipidemia (Riserus *et al.*, 2008; Sprecher *et al.*, 2007), however, the cardiovascular safety and outcome of PPARδ ligands is still under investigation. In conclusion, the present study demonstrates that PPARδ activation can effectively improve endothelial function in diabetic and obese mice through the activation of PI3K/Akt. These novel findings may help to enhance the prospective of the use of safe PPARδ agonistic ligands in combating against vascular dysfunction in diabetes and obesity.

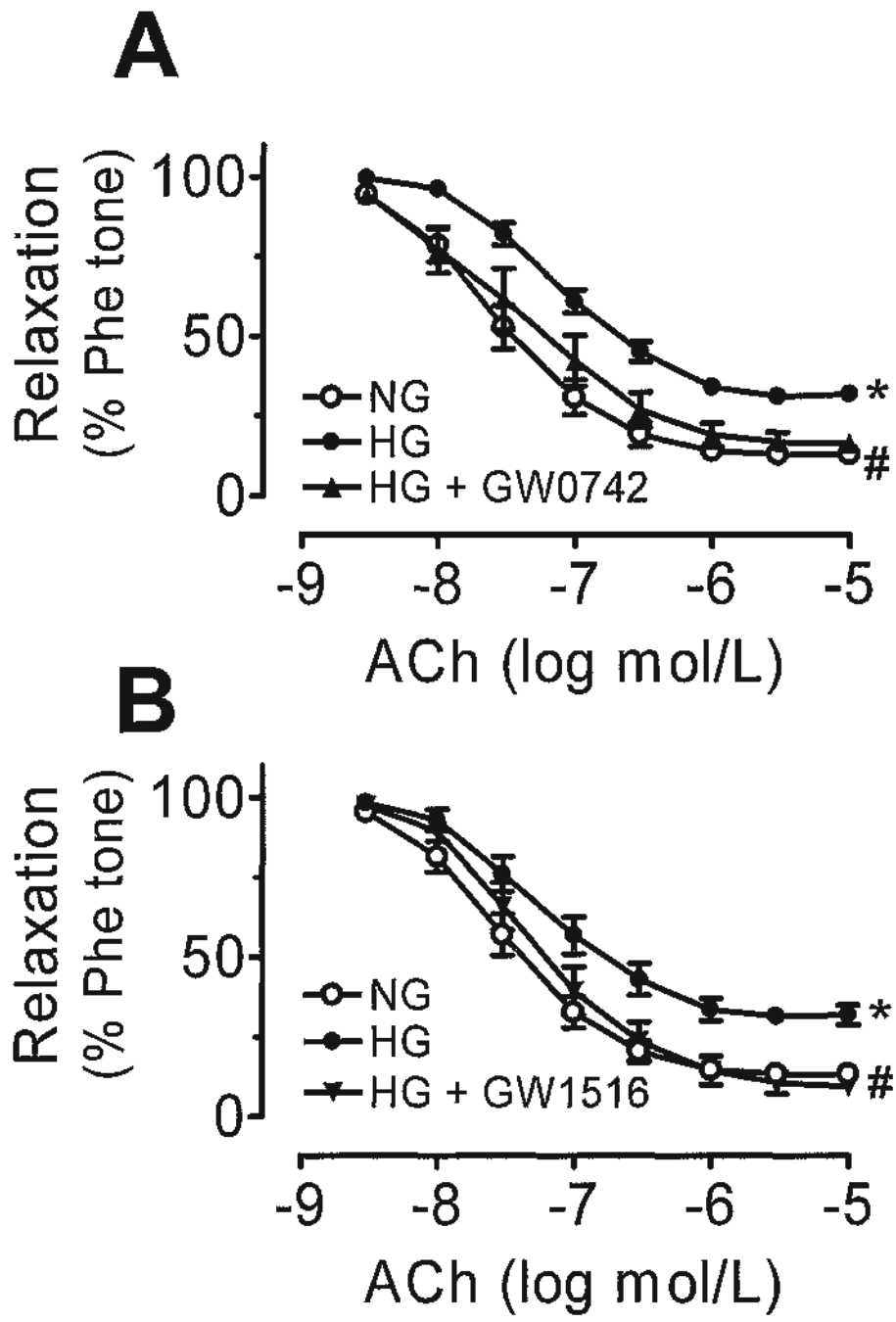

Figure 5.1. PPARδ agonists improved endothelium-dependent relaxations in mouse aortas exposed to high glucose. (A) Endothelium-dependent relaxation (EDRs) was significantly reduced after exposure to high glucose medium (HG, 30 mmol/L glucose, 36 hrs) compared to normal glucose (NG, 25 mmol/L mannitol, 36 hrs) in aortas from C57BL/6J mice. Co-incubation with GW0742 (PPARδ agonist, 0.1 μmol/L) (A) or GW1516 (PPARδ agonist, 0.1 μmol/L) (B) improved EDRs in aortas exposed to HG. Results are means ± SEM of 6 experiments. *$p<0.05$ vs NG. #$p<0.05$ vs HG.

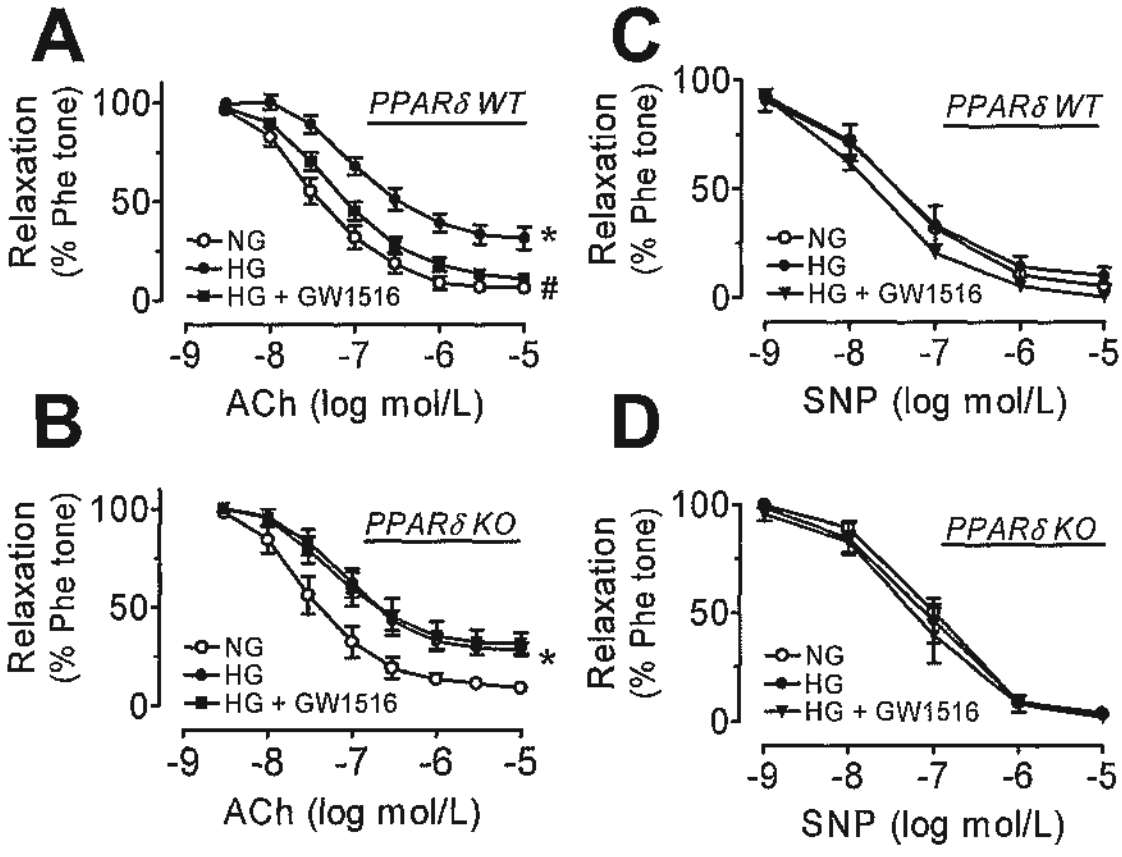

Figure 5.2. The effect of GW1516 on aortas from *PPARδ WT* and *KO* mice exposed to high glucose. (A) Co-incubation with GW1516 (0.1 μmol/L) significantly improved EDRs after exposure to high glucose medium (HG, 30 mmol/L glucose, 36 hrs) as compared with normal glucose (NG, 25 mmol/L mannitol, 36 hrs) in aortas from *PPARδ WT* mice. (B) Co-incubation with GW1516 (0.1 μmol/L) did not affect EDRs after exposure to high glucose medium (HG, 30 mmol/L glucose, 36 hrs) compared to normal glucose (NG, 25 mmol/L mannitol, 36 hrs) in aortas from *PPARδ KO* mice. Sodium nitroprusside (SNP)-induced endothelium-independent relaxations were similar among all groups in both *PPARδ WT* (C) and *PPARδ KO* mice. Results are means ± SEM of 6 experiments. *p<0.05 vs NG from each group. #p<0.05 vs HG from each groups.

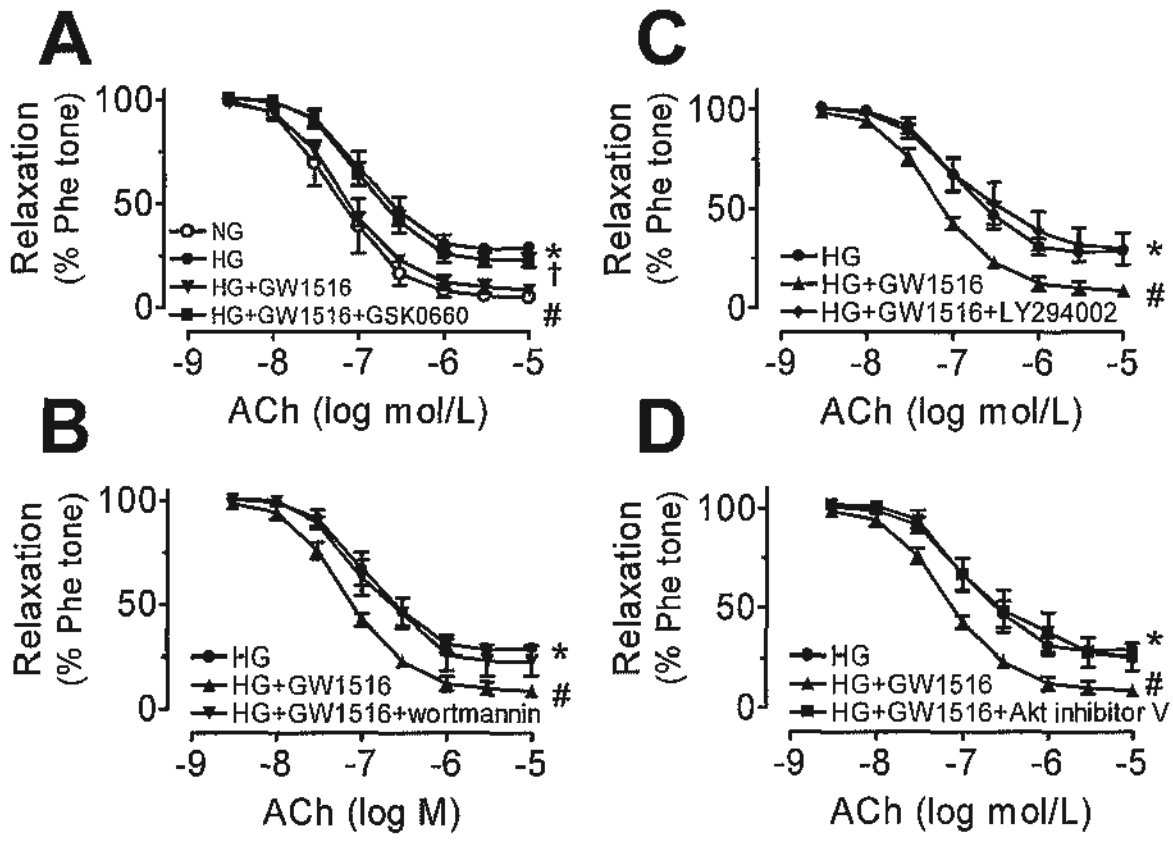

Figure 5.3. The effect of GW1516 with pharmacological inhibitors on EDRs in mouse aortas exposed to high glucose. (A) Co-incubation with GSK0660 (PPARδ antagonist, 1 μmol/L) inhibited the beneficial effect of GW1516 (0.1 μmol/L) in improving the high glucose-impaired relaxations (HG, 30 mmol/L, 36 hrs). Co-incubation with LY294002 (PI3K inhibitor, 5 μmol/L) (B), wortmannin (PI3K inhibitor, 0.1 μmol/L) (C) and Akt inhibitor V (Akt inhibitor, 5 μmol/L) (D) also inhibited the beneficial effect of GW1516 (0.1 μmol/L) in improving the high glucose-impaired relaxations (HG, 30 mmol/L, 36 hrs). Results are means ± SEM of 6 experiments. *p<0.05 vs NG. #p<0.05 vs HG. †p<0.05 vs HG+GW1516.

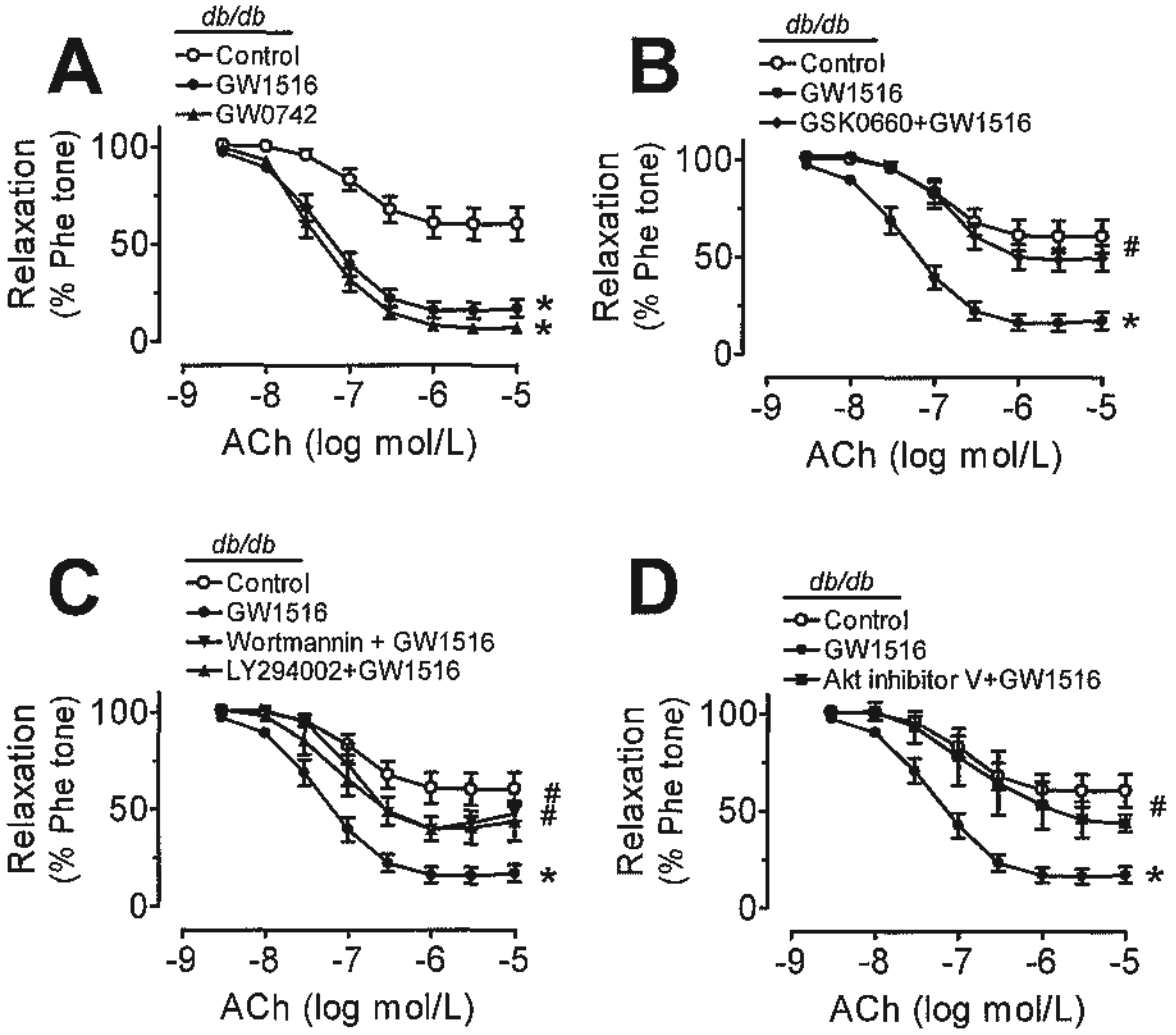

Figure 5.4. The effect of GW1516 with pharmacological inhibitors on EDRs in aortas from *db/db* mice. (A) PPARδ agonist GW0742 (0.1 μmol/L, 24 hrs) and GW1516 (0.1 μmol/L, 24 hrs) improved EDRs in aortas from *db/db* mice. (B) Co-incubation with GSK0660 (PPARδ antagonist, 1 μmol/L) antagonized the effect of GW1516 on EDRs. Co-incubation with LY294002 (PI3K inhibitor, 5 μM) and wortmannin (PI3K inhibitor, 0.1 μmol/L) (C) or Akt inhibitor V (Akt inhibitor, 5 μmol/L) (D) inhibited the improved EDRs induced by GW1516. Results are means ± SEM of 6 experiments. *p<0.05 vs Control. #p<0.05 vs GW1516.

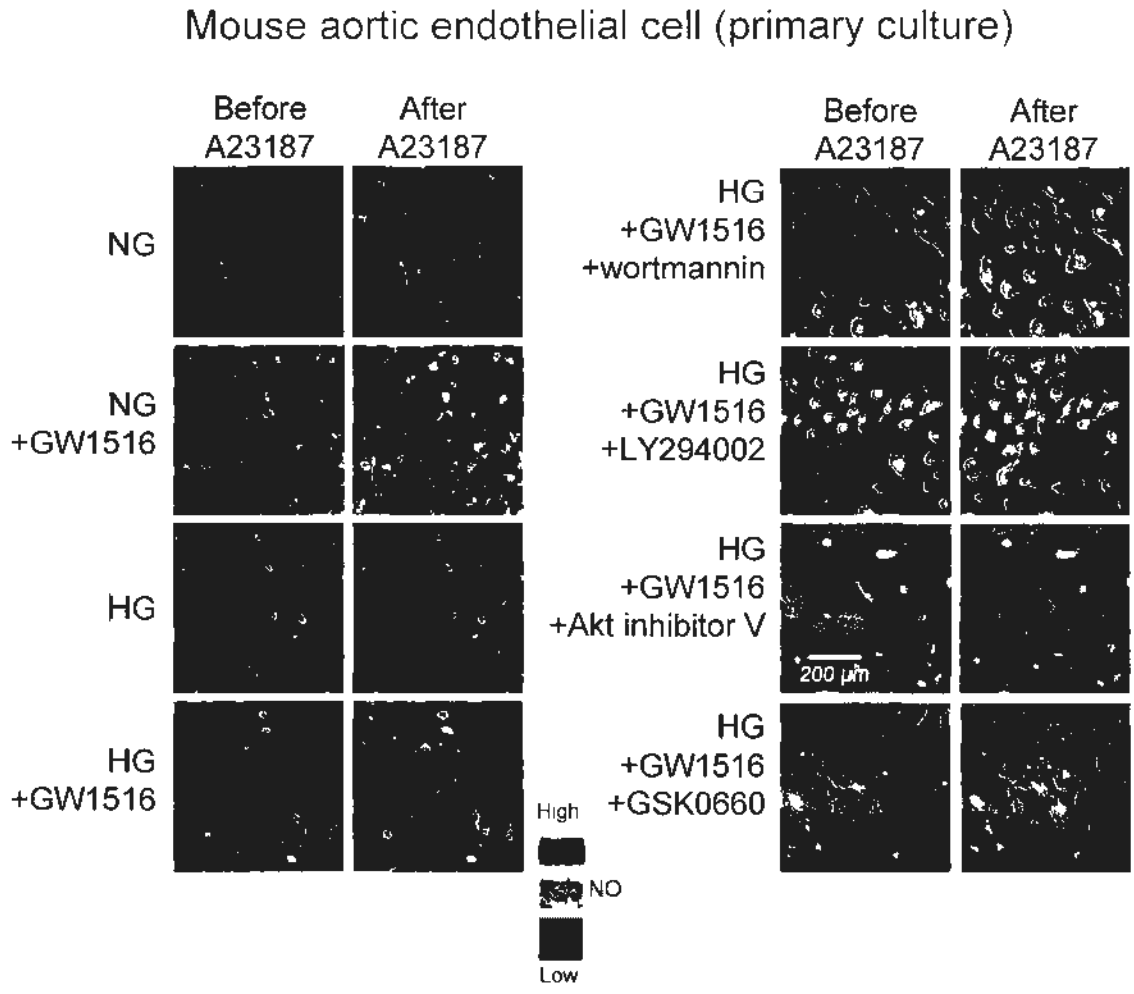

Figure 5.5. The effect of GW1516 on nitric oxide (NO) production in endothelial cells. Representative images of DAF-FM fluorescence signal in endothelial cells that responded to A23187 in different treatment groups. Primary mouse aortic endothelial cells (MAECs) were grown on glass coverslips and mounted in a chamber in NPSS. NO production stimulated by A23187 (Ca^{2+} ionophore, 0.1 μmol) was measured by DAF-FM diacetate fluorescence under a confocal microscope and analyzed by comparing fluorescence intensity before and after the addition of A23187.

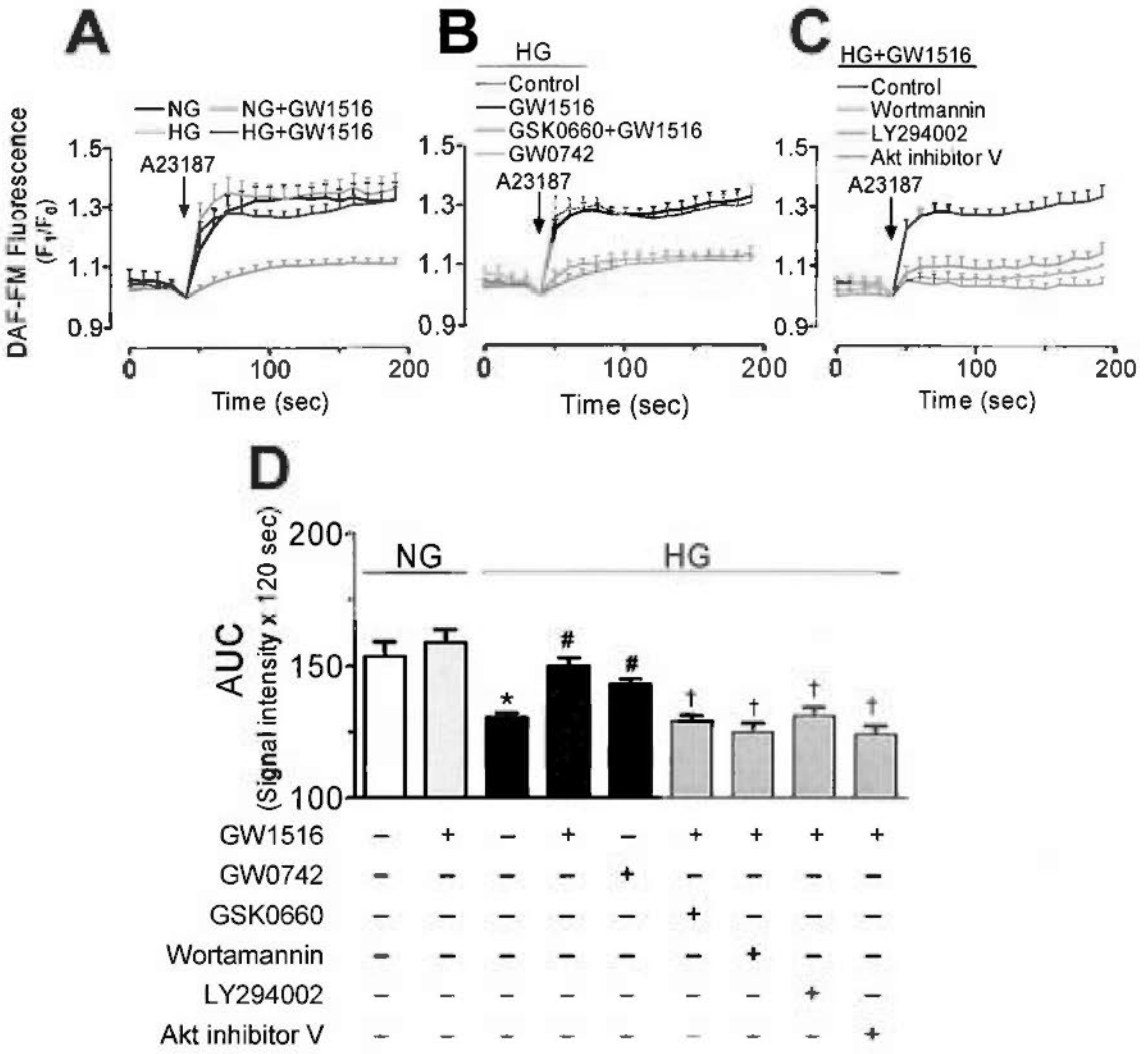

Figure 5.6. The levels of NO production in MAECs treated with GW1516 and under various pharmacological interventions. (A) Exposure to high glucose (HG, 30 mmol/L, 36 hrs) significantly reduced the NO production in response to A23187 in MAECs. Co-incubation with GW1516 (0.1 µmol/L) increased the NO production in MAECs exposed to HG, without affecting those in normal glucose group (NG+GW1516). (B) GW0742 (0.1 µmol/L) increased the NO production in MAECs exposed to HG and GSK0660 (PPARδ antagonist, 1 µmol/L) inhibited the effect of GW1516. (C) Co-treatment with LY294002 (PI3K inhibitor, 5 µmol/L), wortmannin (PI3K inhibitor, 0.1 µmol/L), or Akt inhibitor V (Akt inhibitor, 5 µmol/L) inhibited the effect of GW1516 to improve NO production in MAECs after exposure to HG. (D) Summarized data using area under curve (AUC) starting from the addition of A23187 for 120 sec of Figure 5.6A-C. Results are means ± SEM of 6-8 experiments. *$p<0.05$ vs NG. #$p<0.05$ vs HG. †$p<0.05$ vs HG+GW1516.

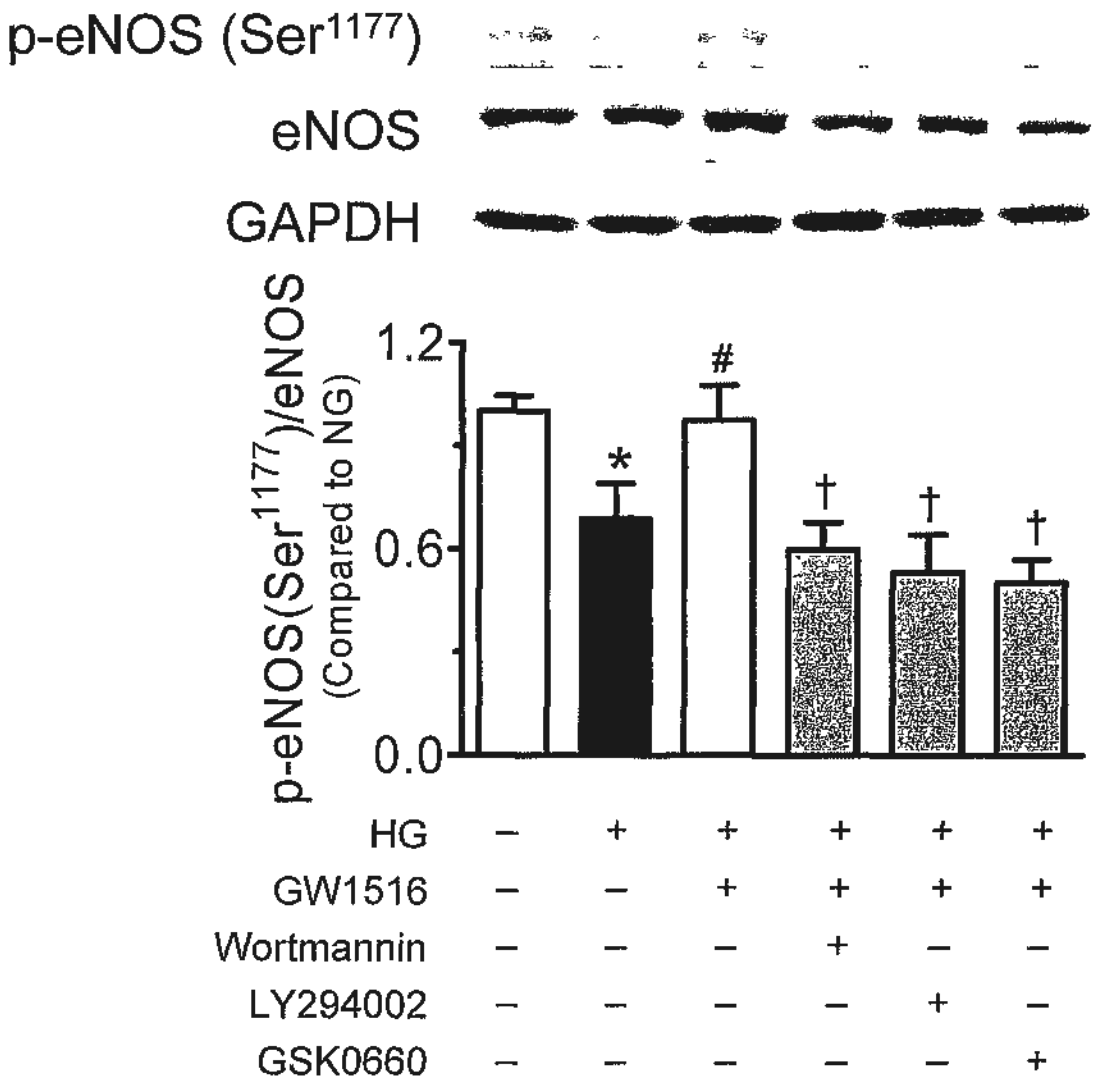

Figure 5.7. The effect of GW1516 on eNOS phosphorylation in MAECs. GW1516 restored eNOS phosphorylation at Ser^{1177} in MAECs which was inhibited by high glucose (HG, 30 mmol/L, 36 hrs) without affecting the total amount of eNOS expression. Co-treatment with LY294002 (PI3K inhibitor, 5 μmol/L), wortmannin (PI3K inhibitor, 0.1 μmol/L), or GSK0660 (PPARδ antagonist, 1 μmol/L) inhibited the effect of GW1516 to increase p-eNOS levels. Representative Western blots showing p-eNOS (140 kDa) and eNOS (140 kDa). Results are means ± SEM of 4-6 experiments. *p<0.05 vs NG. #p<0.05 vs HG. †p<0.05 vs HG+GW1516.

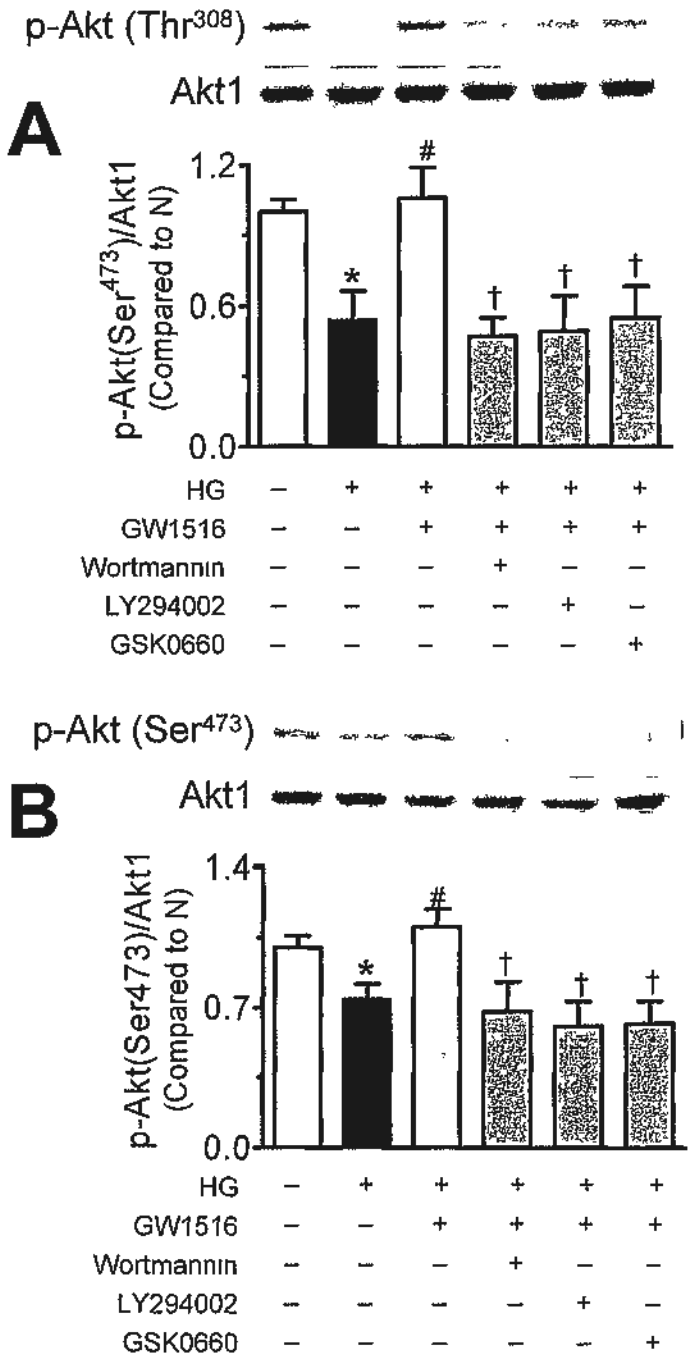

Figure 5.8. The effect of GW1516 on Akt phosphorylation in MAECs. GW1516 restored the reduced Akt phosphorylation at Thr^{308} and Ser^{473} in MAECs exposed to high glucose (HG, 30 mmol/L, 36 hrs) without affecting the total amount of eNOS expression. Co-treatment with LY294002 (PI3K inhibitor, 5 μmol/L), wortmannin (PI3K inhibitor, 0.1 μmol/L), or GSK0660 (PPARδ antagonist, 1 μmol/L) inhibited the effect of GW1516 to increase p-Akt at both phosphorylation sites. Representative Western blots showed p-Akt at Thr^{308} (A) and Ser^{473} (60 kDa) (B) and total Akt1 (60 kDa). Results are means ± SEM of 4-6 experiments. *p<0.05 vs NG. #p<0.05 vs HG. †p<0.05 vs HG+GW1516.

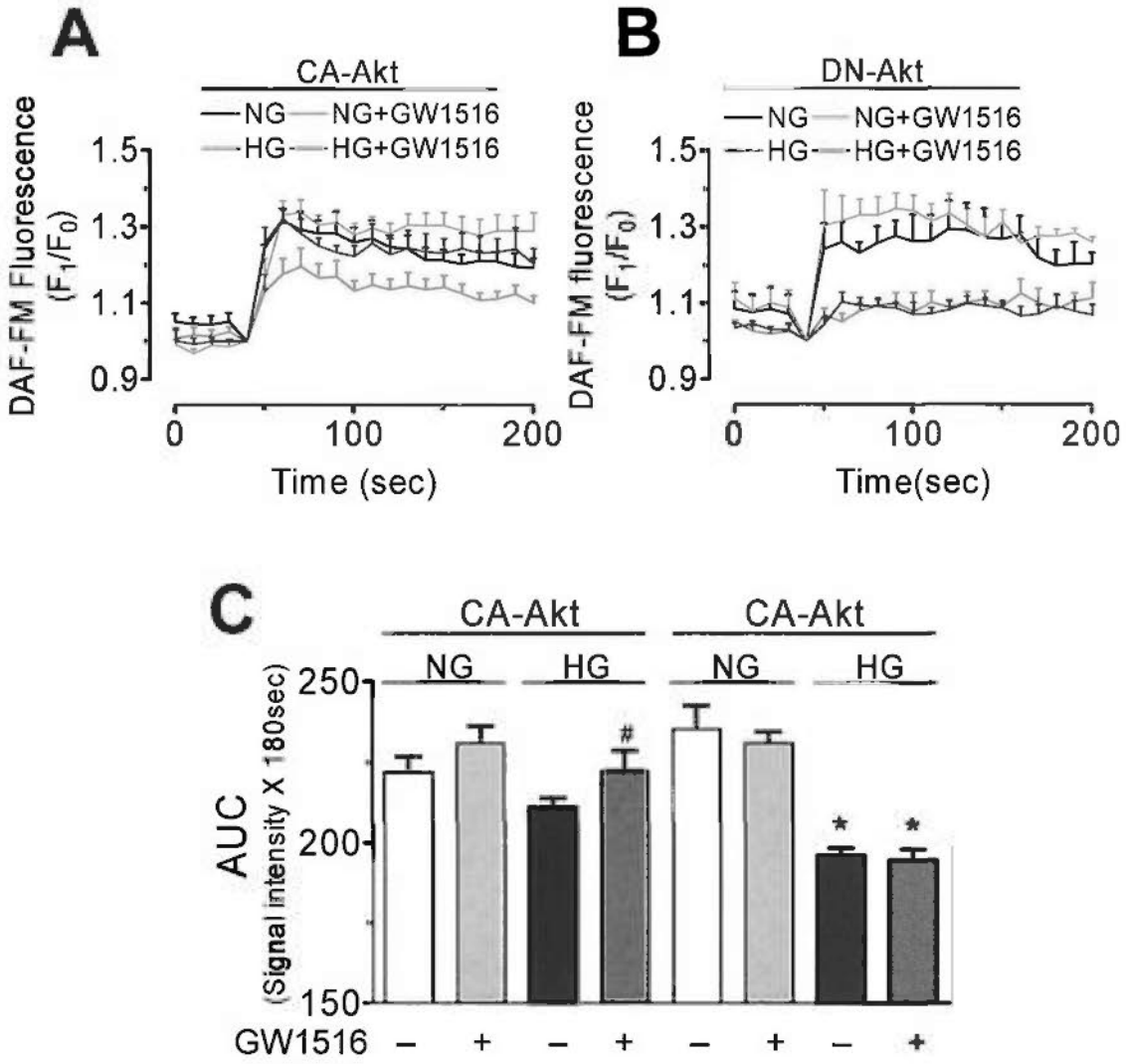

Figure 5.9. The effect of Akt activity inhibition on the NO production in GW1516-treated MAECs. MAECs were transfected with constitutively active Akt (CA-Akt) or dominant negative Akt (DN-Akt) by electroporation. (A) Co-incubation with GW1516 (0.1 μmol/L) increased the NO production after exposure to high glucose (HG, 30 mmol/L, 36 hrs), without affecting those exposed to normal glucose (NG/GW1516) in MAECs transfected with CA-Akt. (B) The effect of GW1516 to restore NO production in high glucose-treated cells was inhibited by transfection with DN-Akt. Results are means ± SEM of 3-4 experiments. *$p<0.05$ vs NG from each group. #$p<0.05$ vs HG from each group.

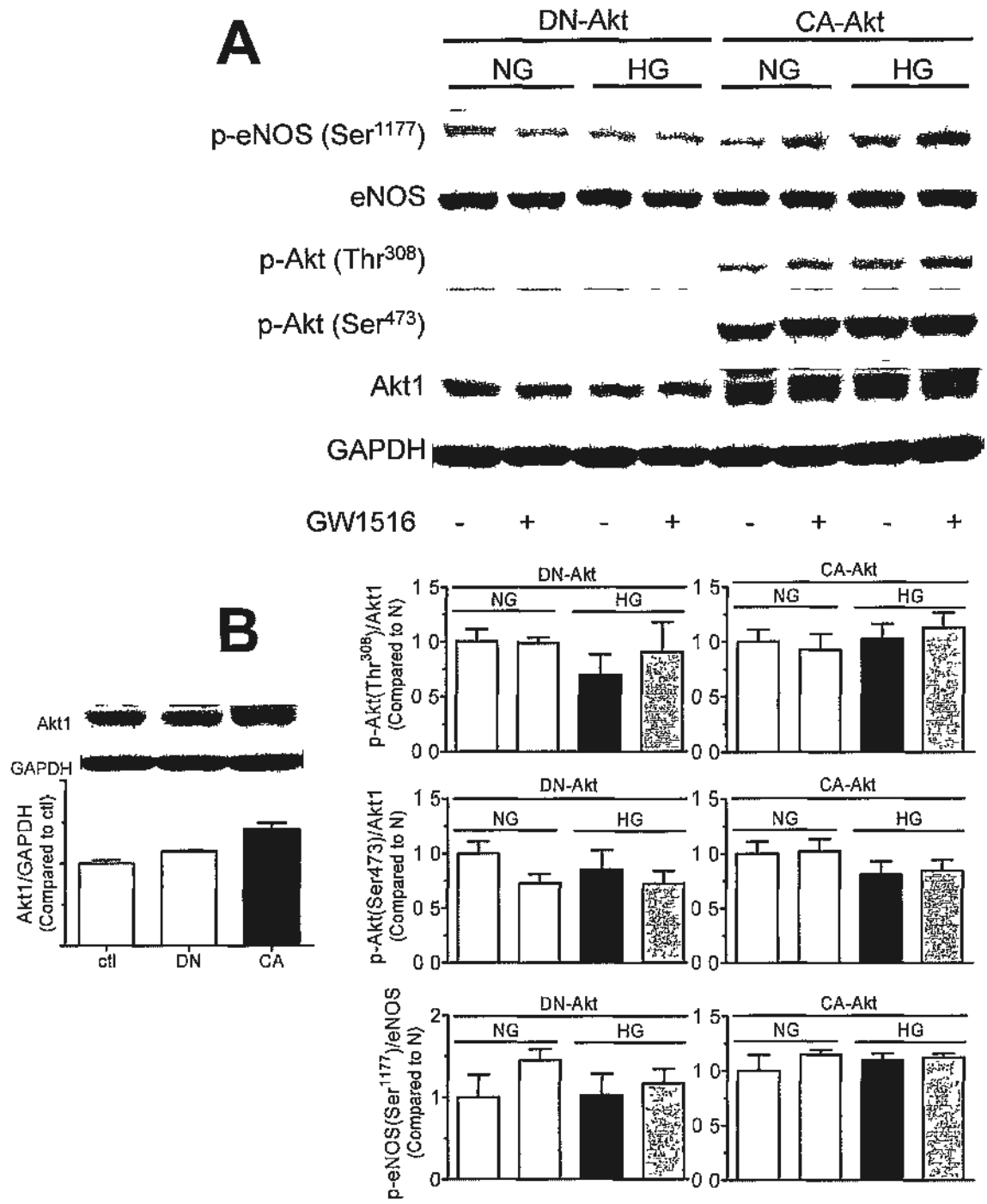

Figure 5.10. The effect of CA-Akt and DN-Akt on Akt and eNOS phosphorylation in HUVECs. HUVECs were transfected with constitutively active Akt (CA-Akt) or dominant negative Akt (DN-Akt) by electroporation. (A) Representative Western blots and summarized data of p-eNOS (Ser^{1177}, 140 kDa), p-Akt (Thr^{308} and Ser^{473}, 60 kDa) in cells transfected with DN-Akt or CA-Akt (B) The total amount of Akt1 (60 kDa) expression in cells transfected with control plasmid (pcDNA vector), DN-Akt, or CA-Akt.

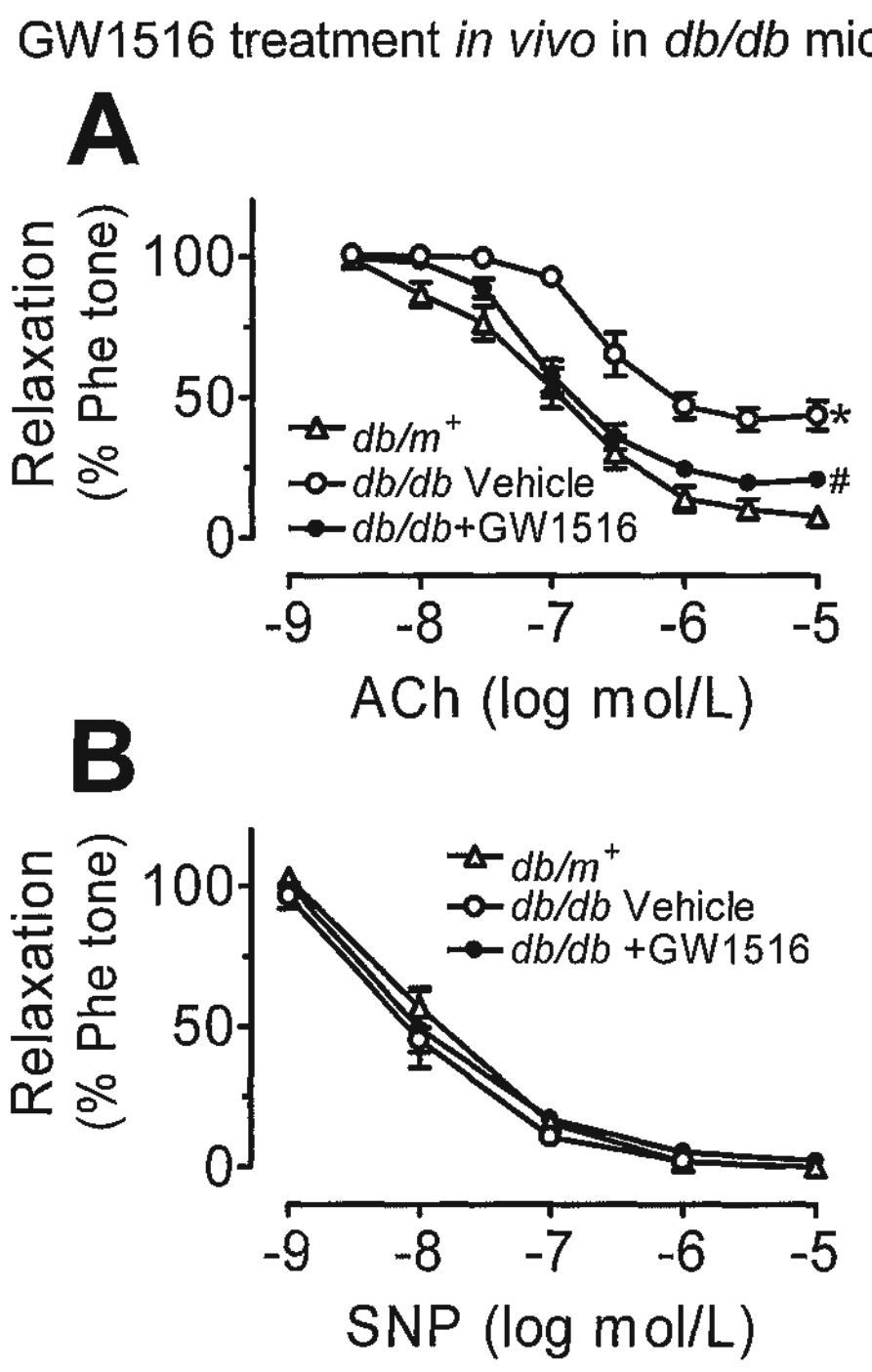

Figure 5.11. The effect of GW1516 treatment *in vivo* on EDRs in aortas from *db/db* mice. GW1516 was administered by oral gavage (5 mg/kg/day, 7-10 days) to *db/db* mice. EDRs in response to ACh in aortas from *db/db* mice significantly increased after GW1516 chronic treatment (A) while endothelium-independent relaxation to SNP was unaltered (B). Results are means ± SEM of 4 experiments. *$p<0.05$ vs *db/m*$^+$. #$p<0.05$ vs *db/db*.

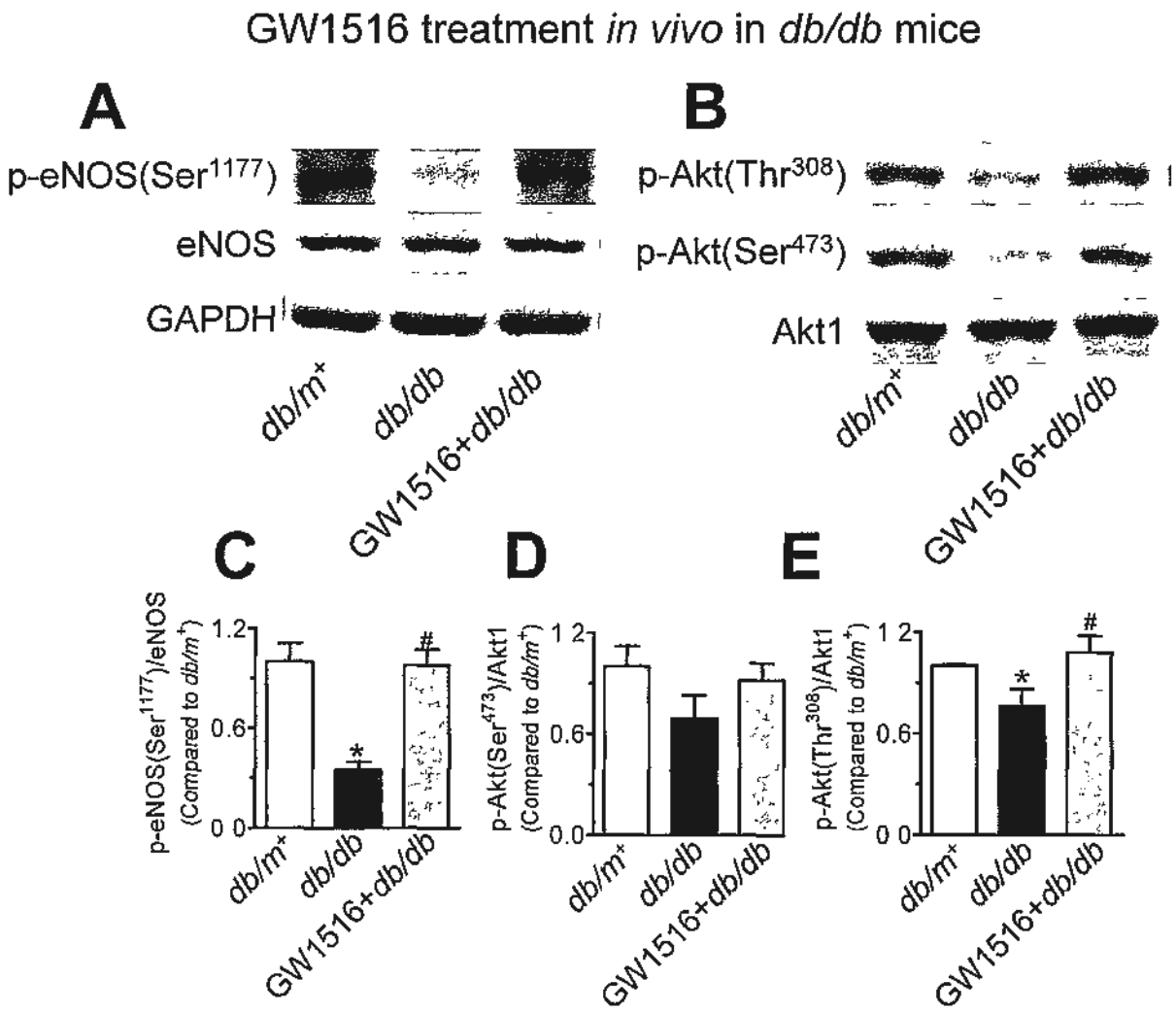

Figure 5.12. The effect of GW1516 treatment *in vivo* on Akt and eNOS phosphorylation in *db/db* mouse aortas. GW1516 was administered by oral gavage (5 mg/kg/day, 7-10 days) to *db/db* mice. Representative Western blots and summarized data showing p-eNOS (Ser^{1177}, 140 kDa) as compared with the total amount of eNOS (A and C), p-Akt (Thr^{308} and Ser^{473}, 60 kDa) (B, D, and E) in aortas from *db/m*$^+$, *db/db*, and *db/db* treated with GW1516. Results are means ± SEM of 4 experiments. *p<0.05 vs *db/m*$^+$. #p<0.05 vs *db/db*.

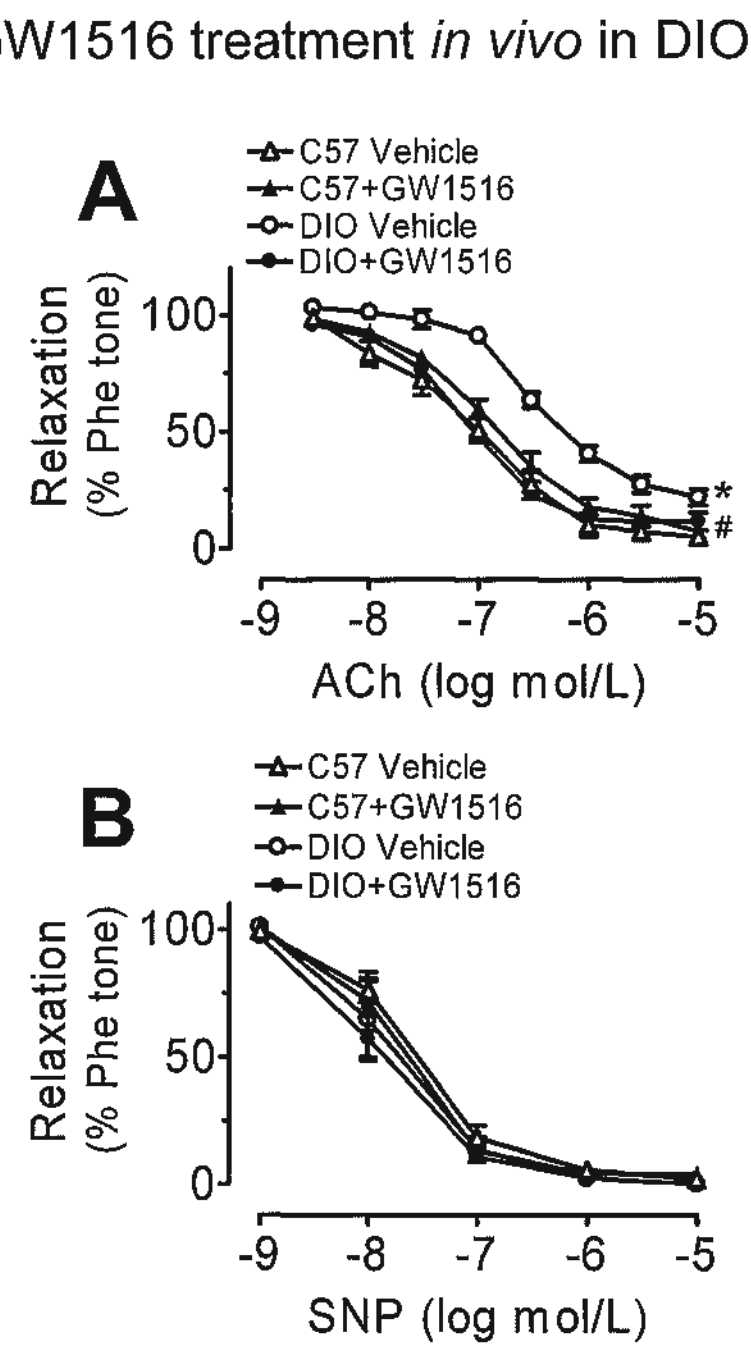

Figure 5.13. The effect of GW1516 treatment *in vivo* on EDRs in aortas from diet-induced-obese mice. GW1516 was administered by oral gavage (5 mg/kg/day, 7-10 days) to diet-induced obese mice (DIO) and age-matched C57 control mice. (A) EDRs were improved in aortas from DIO mice after GW1516 treatment without changes of EDRs in aortas from control C57 mice. (B) Endothelium-independent relaxations to SNP were unaffected in all groups. Results are means ± SEM of 4 experiments. *$p<0.05$ vs C57 Vehicle. #$p<0.05$ vs DIO.

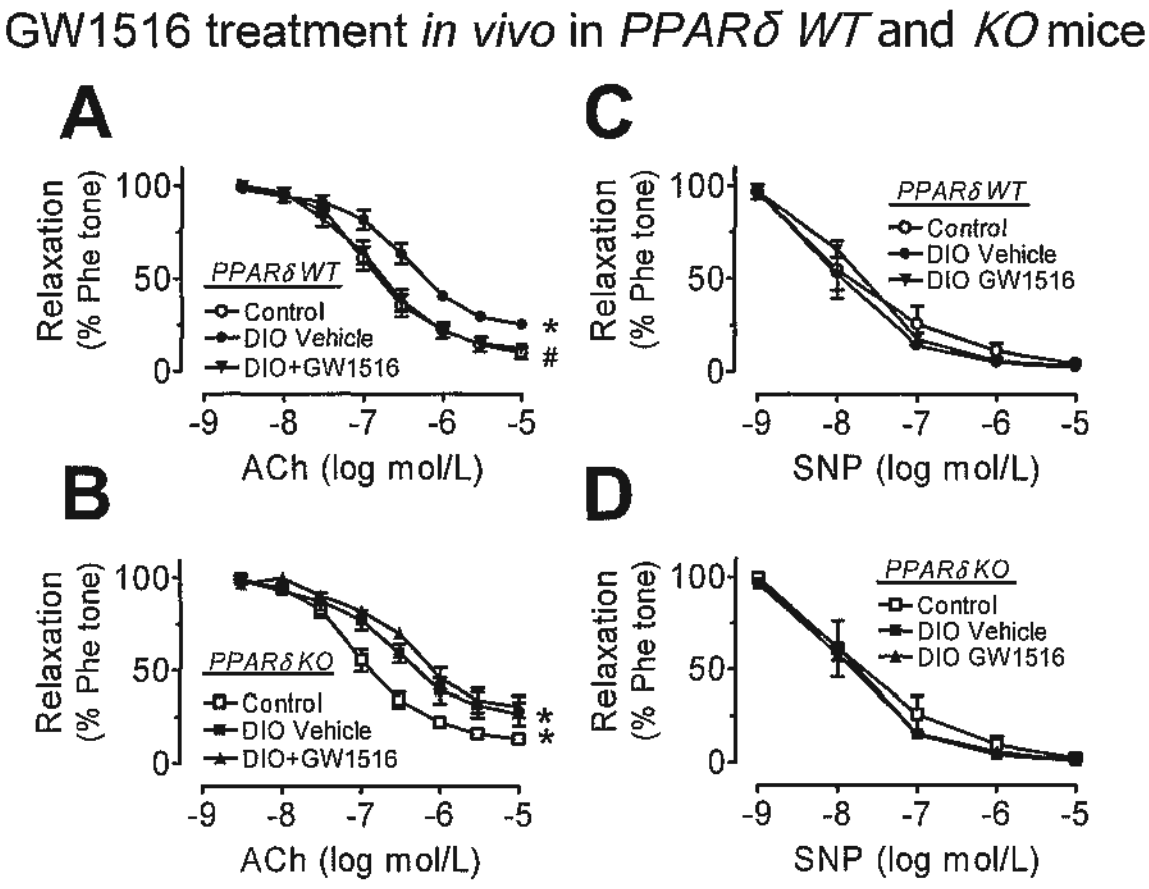

Figure 5.14. The effect of GW1516 treatment *in vivo* was abolished in *PPARδ KO* mice after high-fat diet (DIO). GW1516 was administered by oral gavage (5 mg/kg/day, 7-10 days) to DIO *PPARδ KO* and age-matched *PPARδ WT*. (A and B) EDRs were significantly impaired in aortas from both types of mice after high-fat diet induced obesity. GW1516 treatment *in vivo* improved EDRs in aortas from *PPARδ WT* mice, but not in those from *PPARδ KO* mice. (C and D) Endothelium-independent relaxations to SNP were not affected in all groups. Results are means ± SEM of 4 experiments. *$p<0.05$ vs Control from each group. #$p<0.05$ vs DIO from each group.

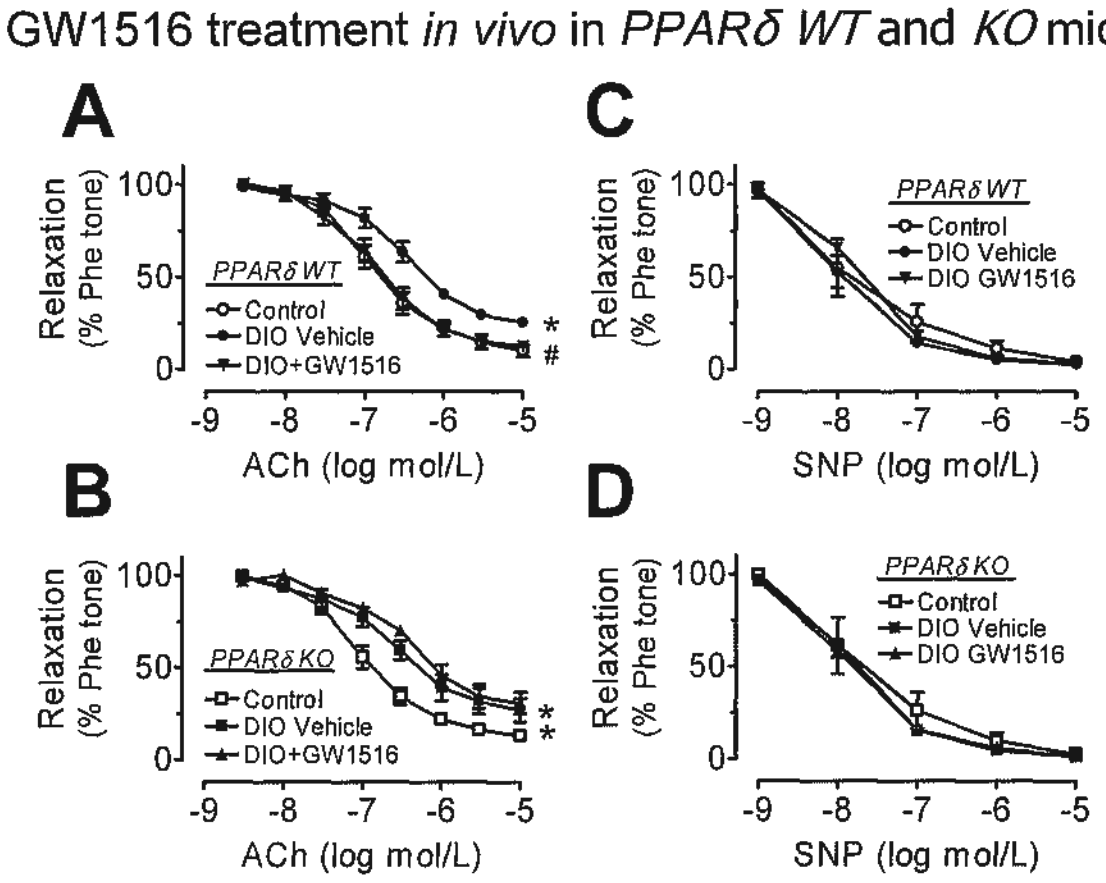

Figure 5.15. The effect of GW1516 treatment *in vivo* on eNOS phosphorylation was inhibited in *PPARδ KO* mice. GW1516 was administered by oral gavage (5 mg/kg/day, 7-10 days) to diet-induced obese (DIO) *PPARδ KO* and age-matched *PPARδ WT*. Reduced p-eNOS (Ser^{1177}, 140 kDa) in aortas was restored after GW1516 treatment in *PPARδ WT*, but not in *PPARδ KO* mice. Results are means ± SEM of 4 experiments. *p<0.05 vs Control from each group. #p<0.05 vs DIO from each group.

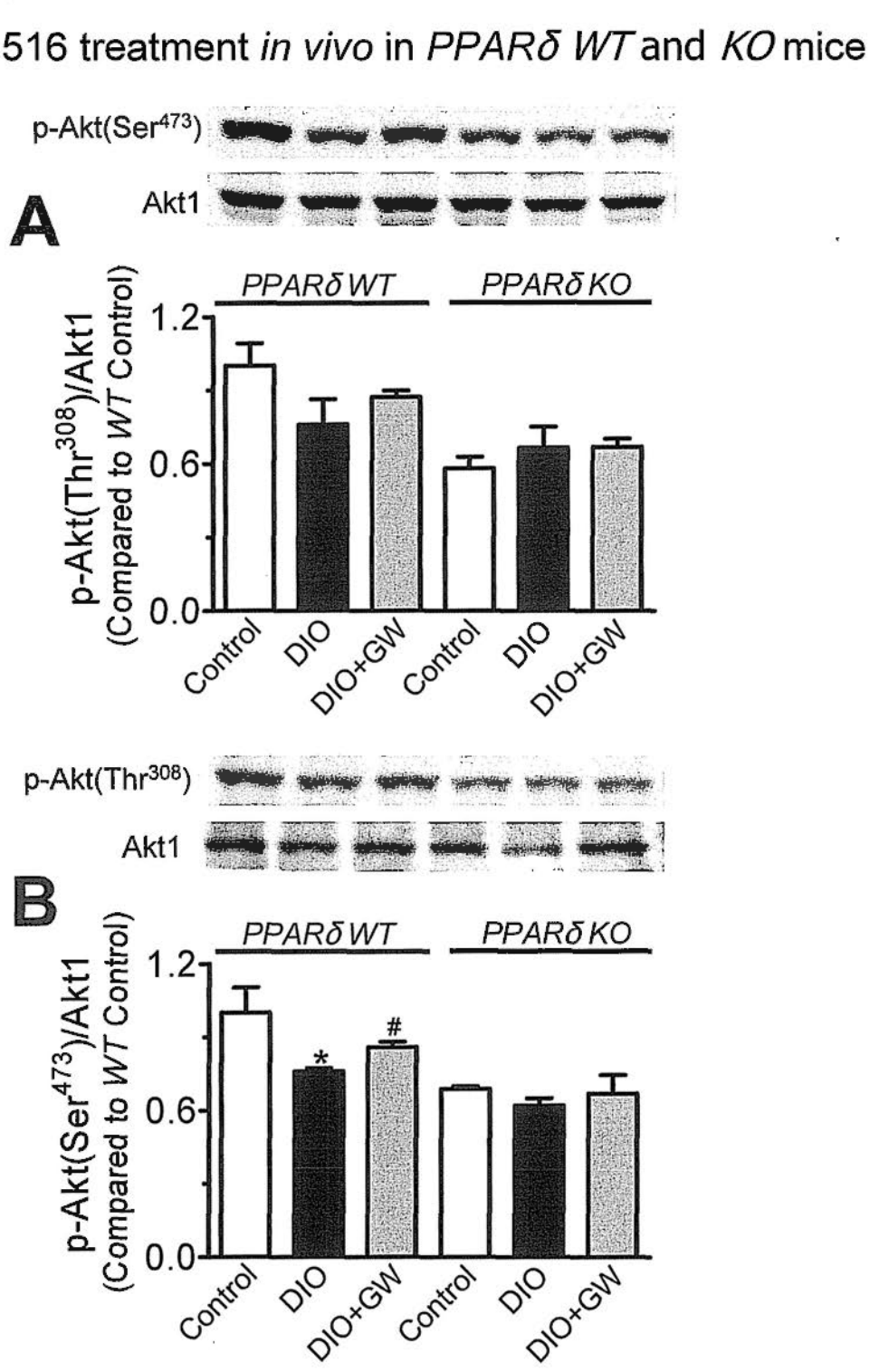

Figure 5.16. The effect of GW1516 treatment *in vivo* on Akt phosphorylation was inhibited in *PPARδ KO* mice. GW1516 was administered by oral gavage (5 mg/kg/day, 7-10 days) to DIO *PPARδ KO* and age-matched *PPARδ WT*. Reduced p-Akt (Thr^{308} and Ser^{473}, 60 kDa) in aortas was restored after GW1516 treatment in *PPARδ WT*, but not in *PPARδ KO* mice. Results are means ± SEM of 4 experiments. *p<0.05 vs Control. #p<0.05 vs DIO.

CHAPTER VI

General conclusion

The present study highlights the therapeutic potential of peroxisome proliferators activated receptor (PPAR) agonists in improving endothelial function in diabetes.

In the first part, I focused on the effect of PPARγ activation to improve endothelial function in diabetic mice which is mediated through adiponectin from adipose tissue, subcutaneous fat depots, in particular. Adipocyte-derived adiponectin is the primary mediator that increases nitric oxide (NO) production, inhibits oxidative stress, and improves endothelium-dependent relaxation through the activation of AMPK and PKA signaling pathways. PPARγ expression and adiponectin synthesis in adipose tissues correlate with the degree of improvement of endothelium-dependent relaxation in aortas from diabetic *db/db* mice. PPARγ agonist rosiglitazone increases the adiponectin release and restores the impaired endothelium-dependent relaxation *ex vivo* and *in vivo*, in arteries from both genetic and diet-induced diabetic mice, confirmed with the use of selective pharmacological inhibitors and *adiponectin*$^{-/-}$ or *PPARγ*$^{+/-}$ mice. In addition, the benefit of PPARγ activation *in vivo* can be transferred by transplanting subcutaneous adipose tissue from rosiglitazone-treated diabetic mouse to un-treated diabetic mouse. The present findings suggest that adipose tissue can be an important therapeutic target in the protection of vascular dysfunction in diabetes through the production and release of anti-inflammatory vaso-active hormones among which adiponectin plays an indispensable role in

protecting vascular function.

In the second part, I have demonstrated for the first time that PPARγ agonist rosiglitazone up-regulates endothelin B receptor (ET_BR) expression in mouse aortas and attenuates endothelin-1 (ET-1)-induced vasoconstriction through an endothelial ET_BR-dependent NO-related mechanism. ET-1-induced vasoconstrictions in conduit and resistance arteries are mediated through ET_AR, while activation of ET_BR induces NO production and produces endothelium-dependent relaxations in resistance arteries. PPARγ directly bound to the PPRE site of the ET_BR gene, indicating that ET_BR is a direct target of PPARγ-activated transcription. Taken together, PPARγ agonist increases ET_BR expression and enhances NO bioavailability in endothelial cells, which provide a possible explanation for the vasoprotective effects of PPARγ ligands.

Finally, I have revealed that PPARδ activation improves endothelial function in diabetic mice through the activation of PI3K/Akt. Firstly, I showed that PPARδ agonists GW501516 and GW0742 can cause direct improvement of endothelium-dependent relaxation in mouse aortas impaired by high glucose. The effects of PPARδ ligands are PPARδ-specific, which is verified by two selective PPARδ agonists, PPARδ antagonist, and *PPARδ KO* mice in mouse aortas and endothelial cells. In addition, stimulation with PPARδ agonists also improves vasodilator response in aortas from *db/db* mice or diet-induced obese mice. The effect of PPARδ activation in mouse aortas and in cultured mouse aortic endothelial cells appears to be mediated through PI3K/Akt signaling

cascade, which is tested by the use of pharmacological inhibitors of both PI3K and Akt on relaxations in aortas, NO release in endothelial cells, and eNOS and Akt phosphorylation, and also by the use of transient transfection to modulate Akt activity, which all showed similar results. Finally, I have confirmed the *in vivo* beneficial effect of PPARδ ligand GW1516 in *db/db* mice and diet-induced obese mice, which are also related to the activation of PI3K/Akt. These findings may help to enhance the prospective of the use of safe PPARδ agonistic ligands in combating against vascular dysfunction in diabetes and obesity.

To summarize, the present investigation has demonstrated the beneficial effect of PPARγ to improve endothelial function through two independent mechanisms: the stimulation of adiponectin release from adipocyte which increases NO bioavailability through the activation of AMPK and reduces oxidative stress through PKA; and increases NO production through upregulation of ETBR in the endothelial cells directly. Secondly, PPARδ activation protects endothelial function in diabetes through PI3K/Akt. These studies provide a few lines of novel mechanistic evidence in support for the positive roles of PPARγ and PPARδ activation as potentially therapeutic targets to combat against diabetic vasculopathy.

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