several structural, functional and molecular changes from occurring in the blood vessel wall. We demonstrated that supplementation with BH4 protects against Ang-II-induced cardiovascular changes by suppressing Ang II-induced oxidative and nitrosative stress.

It has been reported that pterins, including BH4, have scavenging activity for relative oxygen species and antioxidant activity [22]. In the present study, long-term concomitant treatment with BH4 reduced Ang II-induced vascular superoxide production. Denuded endothelium demonstrated very little induction of superoxide production in response to Ang II in this study. We therefore propose that the main source of superoxide production is the endothelium. Within the vasculature, the key enzyme of superoxide production is NAD(P)H oxidase [23–26]. Angiotensin II appears to induce formation of the NAD(P)H oxidase complex, as well as translocation of several cytosolic NAD(P)H oxidase subunits (p47phox, p67phox and p40phox), which associate with membrane-bound subunits (p22phox and gp91phox), resulting in activation of NAD(P)H oxidase and generation of superoxide anions [27]. Increased endothelial production of superoxide anions in Ang II-infused rats was limited by concurrent administration of apocynin [16,17], indicating increased endothelial NAD(P)H oxidase activity in Ang II-infused animals. We propose that the increased endothelial production of superoxide anions is primarily due to an Ang II-induced upregulation of NAD(P)H oxidase subunits, since it has been demonstrated that Ang II-induced NAD(P)H oxidase activation is closely related to increased NAD(P)H oxidase enzyme expression in rats [24]. Thus, our results show that BH4 markedly suppresses Ang II-induced upregulation of NAD(P)H oxidase subunits, but it might be also possible that supplementation with BH4 reversed endothelial dysfunction partially as a result of its direct antioxidant effect.

BH4 was also observed to inhibit Ang II-induced vascular NO production. Ang II can activate nuclear factor kappaB (NF-κB) directly and/or through induction of superoxide production, which leads to induction of iNOS, resulting in considerable production of NO. BH4 supplementation might suppress NF-κB activation by reducing superoxide production, thereby inhibiting iNOS induction. Indeed BH4 was observed to markedly suppress iNOS induction in this study. It is possible that induction of superoxide and NO by Ang II might lead to the production of peroxynitrite, the result of which oxidation of BH4 might lead to 'uncoupling' of the NOS reaction [28,29]. Thus, BH4 supplementation might reduce Ang II-induced vascular peroxynitrite production, thereby preventing NOS uncoupling.

Another possibility is that BH4 enhances the expression and activity of endothelial NO synthase. Greater eNOS expression was observed in the aortas of Ang II + BH4 rats, compared with other groups. In addition, endothelium-dependent relaxation was enhanced at lower concentration of ACh (10^{-8} mol/l) by treatment Ang II with BH4, which occurred via a NO-dependent mechanism, in this study. The mechanism of enhanced eNOS expression in Ang II + BH4 rats remains to be elucidated.

Thus, BH4 may improve endothelial NOS function in Ang II-infused rats by functioning as a co-factor in NO production, and preferentially functions as a superoxide and peroxynitrite scavenger *in vivo*, thereby decreasing the induction of NADPH oxidase and iNOS to reduce peroxynitrite production. BH4 has been shown to scavenge superoxide with a rate constant of 10^5/mole per second and has also been shown to react with peroxynitrite [6,28,29]. The beneficial effect of BH4 supplementation implies that there might be an insufficient amount of BH4 in diseased aortic and heart tissue. However, BH4 levels were not significantly reduced in the aortic and heart tissue of Ang II-infused rats. Similarly, significant differences in plasma BH4 levels were not observed among the various groups included in this study; however, decreased ratios of BH4 to total biopterin were observed in Ang II-infused rats (~81%), while concomitant treatment with BH4 caused a return to normal levels (~90%). The reduced BH4 to total biopterin ratio might reflect Ang II-induced oxidative stress, and administration of BH4 might relieve this stress, resulting in normalization of the BH4 to total biopterin ratio.

In insulin-resistant rats, oral administration of 10 mg/kg BH4 for a longer period (8 weeks) has been demonstrated to prevent endothelial dysfunction and vascular oxidative stress in the aorta [30]. The dose of BH4 (20 mg/kg) was found to effectively reduce plasma phenylalanine concentrations in BH4-responders (BH4-deficient patients and patients with mild phenylananine hydroxylase deficiency) [31]. Thus, the dose of 20 mg/kg BH4 was used in the present study. It has been demonstrated that BH4 restores endothelial function in hypercholesterolemia [32]. Moreover, BH4 has also been shown to improve endothelium-dependent vasodilation in chronic smokers and patients with diabetes mellitus [33,34]. The present study found that BH4 not only improves endothelial function, but also prevents the development of hypertension and cardiomegaly in Ang II-infused rats. This effect may be mediated through a reduction in oxidative and nitrosative stress. Although this experimental approach is very useful, these animal models also have their limitations. However, BH4 may provide a new therapeutic approach in the treatment of oxidative/nitrosative stress-induced cardiovascular disease.

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Stimulated HSP90 binding to eNOS and activation of the PI3–Akt pathway contribute to globular adiponectin-induced NO production: Vasorelaxation in response to globular adiponectin

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Abstract

The present study examined potential interactions between endothelial NO synthase (eNOS), heat shock protein (HSP)90, and Akt in vascular endothelial cells stimulated with globular adiponectin to produce nitric oxide (NO). Globular adiponectin-induced eNOS phosphorylation was accompanied by eNOS–HSP90–Akt complex formation, resulting in a dose-dependent increase in NO release. Globular adiponectin stimulated binding of HSP90 to eNOS, and inhibition of HSP90 significantly suppressed globular adiponectin-stimulated NO release. Globular adiponectin also caused Akt phosphorylation, and inhibition of PI3 kinase significantly suppressed globular adiponectin-stimulated NO release. This study also examined whether globular adiponectin really induces endothelial-dependent vasodilation using rings from rat thoracic aorta. It was observed that globular adiponectin caused dose-dependent vasorelaxation in the aorta. These results indicate that stimulated HSP90 binding to eNOS and activation of the PI3–Akt pathway contribute to globular adiponectin-induced eNOS phosphorylation and NO production, and to endothelium-dependent vasorelaxation.
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Keywords: Heat shock protein 90; Akt; eNOS; Adiponectin

Adiponectin is an important adipocytokine specifically secreted by adipocytes that circulates at relatively high levels in the bloodstream [1]. Adiponectin has potent anti-inflammatory and atheroprotective effects on vascular tissue, and has an insulin-sensitizing effect on tissue involved in glucose and lipid metabolism [1–4]. Adiponectin is reduced in patients with increased insulin resistance, such as obesity, type 2 diabetes, coronary artery disease, and hypertension [1–4]. Vascular endothelial dysfunction plays a pivotal role in the pathogenesis of atherosclerosis and increases the risk of future cardiovascular events [5,6]. Adiponectin has been shown to stimulate nitric oxide (NO) production in vascular endothelial cells [7,8] and additionally, endothelial dysfunction in humans has been linked with hypoadiponectinemia [9,10]. Thus, the observed relationship between insulin resistance and vascular endothelial cell dysfunction may be related to decreased levels of adiponectin.

A proteolytic cleavage product of adiponectin containing its globular head, known as globular adiponectin, has been found to circulate in human plasma [11]. Recent studies have shown that recombinant globular adiponectin is pharmacologically active and induces free fatty acid oxidation in incubated mouse muscle and cultured muscle cells [11]. In addition, when administered to mice fed a high fat meal, globular adiponectin was observed to cause a modest decline in plasma-free fatty acid and glucose levels, while chronic administration

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was observed to cause weight loss without diminished food intake [11]. Yamauchi et al. [12] have reported significantly greater potency of globular adiponectin in reversing insulin resistance than uncleaved adiponectin. We also reported that globular adiponectin potently upregulates NO production in vascular endothelial cells [8].

Recent studies indicate that eNOS is highly regulated by post-translational modifications, such as Akt-induced phosphorylation [13,14] and interaction with several regulatory proteins such as heat shock protein 90 (HSP90) [15–17]. Binding of HSP90 ensures recruitment of activated Akt to the eNOS–HSP90 complex and phosphorylation of eNOS [15]. HSP90 inhibitors, such as radicicol and geldanamycin, can increase eNOS-dependent superoxide anion production by uncoupling eNOS [18,19]. These observations suggest that the association of HSP90 and Akt with eNOS is critical in eNOS-associated NO production.

The present study examines potential interactions between eNOS, HSP90, and Akt in vascular endothelial cells stimulated to produce NO by globular adiponectin. We also examined whether globular adiponectin is capable of inducing endothelial-dependent vasodilation. For this, rings from rat thoracic aorta were exposed to globular adiponectin and endothelial vasodilator function was evaluated.

Materials and methods

Cell culture. Bovine aortic endothelial cells (BAECs) were allowed to grow to confluence in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and 10% fetal bovine serum. These cells exhibited immunohistochemical and morphological characteristics of endothelial cells. All the cells in this experiment were used within 3–5 passages and were examined to ensure that they demonstrated the specific characteristics of endothelial cells.

NO_x measurement. To detect NO production within endothelium monolayers, BAECs were cultured in 24-well dishes and studied 1 day after confluence. After 1 h of incubation, nitrite and nitrate levels (NO_2^- and NO_3^-) in the medium were measured with an automated NO detector/high-performance liquid chromatography system (ENO10, Eicom).

Determination of eNOS activity. Citrulline synthesis was measured by modification of a previously described technique [20,21]. Cell monolayers were incubated at 37 °C for 30 min in Hanks' balanced salt solution (pH 7.4) containing 0.5% FBS. Subsequently, cells were incubated with globular adiponectin and/or Ca ionophore in the presence of 10 μM L-arginine and 3.3 μCi/ml L-[^{3}H]arginine. After 15 min, the reaction was stopped with cold phosphate-buffered saline (PBS) containing 5 mM L-arginine and 4 mM EDTA, after which the cells were denatured with 96% ethanol. After evaporation, the soluble cellular components were dissolved in 50 mM Hepes, 5 mM EDTA (pH 5.5), and applied to 2-ml columns of Dowex AG50WX-8 (Na^+ form). Radioactivity corresponding to [^{3}H]citrulline within the eluate was quantified by liquid scintillation counting. This was expressed as femtomoles/mg of cell protein. Basal [^{3}H]citrulline synthesis was determined from L-NAME (1 mM, 30-min preincubation)-inhibitable radioactivity in unstimulated cells, which was not always detectable.

Immunoprecipitation and immunoblotting of eNOS, HSP90, and Akt. eNOS complex was incubated with anti-eNOS antibody in the corresponding NOS reaction buffer at 4 °C for 2 h, and then with protein G–agarose beads at 4 °C overnight. The immunoprecipitates were subjected to SDS–PAGE and then blotted onto polyvinylidene difluoride membranes. The blots were incubated with the primary antibody at 4 °C overnight and then probed with secondary antibody linked to peroxidase. Immunoreactive proteins were visualized on X-ray film by an enhanced chemiluminescent method. Anti-eNOS antibody and anti-HSP90 antibody were obtained from BD Transduction Laboratories (San Diego, CA), and anti-phospho-Ser-1179 eNOS polyclonal antibody and anti-Akt antibody, anti-phospho-Ser-473 Akt antibody were obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology.

Organ chamber experiments. Organ chamber experiments were performed as previously described [22]. Animals were anesthetized with pentobarbital and exsanguinated. The thoracic aortas were carefully dissected and all perivascular tissue was removed under a microscope in a physiological salt solution (PSS) of the following composition (in mmol/L): NaCl 121, KCl 4.7, $NaHCO_3$ 24.7, $MgSO_4$ 12.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, and glucose 5.8, aerated with 95% O_2 and 5% CO_2. The rings from each thoracic aorta (5 mm in length) were mounted vertically between two hooks in organ chamber myographs (Medical Supply), which were filled with PSS and kept at 37 °C. Isometric tension was measured with force transducers (Nihon Kohden). Each preparation was stretched in a stepwise manner to an optimal length, at which point the force induced by 118 mmol/L KCl became maximal and constant. After equilibration for at least 30 min, the rings were then pre-contracted with prostaglandin F2 (3–10 μmol/L). After a stable contraction was achieved, the rings were exposed to acetylcholine (ACh, 10^{-10} to 10^{-5} mol/L) and globular adiponectin to evaluate endothelial vasodilator function. The effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis, were also evaluated to show that vasodilatation is caused by NO.

Materials. Globular adiponectin was obtained from PeproTech EC. (London, UK). This recombinant protein is derived from mouse globular domain ACRP30 cDNA and is endotoxin-free according to the limulus test (Sigma; sensitivity, 0.06 U/ml).

Statistical analysis. Data are presented as means ± SEM. Multiple comparisons were evaluated by ANOVA followed by Fisher's protected least significant difference test. A value of $P < 0.05$ was considered statistically significant.

Results

Incubation of BAE with globular adiponectin increased the concentration of bioactive NO in the supernatant of the cells (as measured by NO_2^- and NO_3^- levels). Examination of the time course showed a substantial increase in NO production for 1 h, after which only a modest elevation in NO production was observed (3–8 h). As shown in Fig. 1A, incubation of BAE with globular adiponectin (0.01–2.5 μg/ml) increased NO production in a concentration-dependent manner. To measure eNOS activity, BAE were incubated with globular adiponectin (1 μg/ml) for 15 min, after which citrulline synthesis in the cells was measured. eNOS activity, measured in terms of citrulline production, was observed to significantly increase with globular adiponectin treatment (Fig. 1B).

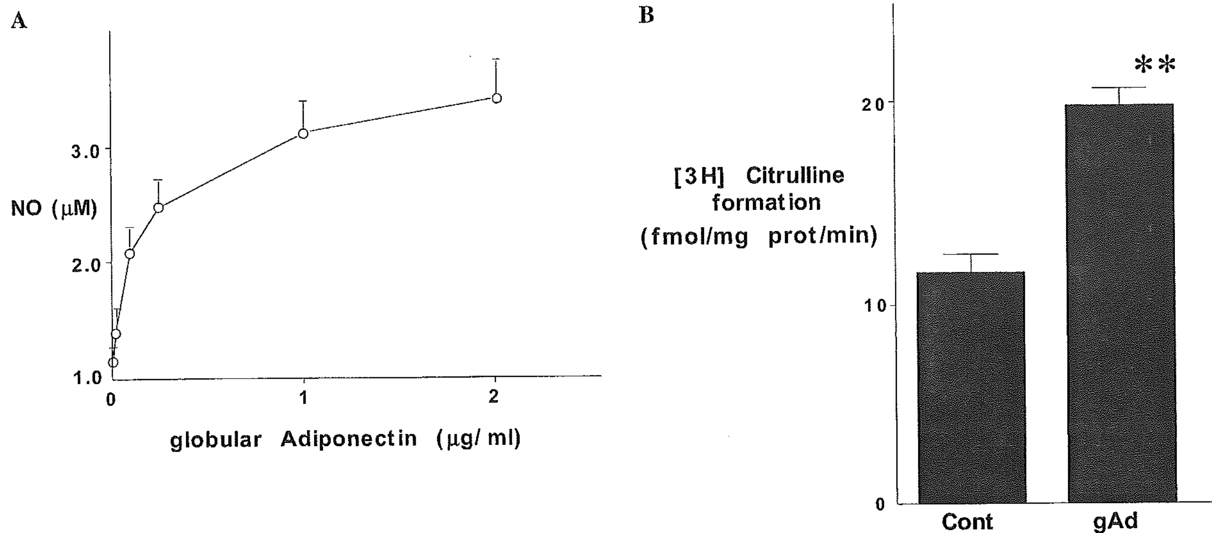

Fig. 1. (A) Effect of globular adiponectin on NO production. BAE were incubated with different concentrations of globular adiponectin for 60 min. NO_x in the medium was measured with an automated NO detector/high-performance liquid chromatography system. (B) Effects of globular adiponectin on eNOS activity. BAE were incubated with globular adiponectin for 15 min, after which eNOS activity was assayed by measuring the formation of L-[^{3}H]citrulline from L-[^{3}H]arginine. Values are means ± SE ($n = 3$). **$P < 0.01$ compared with basal.

The time course of globular adiponectin-induced eNOS–HPS90–Akt complex formation is shown in Fig. 2A. HSP90 increased 5 min after globular adiponectin and remained high for 20 min, while Akt was maximal at 5–10 min after globular adiponectin administration and had returned nearly to baseline by 15 min. Fig. 2B shows globular adiponectin-induced eNOS activation, eNOS–HSP90–Akt complex formation, and the effects of gelnadamycin (GA). Globular adiponectin-induced eNOS activation concomitant with formation with HP90 and activated Akt. GA disrupted association of HSP90, activated Akt with eNOS.

Next, the effects of GA and wortmannin on globular adiponectin-induced NO production in BAE were examined. Cells were pretreated with GA (1 μg/ml) or wortmannin (500 nM) for 30 min followed by globular adiponectin for 60 min. Pretreatment with GA or wortmannin significantly suppressed globular adiponectin-induced NO release (Fig. 3).

We then examined whether globular adiponectin is capable of inducing endothelial-dependent vasodilation. For this, the rings from rat thoracic aorta were exposed to globular adiponectin to evaluate endothelial vasodilator function. First, the rings were exposed to acetylcholine (ACh) as control, where ACh induced relaxation of aortic rings in a dose-dependent manner (Fig. 4). Then globular adiponectin was examined to determine whether it causes vasorelaxation of aortic rings. It was found that globular adiponectin caused a dose-dependent vasorelaxation (Fig. 4). Thirty-eight percent vasorelaxation was elicited at 2 μg/ml globular adiponectin, which corresponds to an Ach dose of ~30 nmol/L.

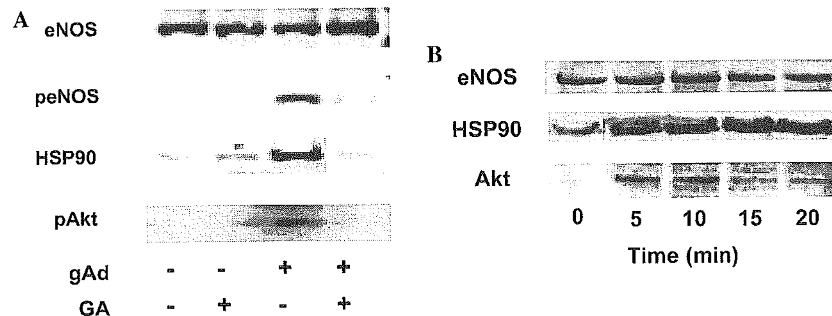

Fig. 2. (A) Time course of eNOS–HSP90–Akt complex formation. BAE were stimulated with globular adiponectin for the indicated times. eNOS was immunoprecipitated at each time point, and eNOS, HPS90, and Akt were evaluated by immunoblotting. (B) Globular adiponectin-induced eNOS activation and eNOS–HSP90–Akt complex formation in BAE. BAE were pretreated with GA and then stimulated with globular adiponectin for 10 min. eNOS was immunoprecipitated, and eNOS, peNOS, HSP90, and pAkt were evaluated by immunoblotting.

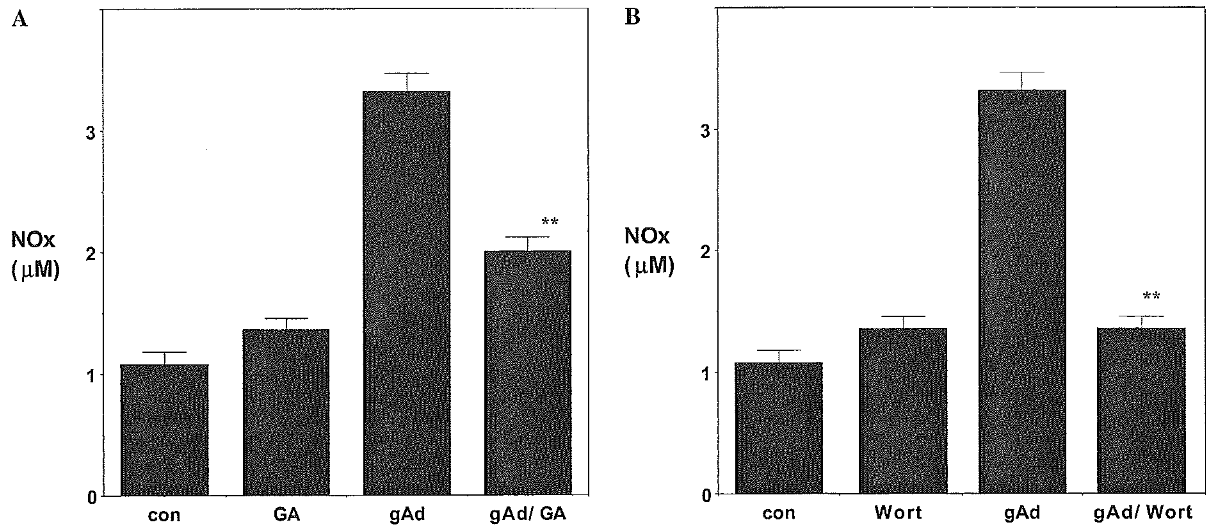

Fig. 3. Effect of PI3 kinase and HSP90 inhibitors on globular adiponectin-stimulated NO production. Cells were pretreated with geldanamycin (GA, 1 μg: A) or wortmannin (Wort, 50 nM: B) for 30 min followed by globular adiponectin for 60 min. NO_x in the medium was measured with an automated NO detector/high-performance liquid chromatography system. Data are represented as means ± SE ($n = 4$). $^{**}P < 0.01$ compared with globular adiponectin treatment.

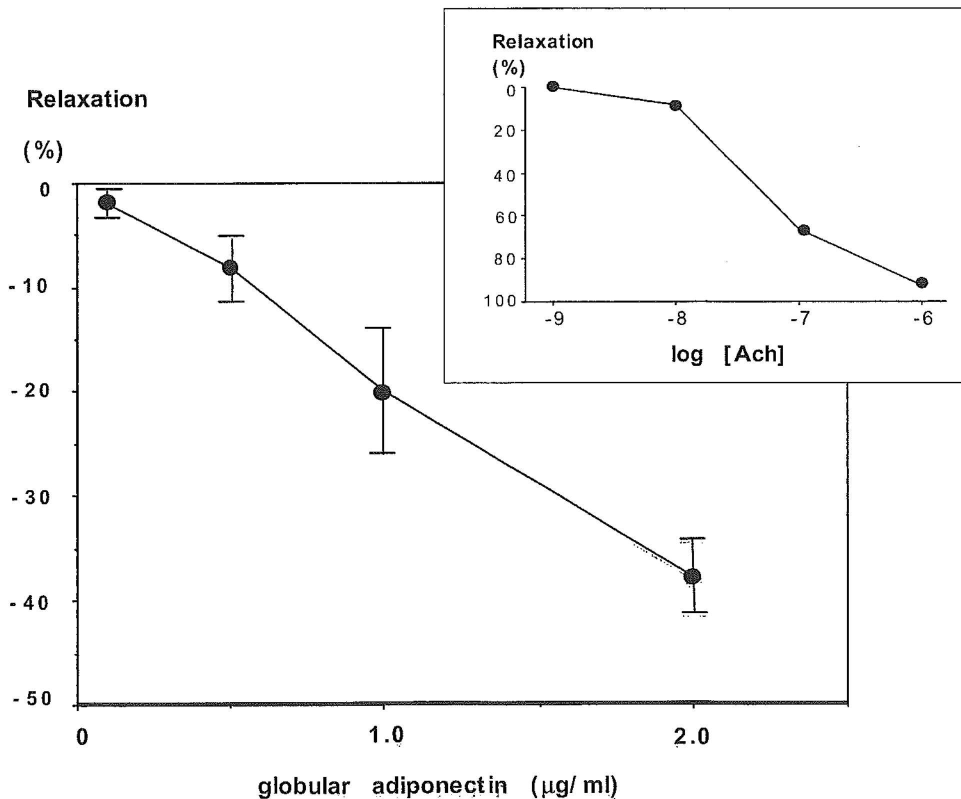

Fig. 4. Endothelium-dependent relaxation in response to acetylcholine (ACh) and globular adiponectin. The rings were exposed to ACh (10^{-10} to 10^{-5} mol/L) and globular adiponectin (0 to 2.0 μg/ml) to evaluate endothelial vasodilator function. Data are represented as means ± SE of 6–8 vascular rings.

This globular adiponectin-mediated vasodilation was abolished by administration of L-NAME (100 μmol/L). Further, vasorelaxation was not caused by globular adiponectin in aortic rings without endothelium (data not shown).

Discussion

Endothelial nitric-oxide synthase (eNOS) is the enzyme responsible for physiological production of NO in the vasculature [23]. eNOS is regulated by subcellular

localization, post-translational modification such as phosphorylation by Akt [24–26], and interactions with several regulatory proteins, such as heat shock protein 90 (HSP90) [14,27,28]. The present study demonstrates that globular adiponectin stimulates eNOS phosphorylation and that this phosphorylation is accompanied by eNOS–HSP90–Akt complex formation, resulting in a dose-dependent increase in NO release. Further, we show that globular adiponectin stimulated binding of HSP90 to eNOS and that inhibition of HSP90 significantly suppressed globular adiponectin-stimulated NO release. Globular adiponectin also caused Akt phosphorylation and blockade of this with a PI3 kinase inhibitor significantly suppressed globular adiponectin-stimulated NO release. These results indicate that activation of the PI3–Akt pathway and HSP90 binding to eNOS contribute to globular adiponectin-induced eNOS phosphorylation and NO production. It has recently been shown that HSP90 and Akt synergistically increase eNOS activity both in vitro and in intact endothelial cells at physiological Ca^{2+} concentrations. This is accompanied by the formation of a ternary complex comprised of HSP90, Akt, and calmodulin-bound eNOS [29]. GA abolishes this cooperative activation of eNOS by HSP90 and Akt, as does substitution of inactive Akt in eNOS activity assays, supporting the idea that HSP90 binding to eNOS and eNOS Ser-1179 phosphorylation by Akt are both essential for their synergistic effects on eNOS activity [29]. Our data therefore support the importance of HSP90 and Akt working together to activate eNOS when cells are stimulated by globular adiponectin.

We also demonstrated that globular adiponectin caused a dose-dependent vasorelaxation in rat aorta. This globular adiponectin-mediated vasodilation was abolished by administration of L-NAME (100 μmol/L). Vasorelaxation was not caused by globular adiponectin in aortic rings without endothelium, indicating that the effect of globular adiponectin on endothelial function was mediated in a NO-dependent mechanism. Thirty-eight percent vasorelaxation was elicited at 2 μg/ml globular adiponectin (120 nmol/L), which corresponds to approximately 30 nmol/L of Ach. Adiponectin is abundantly present in human plasma (range 3–30 μg/ml) and is present in serum as a trimer, hexamer, or high molecular weight form. Levels of globular adiponectin are normally low in serum [11,30] but could be higher at sites of inflammation. Indeed, generation of globular adiponectin by leukocyte elastase secreted by monocytic cell lines has recently been shown [31]. Adiponectin cleavage by leukocyte elastase could be one potential mechanism for the generation of globular adiponectin in plasma [31]. Circulating globular adiponectin may act on endothelial cells and induce vasorelaxation under such conditions.

Recently, it was demonstrated that adiponectin can directly stimulate the production of NO through AMP-activated protein kinase (AMPK)–PI3K–Akt–eNOS signalling axis [7,32]. It was also shown that adiponectin dose-dependently suppressed apoptosis and caspase-3 activity in human umbilical vein endothelial cells [30]. Further, transduction with dominant-negative AMPK abolished the suppressive effect of adiponectin [30]. We confirmed that mRNA of AdipoR1, which has a high affinity for globular adiponectin, is predominantly expressed in endothelial cells, while AdipoR2 mRNA levels are very low. Thus, globular adiponectin exerts its effect at least partly through AMPK activation, which leads to HSP90 binding to eNOS and activation of the PI3–Akt pathway, resulting in eNOS phosphorylation and NO production by globular adiponectin-stimulated endothelial cells bringing about endothelial-dependent vasorelaxation.

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Hypoadiponectinemia is caused by chronic blockade of nitric oxide synthesis in rats

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Abstract

Adiponectin is an adipocyte-derived anti-atherogenic protein. Adiponectin levels are decreased in patients and animal models with obesity, diabetes, and coronary artery disease. However, the mechanism by which adiponectin levels are reduced remains unknown. Since hypoadiponectinemia is closely linked to endothelial dysfunction, we examined the regulation of adiponectin in a rat model of chronic blockade of nitric oxide (NO) synthesis by N^{ω}-nitro-L-arginine methyl ester (L-NAME). Decreased production of NO and increased production of O_2^- were observed in aorta from L-NAME-treated rats. Plasma adiponectin levels and adiponectin mRNA levels of adipose tissue were markedly decreased in L-NAME-treated rats. Cotreatment of pioglitazone (PIO) or allopurinol (ALL) with L-NAME restored plasma adiponectin concentration and fat adiponectin mRNA levels to control levels. Thus, adiponectin levels were decreased in L-NAME-treated rats, however, they returned to normal following administration of PIO due to transcriptional activation of the adiponectin gene, as well as administration of ALL, likely due to elimination of oxidative stress. Oxidative stress appears to be an important cause of hypoadiponectinemia.

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1. Introduction

Adiponectin is abundant in human plasma, and decreased levels are observed in patients and animal models with obesity, diabetes, and coronary artery disease [1-5]. Adiponectin has a number of vascular protective effects [6-10]. These findings suggest that decreased plasma adiponectin levels in the context of obesity and diabetes may contribute to vascular disease in these patients. However, the mechanism by which adiponectin levels are decreased remains unknown. Vascular endothelial dysfunction plays a pivotal role in the pathogenesis of atherosclerosis and enhances the risk of future cardiovascular events [11,12]. Adiponectin has been shown to stimulate nitric oxide (NO) production in vascular endothelial cells [13,14]. A strong link between hypoadiponectinemia and endothelial dysfunction has been reported in man [15,16]. Thus, the established relationship between insulin resistance and vascular endothelial cell dysfunction might be partly explained by decreased levels of adiponectin. Therefore, it is important to investigate the regulation of adiponectin levels in man or animals with endothelial dysfunction. The present study examined plasma adiponectin levels in a rat model with blockade of NO synthesis by administration of an inhibitor of NO synthesis, N^{ω}-nitro-L-arginine methyl ester (L-NAME). We found decreased plasma levels of adiponectin in rats treated with L-NAME. To elucidate the mechanism of the observed effects of L-NAME, we then examined the expression of adipose tissue adiponectin mRNA in these rats. We also examined whether antioxidant drugs might reverse the effects of L-NAME on decreased adiponectin levels.

2. Materials and methods

2.1. Animal model of inhibition of NO synthesis

The present experiments were reviewed and approved by the Committee on Ethics of Animal Experiments, and were conducted according to the Guidelines for Animal Experiments, at Dokkyo University Faculty of Medicine.

Fourteen-week-old male Wistar-Kyoto rats were housed singly in a pyrogen-free facility. Four groups of rats were studied. The first (control) group received untreated laboratory chow and drinking water. The second group (L)

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received L-NAME in the drinking water (1 mg/mL). At this concentration, the daily intake of L-NAME was 100 mg/kg per day [17,18]. The third group (P) received pioglitazone (20 mg/kg) in the chow. The fourth group (L+A) received L NAME in the drinking water and allopurinol by intraperitoneal injection (30 mg/kg per day).

2.2. Vessel Harvesting and preparation

On day 5 of treatment, we measured heart rate as well as systolic blood pressure by the tail-cuff method. The rats were anesthetized with intraperitoneally administered pentobarbital, and the chest was opened. With the heart still beating, heparin (150 IU) was given via intracardiac injection. The thoracic aorta was removed en bloc and placed in cold Krebs-Henseleit solution. Extravascular tissue was removed rapidly, and the vessel lumen was flushed with the solution. In some rats, the aorta was cut into three 5-mm ring segments for use in studies of NO production, as well as superoxide anion production.

2.3. Measurement of NO production

The 5-mm ring segments of the aorta were incubated in 2 mL of Hanks' balanced salt solution containing a calcium ionophore A23187 (1 μmol/L) and L-arginine (100 μmol/L), as previously described. A chemiluminescence-based NO analyzer (270B, Sievers) was used to measure NO production. Specific NO-generating capacity was expressed as nanomoles per hour per dry weight.

2.4. Measurement of plasma NO

Plasma nitrite and nitrate levels (NO_2^- and NO_3^-) were measured with an automated NO detector/high-performance liquid chromatography system (ENO10, Eicom Co, Kyoto, Japan). Nitrite and nitrate in the dialysate were separated using a reverse-phase separation column, and nitrate was reduced to nitrite in a reduction column. Nitrite was mixed with a Griess reagent, after which the absorbance at 540 nm was measured by flow-through spectrophotometry.

2.5. Measurement of vascular superoxide anion production

Superoxide anion production was measured using lucigenin chemiluminescence, as previously described [19]. Briefly, the thoracic aortas were carefully dissected and cleared of perivascular tissue and blood contaminants under a microscope, after which they were placed in HEPES-buffered physiological salt solution (in mmol/L: NaCl 121, KCl 4.7, $NaHCO_3$ 24.7, $MgSO_4$ 12.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, and glucose 5.8, aerated with 95% O_2 and 5% CO_2). In a preliminary study, we confirmed no adhesion of inflammatory cells to the endothelium (data not shown). Scintillation vials containing 1 mL HEPES-buffered PSS with 5 μmol/L lucigenin (bis-N-methylacridinium nitrate) were placed into a scintillation counter (Luminescence Reader BLR 301, ALOKA, Tokyo, Japan). We used Tiron (10 mmol/L; Sigam, Tokyo, Japan), a superoxide scavenger, in all experiments to confirm the validity of our technique with lucigenin. After dark adaptation, background counts were recorded for 3 minutes and then 3 vascular segments (5 mm in length) from each thoracic aorta were added to the vial. Scintillation counts were then recorded every minute for 10 minutes and the respective background counts were subtracted. The vessels were then dried for determination of dry weight. Lucigenin counts were expressed as counts per minute per milligram of dry weight.

2.6. Measurement of adiponectin levels in plasma

The plasma concentration of adiponectin was determined by ELISA using a kit for measurement of rat/mouse adiponectin (Otsuka Phamaceuticals, Tokyo, Japan).

2.7. Measurement of adiponectin mRNA levels in adipose tissue

Standard Northern blotting was used to investigate the mRNA expression of adiponectin in adipose tissue, as previously described [20]. After probing for adiponectin, filters were stripped and re-probed for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Expression of eNOS, iNOS, p22phox, gp91phox, p47phox, Rac1 and GAPDH mRNA was also analyzed by reverse transcription-polymerase chain reaction (RT-PCR), as previously described [20].

2.8. Statistical analysis

Data are expressed as mean values ± SEM. Differences between two experiments were compared by Student *t* tests. Differences between three experiments were determined by 2-way ANOVA and Bonferroni's multiple comparison test. A *P* value of .05 was considered statistically significant.

3. Results

3.1. Body weight and hemodynamic parameters

During the 5-day treatment period, body weights in the control and L groups did not change, and weights did not differ significantly between the 2 groups. During

Table 1

Groups	Body weight, g	Systolic blood pressure, mmHg	Heart rate, bpm
Control group (n = 9)			
Day 0	302 ± 10	126 ± 11	398 ± 11
Day 5	313 ± 12	130 ± 8	402 ± 12
L group (n = 9)			
Day 0	312 ± 7	128 ± 9	408 ± 15
Day 5	314 ± 12	169 ± 13*,**	339 ± 11*,**

Data are mean ± SE.

* $P < 0.01$ vs control group.

** $P < 0.01$ vs day 0.

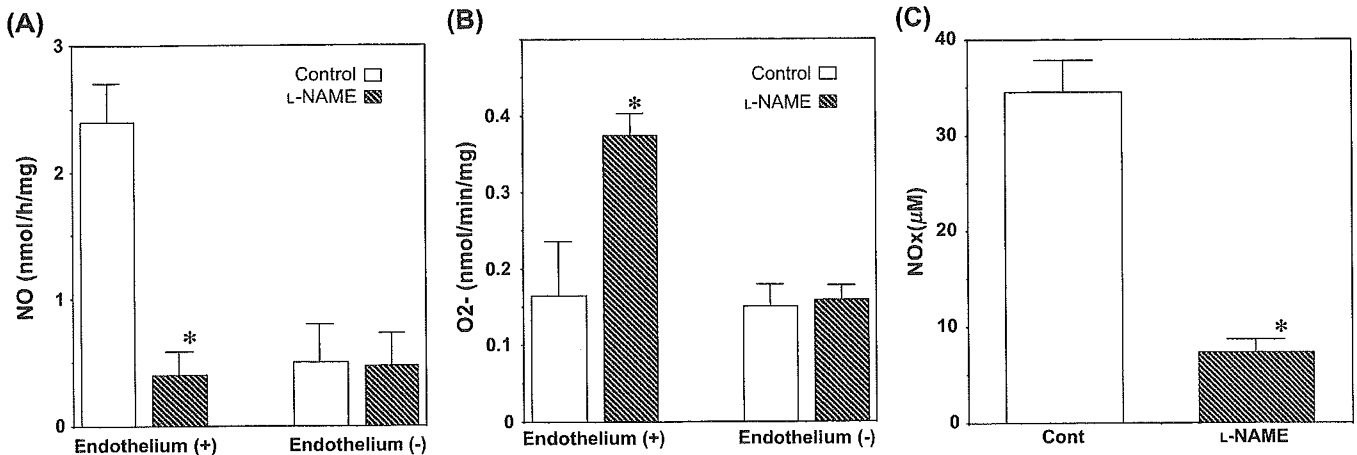

Fig. 1. NO (A) (nanomoles per hour per milligram tissue) and O_2^- (B) (nanomoles per 10 minutes per milligram tissue) production in rat aortic segments and plasma NOx concentration (C) in rats. *$P < .01$ vs control group. Each bar represents n = 6. NOx indicates nitrate + nitrite.

this period, the L group exhibited a significant rise in systolic arterial pressure, compared with the control group (Table 1). While heart rate did not change significantly in the control group, a reduction in heart rate was seen in the L group.

3.2. Aortic NO and O_2^- production and plasma NOx concentration

NO production was much lower in the L group than in the control group (Fig. 1A). Removal of the endothelium markedly decreased aortic NO production in the control group to the same level as the L group with intact endothelium (Fig. 1A). Removal of the endothelium did not affect NO production in the L group.

Production of O_2^- in the aortic segments with intact endothelium was greater in the L group than in the control group (Fig. 1B). In the L group segments without endothelium, O_2^- production was similar to that of the control group segments without endothelium (Fig. 1B).

Plasma NOx was markedly reduced in the L group, compared with the control group (Fig. 1C).

3.3. Plasma adiponectin and adiponectin mRNA levels in adipose tissue

Plasma adiponectin in the control group was 5.90 ± 0.64 μg/mL, while that of the L group was significantly lower at 2.70 ± 0.42 μg/mL ($P < .005$) (Fig. 2A).

There was an abundance of adiponectin mRNA in the abdominal adipose tissue of control rats, while mRNA expression was clearly reduced in the L group (Fig. 2B).

There observed no difference of mRNA expression in eNOS, p22phox, gp91phox, p47phox, and Rac1 in adipose tissue between the control and the L group. iNOS mRNA did not expressed in adipose tissue from the control and the L group.

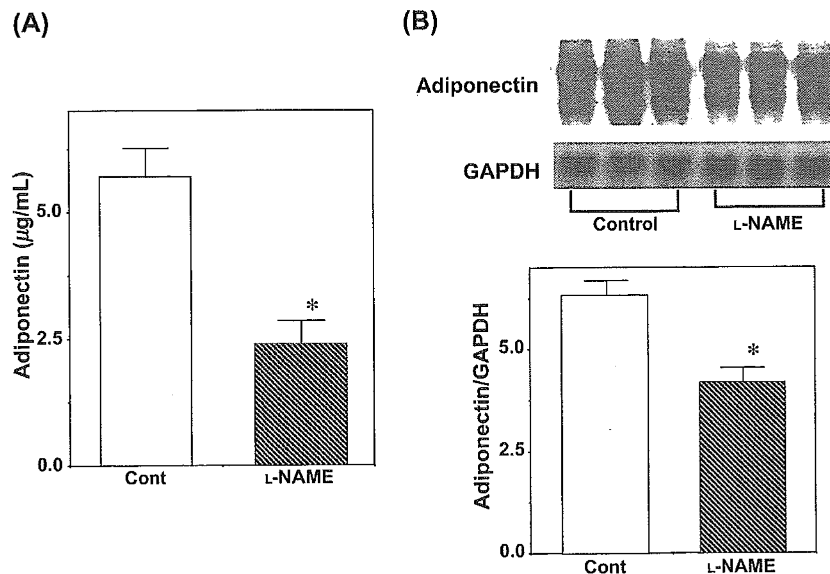

Fig. 2. Plasma adiponectin concentration (n = 8) (A) and adiponectin mRNA levels in adipose tissue (n = 3) (B). *$P < .01$ vs control group.

3.4. Effect of pioglitazone and allopurinol on plasma adiponectin concentration and adiponectin mRNA levels in adipose tissue

Plasma adiponectin levels were significantly lower in the L group. To examine the effects of pioglitazone and allopurinol on plasma adiponectin levels, pioglitazone or allopurinol was administered to rats with and without L-NAME for 5 days. Pioglitazone increased rat adiponectin levels, while no changes were observed with allopurinol. However, both pioglitazone (L+P) and allopurinol (L+A) restored rat adiponectin levels to control levels in rats treated with L-NAME (Fig. 3A). Similarly, pioglitazone and allopurinol recoverd adiponectin mRNA levels in adipose tissue from L-NAME-treated rats (Fig. 3B and C).

4. Discussion

This study showed reduced plasma adiponectin levels in an animal model of inhibition of NO synthesis. Adiponectin mRNA levels were also reduced, suggesting that the observed reduction in adiponectin levels was due to decreased production. The recovery of adiponectin levels in

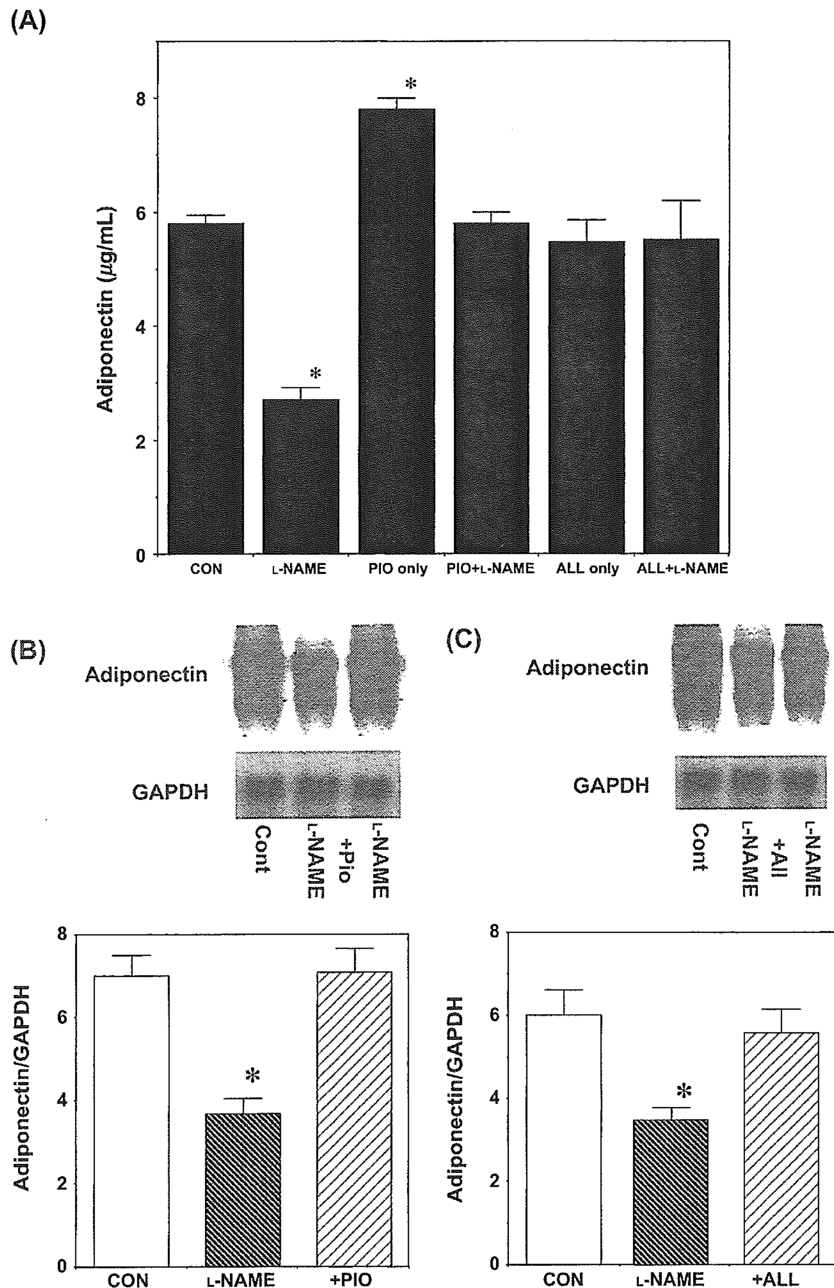

Fig. 3. Effects of pioglitazone and allopurinol on plasma adiponectin concentration (n = 8) (A) and adiponectin mRNA levels in adipose tissue (B and C). *$P < .01$ vs control group.

animals treated with pioglitazone and allopurinol suggests that stimulation of adiponectin production and/or a reversal of inhibition of adiponectin production might occur, thereby reversing the decline in adiponectin levels. Importantly, this suggests that oxidative stress might reduce adipopectin production.

It has been show that peroxisome proliferator-activated receptor (PPAR)-γ plays a significant role in transcriptional activation of the adiponectin gene via PPAR response element (PPRE) and its promoter [21]. Indeed, treatment with PPAR-γ agonists clearly increases adiponectin levels in humans [22,23]. We confirmed that pioglitazone increases adiponectin levels and allows recovery of adiponectin levels to control levels in L-NAME-treated rats. This might be due to transcriptional activation of the adiponectin gene in adipose tissue. This normalization of adiponectin levels by pioglitazone in L-NAME-treated rats might explain the results of previous reports in which prevention of L-NAME-induced coronary inflammation and arteriosclerosis by pioglitazone was observed [24].

Oxidative stress appears to reduce adiponectin levels. We confirmed decreased adiponectin levels in L-NAME-treated rats, as well as in rats infused with angiotensin II (unpublished data). In both groups of rats, hypertensive and cardiovascular effects were observed due to oxidative stress. Insulin resistance was also induced in both groups of rats [25,26]. Treatment of the animals with antioxidant drugs resulted in partial to total recovery of impairment [27,28]. Thus, reduced adiponectin production might play a pathogenic role in the development of cardiovascular effects in these rats. Furthermore, reduced adiponectin levels are closely associated with oxidative stress.

We examined the mRNA levels of NADPH oxidase components, as well as NO synthases, in the adipose tissue of L-NAME-treated rats in order to clarify the role of oxidative stress in fat, which is the source of adiponectin. Angiotensin II activation and angiotensin-converting enzyme activation have been demonstrated in L-NAME treated rats [27,29], thus suggesting a pathogenic role of oxidative stress. Angiotensin II is thought to induce oxidative stress mainly via induction/activation of NADPH oxidase [30,31]. However, we did not observe an increase in the mRNA levels of NADPH oxidase components or NO synthases in adipose tissue from L-NAME treated rats, even though increased mRNA levels were observed in angiotensin II-treated rats (data not shown). Furthermore, allopurinol recovered mRNA levels in L-NAME treated rats, indicating that xanthine oxidase, rather than NADPH oxidase or uncoupled NOS, might play a role in the L-NAME-mediated induction of oxidative stress in rat adipose tissue.

Our present findings suggest that oxidative stress reduces adiponectin production. Adiponectin has been shown to suppress proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL [32]. It has also been demonstrated that angiotensin II-induced apoptosis in human endothelial cells is inhibited by adiponectin through restoration of the association between endothelial nitric oxide synthase and heat shock protein 90 [33]. Adiponectin levels are decreased in patients and animal models with obesity, diabetes, and coronary artery disease [1-5], in which oxidative stress appears to affect adiponectin production in adipose tissue. Thus, relief of oxidative stress, requiring different strategies depending on the underlying cause, might have a role in the treatment of these diseases by normalizing adiponectin levels and thus preventing associated pathology.

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Smoking, Endothelial Function, and Rho-Kinase in Humans

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Objective—Smoking is associated with endothelial dysfunction and activated Rho-kinase in vascular smooth muscle cells (VSMCs) in humans. The purpose of this study was to elucidate the relationship between endothelial function and Rho-kinase activity in forearm VSMCs in healthy young men.

Methods and Results—We evaluated the forearm blood flow (FBF) responses to acetylcholine (ACh), fasudil, a Rho-kinase inhibitor, and sodium nitroprusside (SNP) in male smokers (n=10) and nonsmokers (n=14). FBF was measured by using a strain-gauge plethysmography. The vasodilatory effect of ACh was significantly smaller in smokers than that in nonsmokers. The vasodilatory effect of fasudil was significantly greater in smokers than that in nonsmokers. The vasodilatory effects of SNP in the 2 groups were similar. There was a significant correlation between the maximal FBF response to fasudil and that to ACh ($r=-0.67$; $P<0.01$). There was no significant correlation between the maximal FBF response to fasudil and that to SNP. The intra-arterial coinfusion of fasudil significantly increased the FBF response to ACh in smokers but not in nonsmokers. There were no significant differences between FBF response to fasudil alone and that in combination with N^G-monomethyl-L-arginine in smokers and in nonsmokers. The intra-arterial coinfusion ascorbic acid did not alter the FBF response to fasudil in both groups.

Conclusions—These findings suggest that smoking is involved in not only endothelial dysfunction but also activation of Rho-kinase in VSMCs in forearm circulation, and that there is a significant correlation between endothelial function and Rho-kinase activity in VSMCs. ***(Arterioscler Thromb Vasc Biol.* 2005;25:2630-2635.)**

Key Words: smoking ■ Rho-kinase ■ endothelial function ■ vascular smooth muscle cell ■ healthy young man

Cigarette smoking is a major risk factor for the development of atherosclerosis. Although several lines of evidence have indicated the mechanisms for endothelial dysfunction by smoking,[1,2] the underlying mechanisms are not completely understood. Smoking causes endothelial dysfunction in smokers and passive smokers,[3,4] leading to cardiovascular and cerebrovascular complications.[5]

Recent in vitro and in vivo studies suggested that the Rho-associated kinase (Rho-kinase/ROK/ROCK) family, one of several putative small GTPase Rho effectors, plays major roles in actin cytoskeleton, organization,[6,7] smooth muscle contraction,[8] and gene expression,[9] all of which may be involved in the pathogenesis of atherosclerosis. Results of previous studies have shown that Rho-kinase plays a key role in the contraction of vascular smooth muscle cells (VSMCs). Rho-kinase activates myosin light chain (MLC) kinase (MLCK) by phosphorylation of the myosin-binding subunit (MBS) in MLC phosphatase (MLCPh), leading to contraction of VSMCs.[10-12] Smooth muscle dysfunction has been found in subjects with atherosclerosis.[13] VSMC dysfunction may be partly attributable to the activation of Rho-kinase in VSMCs. It is thought that Rho-kinase activity also interacts endothelial function in humans. However, there is no information on the relationship between endothelial function and Rho-kinase activity in humans.

To evaluate the effects of smoking on endothelial function and Rho-kinase activity, and to determine the relationship between endothelial function and Rho-kinase activity in humans, we measured vascular responses to acetylcholine (ACh), fasudil, a specific inhibitor of Rho-kinase, and sodium nitroprusside (SNP), a direct vasodilator of VSMCs, in healthy young men.

Methods

Subjects

The subjects were 10 healthy young male smokers (mean age 24.9±5.3 years) and 14 healthy age-matched young male nonsmokers (mean age 25.1±4.6 years). All of the subjects were recruited from healthy volunteers. Normal blood pressure was defined as systolic blood pressure of <130 mm Hg and diastolic blood pressure of <80 mm Hg. The results of physical and routine laboratory examinations in all subjects were normal. None of the subjects had a family history of premature cardiovascular disease, and none of the

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subjects were taking oral contraceptives, antioxidant vitamins, or cardioactive drugs. The study protocol was approved by the ethical committee of Hiroshima University Graduate School of Biomedical Sciences. Informed consent for participation in the study was obtained from all subjects. The definition of smokers was those who fulfilled the prespecified entry criteria: regular smoking history >5 pack years. One pack year was equivalent to 20 cigarettes smoked per day for 1 year. All of the smokers (11.4±13.2 pack years) had a smoking history of >5 years and abstained from smoking for ≥3 hours before the forearm blood flow (FBF) measurements. We defined nonsmokers as those who had never smoked.

Measurements of FBF

FBF was measured with a mercury-filled Silastic strain-gauge plethysmography (EC-5R; D.E. Hokanson, Inc.), as described previously.[14,15]

Procedures

The forearm vascular responses to ACh (Daiichi Pharmaceutical Co) were evaluated in 10 smokers and 14 nonsmokers, and fasudil (Asahi Chemical Industries) and SNP (Maluishi Pharmaceutical Co) were evaluated in all subjects. The infusions of ACh, fasudil, and SNP were performed in a randomized fashion. The study began at 8:30 AM with the subjects in the fasting condition. A 23-gauge polyethylene catheter (Hokkow Co) was inserted into the left brachial artery for the infusion of ACh, fasudil, and SNP for the recording of arterial pressure with an AP-641G pressure transducer (Nihon Kohden Co) under local anesthesia (1% lidocaine). Another catheter was inserted into the left deep antecubital vein to obtain blood samples.

After 30 minutes in the supine position, we measured basal FBF and arterial blood pressure. Then, forearm vascular response to ACh, endothelium dependent vasodilator, fasudil, a specific Rho-kinase inhibitor, and SNP, a direct vasodilator of smooth muscle cells, on forearm hemodynamics were measured. ACh (3.75 and 7.5 μg/min), fasudil (3, 10, 30, and 100 μg/min), and SNP (0.75, 1.5, and 3.0 μg/min) were infused intra-arterially for 5 minutes at each dose. Each study proceeded after the FBF returned to baseline.

To determine the coinfusion effect of fasudil on ACh-induced vasodilation, the forearm vascular response to ACh (3.75 and 7.5 μg/min) in combination with fasudil (10 μg/min) was evaluated in 6 smokers and 8 nonsmokers. Furthermore, after a 30-minute rest period, N^G-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor, was infused intra-arterially at a dose of 8 μmol/min for 5 minutes while the basal FBF and arterial blood pressure were recorded and fasudil (3, 10, 30, and 100 μg/min) was administered.

On another day, to determine the effect of fasudil after inhibition of reactive oxygen species (ROS), the forearm vascular responses to fasudil (3, 10, 30, and 100 μg/min) alone and in combination with ascorbic acid (24 mg/min) were evaluated in 7 smokers and 7 nonsmokers.

Analytical Methods

Routine chemical methods were used to determine serum concentrations of total cholesterol, high-density lipoprotein cholesterol, and triglycerides. Serum concentrations of low-density lipoprotein (LDL) were determined using Friedewald's methods. The concentration of angiotensin II was assayed by radioimmunoassay. The plasma concentrations of norepinephrine were measured by high-performance liquid chromatography.

Statistical Analysis

Results are presented as the means±SD. Values of $P<0.05$ were considered to indicate statistical significance. The Mann–Whitney *U* test was used to evaluate differences between current smokers and nonsmokers concerning parameters at baseline. Comparisons between the 2 groups with respect to changes in parameters were performed with adjusted means on an ANCOVA, with baseline data used as the covariates. Comparisons of dose-response curves of parameters during infusion of the drug were analyzed by repeated-measures ANOVA. For the analysis of FBF response to ACh in combination with fasudil and that to fasudil in combination with L-NMMA, the absolute FBF changes from baseline values were used to compare the dose-response curves. Each FBF response to the vasoactive drugs was compared with that in the other group by Bonferroni correction. Spearman's rank correlation was used to compare the maximal FBF response to ACh with that to fasudil and that to SNP. The data were analyzed using the software package StatView V (SAS Institute Inc.) and Super ANOVA (Abacus Concepts).

Clinical Characteristics of Smokers and Nonsmokers

Variables	Smoker (n=10)	Nonsmoker (n=14)
Age, y	24.9±5.3	25.1±4.6
Body mass index, kg/m²	22.7±2.5	24.6±3.0
Systolic blood pressure, mm Hg	118.0±9.3	123.0±6.4
Diastolic blood pressure, mm Hg	61.8±6 8	64.6±6 6
Mean blood pressure, mm Hg	80.5±7.0	84.1±5.2
Heart rate, bpm	65.5±8.1	64.1±8.5
Total cholesterol, mmol/L	4.30±0 80	4.42±0 71
Triglyceride, mmol/L	1.06±0 34	1.07±0.37
HDL cholesterol, mmol/L	1 37±0.36	1.31±0.38
LDL cholesterol, mmol/L	2.45±0.83	2.62±0.55
Mean fasting plasma glucose, mmol/L	5.0±0.4	5.2±0.4
Plasma NE, ng/mL	0.17±0.10	0.20±0.13
Plasma Ang II, pg/mL	7.00±2.31	6.00±3.57
FBF, mL/min per 100 mL tissue	6.9±1.9	7.1±2.8

Ang II indicates angiotensin II; FBF, forearm blood flow; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NE, norepinephrine. All results are presented as mean±SD.

Results

Baseline Clinical Characteristics

The clinical characteristics of the 10 smokers and 14 nonsmokers are summarized in the Table. All of the parameters, including plasma insulin, plasma angiotensin II, norepinephrine, and lipid profiles, were similar in smokers and nonsmokers. Systemic and forearm hemodynamics such as baseline FBF were also similar in the 2 groups.

FBF Responses to ACh in Smokers and Nonsmokers

The intra-arterial infusion of ACh significantly increased FBF in a dose-dependent manner in smokers and nonsmokers. The FBF response to ACh was significantly smaller in smokers than in nonsmokers (maximal FBF 12.3±3.3 versus 21.4±6.8 mL/min per 100 mL tissue; $P<0.01$; Figure 1, top). No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of ACh in either.

FBF Responses to SNP in Smokers and Nonsmokers

The intra-arterial infusion of SNP significantly increased FBF in a dose-dependent manner in smokers and nonsmokers. There was no significant difference between FBF responses to SNP in the 2 groups (Figure 1, bottom). No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of SNP in either.

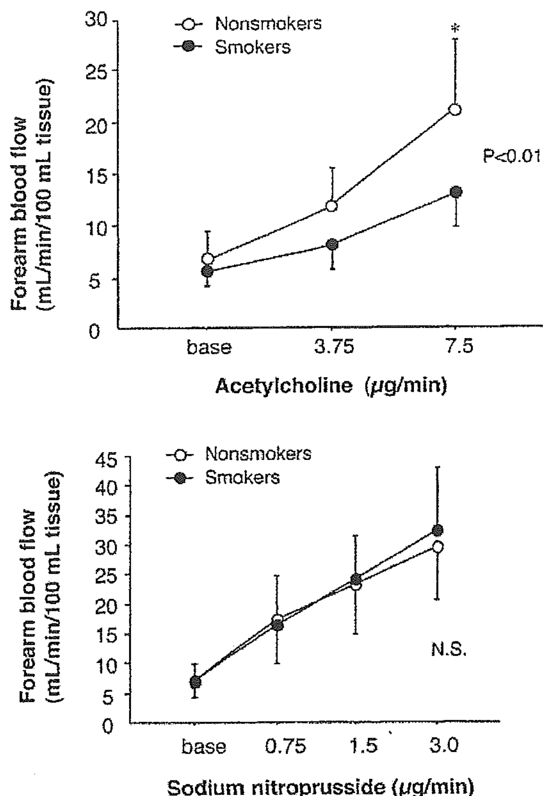

Figure 1. Effects of ACh on FBF in smokers (●) and nonsmokers (○; top). *$P<0.01$ vs smokers. Effects of SNP on FBF in smokers (●) and nonsmokers (○; bottom). Results are presented as mean±SD. The P value refers to a comparison of time course curves by ANOVA for repeated measurements.

FBF Responses to Fasudil in Smokers and Nonsmokers

The FBF response to fasudil was significantly greater in smokers than in nonsmokers (maximal FBF 23.4±6.1 versus 14.6±5.1 mL/min per 100 mL tissue; $P<0.01$; Figure 2). No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of fasudil in either.

There was a significant relationship between the maximal FBF response to ACh and that to fasudil ($r=-0.67$; $P<0.01$).

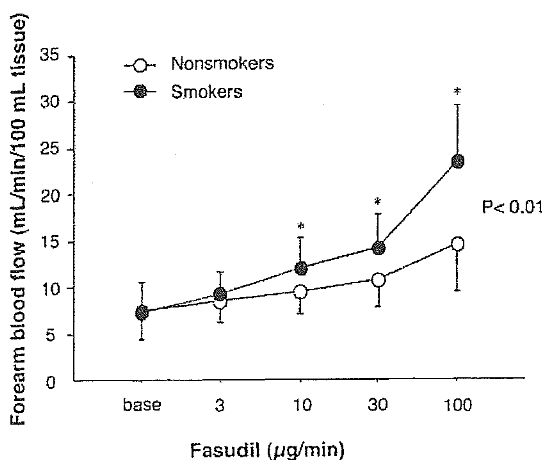

Figure 2. Effects of fasudil on FBF in smokers (●) and nonsmokers (○). *$P<0.05$ vs nonsmokers. Results are presented as mean±SD. The P value refers to a comparison of time course curves by ANOVA for repeated measurements.

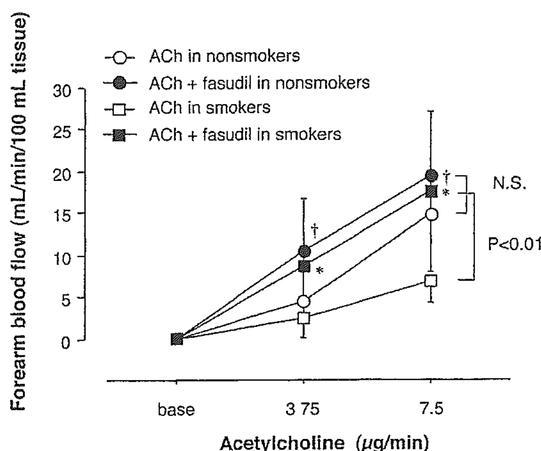

Figure 3. Effects of ACh alone (○) and in combination with fasudil (●) on FBF in smokers and nonsmokers. *$P<0.05$ vs before fasudil in smokers. †$P<0.05$ vs before fasudil in nonsmokers. Results are presented as mean±SD. The P value refers to a comparison of time course curves by ANOVA for repeated measurements.

However, there was no significant relationship between the maximal FBF response to ACh and that to SNP ($r=0.08$; P=NS) or between the maximal FBF response to fasudil and that to SNP ($r=0.28$; P=NS).

FBF Responses to ACh Alone and in Combination With Fasudil in Smokers and Nonsmokers

The intra-arterial coinfusion of fasudil significantly augmented FBF response to ACh in smokers ($P<0.01$; Figure 3) but not in nonsmokers (P=NS; Figure 3). During coinfusion of fasudil, there was no significant difference in ACh-induced vasodilation between the 2 groups. No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of ACh alone and in combination with fasudil in either.

FBF Responses to Fasudil Alone and in Combination With L-NMMA in Smokers and Nonsmokers

The intra-arterial infusion of L-NMMA significantly decreased basal FBF from 7.3±2.8 to 5.1±2.1 mL/min per 100 mL tissue ($P<0.05$) in smokers and from 8.4±3.0 to 5.1±2.1 mL/min per 100 mL tissue ($P<0.05$) in nonsmokers. Changes in basal forearm vascular responses to L-NMMA infusion were similar in the 2 groups. There were no significant differences between FBF response to fasudil alone and that in combination with L-NMMA in smokers and in nonsmokers (Figure 4). There was a significant difference between the changes in FBF response to fasudil after coinfusion of L-NMMA in the 2 groups ($P<0.01$). No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of fasudil alone and in combination with L-NMMA in either.

FBF Responses to Fasudil Alone and in Combination With Ascorbic Acid in Smokers and Nonsmokers

Ascorbic acid did not alter the FBF response to fasudil in smokers and in nonsmokers (Figure 5). No significant change

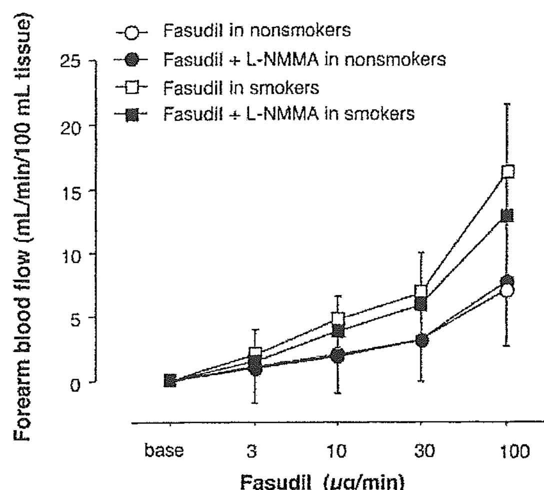

Figure 4. Effects of fasudil alone (□) and in combination with L-NMMA (■) on FBF in smokers and those of fasudil alone (○) and in combination with L-NMMA (●) on FBF in nonsmokers. Results are presented as mean±SD. The *P* value refers to a comparison of time course curves by ANOVA for repeated measurements.

was found in arterial blood pressure or heart rate with intra-arterial infusion of fasudil alone and in combination with ascorbic acid in either.

Discussion

In the present study, we demonstrated that not only endothelial dysfunction but also activated Rho-kinase in VSMCs were found in healthy young male smokers compared with nonsmokers. The results of the present study also showed for the first time that there is a significant correlation between endothelial function and Rho-kinase activity in forearm resistance arteries.

Endothelium-dependent vasodilation was impaired even in healthy young smokers compared with nonsmokers. Our findings are supported by results of previous studies showing

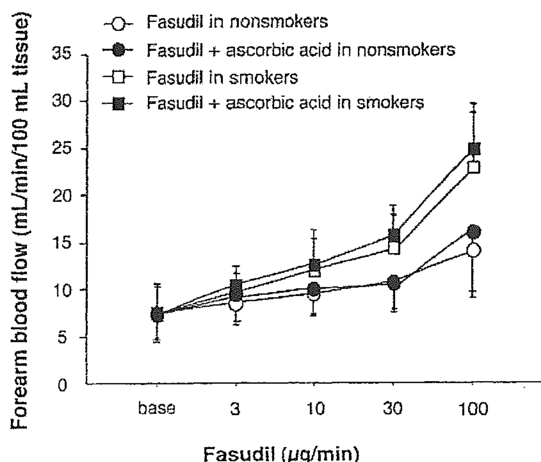

Figure 5. Effects of fasudil alone (□) and in combination with ascorbic acid (■) on FBF in smokers, and those of fasudil alone (○) and in combination with ascorbic acid (●) on FBF in nonsmokers. Results are presented as mean±SD. The *P* value refers to a comparison of time course curves by ANOVA for repeated measurements.

that smoking is significantly associated with endothelial dysfunction and cardiovascular disease.[3,5,16,17] Structural damage,[18] a direct toxic effect,[19] a decreased production or bioavailability of endothelial NO,[1,2] and a superoxide anion by containing of a large number of free radicals and pro-oxidants in cigarette smoke[20] have been proposed as mechanisms of smoking-induced vascular damage.

The main findings of the present study are that the forearm vasodilatory effect evoked by fasudil was greater in smokers than in nonsmokers, whereas SNP-induced vasodilation was similar in the 2 groups, and that there was a significant correlation between the forearm vasodilatory effect evoked by ACh and that by fasudil. Although the precise mechanism of the interaction between endothelial function and Rho-kinase activity remains to be cleared, our results suggest that smoking may contribute to the activation of Rho-kinase in VSMCs as well as endothelial dysfunction. Several lines of evidence have demonstrated that eNOS expression is upregulated by inhibition of Rho-kinase via increase of eNOS mRNA stability and eNOS phosphorylation.[21,22] Hernandez-Perera et al[23] reported that Rho is required for the basal expression of preproendothelin-1 in vascular endothelial cells, which gives rise to endothelin-1. In addition, several investigators demonstrated an interaction between NO and Rho/Rho-kinase in VSMCs.[24,25] Sauzeau et al[24] have shown that exogenous NO attenuates RhoA-dependent Ca^{2+} sensitization of blood vessel contraction by inhibiting RhoA translocation from the cytosol to membrane in VSMCs through activation of the cyclic GMP-dependent kinase pathway. These findings suggest that endothelial dysfunction may result in Rho-kinase activation in VSMCs through a decrease in NO production from the endothelium, and that activated Rho-kinase may inhibit eNOS expression in the endothelium. Consequently, endothelial dysfunction and activation of Rho-kinase in VSMCs may be evoked by smoking.

Recent studies have shown that Rho-kinase plays important roles in various cellular functions, including vascular smooth muscle contraction.[8,11,12,26] Uehata et al[27] reported that systemic administration of a Rho-kinase inhibitor, Y-27632, induced significant and persistent decreases in blood pressure in hypertensive rat models. In clinical studies, several investigators reported that hypertension, stable angina pectoris, and coronary vasospasm are associated with activation of Rho-kinase.[28–30] These findings suggest that activation of Rho-kinase in VSMCs is involved in the development and progression of the atherosclerotic process. Furthermore, recent studies demonstrated the partial contribution of Rho-kinase to VSMC contraction. VSMC contraction is modulated in a dual manner by MLCK and MLCPh, so that the phosphorylation of MBS on MLCPh by Rho-kinase results in the phosphorylation of MLC and subsequent contraction of VSMCs.[31] Moreover, MLC diphosphorylation as well as MLC monophosphorylation were found in impaired VSMCs.[26,32] It is postulated that smoking is associated with Rho-kinase activity.

In the present study, we evaluated endothelial function by using ACh, which is well established as an endothelial dependent vasodilator,[14] and we evaluated Rho-kinase activity in VSMCs by using fasudil, a Rho-kinase inhibitor.[15]

Fasudil, which is currently used for prevention and treatment of cerebral vasospasm after subarachnoid hemorrhage, has been shown recently to be a potent and specific inhibitor of Rho-kinase.[33,34] In addition, fasudil is used for assessment of Rho-kinase activity in humans.[28–30] However, we cannot deny the possibility that fasudil, especially at high doses, has nonspecific effects on vasculature.

In the present study, L-NMMA did not alter the FBF response to fasudil in smokers or nonsmokers. Interestingly, coinfusion of fasudil significantly augmented the FBF response to ACh in smokers but not in nonsmokers. These results may be attributable to decreased Ca^{2+} sensitivity by inhibition of Rho-kinase in VSMCs in smokers. These findings support our hypothesis that Rho-kinase in VSMCs is activated in smokers compared with nonsmokers, although it remains to be clarified whether endogenous NO inhibits Rho-kinase activity in humans. Of additional interest, there was no significant difference between FBF response to ACh in combination with fasudil in smokers and that in nonsmokers in the present study. This may be explained by a decrease in Ca^{2+} sensitivity attributable to inhibition of Rho-kinase in VSMCs and by an increase in phosphorylation of eNOS attributable to inhibition of Rho-kinase in endothelial cells in smokers.[22] Wolfrum et al[35] demonstrated in a rat model of myocardial infarction that acute administration of fasudil leads to rapid activation of eNOS through the phosphatidylinositol 3-kinase/Akt pathway, resulting in increased NO production and subsequent cardiovascular protection. On the other hand, several investigators have shown that inhibition of Rho-kinase upregulates eNOS expression through increase in eNOS mRNA stability and eNOS phosphorylation.[23,35] We cannot deny the possibility that fasudil improves endothelial function via upregulation of eNOS expression in smokers.

Recently, Higashi et al[36] demonstrated that Rho-kinase is substantially involved in production of ROS through NAD(P)H oxidase upregulation. Moreover, several investigators have shown a possible interaction between Rho/Rho-kinase and ROS.[37,38] In the present study, coinfusion of antioxidant ascorbic acid had no effect on FBF response to fasudil in smokers or nonsmokers. It is unlikely that ROS has effects on Rho-kinase activity in healthy young male smokers.

Several methods have been used to assess endothelial function in humans. Recently, several investigators, including us, evaluated the effects of intra-arterial infusion of NO agonists, such as ACh, methacholine, and bradykinin, and the effects of intra-arterial infusion of NO antagonists on FBF. The responses to intra-arterial infusion of vasoactive agents should be considered the gold standard for assessing endothelial function because the use of agonists to stimulate NO release and the use of antagonists of NO allow us to draw more specific conclusions concerning the role of basal and stimulated NO release. Measurement of flow-mediated vasodilation (FMD) in the brachial artery using ultrasound also reflects NO production well. It is accepted that measurement of FBF responses to vasoactive agents is an index of resistance artery endothelial function and that measurement of FMD is an index of conduit artery endothelial function. Both measurements of FBF responses to vasoactive agents and FMD would enable more specific conclusions concerning the relationship between Rho-kinase activity and endothelial function to be drawn. Unfortunately, we were not able to perform measurement of FMD as an index of conduit artery endothelial function in the present study.

Endothelial dysfunction and activation of Rho-kinase may play a critical role in the pathogenesis of atherosclerosis in smokers, leading to cardiovascular and cerebrovascular complications. Further studies on the mechanisms underlying the interaction between endothelial function and Rho-kinase, not only in smokers but also in other subjects who have cardiovascular risk factors such as hypercholesterolemia and diabetes mellitus, are awaited for future therapeutic benefits.

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