# *Functional changes in the vascular endothelium and smooth muscle in the hyperinsulinemic conditions and its improvement*

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## List of abbreviations

本論文では、以下の略語を用いた。

AGE : advanced glycation end product、 糖化最終産物

ACE : angiotensin-converting enzyme、 アンギオテンシン変換酵素

ACh : acetylcholine、 アセチルコリン

Ang II : angiotensin II、 アンギオテンシン II（ 血管収縮性ペプチド ）

ANOVA : analysis of variance、 分散分析

ARBs : Ang II receptor blockers、 アンギオテンシン受容体阻害薬

cGMP : guanosine 3' , 5'-cyclic monophosphate、 環状グアノシン一リン酸化物（ セカンドメッセンジャー ）

ECM : extracellular matrix、 細胞外マトリックス

eNOS : endothelial NO synthase、 内皮型 NO 合成酵素

GK rat : Goto-Kakizaki rat、 2 型糖尿病モデルラット

GPCR : G-protein coupled receptor、 G タンパク共役型受容体

$H_2O_2$ : hydrogen peroxide、 過酸化水素

HDL : high-density lipoprotein、高比重リポタンパク

HMG-CoA reductase : 3-hydroxyl-3-methyl coenzyme A reductase、 HMG-CoA 還元酵素

ICAM-1 : intercellular adhesion molecule-1、細胞間接着分子

IGF-1 : insulin-like growth factor 1、 インスリン様成長因子

IRS : insulin receptor substrate、 インスリン受容体基質

LDL : low-density lipoprotein、 低比重リポタンパク

L-NNA : $N^G$-nitro-L-arginine、 NO 合成酵素阻害薬

KHS : Krebs-Henseleit solution、 （ 栄養液 ）

MLC : myosin light chain、 ミオシン軽鎖

NADH/NADPH oxidase : NADH/NADPH オキシダーゼ（ 活性酸素産生酵素 ）

NE : norepinephrine、 ノルエピネフリン( 交感神経興奮薬 )

NO : nitric oxide、 一酸化窒素

NOx : nitric oxide metabolites、 NO 代謝物

$O_2^-$ : superoxide、 活性酸素

$ONOO^-$ : peroxynitrite、ペルオキシ亜硝酸イオン（$O_2^-$ と NO の反応物 ）

PDK : PI-dependent kinase、 タンパクリン酸化酵素

$PGF_{2\alpha}$ : prostaglandin $F_{2\alpha}$、 プロスタグランジン $F_{2\alpha}$

PI3-K : phosphatidylinositol-3-kinase、 イノシトールリン脂質 3-リン酸化酵素

PIP3 : phosphatidylinositol-3, 4, 5-trisphosphate、 ホスファチジルイノシトール -3, 4, 5-三リン酸

PKB : protein kinase B、 プロテインキナーゼ B (Akt とも呼ばれる)

PMSF : phenylmethylsulfonyl fluoride、フェニルメチルスルホニルフッ化物

PTEN : phosphatase and tensin homolog deleted on chromosome Ten、 癌抑制遺伝子産物

ROS : reactive oxygen species、 活性酸素種

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis、 ドデシル硫酸ナトリウム・ポリアクリルアミド電気泳動 ( タンパク質分析手法 )

SEM : standard error of the mean、 標準誤差平均

SER : sarco/endoplasmic reticulum、筋小胞体

SHR : spontaneously hypertensive rat、 自然発症高血圧ラット

SNP : sodium nitroprusside、NO 供与体

SOD : superoxide dismutase、スーパーオキシドジスムターゼ( ラジカルスカベンジャー )

STZ : streptozotocin、 ストレプトゾトシン ( 糖尿病誘発薬 )

SERCA : sarco/endoplasmic reticulum $Ca^{2+}$ pumps、 筋小胞体カルシウムポンプ

TG : thapsigargin、 SERCA 阻害薬

VEGF : vascular endothelial growth factor、 血管内皮成長因子

VSMC : vascular smooth muscle cell、 血管平滑筋細胞

## General Introduction

It has been reported that more than 80% is type 2 among diabetic patients [1] ,and this disease has recently been confirmed as an independent risk factor for cardiovascular disease [2]. Insulin resistance is a common feature associated with type 2 diabetes and cardiovascular diseases. The degree of insulin resistance relates directly to increasing rates of myocardial infarction [3] and stroke [4, 5]. Insulin resistance leads to endothelial dysfunction, it precedes the development of type 2 diabetes, and is associated with increased plasma concentrations of endothelin-1 and von Willebrand factor, even in the absence of diabetes [6]. The insulin resistance syndrome encompasses more than a subnormal response to insulin-mediated glucose disposal. Patients with this syndrome also frequently display elevated blood pressure, hyperlipidemia and dysfibinolysis even without any clinically demonstrable alteration in plasma glucose concentrations. Endothelial dysfunction also has been demonstrated in patients with hypertension [7-11], which is one of the features of the insulin resistance syndrome. It is tempting to speculate that loss of endothelium-dependent vasodilation and increased vasoconstrictors might be etiological factors of hypertension. Moreover loss of activity and/or quantity of endothelium-bound protein lipase activity may contribute to hyperlipidemia, which is typical of the insulin resistance syndrome. Insulin resistance has a well-known but not completely defined genetic influence, frequently transmitted along generations in any given family. Endothelial dysfunction has been demonstrated in insulin-resistant states in animals and humans and may represent an important early event in the development of atherosclerosis. Insulin resistance may be linked to endothelial dysfunction by a number of mechanisms, including disturbances of subcellular signaling pathways common to both insulin action and NO production. Other potential unifying links include the roles of oxidant stress, endothelin-1, the renin-angiotensin system and the secretion of hormones and cytokines by adipose tissue. Individuals who advance toward the development of type 2 diabetes experience progressive deterioration of glucose tolerance over time. In addition, obesity, which also has an important genetic component, invariably exacerbates any degree of insulin resistance [12]. Thus, obesity and insulin resistance are usually present for many years

before the appearance of other abnormalities such as hypertension, dyslipidemia, type 2 diabetes and cardiovascular disease. In certain individuals, obesity and insulin resistance may be present during childhood and adolescence [13]. Increasing evidence suggest that hyperinsulinaemia is linked with the development of atherosclerosis in patients with diabetes. Whether hyperinsulinaemia directly affects neutrophil transendothelial migration and surface expression of related endothelial adhesion molecules were studied on healthy volunteers and from patients with non-insulin-dependent diabetes mellitus across human umbilical vein. Endothelial cells cultured in insulin-rich medium using cell-culture inserts high insulin (over 50 microU/mL for 24 h) enhanced neutrophil transendothelial migration in a dose-dependent manner. This was associated with increased expression of but not of intercellular adhesion molecule-1 (ICAM-1), P-selectin or E-selectin [14]. Multiple, interrelated mechanisms contribute to endothelial cell dysfunction in insulin resistance. The exact mechanism by which dyslipidemia contributes to endothelial dysfunction is unknown. It is known that endothelial NOS infiltrates into caveolae, which are cholesterol-rich invaginations present in endothelial cells and vascular smooth muscle (VSMC) that decrease vasoconstrictive responses to angiotensin II (Ang II), endothelin-1 and constitutive endothelial NOS activity in animals [15]. Addition of oxidized low-density lipoprotein (LDL) to cultured endothelial cells disrupts the caveolae complex and is thought to be associated with decreased endothelial NOS activity and endothelial dysfunction [16, 17]. high-density lipoprotein (HDL) cholesterol can prevent the oxidized LDL–mediated decrease in cholesterol in caveolae, prevent the translocation of endothelial NOS and caveol in from caveolae, and prevent the decrease in responsiveness to acetylcholine [18]. These effects occur because HDL cholesterol donates cholesterol to the caveolae complex. These cellular events are consistent with the proatherogenic effects of LDL cholesterol and oxidized LDL cholesterol and the protective effects of HDL cholesterol. The presence of hypertension and other atherosclerotic risk factors is associated with increased vascular Ang II generation and activity [19]. Because Ang II and insulin activate a common signaling pathway, increased sensitivity to Ang II may occur in the hyperinsulinemic, insulin-resistant state [20]. In addition, Ang II stimulates ICAM-1 and monocyte chemo-attractant protein–1 (MCP-1) through the MAPK pathway in endothelial cells and VSMC [21-23]. It is likely that

many of the components of the metabolic syndrome directly alter endothelial vasoreactivity. However, these factors may decrease NO activity through oxidation pathways; the role of oxygen free radical is discussed bellow in oxidative stress and endothelial cell dysfunction.

Endothelial dysfunction may be functionally defined as the failure of the vascular endothelium to subserve its normal role in vasodilatation and/or vascular homeostasis. The predominant effect of endothelium stimulation is vasodilatation. Other regulatory functions involve vasorelaxation, vasoconstriction, and antiplatelet and anticoagulant effects [24]. It emerges as a key component in the pathophysiology of diverse cardiovascular abnormalities associated with atherosclerosis, diabetes, hypertension and aging [25, 26].

Endothelial dysfunction, present at disease onset, is the prime focus of atherosclerotic lesions that present throughout the course of diabetes and is associated with late-stage adverse outcomes. Diabetes-related endothelial dysfunction precedes morphologic and structural vascular changes [27], which includes accelerated disappearance of capillary endothelium [28], weakening of intercellular junctions [29], altered protein synthesis and altered expression/production of adhesion glycoproteins on endothelial cells [28-31], promoting attachment of monocytes and leucocytes, and their transendothelial migration [29].Endothelial dysfunction results from the imbalance between endothelium-derived contracting and relaxing factors. Unlike normal endothelium, it produces abnormal response when exposed to endogenous or exogenous vasodilators. Due to endothelium's strategic location between the circulating blood and vascular smooth muscle, it is a primary target and mediator of cardiovascular diseases. Endothelium modulates the activity of vascular smooth muscle and therefore regulates vascular tone [32]. Endothelial cells release humoral factors that control relaxation and contraction, thrombogenesis and fibrinolysis, and platelet activation and inhibition [24]. These cells provide a metabolically active interface between blood and tissue that modulates blood flow, nutrient delivery and leucocyte diapedesis [33]. The endothelial cells also synthesize important bioactive substances, for example nitric oxide (NO), other reactive oxygen species (ROS), prostaglandins, endothelin-1 and Ang II, which regulate blood vessel structure and function. NO potently dilates vessels and mediates much of the endothelium's control of vascular relaxation [34]. Endothelial cell damage, with the loss of

the vascular protective effects of NO, is likely the early step in atherosclerosis. An Impairment of endothelium-dependent vasodilatation has been described in patients with diabetes and the degree of impairment may correlate with glycemic control [35]. This is also seen with other associated conditions that lead to endothelial dysfunction, for example hypertension and dyslipidemia [35]. This may be an effect of hyperglycemia itself leading to an increase in oxidative stress and release of mediators (e.g. cytokines) by adipocytes in the presence of insulin resistance [35]. High glucose concentration is associated with increased oxidative stress [36], enhanced leucocyte-endothelial interaction [37] and glycosylation of protein in the body, including lipoproteins, apolipoproteins and clotting factors. Hyperglycemia also enhances endothelial cell matrix production, which may contribute to basement membrane thickening [38]. It also increases enzymes involved in collagen synthesis [38] and specifically enhances endothelial cell collagen IV and fibronectin synthesis [38].

Hyperglycaemia enhances the formation of AGEs through a complex series of dehydrogenation and oxidation reactions [39]. These end products are found in plasma, vessel wall and tissues and are linked to the development of diabetic complications [30, 40-44]. These end products can also induce excessive cross-linking of collagen and extracellular matrix (ECM) proteins in the vascular wall, which in turn could lead to accumulation of LDL particles of prolonged half-life. Such particles are more susceptible to oxidative modification, impairment of endothelial function, stimulating inflammation and adhesion, and promoting VSMC changes [45]. Atherosclerotic vascular disease is the leading cause of death in patients with diabetes, which mainly occurs due to endothelial dysfunction [46, 47]. An Improvement of metabolic control in patients with diabetes is associated with near normalization or restoration of normal endothelial function [48].

Progression of atherosclerotic lesion or alteration of vasculature is the characteristic feature of diabetic complications [49]. Diabetes accelerates these processes by stimulating the atherogenic activity of VSMC-the integral part in the development of atherosclerosis [46]. The process begins as a response to chronic minimal injury to the endothelium leading to it being dysfunctional. A dysfunctional endothelium is found to be more porous, which allows macrophages and LDL to penetrate into the medial layer of

arteries heralding the formation of foam cells. The vessels are then on the way to develop atheroma. Once the macrophage-rich fatty streak forms, VSMCs in the medial layer of the arteries migrate into the nascent intimal lesion. Here VSMCs replicate and lay down a complex ECM, important steps in the progression to advanced atherosclerotic plaque [46]. These cells, being the source of collagen, strengthen the atheroma, making it less likely to rupture and cause thrombosis. Lesions that have disrupted and caused fatal thrombosis tend to have few VSMCs [50]. Fewer VSMCs are also found in patients with diabetes with advanced atherosclerotic lesions [51]. Hyperglycemic lipid modifications of LDL regulate the increased migration and cell death of VSMCs in atherosclerotic lesions. LDL that has undergone non-enzymatic glycation induces VSMC migration *in vitro*, while oxidized glycated LDL can induce cell death of VSMCs [52]. High glucose concentrations promote necrotic cell death through hydrogen peroxide ($H_2O_2$) formation, which may participate in the development of diabetic vasculopathy [53]. Thus, diabetes alters vascular smooth muscle function in ways that promote atherosclerotic lesion formation, plaque instability and clinical events.

Insulin exerts important biological effects on cardiovascular tissue as well as conventional Ins tissues such as skeletal muscle and adipose tissue [54-60]. For example, insulin induce vasorelaxation by mechanisms that include stimulation of NO production and reductions in VSMC intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and $Ca^{2+}$-myosin light chain (MLC) sensitization [54-61]. Many of the metabolic and vasomotor effects of insulin is mediated by activation of the phosphatidylinositol 3-kinase (PI3-K) and downstream signaling pathways, including protein kinase B (Akt) [60, 62]. The serine-threonine kinase Akt interacts with the phospholipids produced by PI3-K, thereby undergoing phosphorylation of $Thr^{308}$ and $Ser^{473}$, which results in its activation [62]. The activation of Akt is necessary but not definitive requirement for insulin to exert their metabolic and vascular effects. Vascular relaxation in response to activation of PI3-K/Akt signaling is mediated in part by endothelial cell production of NO [59]. The effect of insulin on endothelial NO formation is mediated via PI3-K-dependent Akt activation, involving phosphorylation of endothelial NO synthase at $Ser^{1179}$ [56]. Insulin exert their metabolic effects by binding to their cell surface heterotetrameric receptors, thus stimulating receptor autophosphorylation and activation of

several cytosolic docking proteins termed insulin receptor substrates (IRSs) [56]. Tyrosine phosphorylation of IRS-1 and IRS-2 induces their binding to Src homology 2-domain containing molecules, including PI3K. The interaction between the IRSs and PI3K increases the catalytic activity of the p110 subunit of this enzyme. Activated PI3-K, in turn, activates the Ser/Thr kinase Akt by binding phosphatidylinositol-3,4,5-triphosphate to its pleckstrin domain and consequent Ser/Thr phosphorylation [56]: the two major positive regulatory phosphorylation sites in Akt are $Thr^{308}$ and $Ser^{473}$. Another effect of insulin stimulation of the PI3-K/Akt signaling pathway is a reduction in VSMC $[Ca^{2+}]_i$ and $Ca^{2+}$-MLC sensitization [57]. Insulin reduce VSMC $[Ca^{2+}]_i$ by inhibiting agonist-induced inward $Ca^{2+}$ currents and intracellular organelle release of $Ca^{2+}$ [57, 63,64]. Insulin also reduce $[Ca^{2+}]_i$ by stimulating the activity of the $Na^+$-$K^+$-ATPase pump in VSMC, a process that is dependent on PI3-K/Akt signaling [58]. Thus insulin induce vascular relaxation by stimulation of endothelial cell production of NO and by reducing VSMC $[Ca^{2+}]_i$ and $Ca^{2+}$-MLC sensitization. These effects are mediated, in part, by activation of PI3-K/Akt signaling pathways. This signaling pathway is also necessary for insulin stimulation of glucose transport in vascular skeletal muscle and adipose tissue [62, 64].

Resent clinical trials of statins, angiotensin-converting enzyme (ACE) inhibitors and Ang II receptor blockers (ARBs) suggest that beneficial effects of endothelial function in patients with diabetes. Insulin sensitizers may have a beneficial effect in the short term, but the virtual absence of trials with cardiovascular end-points precludes any definitive conclusion. Several clinical trials have demonstrated that statin treatment not only reduces serum cholesterol levels in hypercholesterolemia patients, but also substantially decreases the risk of cardiovascular disease [65]. In current clinical use, statins can reduce LDL cholesterol levels by an average of 20%-35%, with a corresponding 30%-35% reduction in major cardiovascular outcomes. Decreases in serum cholesterol levels could account for the observed risk reduction, since LDL cholesterol has a strong, well-documented association with cardiovascular risk, and since plasma LDL apheresis has been shown to improve both endothelium-dependent vasodilation and cardiovascular risk in hypercholesterolemic patients [66, 67]. ACE inhibitor and ARB are known to improve

endothelial dysfunction, since Ang II has several pro-oxidative effects on the vasculture, decreasing NO bioavailability and resulting in vascular injury [68, 69]).

Although the insulin resistance may make important contributions to the development of cardiovascular disorders, there is a few evidence that the elevation of insulin alone in patients with insulinomas and its therapeutic strategy. In the present study, thus, I have investigated the vascular function in insulin resistance by use of culturing diabetic aortas with insulin and type 2 diabetic model mice (streptozotocin plus nicotinamide-induced diabetic mice). By these experiments, the therapeutic target of the vascular dysfunction seen in insulin resistance may be clarified.

## Chapter 1

## Insulin-induced impairment via peroxynitrite production of endothelium-dependent relaxation and sarco/endoplasmic reticulum $Ca^{2+}$-ATPase function in aortas from diabetic rats

## Introduction

Numerous epidemiological studies have indicated that the insulin resistance and hyperinsulinemia associated with diabetes make important contributions to the development of hypertension and cardiovascular diseases, and impaired endothelium-dependent vasodilation has been described both in humans and in animal models of the disease [1-8]. It is widely believed that increased superoxide ($O_2.^-$) production may underlie impaired vascular dilation [1-8]. We previously reported (a) enhancement of $O_2.^-$ production and of nitrate ($NO_3^-$) formation [nitric oxide (.NO) is metabolized by $O_2.^-$ to $NO_3^-$] [9], (b) a decreased activity and expression of the Mn-SOD [8], and (c) an enhanced expression of the subunit mRNA of NAD(P)H oxidase in diabetic arteries [10, 11].

It is possible that in diabetic states, hyperinsulinemia initiates oxidant stress, leading to endothelial dysfunction at a later stage. We and others have demonstrated that in models with hyperinsulinemia and hyperglycemia, .NO productions and/or .NO responsiveness are impaired in aortic strips from either spontaneously type 2 diabetic Goto-Kakizaki (GK) rats or type 2 diabetic mice [12-14]. Interestingly, our study in GK rats revealed that (a) at an early stage in the diabetes, there is enhanced aortic relaxation due to increased .NO production via over-expression of endothelial .NO synthase (eNOS), whereas (b) at a later there are impairments of endothelium-dependent and endothelium-independent relaxations that appear to be due to a decrease in .NO responsiveness in vascular smooth muscle cells [13]. On the downside, excessive increases in eNOS expression, .NO production, and plasma insulin could be key events in the initiation of endothelial dysfunction in diabetes. Furthermore, Piper et al have shown that that long-term administration of NOX-101, a .NO scavenger, prevents the impairment of endothelial function otherwise seen in aortas from streptozotocin (STZ)-induced diabetic rats [15]. Since a .NO scavenger might act by preventing peroxynitrite ($ONOO^-$)-mediated endothelial injury being caused by excess .NO production in the presence of $O_2.^-$, we hypothesized that increased $ONOO^-$ formation might be related to the impairment of endothelial function previously observed in diabetic rats (see above).

When insulin is administered in vitro or in vivo, it enhances endothelial vasorelaxation by potentiating .NO synthase and increasing the expression of its mRNA, suggesting that insulin itself might be a factor related to .NO production [16, 17]. On the other hand, insulin stimulates NADPH-dependent $O_2^{\cdot -}$ generation in human skin fibroblasts, and also increases $O_2^{\cdot -}$ production in aortic segments from exogenously hyperinsulinemic rats [18, 19]. NO rapidly reacts with $O_2^{\cdot -}$ to form $ONOO^-$. Interestingly, $ONOO^-$ can modify tyrosine residues in various proteins to form nitrotyrosine, and nitration of protein tyrosine residues can lead to damage that alters protein function and stability [20]. Therefore, nitrotyrosine is a marker of oxidative stress [21, 22], a condition present in such diseases as human atherosclerosis and diabetes, and nonobese diabetes in mice [23-25]. Several recent studies have shown that formation of nitrotyrosine and/or $ONOO^-$ impairs vascular .NO responsiveness and .NO production [26-28]. Further, the observation of nitrotyrosine formation in endoplasmic reticulum (ER) protein within early atherosclerotic lesions suggests that $ONOO^-$ contributes to atherogenesis through a mechanism involving ER stress [29]. In high-cholesterol-fed rabbits, sarco/endoplasmic reticulum calcium ATPase (SERCA) function and endothelium-dependent and -independent relaxations are reportedly inhibited by nitrotyrosine formation [26]. However, the mechanisms by which in the diabetic state, insulin and $ONOO^-$ formation contribute to endothelial dysfunction remains uncertain.

Although the hyperinsulinemia associated with diabetes may make important contributions to the development of cardiovascular disorders, as described in several models of the disease, there is evidence that the elevation of insulin alone found in patients with insulinomas or in control rats subjected to high-dose insulin treatment do not cause hypertension or an impairment of endothelial function [30, 31]. We postulated that a perturbation of the activity and/or function of the insulin system in diabetes could be a key event in the development of vascular disease, by an unknown mechanism. Such (a) $ONOO^-$ production and nitrotyrosine formation are associated with an impairment of endothelial function, (b) a high insulin level in a diabetic state lead to $O_2^{\cdot -}$ production, and (c) the formation of nitrotyrosine in SERCA protein may lead to an impairment of endothelium-dependent relaxation via SERCA dysfunction, we attempted to clarify whether

the co-existence of a high insulin level and an established diabetic state might lead to an excessive generation of $ONOO^-$, which might then trigger an impairment of endothelium-dependent relaxation via a decrease in SERCA function.

## Experiment

### *Reagents*

Insulin, nitro blue tetrazolium (NBT), 3-morpholinosydnomine hydrochloride (SIN-1), norepinephrine (NE), $N^G$-nitro-L-arginine (L-NNA), phenylmethylsulfonyl fluoride (PMSF), protease-inhibitor cocktail, superoxide dismutase (SOD), STZ, thapsigargin (TG) and uric acid were all purchased from Sigma Chemical Co. (St. Louis, MO, USA), while acetylcholine chloride (ACh) was from Daiichi Pharmaceuticals (Tokyo, Japan). Angeli's Salt was from Cayman Chemical Co, (Ann Arbor, MI, USA). All drugs were dissolved in saline, except where otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath.

### *Animals and experimental design*

Male Wistar rats were randomly allocated to diabetic and control groups. The rats (8 weeks old) in the former group received a single injection via the tail vein of STZ 65 mg/kg dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given ad libitum. Plasma parameters were measured as previously described [9, 13]. The experimental design was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with "Guide for the Care and Use of Laboratory Animals", published by the US National Institute of Health, and with "Guide for the Care and Use of Laboratory Animals" adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

### *Organ-culture procedure*

We used organ culture of the entire vascular wall, as previously described [32-34]. Rats were anestheized with diethyl ether and euthanized by decapitation 10 weeks after treatment with STZ or buffer. The aorta was then placed in a bath containing 10 mL modified Krebs-Henseleit solution (KHS ; bubbled with 95 % $O_2$, plus 5 % $CO_2$, and kept at 37 ℃). The aorta was cleaned of loosely adhering fat and connective tissue, and helical strips 3

mm in width and 20 mm in length were placed in 5mL of serum-free Leibovitz's L-15 medium supplemented with penicillin-streptomycin containing one of the following: 50 ng/mL insulin, 50 ng/mL insulin plus SOD (180 U/mL), 50 ng/mL insulin plus uric acid (0.5 mM), or SIN-1 alone (1 mM). Arterial preparations were maintained at 37℃ for 16 h except where otherwise noted.

***Measurement of isometric force***

After incubation, the tissue was placed in a bath of KHS at 37 °C with one end connected to a tissue holder and the other to a force-displacement transducer, as previously described [34, 35]. For the relaxation studies, the aortic strips were precontracted with an equieffective concentration of NE ($10^{-8}$ - 5 x $10^{-8}$ M). For the Angeli's salt-induced relaxation studies, the endothelium was removed (see Results) by rubbing the intimal surface with a cotton swab, successful removal being functionally confirmed by the absence of a relaxation to $10^{-5}$ M acetylcholine. When the NE-induced contraction had reached a plateau level, ACh ($10^{-9}$ - $10^{-5}$ M) or Angeli's salt ($10^{-9}$ - $10^{-5}$ M) was added in a cumulative manner. When the effect of TG (10 μM) on the response to a relaxant agent was to be examined in the diabetic aorta, this agent was added to the bath 60 min before the administration of NE.

***Quantification of superoxide anion by measurement of the amount of NBT reduced***

Cultured aortic rings were incubated with NBT to allow the $O_2^{\cdot -}$ generated by the tissue to reduce the NBT to blue formazan. The details of this assay have been published previously [36]. Briefly, cultured aortas cut into transverse rings 10 mm in length were kept for 1.5 h in 5 ml buffer containing NBT (100 μmol/L) in the presence or absence of L-NNA. After this incubation, they were minced and homogenized in a mixture of 0.1 N NaOH and 0.1 % SDS in water containing 40 mg/L of diethylenetriaminepentaacetic acid. The mixture was centrifuged at 20,000 g for 20 min, and the resultant pellet resuspended in 1.5 mL of pyridine during heating at 80 °C for 1.5 h to extract formazan. The mixture was then subjected to a second centrifugation at 10,000 g for 10 min. The absorbance of the formazan was determined spectrophotometrically at 540 nm. The amount of NBT reduced (= quantity of formazan) was calculated as follows: amount of NBT reduced = A x V/(T x Wt

x ε x l), where A is the absorbance, V is the volume of the solution, T is the time for which rings were incubated with NBT, Wt is the blotted wet weight of the aortic ring, ε is the extinction coefficient (0.7 L /mmol per mm), and l is the length of the light path. The results are reported in pmol/min per Wt mg.

***Measurement of SOD activity in aortic tissue***

Cultured aortic rings were homogenized in 200 mL ice-cold Tris-sucrose buffer containing 10 mM Tris-HCl (pH 7.2), 340 mM sucrose, 1 mM EDTA, 1 mM PMSF, and 10 mg/ml aprotinin, then incubated for 30 min. Samples were centrifuged (16,000 g, 10 min, 4 ˚C), and the supernatant was used for the measurement of SOD activity. SOD activity was assayed by means of a SOD Assay Kit-WST (Dojindo Lab., Kumamoto, Japan), in which xanthine and xanthine oxidase serve as a superoxide generator, and the highly water-soluble tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) is used as a $O_2^{\cdot -}$ indicator. SOD activity was calculated using the standard curve, according to the manufacturer's instructions.

***Measurement of NOx***

The concentration of nitrite plus nitrate (NOx) in the culture medium or in ACh-stimulated samples of the medium was measured using a method described previously [9]. In case of ACh-stimulated samples, the incubated strips were placed in 0.5 mL KHS. Samples of medium were collected as follows: sample 1, for a 20-min period after application of $10^{-6}$ M ACh; sample 2, for a 20-min period after non-stimulation. The amount of NOx was calculated as follows: agonist-stimulated NOx ($10^{-7}$ mol $min^{-1}$ $g^{-1}$) = [sample 1 - sample 2] / 20 (min) x g (wet weight of the aorta). The concentrations of $NO_2^-$ plus $NO_3^-$ in KHS and the reliability of the reduction column were examined in each experiment.

***Immunohistochemistry for nitrotyrosine***

After incubation, some strips from the aortas were embedded in O. C. T. compound (Sakura, Torrance, CA, USA). After a washout of the compound, slides were treated with

10 mmol/L citric acid, then microwave-heated (for 1 min) to recover antigenicity. Nonspecific binding was blocked with a drop of normal horse serum in Block ace (Dainippon-Pharm., Osaka, Japan) for 20 min before overnight incubation at 4 °C with polyclonal anti-nitrotyrosine antibody (1: 100; Chemicon, Temecula, CA, USA) in Block ace Tissue sections were then incubated for 30 min at room temperature with a biotinylated anti-rabbit IgG (1: 800) secondary antibody, using a VECTASTAIN Universal ABC-AP kit (Vector Laboratories, Burlingame, CA, USA). Alkaline Phosphatase Substrate Kit I (Vector Laboratories) was used to visualize positive immunoreactivity for nitrotyrosine. Sections of rat aorta enclosed in Entellan new (Merck, Darmstadt, Germany) were imaged using a light microscope.

***Measurement of the expressions of nitrotyrosine by Western blotting***

Aortas (two pooled vessels per group) were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Triton X-100, and protease-inhibitor cocktail. Homogenates were centrifuged at 13,000 g for 5 min. The supernatant was sonicated at 4 °C, and the proteins were solubilized in Laemmli's buffer containing mercaptoethanol, and boiled for 5 min at 95 °C. The protein concentration was determined by means of a BCA protein assay reagent kit (PIERCE, IL, USA). Samples (40 µg/lane) were resolved by electrophoresis on 7.5% SDS-PAGE gels and transferred onto PVDF membranes. Briefly, after blocking the residual protein sites on the membrane with Block ace (Dainippon-Pharm., Osaka, Japan), the membrane was incubated with anti-nitrotyrosine (1:1000; Chemicon) or ß-actin (1:5000; Sigma), in blocking solution. Horseradish peroxidase-conjugated, anti-rabbit antibody (Vector Laboratories) was used at a 1:4000 dilution in Tween PBS, followed by detection using SuperSignal (PIERCE).

***Immunoprecipitation with anti-nitrotyrosine or anti-SERCA2 antibody***

The immunoprecipitation procedure involved a method described elsewhere [26]. Briefly, 1 mg protein extract was diluted in 500 µL lysis buffer. After preclearing with protein A or G agarose, the supernatant was mixed with 10 µL polyclonal anti-nitrotyrosine or SERCA2 antibody, then incubated for 4 h at 4 °C. Prewashed protein A or G agarose (50 µL) was

added to the samples, and after 1 h further incubation, the immunocomplex was resuspended in Laemmli's buffer containing mercaptoethanol, boiled for 5 min at 95 °C, loaded onto SDS-PAGE gels, and transferred onto PDVF membranes. Briefly, after blocking the residual protein sites on the membrane with Block ace (Dainippon-Pharm., Osaka, Japan), the membrane was incubated with anti- nitrotyrosine (1:1000), SERCA2 (1:2500, Sigma), or ß-actin (1:5000), in blocking solution. Horseradish peroxidase-conjugated, anti-rabbit antibody (Vector Laboratories) was used at a 1: 4000 dilution in Tween PBS, followed by detection using SuperSignal.

***Statistical analysis***

The contractile force developed by aortic strips from control and diabetic rats is expressed in milligrams of tension per milligrams of tissue, the data being given as mean ± SE. When appropriate, statistical differences were assessed using Dunnett's test for multiple comparisons after a one-way analysis of variance, a probability level of $P < 0.05$ being regarded as significant. Statisitcal comparisons between concentration-response curves were assessed using a two-way ANOVA with a Bonferroni correction performed post hoc to correct for multiple comparisons; again, $P < 0.05$ was considered significant.

## Abbreviations

ACh, acetylcholine chloride; BHT, *t*-butylhydroxytoluene; eNOS, endothelial NO synthase; ER, endoplasmic reticulum; GK, Goto-Kakizaki; KHS, Krebs-Henseleit solution; L-NNA, $N^G$-nitro-L-arginine; NBT, nitro blue tetrazolium; NE, norepinephrine; $NO_3^-$, nitrate; .NO, nitric oxide; NOx, nitrite plus nitrate; $O_2.^-$, superoxide; $ONOO^-$, peroxynitrite; PMSF, phenylmethylsulfonyl fluoride; SERCA, sarco/endoplasmic reticulum calcium ATPase function; SIN-1, 3-morpholinosydnomine hydrochloride; SOD, superoxide dismutase; STZ, streptozotocin; TG, thapsigargin.

## Results

### *General parameters*

As in our previous study [9, 35], at the time of the experiment all diabetic rats exhibited hyperglycemia, their blood glucose concentrations (527.6 ± 27.35 mg $dL^{-1}$, *n* = 12) being significantly higher ($P<0.001$) than those of the age-matched, non-diabetic control rats (109.21 ± 5.9 mg $dL^{-1}$, *n* = 12). Plasma insulin levels were significantly lower ($P<0.001$) in the diabetics (0.21 ± 0.08 ng $mL^{-1}$, *n* = 12) than in the controls (4.25 ± 0.15 ng $mL^{-1}$, *n* = 12).

### *Effects of insulin on relaxation responses to ACh and Angeli's salt*

In order to demonstrate relaxation responses in diabetic animals and their modulation by insulin treatment, I examined the vasodilator responses to the endothelium-dependent activator ACh and the endothelium-independent activator Angeli's Salt. I used organ culture of the entire vascular wall (a) because in this way it is possible to incubate the tissue with a constant concentration of insulin for a prolonged period of time and (b) because direct interactions between vascular smooth muscle cells and endothelial cells can easily be examined [32-34]. When the NE ($10^{-8}$ -5 x $10^{-8}$ M)-induced contraction had reached a plateau, ACh ($10^{-9}$-$10^{-5}$ M) was added cumulatively. The results are summarized in Fig 1. In aortas incubated in serum-free medium, ACh caused a concentration-dependent relaxation with the maximum response at $10^{-5}$ M, and a comparison of the entire curves revealed that this relaxation was significantly weaker in diabetic aortas than in the controls ($p=0.048$). Diabetic aortas incubated with insulin (50 ng/mL, for 16h) exhibited marked reductions both in the ACh-induced a concentration-dependent relaxation as a whole and in the maximal relaxation to ACh (Fig 1 B). In contrast, incubating control aortas with insulin had no significant effect (Fig 1 A). These results suggest that prolonged treatment of the diabetic aorta with insulin caused an impairment of endothelium-dependent relaxation.

Relaxation responses to Angeli's salt, which generates nitroxyl anion and .NO [37], were examined using aortic preparations denuded of endothelium after the incubation. When the NE-induced contraction had reached a plateau, Angeli's salt ($10^{-9}$-$10^{-5}$ M) was added

cumulatively. The results are summarized in Fig 1C and D. The Angeli's salt-induced relaxation was not significantly different between controls incubated with insulin and those incubated without insulin (Fig 1C). However, this relaxation was significantly impaired in diabetic aortas incubated with insulin compared with those incubated without insulin (Fig 1D). The maximal response for the Angeli's salt-induced relaxation exhibited no significant difference among the groups (Fig 1C, 1D). In contrast to the lack of effect of 50 ng/mL insulin on relaxation responses in control aortas (see above), incubating control aortas with high-dose insulin (500 ng/mL, for 16 h) led to a relaxation that was significantly greater than that observed in control aortas incubated in serum-free medium (data not shown). These results suggest that the diabetic aorta cultured with insulin underwent an impairment of .NO-induced relaxation. Incubating diabetic aortas for only 1 h in culture medium (Fig 2A) containing insulin had no effect on the relaxation induced by ACh. In contrast to the above results obtained in aortas denuded of endothelium *after* incubation (Fig 1D), incubation with insulin had no effect on the relaxation induced by Angeli's salt in strips in the diabetic group denuded of endothelium *before* incubation (Fig 2B).

Taken together, these results suggest that prolonged treatment of the diabetic aorta with insulin caused impairments of both endothelium-dependent and .NO-dependent responses through a mechanism that is mediated by a factor produced from endothelial cells (since its effects were evident only when the endothelium was intact during the incubation).

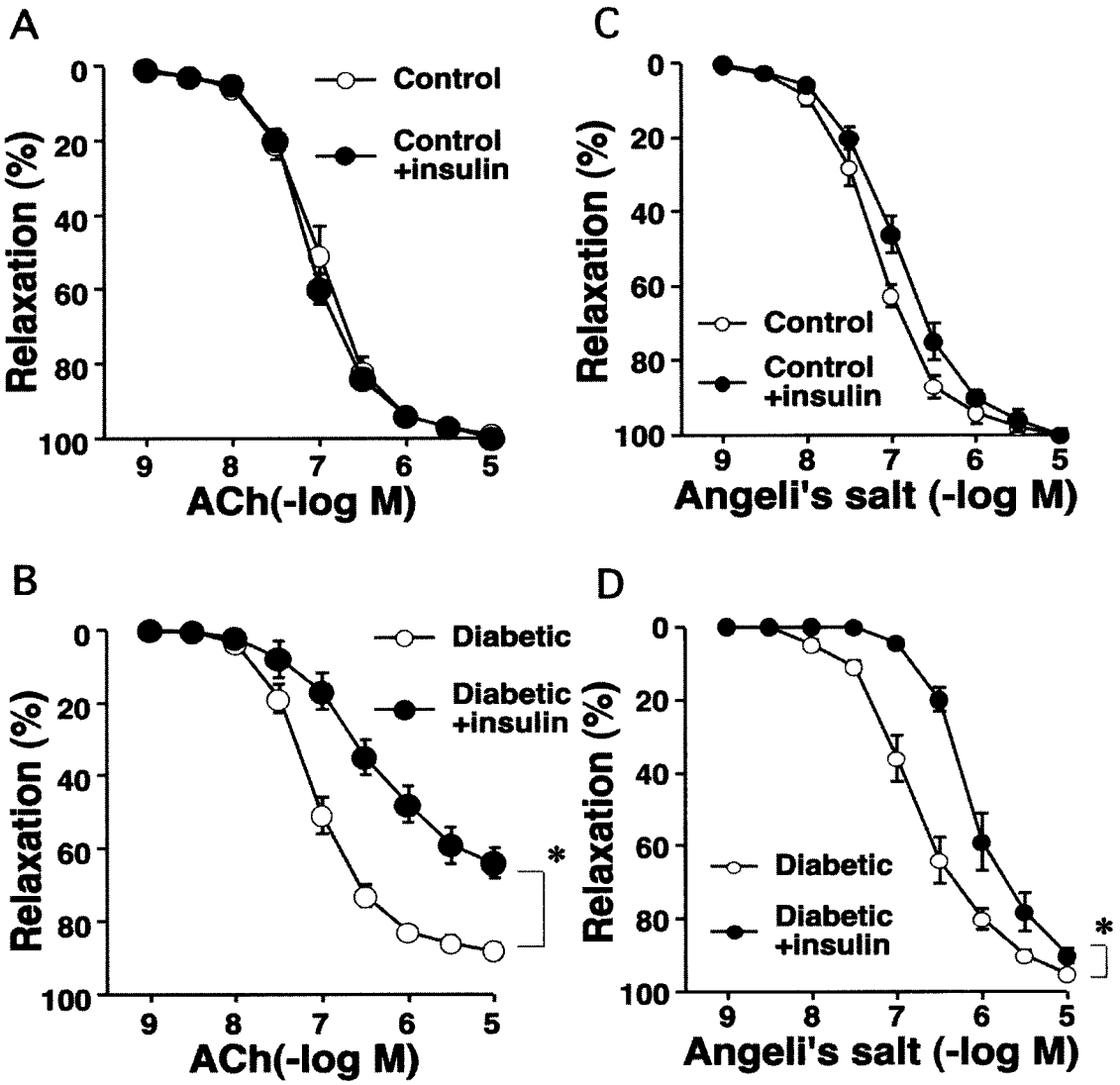


**Figure. 1.** Concentration-response curves for the ACh (A, B)- and Angeli's salt (C, D)-induced relaxations of aortic strips after organ culture. Strips obtained from control rats (A, C) and diabetic rats (B, D) were cultured in serum-free medium or in the presence of insulin (50 ng/mL) In C and D, the endothelium was removed *after* culture. Ordinates show relaxation as a percentage of the contraction induced by an equieffective concentration of NE ($10^{-8}$ – 5 x $10^{-8}$ M). Each data-point represents the mean±S.E. from 8-10 experiments (S.E. is included only when it exceeds the dimension of the symbol used). *P< 0.05 vs diabetic aortas cultured in serum-free medium.

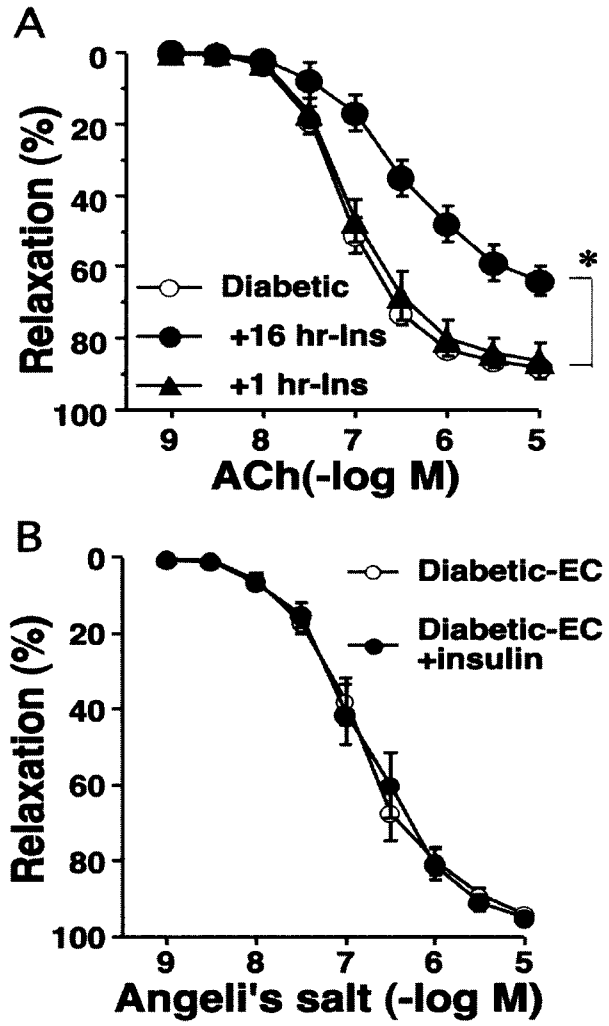


**Figure. 2.** Effects of duration of insulin-exposure (A) and denudation of endothelium (B) on concentration-response curves for ACh-induced and Angeli's salt-induced relaxations of aortic strips from diabetic rats. (A), Aortas from diabetic rats were cultured either in serum-free medium for 16 h or in medium containing insulin (Ins, 50 ng/mL) for 1 h or 16 h. (B), Aortas were cultured either in serum-free medium or in the presence of insulin (for 16 h), the strips used having had their endothelium removed (-EC) *before* culture. Ordinates show relaxation as a percentage of the contraction induced by an equieffective concentration of NE ($10^{-8}$ – 5 x $10^{-8}$ M). Each data-point represents the mean±S.E. from 8-10 experiments (S.E. is included only when it exceeds the dimension of the symbol used). *P< 0.05 vs diabetic aortas cultured in presence of insulin for 1 h.

***Effects of oxidative stress on insulin-induced impairment of relaxation***

To examine the effects of $O_2.^-$ and $ONOO^-$ on the insulin-induced impairment of relaxation, cultured aortas were co-incubated with SOD [$O_2.^-$ scavenger (180 U/mL)], or with uric acid [$ONOO^-$ scavenger (0.5 mM)] [38]. As shown in Fig 3, co-incubation with insulin plus SOD or with insulin plus uric acid prevented the insulin-induced impairment of both the ACh-induced (Fig 3A) and Angeli's salt-induced (Fig 3B) relaxations. In diabetic strips incubated with SIN-1 [$ONOO^-$ generator (1 mM)], the Angeli's salt-induced relaxation was significantly impaired (versus that observed without SIN-1) (Fig 3C). These results suggest that the impairments observed following insulin-treatment of the diabetic aorta are due to increases in $ONOO^-$ and $O_2.^-$ production.

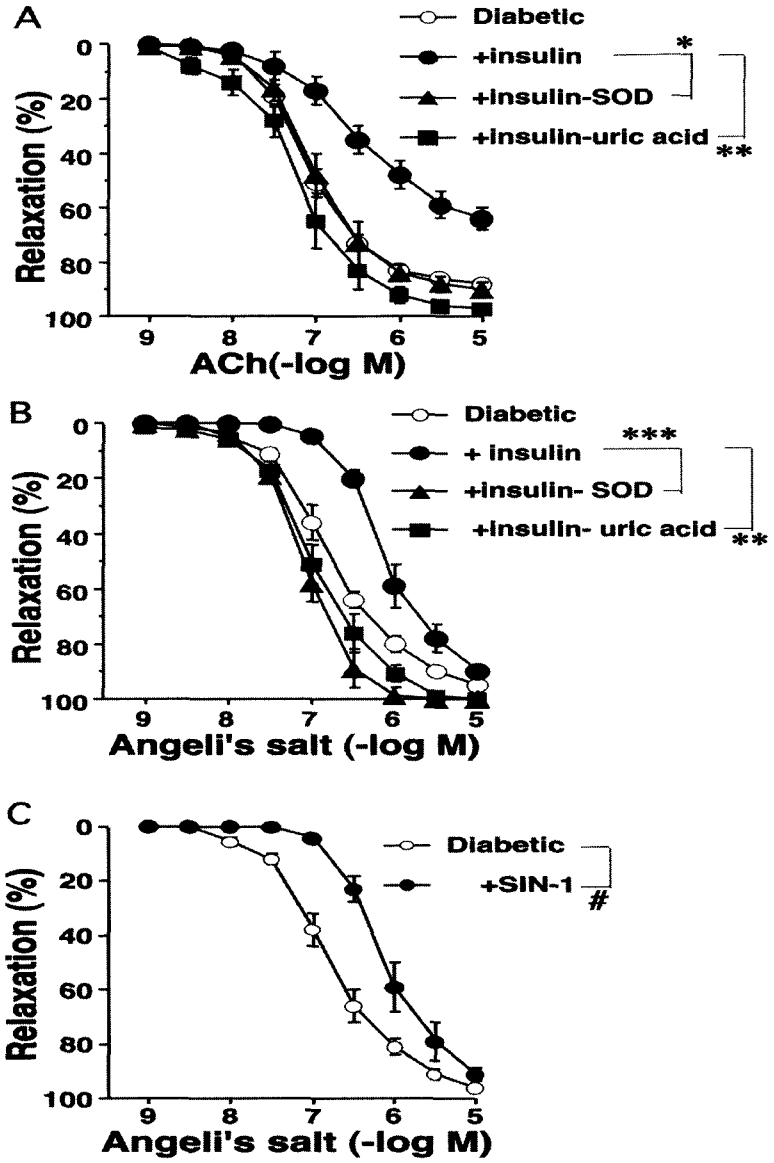


**Figure. 3.** Effects of SOD, uric acid, and SIN-1 on concentration-response curves for ACh (A)-induced and Angeli's salt (B, C)-induced relaxations of aortic strips from diabetic rats. Strips were cultured for 16 h either in serum-free medium or in the presence of insulin (50 ng/mL), insulin plus SOD (180 U/mL), insulin plus uric acid (0.5mM), or SIN-1 (1mM). In B and C, the endothelium was removed *after* culture. Ordinates show relaxation as a percentage of the contraction induced by an equieffective concentration of NE ($10^{-8}$ – 5 x $10^{-8}$ M). Each data-point represents the mean±S.E. from 7-8 experiments (S.E. is included only when it exceeds the dimension of the symbol used). *P< 0.05, **P< 0.01, ***P< 0.001 vs diabetic aortas cultured in presence of insulin alone. #P<0.05 vs diabetic aortas.

***Effects on SERCA function during insulin-induced impairment of relaxation***

To investigate the SERCA-derived relaxation evoked by Angeli's salt, we performed a series of experiments in which Angeli's salt was added to aortic strips precontracted by NE in the presence of TG [irreversible SERCA inhibitor (10 μM)]. TG significantly inhibited the Angeli's salt-induced relaxation response in control aortas incubated with or without insulin (Fig 4A). In diabetic aortas, TG significantly inhibited the Angeli's salt-induced relaxation response in aortas incubated without insulin, but did not inhibit it significantly in those incubated with insulin (Fig 4B). These observations provide evidence that the contribution of SERCA to the Angeli's Salt-induced relaxation may be decreased in diabetic aortas incubated with insulin.

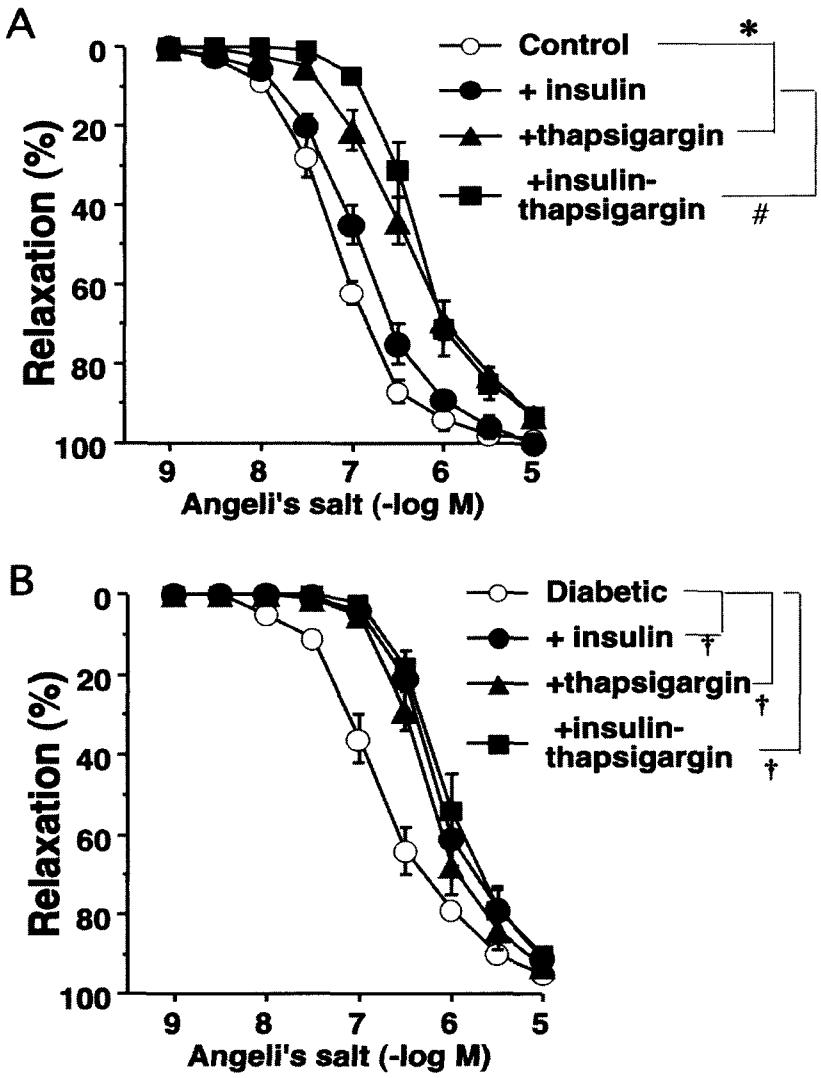


**Figure. 4.** Effect of thapsigargin on Angeli's salt-induced relaxation of aortic strips after culture either in serum-free medium or in the presence of insulin. Strips obtained from control (A) and diabetic (B) rats were cultured for 16 h either in serum-free medium or in the presence of insulin (50 ng/mL). The endothelium was removed *after* culture. When the effects of thapsigargin (10 μM) on the response to the relaxant agent were examined, this agent was added to the bath 60 min before the administration of NE ($10^{-8}$ – 5 x $10^{-8}$ M). Ordinates show relaxation as a percentage of the contraction induced by an equieffective concentration of NE. Each data-point represents mean±S.E. from 8-10 experiments (S.E. is included only when it exceeds the dimension of the symbol used). *P< 0.05 vs control aortas cultured in serum-free medium. #P< 0.05 vs diabetic aortas cultured in the presence of insulin. †P< 0.05 vs diabetic aortas cultured in serum-free medium.

***Measurement of NOx production***

To investigate the involvement of $ONOO^-$, which is derived from .NO and $O_2.^-$, in diabetic aortic dysfunction, we examined whether NOx production [.NO is metabolized to NOx] might be altered in diabetic aortas and/or by insulin treatment. The total NOx level in the culture medium after 16 h incubation was not significantly different between diabetic aortas and the controls. However, NOx was significantly increased in diabetic aortas incubated with insulin (versus diabetic aortas without insulin and versus control aortas with insulin), although it was not different between controls incubation with or without insulin (Fig 5A). These results suggest that in our diabetic aortas, incubated with insulin promoted .NO production.

In view of the published evidence that .NO plays a major role in endothelium-dependent relaxation in the aorta [9, 13], we compared the ACh-stimulated NOx level among our groups. ACh increased the NOx level in the medium from all aortic strips (versus the basal levels), and the ACh-stimulated NOx level was significantly lower in diabetic aortas incubated with insulin than in those incubated without insulin. Co-incubation with insulin plus uric acid prevented the insulin-induced impairment of ACh-induced NOx production (Fig 5B). In contrast, the basal NOx level was significantly increased in diabetic aortas incubated with insulin (versus without insulin)(data not shown). These results suggest that the impaired ACh-induced relaxation seen in such aortas following insulin treatment may be, at least in part, due to a decrease in .NO production by endothelial cells.

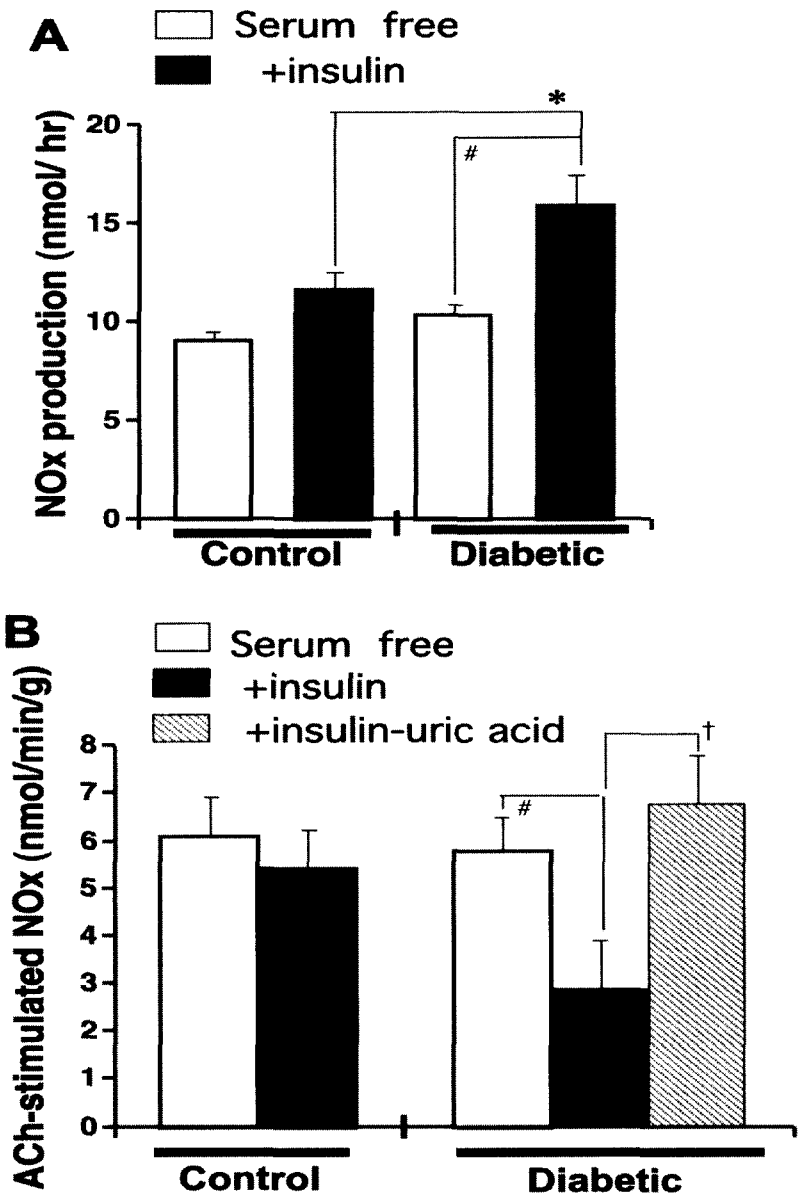


**Figure 5.** Production of $NO_2^-$ plus $NO_3^-$ (NOx) by organ-cultured aortas from control and diabetic rats, without (A) or with (B) ACh-stimulation. (A), Total NOx production during 16 h culture in serum-free medium or in the presence of insulin (50 ng/mL). (B), ACh-stimulated release of NOx in aortic rings after 16 h incubation either in serum-free medium or in the presence of insulin (50 ng/mL) with or without uric acid (0.5 mM). Control or diabetic aortas in serum-free medium (open columns), with insulin (closed columns), with insulin plus uric acid (hatched column). Each column represents the mean±S.E. from 6-8 experiments. *P< 0.05 vs control aortas cultured with insulin. #P< 0.05 vs diabetic aortas in serum-free medium. †P< 0.05 vs insulin-treated diabetic aortas.

***Quantification of superoxide anion by measurement of amount of NBT reduced***

In our experiments to assess $O_2^{\cdot-}$ generation (by measuring the amount of NBT reduced by $O_2^{\cdot-}$), the basal $O_2^{\cdot-}$ level was greater in aortas from diabetic rats than in those from the controls. In control aortas, $O_2^{\cdot-}$ production tended to be slightly enhanced in those incubated with insulin (versus without insulin). In diabetic aortas, $O_2^{\cdot-}$ production was significantly decreased in those incubated with insulin (versus without insulin) (Fig 6A). Since $O_2^{\cdot-}$ is rapidly scavenged by .NO, we also determined the amount of NBT reduced in the presence of L-NNA, a NO synthase inhibitor. Although L-NNA had no effect on $O_2^{\cdot-}$ production in aortas from the control group, the $O_2^{\cdot-}$ level in diabetic aortas incubated with insulin was significantly enhanced by treatment with this agent, the level reached being significantly greater than those seen in the presence of L-NNA in control aortas with insulin or diabetic aortas without insulin (Fig 6A). This may indicate that in the diabetic aorta incubated with insulin, $O_2^{\cdot-}$ production is increased, but it is effectively scavenged by .NO derived from endothelial cells.

***Aortic SOD activity***

Local steady-state levels of $O_2^{\cdot-}$ are dependent both on the rate of production of $O_2^{\cdot-}$ and on the endogenous SOD activity. Hence, we examined whether SOD activity might be altered in the diabetic aorta and/or by insulin treatment (Fig 6B). The aortic total SOD activity was significantly lower in diabetic aortas than in control aortas, and this decreased SOD activity was not altered by insulin treatment (versus without insulin). These results suggest that the increased $O_2^{\cdot-}$ production observed in diabetic aortas incubated either without or with insulin may be, at least in part, due to a decrease in SOD activity.

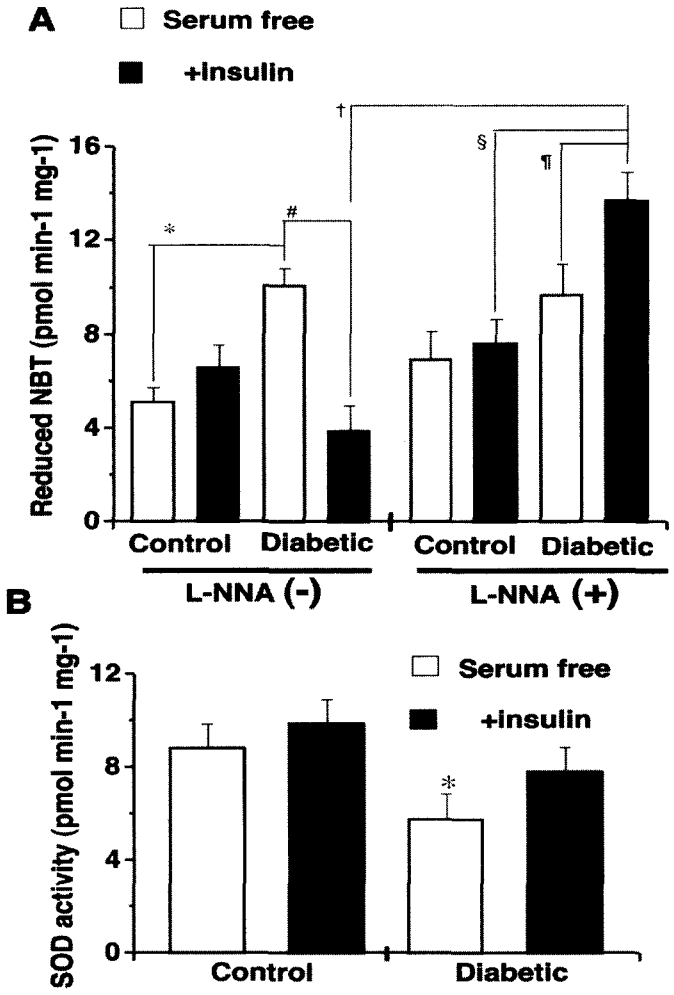


**Figure. 6.** Quantification of superoxide-anion production (A) and endogenous SOD activity (B) by measurement of amount of reduced nitro blue tetrazolium (NBT) in aortic strips after culture either in serum-free medium or in the presence of insulin (50 ng/mL). (A), Strips from controls and diabetic rats were cultured as above, then incubated with NBT, with or without L-NNA, for 1.5 h. Control or diabetic aortas cultured in serum-free medium (open columns) or with insulin (closed columns). L-NNA(-), amount of superoxide anion in the absence of L-NNA. L-NNA(+), amount of superoxide anion in the presence of L-NNA. Each value is the mean+S.E. from 6-8 experiments. *P< 0.05 vs serum-free control in L-NNA(-) condition. #P< 0.05 vs serum-free diabetic in L-NNA(-) condition. †P< 0.01 vs insulin-treated diabetic in L-NNA(-) condition. §P< 0.05 vs. insulin-treated control in L-NNA(+) condition. ¶P< 0.05 vs serum-free diabetic in L-NNA(+) condition. (B), endogenous SOD activity was measured as described in Materials and methods. Each column represents the mean + S.E. from eight experiments. *P < 0.05 vs. serum-free control.

***Levels of nitrotyrosine-containing protein***

The presence of nitrotyrosine is considered indirect evidence of $ONOO^-$ production. So, I performed immunohistochemistry for nitrotyrosine using the aortas of rats from the five groups. Compared with the controls (Fig 7A), the diabetic aortas showed slightly increased nitrotyrosine staining (Fig 7B). Incubating control aortas with insulin led to no change in nitrotyrosine staining (Fig 7C). In diabetic aortas incubated with insulin (Fig 7D), nitrotyrosine staining was markedly increased versus both control aortas (incubated with or without insulin) and diabetic aortas incubated without insulin. This observed increase in the diabetic aorta was considerably attenuated by co-incubation with insulin plus uric acid (Fig. 7F), although the staining was still greater than that seen in control aortas co-incubated with insulin and uric acid (Fig. 7E). These results suggest that diabetic aortas cultured with insulin lead to increases in both $ONOO^-$ formation and nitrotyrosine-containing protein.

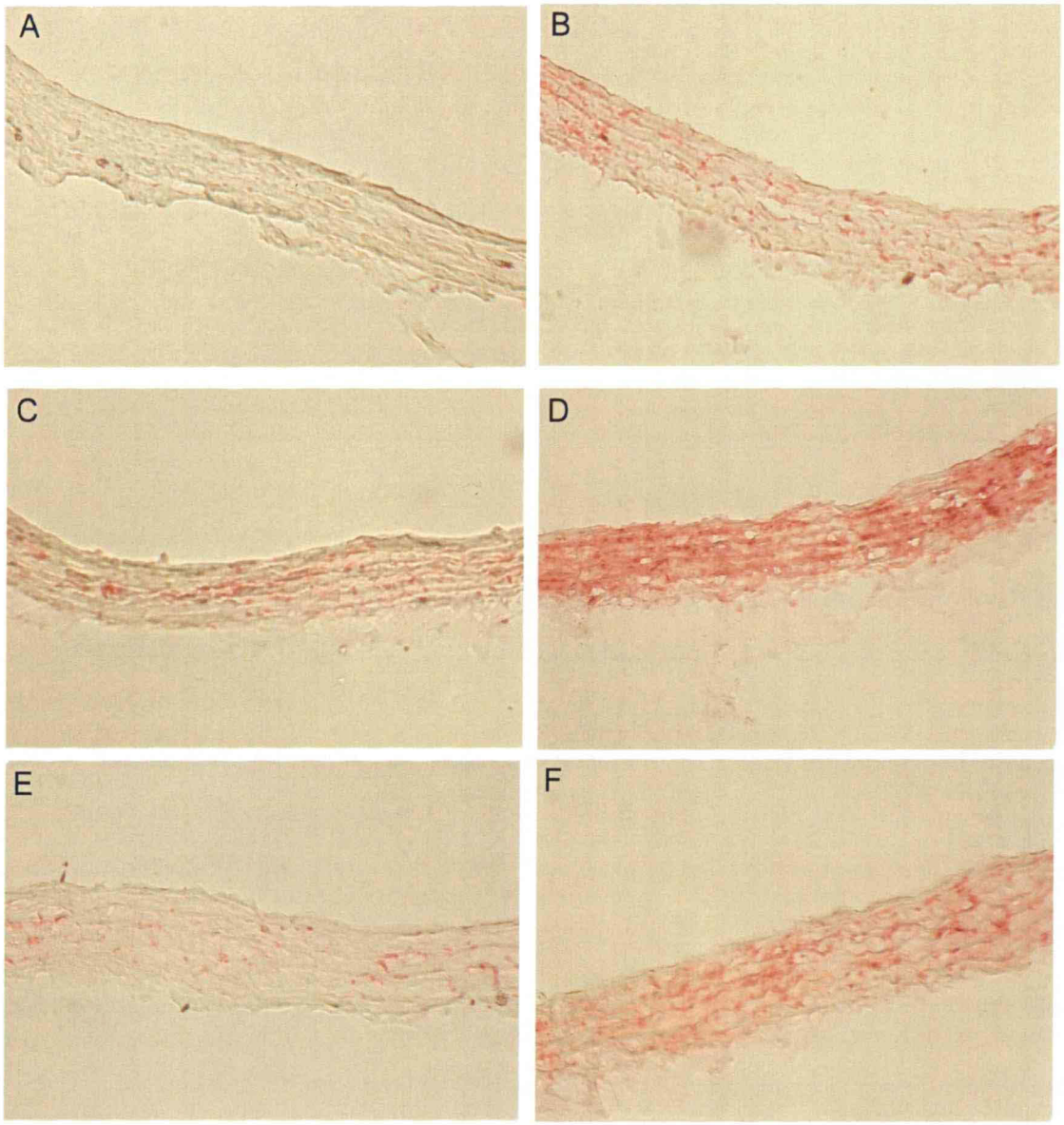


**Figure. 7.** Immunohistochemical staining for nitrotyrosine in aortic sections after culture either in serum-free medium or in the presence of insulin with or without uric acid. Strips obtained from control (A, C, E) or diabetic (B, D, F) rats were cultured in serum-free medium (A, B) or in the presence of insulin (50 ng/mL) without (C, D) or with (E, F) uric acid (0.5 mM). Positive staining is shown as red. Magnification x 100. Representative pictures from 5-6 independent experiments. Upper area, lumen.

***Formation of nitrotyrosine protein in SERCA2***

SERCA pump activity lowers the concentration of $Ca^{2+}$ in the cytoplasm while at the same time raising that of the sarcoplasmic/endoplasmic reticulum. Evidence has been provided that .NO induces relaxation in part by stimulating the refilling of the intracellular $Ca^{2+}$ stores via SERCA [26]. Molecular cloning studies have revealed a family of three genes (SERCA1, SERCA2, SERCA3) that encode SERCA. SERCA2, which is the primary isoform in vascular smooth muscle, is sensitive to oxidative stress, and its activity is known to be inhibited by both $ONOO^-$ and nitrosotyrosine formation [26, 39, 40, 41]. We found, as described above, that the SERCA-derived relaxation was impaired, and that superoxide anion and $ONOO^-$ productions were increased, in diabetic aortas cultured with insulin. Next, the level of nitrotyrosine-containing protein was examined by Western blot (Fig. 8A). Typically, SERCA protein migrates on SDS-PAGE as a series of protein bands with an apparent molecular mass of 110 kDa [26, 41]. Use of an anti-nitrotyrosine antibody allowed detection of immunoreactive protein with a molecular mass of 100-130 kDa. Treating control aortas with insulin did not significantly change the nitrotyrosine-containing protein level (versus control without insulin). In diabetic aortas incubated with insulin, nitrotyrosine was significantly increased (versus untreated control, insulin-treated control, and untreated-diabetic aortas), and this increase was considerably attenuated by co-incubation of diabetic aortas with insulin plus uric acid (Fig. 8B). This Western blot method provides measurements that may include not only nitrotyrosine-containing SERCA protein, but also other nitrotyrosine-containing proteins. To establish whether nitrotyrosine really was formed in SERCA2 protein in the insulin-treated diabetic aorta, immunoprecipitates of aortic proteins obtained using anti-SERCA2 antibody were stained using anti-nitrotyrosine antibody. Using Western blot, the expression of SERCA2 protein was examined (data not shown). Use of anti-SERCA2 antibody allowed detection of immunoreactive protein with a molecular mass of 110 kDa. The expression of SERCA2 protein was similar among the groups (data not shown). Using these immunoprecipitates and Western blot, we found that incubating control aortas with insulin did not significantly change the nitrotyrosine protein present in the SERCA2 immunoprecipitate (versus without insulin). Despite the similar expression of SERCA2 protein among the groups, incubating diabetic aortas with insulin

markedly increased the nitrotyrosine protein present in the SERCA2 immunoprecipitate (versus the controls with insulin and the diabetics without insulin), while co-incubating diabetic aortas with insulin plus uric acid significantly attenuated this increase (Fig. 8 C,D). These results suggest that in aortas from rats with diabetes, insulin causes an impairment of SERCA function through a mechanism that is mediated by an enhancement of nitrotyrosine formation in SERCA2 protein.

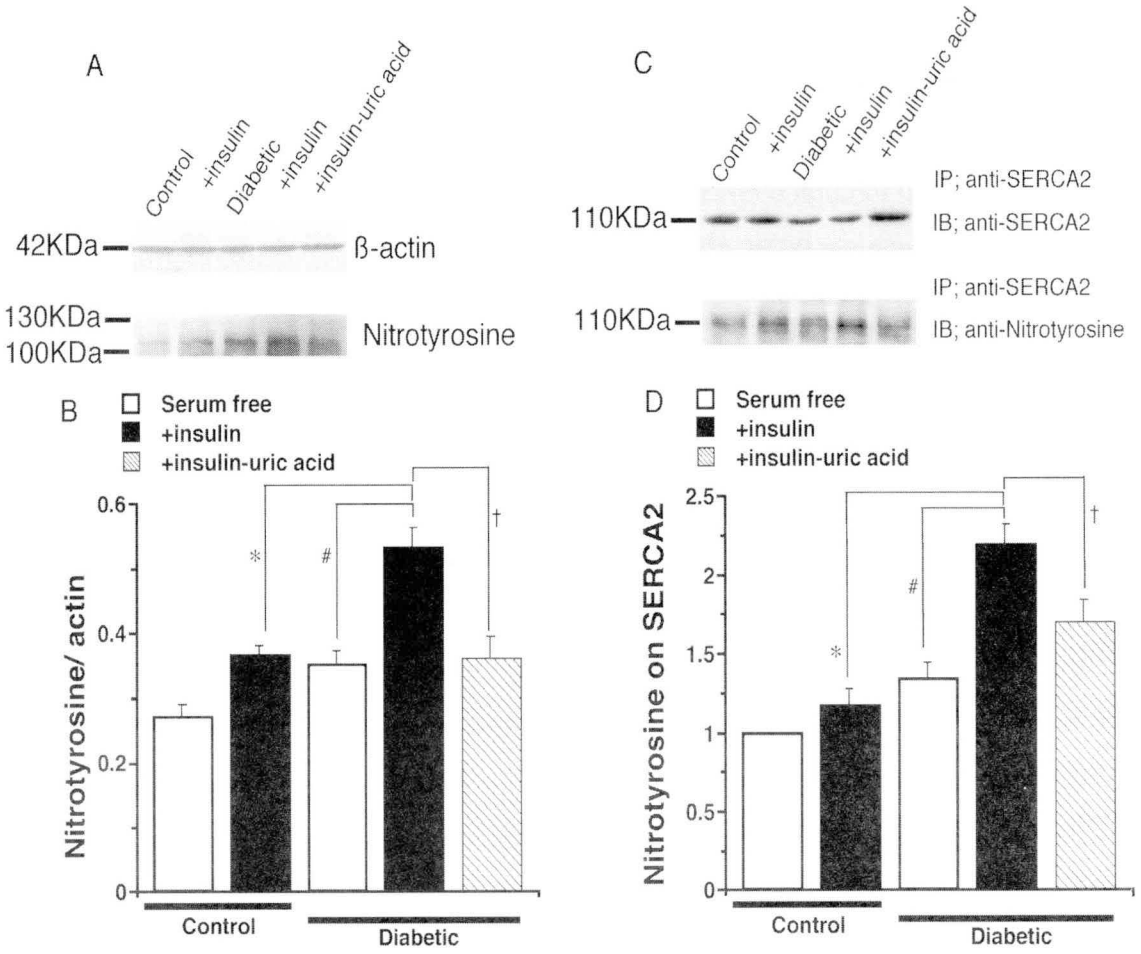


**Figure. 8.** Expression of nitrotyrosine (A, B) or nitrotyrosine in SERCA2 protein (C, D) in aortic strips cultured either in serum-free medium or in the presence of insulin with or without uric acid. Strips obtained from controls and diabetic rats were cultured in one of three ways: in serum-free medium, in the presence of insulin alone (+insulin) (50 ng/mL), or in the presence of insulin plus uric acid (0.5 mM) (+insulin+uric acid). (A, B), Expression of nitrotyrosine assayed by immunoblotting (IB). (C, D), For detection of nitrotyrosine in SERCA protein (C, top), an immunoprecipitate (IP) was obtained using anti-SERCA2 antibody, then immunoblotted with anti-SERCA2 antibody. (C, bottom), an IP was obtained using anti-SERCA2 antibody, then immunoblotted with anti-nitrotyrosine antibody. (B, D), Quantitative analysis of nitrotyrosine by scanning densitometry. Control or diabetic aortas in serum-free medium (open columns), with insulin (closed columns), or with insulin plus uric acid (hatched columns). Each value is mean±S.E. from 6-8 experiments. *$P< 0.05$ vs control aortas cultured in presence of insulin. #$P< 0.05$ vs diabetic aortas cultured in serum-free medium. †$P< 0.05$ vs diabetic aortas cultured in presence of insulin.

## Discussion

The main inferences we can draw from the present findings are that in aortas from rats with established STZ-induced diabetes, insulin leads to an enhanced aortic $ONOO^-$ generation and that this increment causes a dysfunction of endothelium-dependent relaxation, an impairment of SERCA function, and an enhancement of nitrotyrosine formation in SERCA2 protein. Further, we found that addition of a $ONOO^-$ scavenger or a $O_2.^-$ scavenger normalized this impaired relaxation. These are important findings concerning the action of insulin in diabetic arteries, and the present results suggest that an insulin-induced production of $ONOO^-$ may be involved in mediating diabetic complications.

It was suggested long ago that hyperinsulinemia *per se* exerts a detrimental effect on the vasculature by virtue of its growth factor-like and oxidant-stress effects. Recently, we demonstrated that in a model with hyperinsulinemia (diabetic GK rats), both endothelium-dependent and -independent relaxations are impaired in aortic strips [13]. In the present study, culturing diabetic aortas with insulin markedly impaired the relaxations caused by ACh (endothelial-dependent) and Angeli's salt (.NO donor), but only when the diabetic aortas were subjected to long-term (16h) culture. Such impairments were not seen when the aortas were from control rats, when diabetic aortas were subjected to short-term (1 h) culture, or when diabetic aortas were denuded of endothelium before culture. These results suggest that the insulin-induced impairments of endothelium-dependent and -independent responses in diabetic aortas are strongly related. Likewise, a previous study on large conduit arteries in humans showed impairments of endothelial function upon prolonged insulin exposure, an effect involving increased oxidant stress [42]. On the other hand, several laboratories, including ours, have shown that insulin causes vasodilation via a .NO-dependent mechanism, and this in turn has a wide array of antiatherogenic actions [9, 17, 43]. The apparent impairment of this action of insulin in diabetes could be related to the vascular insulin resistance associated with arterial hypertension and endothelial dysfunction. In fact, it has been reported that insulin resistance plays essential roles in the hypertension and impairment of endothelium-dependent relaxation seen in mice lacking insulin-receptor substrate-1 or -2 [44, 45]. On the other hand, there is evidence that an

elevation of insulin alone does not cause either hypertension or an impairment of endothelial function [30, 31]. On the basis of our study, the presence of high insulin may not, by itself, be sufficient to induce endothelial dysfunction in the rat aorta, and we suggest that for such an impairment of relaxation, a high insulin level and an established diabetic state need to exist together. Our data indicate that the established diabetic aorta may be exquisitely sensitive to the detrimental effects of insulin.

It has been shown that in diabetic arteries, increases in the levels of $O_2^{\cdot-}$ and $NO_3^-$ (.NO is metabolized by $O_2^{\cdot-}$ to $NO_3^-$) lead to alterations in endothelial function [1-8]. It is possible that $ONOO^-$, derived from .NO and $O_2^{\cdot-}$, plays an important role in mediating diabetic vascular complications. Indeed, several studies have shown that the formation of nitrotyrosine and $ONOO^-$ has deleterious effects on .NO responsiveness and .NO production in blood vessels [26-28]. In the present study: 1) the insulin-induced impairments were prevented by treatment with either a $O_2^{\cdot-}$ scavenger or $ONOO^-$ scavenger, 2) incubating diabetic aortas with insulin led to a marked increase in nitrotyrosine, and 3) incubating diabetic aortas with a $ONOO^-$ generator impaired .NO-induced relaxation. These results strongly suggest that the impairments observed following insulin treatment of the diabetic aorta are due to increases in $ONOO^-$ production and nitrotyrosine formation. In fact, insulin stimulates both NADPH-dependent $O_2^{\cdot-}$ generation [17,18] and NO synthase-dependent .NO generation [19]. In our diabetic aortas, insulin increased the productions of both $O_2^{\cdot-}$ and .NO and also increased nitrotyrosine formation. Furthermore, in our organ culture, endogenous SOD activity was decreased in the diabetic aorta, and this level was not increased by insulin treatment. These results suggest that the present insulin-induced impairments in the diabetic aorta were due to $ONOO^-$ formation via increased productions of $O_2^{\cdot-}$ (partially decreased SOD activity) and .NO.

The mechanisms through which insulin impairs vascular relaxation via increases in $ONOO^-$ remain unclear. In the aorta, calcium uptake into the intracellular store sites via SERCA activation is normally involved in mediating both the decrease in intracellular $Ca^{2+}$ and the vascular relaxation induced by .NO [46]. In our study, use of an irreversible SERCA inhibitor weakened the .NO donor-induced relaxation response in both the control aortas and the untreated diabetic aortas, but had no such effect in diabetic aortas incubated with insulin,

suggesting that the contribution of SERCA to .NO donor-induced relaxation may be decreased in the insulin-incubated diabetic aorta. It has been reported that in dilated cardiomyopathic hearts, the SR $Ca^{2+}$-pump function is inhibited by SERCA2 nitration, an event that could contribute to the heart-failure state. Further, increased nitration of the SERCA2 isoform of the $Ca^{2+}$ pump with aging has been observed in skeletal muscle and cardiac myocytes, an event associated with decreased $Ca^{2+}$-pump function [40, 41]. Of particular interest in this connection, Adachi *et al.* [26] reported that the marked increase in nitrotyrosine seen in aortic smooth muslce in hypercholesteremic rabbits is dramatically decreased by the antioxidant *t*-butylhydroxytoluene (BHT), and they further found that $ONOO^-$ inhibited SERCA2 activity in aortas isolated from such animals. Here, we detected nitrotyrosine in the SERCA2 immunoprecipitates derived from the rat aorta. Our findings that diabetic aortas incubated with insulin exhibited increased SERCA2-associated nitrotyrosine and that use of a $ONOO^-$ scavenger reduced this level suggest that insulin leads to an increase in aortic $ONOO^-$ generation and an enhancement of nitrotyrosine formation in SERCA protein. These results, which are consistent with insulin's effects on the $ONOO^-$ productions and relaxations induced by ACh and Angeli's salt, suggest that in the established diabetic aorta, insulin may induce $ONOO^-$ generation, and that this may in turn trigger a dysfunction of vascular smooth muscle SERCA.

In this study, I found that after culture with insulin, ACh-stimulated .NO production was decreased in the diabetic aorta. In addition, both the concentration-dependent response as a whole and the maximal response to ACh were impaired in diabetic aortas incubated with insulin. It has been shown that in rat aortas, ACh-induced relaxation is completely abolished by treatment with an inhibitor of nitric oxide synthase [13]. These observations suggest that the impaired endothelium-dependent relaxation seen in such aortas following insulin treatment may be, at least in part, due to a decrease in .NO production by endothelial cells. Moreover, the impairment of the maximal response to ACh may also be due to a decrease in .NO production. Indeed, several studies have shown that eNOS and cofactor tetrahydrobiopterin are important targets for $ONOO^-$, which thereby causes an impairment of eNOS activities and a conseqeunt endothelial dysfunction [27, 28, 47]. I suggest that the effects of insulin on the diabetic aorta are mediated via an increased $ONOO^-$

formation and a subsequent mechanism involving not only a dysfunction of SERCA, but also a reduction in eNOS activities. The mechanisms by which insulin acts on the diabetic aorta to cause a reduction in eNOS activities require further investigation.

Previous studies have provided evidence that NO-induced relaxation in normal vascular smooth muscle occurs via two signal pathways: SERCA-dependent and -independent mechanisms [39, 46]. Interestingly, the present results showed that the concentration-dependent relaxation response to Angeli's salt was impaired in diabetic aortas incubated with insulin, whereas the maximal Angeli's salt-induced relaxation was not decreased. In addition, a SERCA inhibitor reduced the Angeli's salt-induced relaxation response in aortas incubated without insulin, but did not reduce the maximal response. These observations provide evidence that the contribution made by SERCA-dependent relaxation to the response to Angeli's Salt may be greater at low concentrations than at high concentrations of this agent. Hence, it is possible that low concentrations of Angeli's Salt lead to relaxation via SERCA-dependent mechanisms, whereas high concentration lead to relaxation via SERCA-independent ones, and that the latter are not impaired in diabetic aortas incubated with insulin.

In conclusion, the present findings suggest that prolonged treatment of the diabetic aorta with insulin causes a relaxant dysfunction involving both the endothelium and the smooth muscle, and that this is mediated, at least in part, by a mechanism leading to SERCA dysfunction. More importantly, our evidence suggests that the presence of excess insulin in established diabetes may cause both $ONOO^-$ formation and an increase in nitrotyrosine in SERCA protein via increased productions of $O_2.^-$ and .NO. This sequence may represent a major cause of endothelial dysfunction in diabetes with hyperinsulinemia. Indeed, hyperinsulinemia is inevitable during insulin treatment in patients with diabetes mellitus and at several stages of the diabetic state [1, 48]. As far as the possible effects of the co-existence of a diabetic state and hyperinsulinemia are concerned, this study agrees with previous studies conducted on arteries in several diabetic models, in that these studies, too, suggested that enhancement of vascular disease can occur independently through high-insulin and high-glucose levels [49, 50]. Therefore, our results may support the idea that both hyperglycemia and hyperinsulinemia play roles in the development of diabetic

vascular complications and $ONOO^-$-induced endothelial dysfunction. Our data may provide evidence in favor of a novel scenario regarding the actions of insulin in states involving insulin resistance and hyperinsulinemia.

## Chapter 2

## Possible involvement of Akt activity in endothelial dysfunction in type 2 diabetic mice

## Introduction

Several epidemiological studies have indicated that the insulin resistance and hyperglycemia associated with type 2 diabetes make important contributions to the development of hypertension and cardiovascular diseases, and moreover impaired endothelium-dependent vasodilation has been described in humans and in animal models of the disease [1-4]. We and others have demonstrated that both aortic endothelial dysfunction and hypertension are present in type 2 spontaneously diabetic ($db/db^{-/-}$) mice and in fructose-fed insulin-resistant mice [5, 6].

In endothelial cells, the main signal-transduction pathway for agonist-stimulated eNOS activation depends on $Ca^{2+}$/calmodulin/caveolin-1 [4, 7]. In contrast, with other forms of stimuli such as fluid shear stress [8], estrogen [9], and insulin/IGF-1 [10] a rise in $Ca^{2+}$ is not required for NO production. Many stimuli including insulin, vascular endothelial growth factor (VEGF), β-agonists, adrenomedullin, and shear-stress signals have been reported to regulate NO production by phosphlylation of eNOS, which facilitates the association of the enzyme with calmodulin, thus reducing its inhibitory interaction with caveolin-1 [11-15]. There is some evidence that abnormal regulation of the PI3-K/Akt pathway may be one of several factors contributing to vascular dysfunction in diabetes [16]. We previously found [17] that *additon of a PI3-K or Akt inhibitor had no significant effect on either acetylcholine* (ACh)-induced relaxation or NOx /c-GMP production in control mouse aortas, whereas the clonidine- and insulin-induced relaxation responses were completely abolished by each of these inhibitors. These observations suggest that clonidine- and insulin-induced vasorelaxations are regulated by the PI3-K/Akt signal pathway [17].

Normally, there is a close relationship between insulin mediated glucose disposal and the incremental increase in blood flow in response to insulin. This normal response is lost in insulin-resistant states, suggesting a resistance to the action by which insulin induces vascular NO production [18]. It may be insulin-resistance, rather than the hyperisulinemia itself, that is a pathogenic factor for decreased vascular relaxation in diabetes. Jiang et al. found that both vascular insulin-induced phosphorylation and activation of the components of insulin signaling from the receptor level downstream to Akt were blunted in these obese

insulin-resistant rats [19]. Chronically in type II diabetes, a lack of insulin sensitization of the endothelial component of Akt signaling may contribute to a decrease in endothelial function, and hence to the progress of hypertension.

Statins inhibit the activity of 3-hydroxyl-3-methyl coenzyme A (HMG-CoA) reductase, which catalyzes the rate-limiting step in cholesterol biosynthesis [20]. Statins are widely prescribed (to lower cholesterol) to hyperlipidemic patients at risk of cardiovascular disease [21]. Recently, it has been recognized that the protective effects of these drugs can be extended to myocardial-infarction patients with average plasma cholesterol concentrations, and that lipid reduction alone cannot entirely account for the benefits of statin therapy [22]. The reported beneficial effects that are independent of lipid lowering include improvements in endothelial function, which may be due to alterations in endothelial nitric oxide synthase expression and activity as well as to the antioxidant effects of statins [23, 24]. Further, the observation that statin-stimulated NO release and phosphorylation can be reduced by PI3-K/Akt inhibitors suggests that acute statin treatment of endothelial cells results in activation of PI3-K/Akt, indicating that statins directly increase NO by activating Akt [25, 26]. However, whether the impaired endothelial function and reduced Akt activity that are seen in the aorta in type 2 diabetic mice might be improved by chronic simvastatin treatment has not been investigated in detail.

We recently observed that both the relaxation and NO production induced by clonidine via the Akt pathway are impaired in aortic rings from a nicotinamide+STZ- induced type 2 diabetic mouse model [17]. In the present study, we examined clonidine- and adrenomedulin-induced relaxations (which are mediated through the Akt pathway) to determine whether the impairments seen in aortas isolated from type 2 diabetic mice might be improved by chronic simvastatin treatment.

## Experiment

### *Reagents*

STZ, clonidine hydrochloride, L-NNA and (-) adrenomedullin 52 human (AM) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sodium nitroprusside dehydrate (SNP) was from Wako (Osaka, Japan). All drugs were dissolved in saline, except where otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath.

### *Experimental design*

To induce diabetes, Institute of Cancer Research (ICR ; Tokyo Animal Laboratories, Tokyo, Japan) mice (males; 5 weeks old) received an intraperitoneal injection of 1.5g/kg body weight of nicotinamide dissolved in saline 15 min before an injection via the tail vein of STZ 200 mg/kg dissolved in a citrate buffer [17]. At 12 weeks after nicotinamide-STZ administration, systolic blood pressure was measured in each mouse (see below), and mice were then anesthetized with diethyl ether and euthanized by decapitation. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Science, Sports, and Culture, Japan). Mice were randomly allocated to one of three groups as follows. Starting 8 weeks after the nicotinamide+STZ treatment, diabetic mice were fed for 4 weeks on a normal diet either containing or not containing simvastatin (Banyu Tokyo, Japan) (10 mg/kg/day). These mice are referred to as DM-simvastatin and DM groups, respectively. Age-matched control mice (Cont) were fed a normal diet throughout.

### *Measurement of plasma glucose, insulin, and cholesterol, and blood pressure*

Plasma parameters and blood pressure were measured as described previously [25, 26]. Briefly, plasma glucose and cholesterol levels were each determined by the use of a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan). Plasma insulin was measured by enzyme immunoassay (Shibayagi, Gunma, Japan). Systolic blood pressure and diastolic blood pressure were measured by the tail-cuff method using a

blood pressure analyzer (BP-98A; Softron, Tokyo, Japan), while mice were in a constant-temperature box at 37°C.

***Measurement of isometric force***

Each aorta was separated from the surrounding connective tissue and cut into rings, as previously described [5]. For the relaxation studies, rings were precontracted with an equieffective concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)($10^{-6}$ ~ $3x10^{-6}$ mol/L). When the $PGF_{2\alpha}$-induced contraction had reached a plateau level, clonidine ($10^{-9}$ ~ $10^{-5}$ mol/L), adrenomedullin ($10^{-10}$~$3\times10^{-8}$mol/L), or sodium nitroprusside (SNP) ($10^{-10}$ ~ $10^{-5}$ mol/L) was added in a cumulative manner. When the effects of an Akt inhibitor (CALBIOCHEM, Dermstadt, Germany) (7x $10^{-7}$ M), on the response to a given relaxant agent were to be examined, the inhibitor was added to the bath 30 min before the application of $PGF_{2\alpha}$.

***Measurement of $NO_2^-$ and $NO_3^-$***

The concentrations of nitrite and nitrate in the effluent from each tissue were sampled and assayed by the method described previously (ENO-20; Eicom). Each aorta was cut into transverse rings 5 mm in length. These were placed in 0.5 mL Krebs-Henseleit-solution at 37°C. Samples were collected on two occasions as follows: for one 20-min period before and one after application of $10^{-6}$ mol/L clonidine. The amount of $NO_x^-$ was calculated as follows: agonist-stimulated NOx ($10^{-7}$ mol $min^{-1}$ $g^{-1}$) = sample / 20 (min) · g (weight of the frozen aorta). The concentrations of $NO_2^-$ and $NO_3^-$ in the Krebs-Henseleit-solution and the reliability of the reduction column were examined in each experiment.

***Measurement of the protein expressions of Akt, and of Phospho-Akt and Phospho-PTEN (by Western blotting)***

Aortas (3 pooled vessels, total protein 200 μg) were homogenized in ice-cold lysis buffer, as previously described [17, 27, 28]. Samples (20 μg/lane) were resolved by electrophoresis on 7.5% SDS-PAGE gels, then transferred onto PVDF membranes. The membrane was incubated with anti-Akt antibody (1:1000; Cell Signaling Technology), anti-Phospho-Akt (Ser473) antibody (1:1000; Cell Signaling Technology), anti-Phospho-PTEN (Ser380)

antibody (1:1000; Cell Signaling Technology), or β-actin (1:5000; Sigma) in blocking solution. Horseradish-peroxidase-conjugated, anti-rabbit antibody (Vector Laboratories) was used at a 1:4000 dilution in Tween PBS, followed by detection using SuperSignal (PIERCE). To normalize the data, we used β-actin as a housekeeping protein. The optical densities of the bands on the film were quantified using densitometry, with correction for the optical density of the corresponding β-actin band.

***Statistical analysis***

Data are expressed as the mean ± S.E. mean. When appropriate, statistical differences were assessed by Dunnett's test for multiple comparisons after one-way analysis of variance. Statistical comparisons between concentration-response curves were made using one-way ANOVA, with Bonferroni's correction for multiple comparisons being performed post hoc. In each test, $P<0.05$ was regarded as significant.

## Abbreviations

ACh, acetylcholine chloride; c-GMP, guanosine 3',5'-cyclic monophosphate; eNOS, endothelial NO synthase; GPCR, G-protein coupled receptor; HMG-CoA, 3-hydroxyl-3-methyl coenzyme A; IGF-1, insulin-like growth factor 1; KHS, Krebs-Henseleit solution; L-NNA, $N^G$-nitro-L-arginine; $NO_3^-$, nitrate; NO, nitric oxide; NOx, nitrite plus nitrate; PDK, PI-dependent kinase; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PI3-K, phosphatidylinositol-3-kinase; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome Ten; SNP, sodium nitroprusside dehydrate; STZ, streptozotocin; VEGF, vascular endothelial growth factor.

## Results

### *Plasma glucose, insulin, and cholesterol, and systolic blood pressure and body weight*

In the fairly recently devised experimental model employed here (adult mice or rats given streptozotocin [STZ] and partially protected with a suitable dose of nicotinamide) [28-32], the diabetic syndrome shares a number of features with human type 2 diabetes. It is characterized by stable moderate hyperglycemia, glucose intolerance, altered but significant glucose-stimulated insulin secretion, altered in vivo and in vitro responsiveness to tolbutamide, and a reduction in pancreatic ß-cell mass [30, 32]. As shown in Table 1, the nonfasting plasma glucose level and the plasma total cholesterol level were significantly elevated in nicotinamide + STZ-induced diabetic mice (versus age-matched controls). These increases were not affected by chronic (4 week) administration of simvastatin. Although systolic blood pressure was significantly higher in diabetic than in control mice, simvastatin treatment significantly reduced it (back to the control level). Plasma insulin and body weight were not different among the three groups.

Table 1.
Plasma glucose, plasma insulin, systolic blood pressure, diastolic blood pressure, plasma total cholesterol, and body weight in contols, nicotinamide + STZ - induced diabetic, and simvastatin - treated diabetic mice

| parameters | Cont (n=12) | DM (n=12) | DM-simvastatin (n=12) |
|---|---|---|---|
| Glucose (mg/dL) | 151.6±6.5 | 563.6±29.8‡ | 627.8±22.2‡ |
| Insulin (pg/mL) | 771.8±96.8 | 850.5±65.7 | 706.9±24.7 |
| Systolic blood pressure (mmHg) | 106.0±0.9 | 131.8±1.4‡ | 110.3±2.0* |
| Diastolic blood pressure (mmHg) | 69.9±0.9 | 81.1±1.7‡ | 70.6±2.0* |
| Total cholesterol (mg/dL) | 109.4±6.0 | 133.2±5.2# | 133.5±6.2# |
| Body weight (g) | 47.3±0.7 | 46.5±1.0 | 47.0±1.1 |

Values are means ± SE. Number of determinations is shown within parentheses.
‡ $p < 0.001$, #$p<0.01$ vs controls. *$p<0.001$ vs DM (diabetic mice)

***Vascular reactivity in aorta***

When the $PGF_{2\alpha}$ ($10^{-6}$~$3x10^{-6}$M)-induced contraction had reached a plateau, clonidine ($10^{-9}$~$10^{-5}$ M), SNP ($10^{-10}$~$10^{-5}$ M), or adrenomedullin ($10^{-9}$~$3x10^{-7}$ M) was cumulatively added. The clonidine-induced relaxation was significantly weaker (versus the controls) in aortic rings from diabetic mice at 8 weeks after administration of nicotinamide+STZ (Figure. 1A). This attenuated relaxation was significantly improved by chronic simvastatin treatment (closed symbols Figure. 1B). The aortic relaxation induced by clonidine was almost completely abolished by preincubation with the Akt - inhibitor at $7x10^{-7}$ mol/L and L-NNA (NOS inhibitor) at $10^{-5}$ mol/L (open symbols in Figure. 1B and 1C). The relaxation induced by SNP did not differ significantly among the three groups (Figure. 1D). The relaxation induced by adrenomedullin was significantly weaker in rings from diabetic mice (versus the controls), and this impaired relaxation response was recovered by chronic simvastatin treatment (closed symbols in Figure. 2). In the presence of the Akt inhibitor ($7x10^{-7}$M), the aortic relaxation induced by adrenomedullin was not significantly different among the three groups (open symbols in Figure. 2). However, in simvastatin-treated diabetic mice the aortic relaxation induced by adrenomedullin was significantly weaker in the presence of the Akt inhibitor than in its absence (Figure. 2).

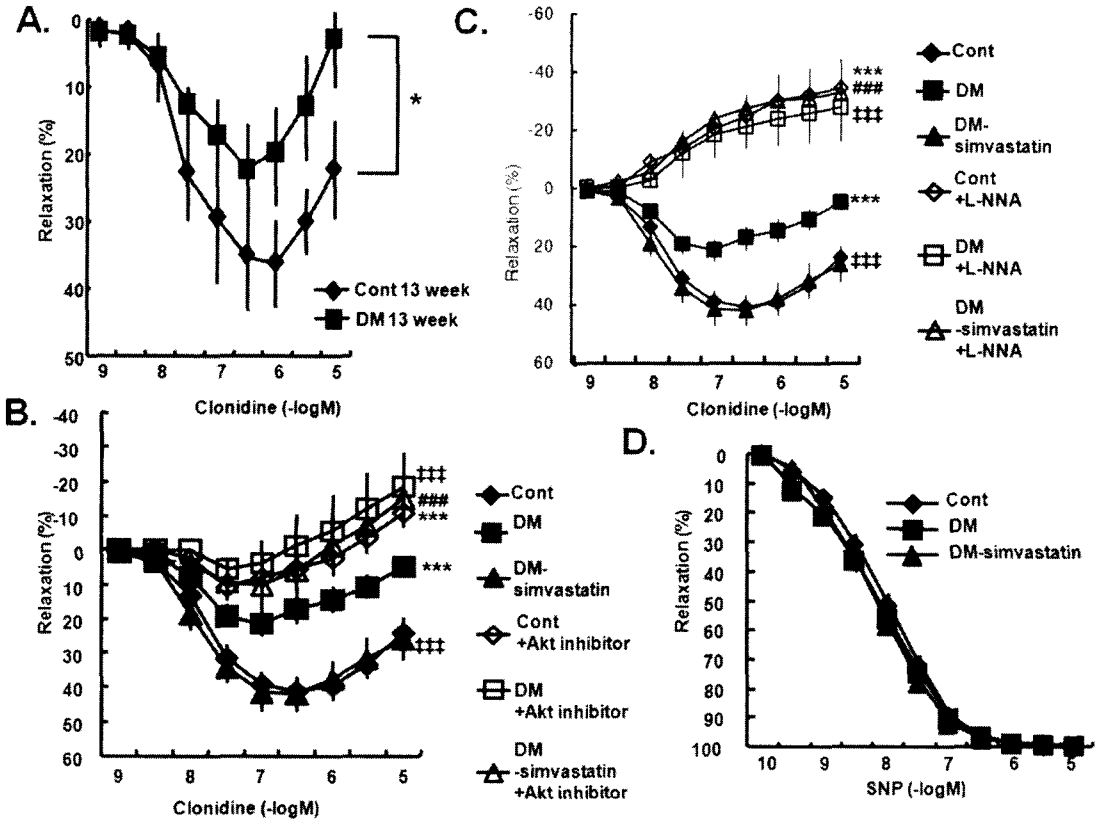


**Figure 1.** (A) Concentration-response curves for clonidine-induced relaxation in isolated thoracic aortic rings from 13-week-old control and diabetic [8 weeks after administration of nicotinamide+STZ] mice. Concentration-response curves for (B) (C) clonidine- and (D) SNP-induced relaxation in isolated thoracic aortic rings from control, diabetic, and simvastatin-treated diabetic mice. Each data-point represents the mean±S.E.M. of 5-8 observations. (A) *p<0.05 (B) (C) ***p<0.001 vs control; ‡‡‡p<0.001 vs DM; ###p<0.001 vs DM-simvastatin

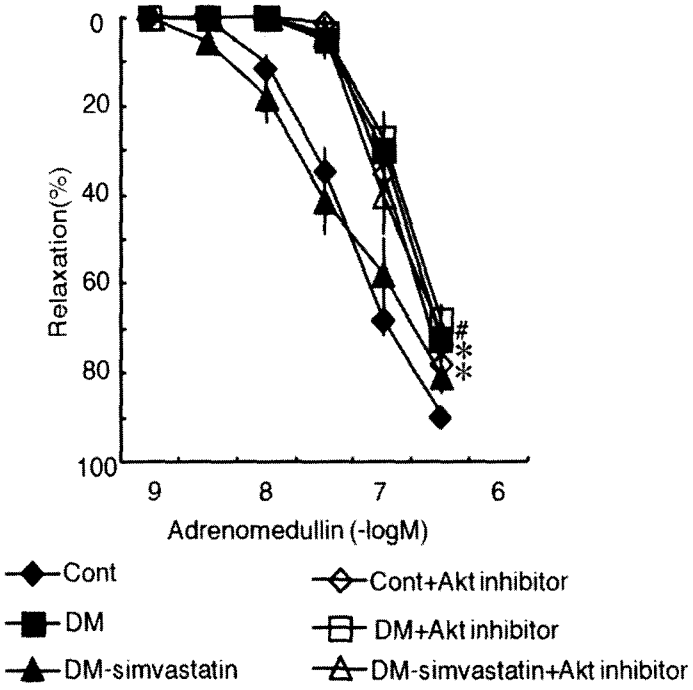


**Figure 2.** Concentration-response curves for adrenomedullin-induced relaxation in isolated thoracic aortic rings from control, diabetic, and simvastatin-treated diabetic mice, together with effects of an Akt inhibitors. Each data-point represents the mean±S.E.M. of 5-7 observations. *p<0.05 vs control; #p<0.05 vs DM-simvastatin

***Measurement of $NO_x^-$ production***

Clonidine increased the $NO_x^-$ ($NO_2^-$ + $NO_3^-$) level in the perfusate from aortic rings, but the increase was significantly smaller in the diabetics (versus the controls) (Figure. 3). This decrease in $NO_x^-$ production was significantly recovered by chronic simvastatin treatment.

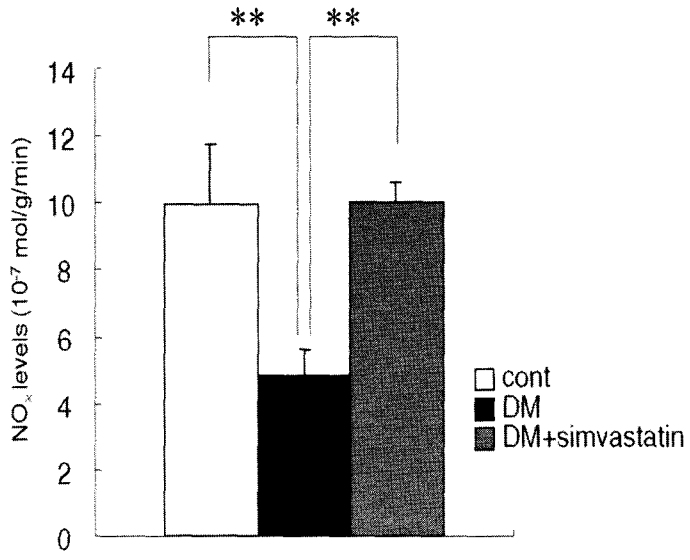


**Figure 3.** Release of $NO_2^-$ plus $NO_3^-$ ($NO_x^-$) from clonidine ($10^{-6}$ mol/L)-stimulated aortic rings in KHS. Each column represents the mean±S.E.M of 8 observations. **p<0.01

***Expressions of Akt Protein and Phosphorylated Akt and PTEN, and effects of clonidine***

Use of anti-Akt antibody allowed detection of an immunoreactive protein with a molecular weight of 60 kDa. The expression of this Akt protein was significantly decreased in aortas from diabetic mice (versus the controls), and this reduction was significantly recovered by chronic simvastatin treatment (Figure. 4A, B). We next evaluated clonidine-induced Akt phosphorylation (clonidine-stimulated aorta / clonidine-stimulated aorta with Akt inhibitor). Clonidine-stimulated Akt phosphorylation was significantly weaker in aortas from diabetic mice (versus the controls), and this reduction was significantly improved by chronic simvastatin treatment (Figure. 4C, D).

Use of anti-phospho-PTEN antibody allowed detection of an immunoreactive protein with a molecular weight of 74 kDa. The expression of phosphor-PTEN protein induced by clonidine was significantly increased in aortas from diabetic mice (versus the controls) (Figure. 5). This enhancement was not affected by chronic simvastatin treatment.

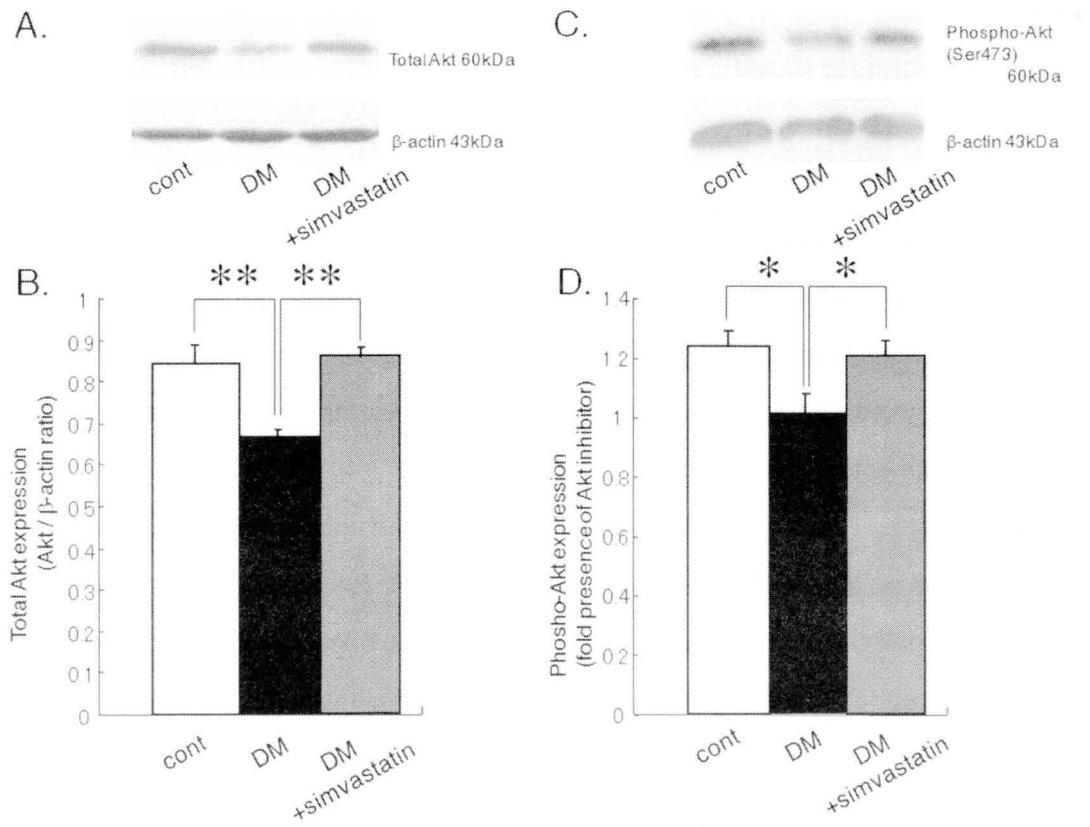


**Figure 4.** (A & B) Protein expression and phosphorylation of Akt in aortas from control, diabetic, and simvastatin-treated diabetic mice. [(A) expression of total Akt assayed by Western blotting; (B) quantitative analysis of total Akt expression by scanning densitometry.] (C & D) Protein expression of phosphorylated Akt in aortas from control, diabetic, and simvastatin-treated diabetic mice. [(C) expression of clonidine-induced phospho-Akt-Ser473 assayed by Western blotting; (D) quantitative analysis by scanning densitometry of clonidine-induced phospho-Akt-Ser473 [clonidine-stimulated aorta / clonidine-stimulated aorta in presence of Akt inhibitor]]. Values are each the mean±S.E.M of 10 determinations. **p<0.01 *p<0.05

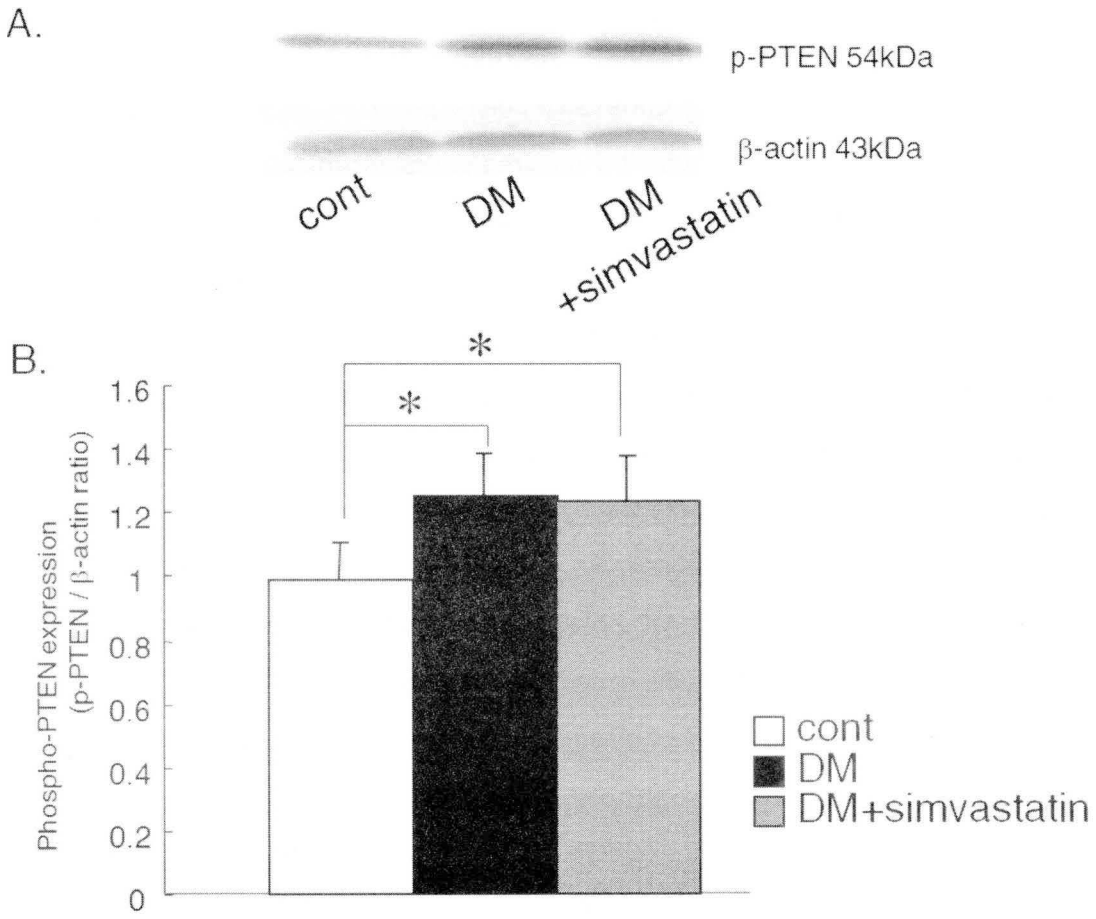


**Figure 5.** Protein expression of phosphorylated PTEN in aortas from control, diabetic, and simvastatin-treated diabetic mice. (A) Expression of clonidine-induced phospho-PTEN assayed by Western blotting. (B) Quantitative analysis of phospho-PTEN expression by scanning densitometry. Values are each the mean±S.E.M of 10 determinations. *$p<0.05$

## Discussion

The novel findings made in the present study were that in type 2 diabetic mice, chronic simvastatin treatment: 1) normalized the raised blood pressure without lowering the plasma cholesterol level, 2) improved the impaired endothelium-dependent aortic vasorelaxation responses to clonidine and adrenomedullin and restored clonidine-induced NO production, 3) improved the decreased expression of Akt protein and Akt activity, but 4) did not affect the clonidine-induced increase in phosphorylated PTEN.

When simvastatin was administered for 4 weeks to our nicotinamide+STZ-induced diabetic mice, there was no significant effect on the plasma glucose, insulin, or cholesterol levels. Thus, its beneficial effects are clearly unrelated to a correction of the hyperglycemia and hypercholesterolemia in these animals. In the present study, the short-term treatment with statins did not lower plasma total cholesterol as usually seen in humans [33]. This result is consistent with the previous results described by Sparrow et al. [34], who showed that simvastatin treatment does not affect plasma cholesterol levels after 6 weeks dosing with 10 or 100mg/kg of simvastatin.

The high blood pressure seen in these diabetic mice was improved by chronic simvastatin treatment, a result consistent with the reported blood pressure-lowering effect of statins. One possible mechanism that may participate in the blood pressure-lowering effect of statins may be a reduction in the extracellular fluid volume. Indeed, young spontaneously hypertensive rats (SHR), lovastatin reportedly attenuated the hypertension and shifted the pressure-natriuretic curve to a lower perfusion pressure [35]. It has been reported that mice lacking the gene for endothelial nitric oxide synthase show hypertension [36], suggesting that the consequential impairment in endothelium-dependent vascular relaxation may be involved in the pathophysiology of their hypertension. Clonidine-induced endothelium-dependent vascular relaxation was impaired in our type 2 diabetic mice, and this endothelial dysfunction was significantly recovered by the chronic administration of simvastatin. Hence, we propose that the anti-hypertensive effect of simvastatin may be due to an improvement in endothelial dysfunction in type 2 diabetes.

The most important finding made in the present study was that the chronic simvastatin treatment improved the endothelial dysfunction seen in our type 2 diabetic mice. We examined the endothelium-dependent and –independent relaxations of the isolated aorta to clonidine, adrenomedullin, and SNP (Figures. 1B, 1C and 2). One well-documented pathway downstream of activated G-protein coupled receptors (GPCRs) includes dissociation of $G_{\alpha\beta\gamma}$ trimers and production of $G_{\alpha}$ monomer and $G_{\beta\gamma}$ dimer, and involvement of the latter proteins in signal transduction events downstream of $\alpha$-adrenoceptors. $G_{\beta\gamma}$ dimmers can initiate intracellular signal transduction events. PI3-K was identified as a major effector of $G_{\beta\gamma}$ in various cell and tissue preparations [37, 38]. There are known to be functional $\alpha_2$-adrenoceptors and insulin receptors on the endothelium, and stimulation of NO production via each receptor has been shown to inhibit the contractile effects of $\alpha$-adrenergic agonists and catecholamines in vascular smooth muscle [16, 39]. In our previous study on the mouse aorta, we found that when a PI3-K or Akt inhibitor was applied to control aortas, there was no significant difference in the ACh-induced relaxation or ACh-induced in $NO_x^-$/cGMP production, whereas the clonidine-induced and insulin-induced relaxation responses were completely abolished by each [17]. Those results indicated that the endothelium-dependent relaxation induced by clonidine, but not that induced by ACh, is regulated by the PI3-K/Akt signal pathway. In addition, adrenomedullin reportedly increases NO production via the PI3-K/Akt/eNOS pathway in the rat aorta [14], and since the adrenomedullin-induced relaxation was significantly decreased by an Akt inhibitor and L-NNA, this relaxation is presumably also regulated by the PI3-K/Akt/eNOS signal pathway. Further, the endothelium-dependent aortic relaxations to clonidine and adrenomedullin were weaker in our diabetic group than in the controls, a dysfunction that was improved by 4 weeks' simvastatin treatment. Moreover, the decreased clonidine-induced $NO_x^-$ level was normalized by such simvastatin treatment. In addition, both Akt expression and the clonidine-induced Akt phosphorylation was decreased in our diabetic mice, and this decrease was normalized by chronic simvastatin treatment, suggesting that the impairment of the endothelium seen in the type 2 diabetic state may be related to decreases in Akt protein expression and activity. Recently (in addition to insulin, VEGF, β-agonists, and shear-stress signals), it has been reported that HMG-CoA reductase inhibitors can activate

PI3-K/Akt and NO production. We suggest that chronic Akt activation by simvastatin induced correct Akt expression and phosphorylation. These results strongly suggest that in type 2 diabetic mice, chronic simvastatin treatment improves endothelial function and normalizes blood pressure via a increase in Akt-associated eNOS activity.

That the PI3-K/Akt pathway plays a crucial role of in cell survival is supported by the observation that the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten), which is inactivated in a number of human cancers, possesses 3'-phosphoinositide-phosphatase activity, and thereby inactivates the PI3-K/Akt pathway [40]. Akt phosphorylation is regulated by PIP3, which recruits PDKs and Akt to the plasma membrane, allowing Akt regulatory residues to be more accessible to PDKs and to be phosphorylated [41, 42]. PIP3 levels are tightly regulated by phosphatidylinositol (PI) 3K and phosphatases, such as PTEN, which has been shown to antagonize PI3-K/Akt signaling by dephosphorylating PIP3 [43, 44]. A very interesting made in the present study was that clonidine-induced level of phosphorylated PTEN was significantly greater in aortas from diabetic mice than in the controls. To our knowledge, this is the first report of an increase in PTEN activity in the diabetic state. This finding strongly suggests that PTEN is also involved in the decreased activity of the PI3-K/Akt pathway, and this decrease may lead to reduction in both eNOS activity and endothelium-dependent relaxation. Chronic administration of simvastatin to our type 2 diabetic mice did not cause a recovery in PTEN phosphorylation, but it did lead to a recovery in endothelial function, suggesting that the improvement in endothelial dysfunction brought about by simvastatin is due to increased Akt expression and phosphorylation, and not directly to Akt dephosphorylation.

In conclusion, the present data shows that 4 weeks' simvastatin treatment improves blood pressure, as well as clonidine- and adrenomedullin-mediated vasorelaxations and NO production in aortas from a type 2 diabetic mouse model. These effects may be accompanied by increases in Akt protein expression and phosphorylation. We propose that simvastatin may improve at least some diabetes-related cardiovascular diseases through increases in Akt expression and Akt activity without lowering the plasma cholesterol level.

## Conclusions

**In the present study, I have investigated the relationships among functional changes in insulin resistance and vascular function in the diabetic state. I also tried the effects of the chronic administration of simvastatin to type 2 diabetic model mice associated with insulin resistance on endothelium-dependent relaxation. I believe that these experimentations play an important role in therapeutic targets for insulin resistance syndrome.**

The above findings lead to the following conclusions;

(Chapter 1) I designed this study to determine whether a high insulin level and a diabetic state do need to exist together to cause an impairment of endothelium-dependent relaxation. In diabetic-rat aortas organ-cultured with insulin [vs. both control-rat aortas cultured with insulin and diabetic-rat aortas cultured in serum-free medium]: 1) the relaxation responses to both ACh and Angeli's salt were significantly weaker, 2) ACh-stimulated NO production was significantly smaller, 3) superoxide and NO productions into the culture medium were greater, and 4) the levels of both nitrotyrosine and tyrosine-nitrated sarco/endoplasmic reticulum calcium ATPase (SERCA) protein were greater. The insulin-induced effects were prevented by co-treatment with either a superoxide scavenger or a peroxynitrite scavenger. After preincubation with an irreversible SERCA inhibitor, the relaxation induced by the nitric oxide donor was significantly impaired in control aortas cultured with or without insulin and in diabetic aortas cultured without insulin, but not in diabetic aortas cultured with insulin. These results suggest that the co-existence of a high insulin level and an established diabetic state may lead to an excessive generation of peroxynitrite, and that this may in turn trigger an impairment of endothelium-dependent relaxation via a decrease in SERCA function.

(Chapter 2) I investigated the effects of chronic simvastatin treatment on the impaired endothelium-dependent relaxation seen in aortas from type 2 diabetic mice associated with

insulin resistance. Starting at 8 weeks of diabetes, simvastatin (10 mg/kg/day) was administered to diabetic mice for 4 weeks. The significantly elevated systolic blood pressure in diabetic mice was normalized by simvastatin. Aortas from diabetic mice, but not those from simvastatin-treated diabetic mice, showed impaired endothelium-dependent relaxation in response to both clonidine and adrenomedullin. After preincubation with an Akt inhibitor, these relaxations were no significant difference among the three Akt inhibitor–treated groups (controls, diabetes, and simvastatin-treated diabetes). Although clonidine-induced $NO_x^-$ ($NO_2^-$ + $NO_3^-$) production was greatly attenuated in our diabetic model, it was normalized by simvastatin treatment. The expression levels of both total Akt protein and clonidine-induced Ser-473-phosphorylated Akt were significantly decreased in diabetic aortas, while chronic simvastatin administration improved these decreased levels. The expression level of clonidine-induced phosphorylated PTEN was significantly increased in diabetic aortas, but chronic simvastatin did not affect it. These results strongly suggest that simvastatin improves the endothelial dysfunction seen in type 2 diabetic mice via increases in Akt protein expression and Akt phosphorylation.

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## List of publications

1) Insulin-induced impairment via peroxynitrite production of endothelium-dependent relaxation and sarco/endoplasmic reticulum Ca(2+)-ATPase function in aortas from diabetic rats. : T. Kobayshi, K. Taguchi, **Y. Takenouchi,** T. Matsumoto, and K. Kamata. Free Radic Biol Med 2007; 43(3): 431-43.

2) Possible involvement of Akt activity in endothelial dysfunction in type 2 diabetic mice. : **Y. Takenouchi,** T. Kobayshi, T. Matsumoto, and K. Kamata. J Pharmacol Sci 2008 ;106(4):600-8.

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