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Atherosclerosis xxx (2005) xxx–xxx

ATHEROSCLEROSIS

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Tetrahydrobiopterin improves aging-related impairment of endothelium-dependent vasodilation through increase in nitric oxide production

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Received 12 January 2005; received in revised form 24 June 2005; accepted 20 July 2005

Abstract

Deficiency of tetrahydrobiopterin (BH_4), an essential cofactor for nitric oxide (NO) synthase, decreases NO production and increases reactive oxygen species. The purpose of this study was to elucidate the effects of aging on endothelial function and to determine whether the degree of BH_4 deficiency is related to aging and oxidative stress. We evaluated forearm blood flow (FBF) responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and isosorbide dinitrate (ISDN), an endothelium-independent vasodilator, before and after co-infusion of BH_4 (500 mg/min) in 37 healthy men (mean age, 41 ± 18 yr; range, 19–81 yr). FBF was measured using strain-gauge plethysmograph. Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and serum malondialdehyde-modified low-density lipoprotein (MDA-LDL) were measured as indices of oxidative stress. Both ACh and ISDN increased the FBF in a dose-dependent manner in all subjects. Co-infusion of BH_4 resulted in a significant increase in ACh-induced vasodilation (from 22.3 ± 6.7 to 30.1 ± 7.5 mL/min/100 mL tissue, $P < 0.05$). Aging was found to be significantly correlated with ACh-induced vasodilation ($r = -0.47$, $P = 0.006$), urinary 8-OHdG ($r = 0.38$, $P = 0.02$), serum MDA-LDL ($r = 0.36$, $P = 0.02$), and the change in ACh-induced vasodilation after co-infusion of BH_4 ($r = 0.45$, $P = 0.007$). The FBF response to ISDN did not correlate with any parameters. Infusion of N^G-monomethyl-L-arginine, an NO synthase inhibitor, abolished the BH_4-induced enhancement of forearm vasorelaxation evoked by ACh. The increase in FBF after ISDN was not altered by BH_4. These findings suggest that a deficiency of BH_4 may be involved in the pathogenesis of disturbances in endothelium-dependent vasodilation related to aging through decrease in NO production and increase in oxidative stress.
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Keywords: Aging; Endothelial function; Nitric oxide; Tetrahydrobiopterin; Oxidative stress

Aging may alter the structure and function of vascular components, such as the endothelium, intimas, and smooth muscle cells, resulting in an increase in the risk of development of cardiovascular and cerebrovascular diseases [1–3]. Several lines of evidence suggest that endothelial dysfunction is involved in the development of atherosclerosis. Disturbances in endothelium-dependent vasodilation have been observed in elderly subjects and animal models [4,5].

Endothelial nitric oxide synthase (eNOS) requires several cofactors, such as heme, FAD, and FMN as well as tetrahydrobiopterin (BH_4), for elicitation of full enzymatic activity

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0021-9150/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2005.07.025

ATH-9155; No. of Pages 6

[6,7]. BH_4 is an allosteric factor in the coupling of the oxidase and reductase domains of eNOS [6]. Recently, it has been reported that a deficiency of BH_4 results in a decrease in NO synthesis and an increase in production reactive oxygen species (ROS) [8]. In animals and humans of advanced age, dysfunctional eNOS with insufficient BH_4 induces production of ROS, resulting in a decrease in NO bioavailiability [9]. Reduced availability of BH_4 may contribute to the maintenance and development of atherosclerosis. In addition, it has been demonstrated that supplementation of BH_4 or folic acid improves endothelial function in smokers and patients with diabetes, hypertension, hypercholesterolemia, and chronic heart failure [10–14]. However, there is no information on the roles of BH_4-related NO production and oxidative stress in elderly subjects.

To determine whether BH_4 restores endothelium-dependent vasodilation in elderly subjects, we studied the effects of BH_4 on forearm vascular responses to the vasoactive agents acetylcholine (ACh), an endothelium-dependent vasodilator, and isosorbide dinitrate (ISDN), an endothelium-independent vasodilator. To further examine the role of BH_4 in the release of NO, the effects of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS, on responses to ACh or ISDN and in combination with BH_4 were studied.

1. Methods

1.1. Subjects

Thirty-seven healthy men (mean age, 41 ± 18 yr; range, 19–81 yr) were enrolled in this study. Subjects with a history of cardiovascular or cerebrovascular disease, hypertension, hypercholesterolemia, diabetes mellitus, liver disease, renal disease, or a smoking habit were excluded. The study protocol was approved by the Ethical Committee of the Hiroshima University School of Medicine. Informed consent for participation in this study was obtained from all subjects.

1.2. Study protocol: effects of BH_4 on endothelium-dependent and -independent vasodilation

Forearm vascular responses to ACh (Daiichi Pharmaceutical Co.) alone and in combination with BH_4 (Sigma Chemical Co.) and in the absence or presence of infusion of L-NMMA (Sigma Chemical Co.) were evaluated in the 32 healthy male subjects. The study began at 8:30 a.m. Subjects fasted the previous night for at least 12 h. Each subject was kept in the supine position in a quiet, dark, and air-conditioned room (temperature maintained in the range of 22–25 °C) throughout the study period. A 23-gauge polyethylene catheter (Hakkow Co.) was inserted into the left brachial artery for infusion of ACh, BH_4, and L-NMMA and for recording of arterial pressure with an AP-641G pressure transducer (Nihon Kohden Co.) under local anesthesia (1% lidocaine), and another catheter was inserted into the left deep antecubital vein to obtain blood samples.

After each subject had remained in the supine position for 30 min, forearm blood flow (FBF) and arterial blood pressure were measured. Then, the effects of the endothelium-dependent vasodilator ACh and endothelium-independent vasodilator ISDN on forearm hemodynamics were determined. ACh (3.75, 7.5, and 15 μg/min) and ISDN (0.15, 1.5, and 3.0 μg/min) were infused intra-arterially for 5 min at each dose using a constant-rate infusion pump (Terfusion STG-523, Termo Co.). The FBF was measured during the last 2 min of the infusion. After a 30 min rest period, ACh (3.75, 7.5, and 15 μg/min) was infused for 5 min at each dose in combination with BH_4 (500 μg/min), and FBFs were measured. In the present study, we used 500 μg/min of BH_4, as in previous studies [12,13].

Furthermore, after a 30 min rest period, L-NMMA was infused intra-arterially at a dose of 8 μmol/min for 5 min, while basal FBF and arterial blood pressure were recorded. In addition, to examine the role of NO release in the effects of BH_4 on forearm hemodynamics, we coadministered ACh (3.75, 7.5, and 15 μg/min) and BH_4 (500 μg/min) after the infusion of L-NMMA (8 μmol/min) for 5 min.

1.3. Measurement of FBF

FBF was measured using a mercury-filled silastic strain-gauge plethysmograph (EC-5R, D.E. Hokanson Inc.) as previously described [15,16].

1.4. Analytical methods

Routine chemical methods were used to determine serum concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, creatinine, insulin, glucose, and electrolytes. The serum concentrations of malondialdehyde-modified low-density lipoprotein (MDA-LDL) were assayed by enzyme-linked immunosorbent assay (ELISA) as previously described [17]. The urinary concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were also assayed by ELISA with the use of 8-OHdG assay kits (Nihon Yushi Co.). The intra- and inter-assay coefficients of variation were 6.8 and 7.7% for MDA-LDL and 5.9 and 6.5% for 8-OHdG.

1.5. Statistical analysis

Results are presented as mean ± S.D. P-values < 05 were considered statistically significant. Comparisons of dose–response curves of parameters during drug infusion were analyzed by analysis of variance (ANOVA) for repeated measures. The data were processed using either the software package StatView IV (Brainpower) or Super ANOVA (Abacus Concepts).

Table 1
Baseline clinical characteristics of subjects

Variables	
Body mass index (kg/m^2)	23.7 ± 2 2
Systolic blood pressure (mmHg)	118.9 ± 11.7
Diastolic blood pressure (mmHg)	69.2 ± 6.6
Heart rate (bpm)	61.0 ± 5.9
Total cholesterol (mmol/L)	4.13 ± 0.43
Triglycerides (mmol/L)	0.97 ± 0.41
High-density lipoprotein cholesterol (mmol/L)	1.36 ± 0.32
Low-density lipoprotein cholesterol (mmol/L)	2.53 ± 0.38
Serum glucose (mmol/dL)	4.6 ± 0.4
Serum insulin (pmol/L)	43.9 ± 9.8
Serum creatinine (umol/L)	89.6 ± 18.7
Plasma norepinephrine (ng/mL)	0.25 ± 0.15
Plasma angiotensin II (pg/mL)	5.4 ± 2.3
Serum malondialdehyde low-density lipoprotein (U/L)	73.8 ± 21.5
Urinary 8-hydroxy-2′-deoxyguanosine (ng/mg of creatinine)	12.4 ± 5.7
Forearm blood flow (mL/min/100 mL tissue)	5.3 ± 1.1

All results are presented as mean ± S.D.

2. Results

2.1. Clinical characteristics

The baseline clinical characteristics of the 37 healthy male subjects are shown in Table 1.

2.2. Effects of co-infusion of BH_4 and ACh or ISDN on forearm hemodynamics

Intra-arterial infusions of ACh and ISDN significantly increased FBF in dose-dependent manners (Figs. 1 and 2). The FBF response to ACh increased significantly with co-infusion of BH_4 (Fig. 1). The increase in FBF during infusion of ISDN was not altered by co-infusion of BH_4 (Fig. 2). No significant change was observed in arterial blood pressure or heart rate after intra-arterial infusion of either ACh or ISDN in combination with BH_4 in all subjects.

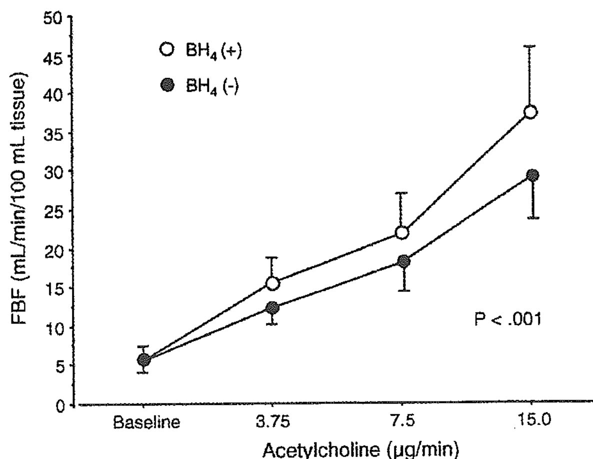

Fig. 1. Effects of acetylcholine on FBF before and after co-infusion of BH_4.

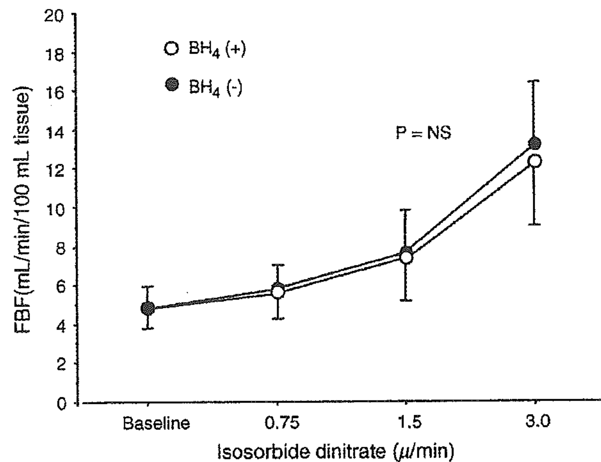

Fig. 2. Effects of isosorbide dinitrate on FBF before and after co-infusion of BH_4.

Aging significantly correlated with ACh-induced vasodilation ($r = -0.48$, $P = 0.006$), urinary 8-OHdG ($r = 0.39$, $P = 0.02$), serum MDA-LDL ($r = 0.36$, $P = 0.03$), and the change in ACh-induced vasodilation after co-infusion of BH_4 ($r = 0.46$, $P = 0.007$) (Fig. 3). ACh-induced vasodilation significantly correlated with urinary 8-OHdG ($r = -0.43$, $P = 0.009$), serum MDA-LDL ($r = -0.41$, $P = 0.01$), The FBF response to ISDN did not correlate with any parameters.

2.3. Effects of L-NMMA on forearm vascular responses to co-infusion of BH_4 and ACh

Intra-arterial infusion of the NOS inhibitor L-NMMA significantly decreased basal FBF from 5.3 ± 1.1 to 2.5 ± 0.4 mL/min/100 mL tissue ($P < 0.01$). No significant change in arterial blood pressure or heart rate was detected during the infusion of L-NMMA in any of the subjects. Intra-arterial infusion of L-NMMA decreased the response to ACh in combination with BH_4. L-NMMA completely abolished the enhanced response of forearm vasorelaxation to ACh in combination with BH_4 (Fig. 4).

3. Discussion

The present findings demonstrate that BH_4 augments ACh-induced vasodilation but not ISDN-induced vasodilation and that aging significantly correlated with FBF response to ACh, urinary excretion of 8-OHdG, serum concentration of MDA-LDL, and the augmentation of FBF response to ACH in combination with BH_4. The infusion of L-NMMA completely abolished the enhancement of ACh-induced vasodilation by BH_4.

In the present study, ACh-induced vasodilation was progressively more impaired with advance of age. Our results

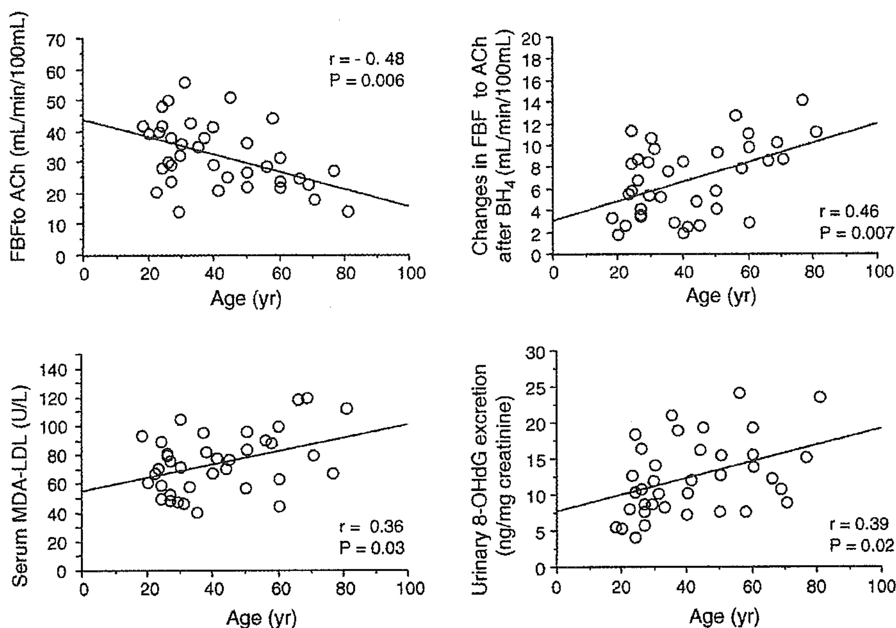

Fig. 3. Relationship between aging and maximal FBF response to acetylcholine, urinary excretion of 8-OHdG, serum MDA-LDL concentrations, and changes in FBF response to acetylcholine after BH_4.

are consistent with results of previous studies showing that aging is associated with endothelium-dependent vasodilation. Also, BH_4 supplementation has been shown to improve impaired endothelium-dependent vasodilation in brachial arteries of patients with hypercholesterolemia [13] and in saphenous vein rings from smokers [11]. Reduced availability of BH_4 may contribute to the maintenance and development of atherosclerosis with aging. In the present study, the NOS inhibitor L-NMMA abolished the FBF response to ACh in combination with BH_4, indicating that the BH_4-induced augmentation of endothelium-dependent vasodilation probably results from increased NO bioavailability.

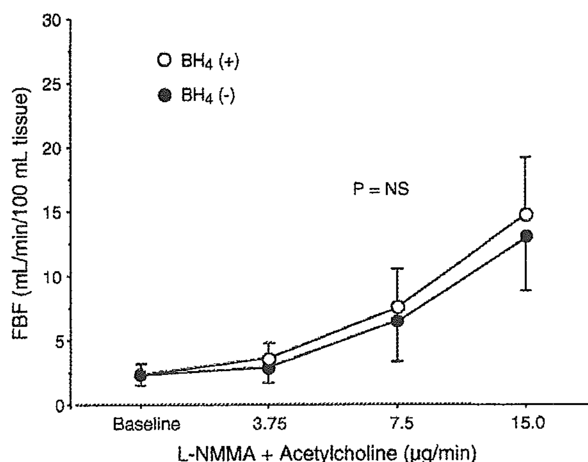

Fig. 4. Effects of acetylcholine in combination with BH_4 before and after L-NMMA on FBF.

A balance between ambient levels of superoxide and NO release plays a critical role in the maintenance of normal endothelial function [16,18]. Both 8-OHdG and MDA-LDL have been used as indices of oxidative stress [16–21]. The 8-OHdG is one of the most common markers for evaluating oxidative DNA damage and is a product formed by the specific attack of a hydroxy radical on DNA [19]. Several lines of evidence have suggested that oxidative DNA damage is increased in non-insulin-dependent diabetes mellitus and aging [18,19]. The level of MDA-LDL has been proposed as a biologic signature of clinical in vivo LDL oxidation [20,21]. Mutlu-Turkoglu et al. [22] reported that plasma MDA levels were increased in elderly subjects compared with those in young subjects. In the present study, aging correlated with urinary 8-OHdG excretion and serum MDA-LDL concentration, suggesting that oxidative stress progressively increases with aging. Several investigators have shown that both oxidized LDL and native LDL down-regulate eNOS mRNA and protein levels in endothelial cells [23,24]. Therefore, although the effect of MDL-LDL on endothelial function in humans is not clear, MDA-LDL is not merely a maker of oxidative stress but also may directly impair endothelial function through a decrease in the expression of eNOS. One possible mechanism by which supplementation of BH_4 augments endothelium-dependent vasodilation is decreasing oxidative stress, which may cause endothelial dysfunction.

While eNOS activation at optimal levels of BH_4 leads to production of NO, the activation of NOS at deficiency or

decreased levels of BH_4 results in generation of superoxide rather than NO in the vascular endothelium [6,7]. Under conditions of BH_4 deficiency, electron flow from the reductase domain to the oxidase domain is diverted to molecular oxygen rather than to L-arginine, a substrate of NO, leading to eNOS uncoupling. In this condition, uncoupled eNOS produces superoxide rather than NO [8]. In aging models, dysfunctional eNOS with insufficient BH_4 causes superoxide generation, resulting in decreased NO bioaviliability [9]. It has recently been reported that degradation of BH_4 by reactive oxygen species, including peroxynitrite, superoxide, and hydrogen peroxide, is associated with down-regulation of eNOS [25]. These findings suggest that BH_4 deficiency and decreased eNOS activity cause endothelial dysfunction in elderly subjects through an increase in oxidative stress. Therefore, BH_4 supplementation may increase the intracellular content of BH_4 and augment ACh-induced vasodilation by inhibition of NO inactivation. However, in the present study, the mechanism by which supplementation of BH_4 increases NO bioavailability was not elucidated.

Supplementation of BH_4 may directly stimulate eNOS activity, leading to an increase in NO production. BH_4 is an important cofactor for activity of all NOS isoforms, including eNOS [6,7]. It is known that cytokine-induced NO production requires an increase in intracellular BH_4 levels and that exogenous BH_4 supplementation enhances NO production [26]. These findings suggest that the cofactor BH_4 alone can regulate NOS activity. In rat aortic rings, administration of exogenous BH_4 causes endothelium-dependent vasorelaxation [27]. This vasorelaxation was abolished by L-NMMA, suggesting that BH_4-evoked endothelium-dependent vasodilation may be due to stimulated eNOS activity and an increase in NO production.

Finally, it is possible that the increase in BH_4 directly causes vasodilation. In the present study, BH_4 did not significantly alter parameters of forearm hemodynamics, including FBF, arterial blood pressure, and heart rate. This finding is consistent with results of previous studies showing that BH_4 does not alter basal FBF in patients with hypercholesterolemia, hypertension or heart failure, or in normal individuals. It is, therefore, unlikely that infusion of exogenous BH_4 directly causes dilation of the brachial artery.

3.1. Perspectives

The augmentation of endothelium-dependent vasodilation induced by BH_4 may be due to an increase in NO bioavailability, and impairment of ACh-induced vasodilation with aging may be due to an increase in production of superoxide due to deficiency of BH_4. The effect of BH_4 on endothelium-dependent vasodilation in elderly subjects was greater than in young subjects. Reduced availability of BH_4 may play a role in the pathogenesis of altered endothelium-dependent vasodilation in elderly subjects. Our results also indicate that BH_4 supplementation may be an effective treatment of impaired endothelial function in elderly subjects.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, Japan Heart Foundation Grant for Research on Hypertension and Metabolism, and a Grant for Research Foundation for Community Medicine.

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Cardiovascular Research 68 (2005) 289 – 298

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Role of the JNK pathway in thrombin-induced ICAM-1 expression in endothelial cells

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Received 28 October 2004; received in revised form 12 May 2005; accepted 31 May 2005

Available online 23 June 2005

Time for primary review 26 days

Abstract

Objective: Thrombin induces leukocyte adherence to endothelial cells via increased expression of intercellular adhesion molecule-1 (ICAM-1). Although ICAM-1 expression is regulated by NF-κB, recent studies have suggested that additional signaling mechanisms may also be involved. The goal of this study was to determine whether mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAP kinase (p38), mediate thrombin-induced ICAM-1 expression in endothelial cells.

Methods: Western blot analysis using anti-ICAM-1 antibody and luciferase assays were performed in cultured endothelial cells after addition of signal transduction inhibitors or transfection of various gene constructs. JNK kinase activity was determined by a kinase assay using c-Jun as a substrate or by Western blot analysis with anti-phospho-JNK antibody.

Results: Treatment of endothelial cells with the JNK-specific inhibitors, SP600125 or JNK inhibitory peptide 1 (JNKI1), resulted in a significant decrease in thrombin-induced ICAM-1 expression as demonstrated by Western blot analysis ($67 \pm 3\%$ and $72 \pm 7\%$, respectively). In contrast, inhibitors of MEK and p38 had only minimal effect. The combination of SP600125 and the NF-κB inhibitor, BAY11-7082, resulted in complete inhibition of thrombin-induced ICAM-1 expression. The $G\alpha_q$ inhibitor, YM-254890, inhibited thrombin-induced JNK activation and ICAM-1 expression. Dominant-negative Ras and Rac1, but not Rho, inhibited thrombin-induced JNK activation and ICAM-1 promoter activity. Finally, thrombin-induced JNK activation and ICAM-1 promoter activity were inhibited by βARK1ct (a Gβγ subunit scavenger) and Csk.

Conclusions: These data suggest that, in concert with NF-κB, JNK regulates thrombin-induced ICAM-1 expression by a mechanism that is dependent on $G\alpha_q$, Gβγ, Ras, Rac1 and the Src kinase family.

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Keywords: G proteins; Gene expression; MAP kinase; Signal transduction

1. Introduction

Thrombin is a procoagulant factor that exerts diverse effects on vascular cells via its ability to bind proteinase-activated receptor 1 (PAR1) [1], a seven transmembrane receptor that couples to heterotrimeric G proteins. Activation of this receptor results in stimulation of various downstream signal transduction elements, including the mitogen-activated protein (MAP) kinase family, nuclear factor-κB (NF-κB), Rho kinase and calcium-dependent kinases, and results in changes in transcription, cell morphology and cell migration. Thrombin may also contribute to the inflammatory state that promotes athero-

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doi:10.1016/j.cardiores.2005.05.029

genesis, as is evidenced by thrombin-induced increases in various proinflammatory cytokines, including interleukin (IL)-1, IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) in endothelial cells, as well as by thrombin-induced increases in expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E- and P-selectin in endothelial cells [2].

ICAM-1 mediates adhesion of inflammatory cells to endothelial cells. Binding of endothelial ICAM-1 to β integrin (LFA-1 and Mac-1) on leukocytes causes firm attachment, enabling leukocytes to transmigrate across the endothelial barrier [3]. Further, studies have demonstrated that upregulation of ICAM-1 on the surface of endothelial cells promotes atherogenesis [4]. ICAM-1 expression is induced by various stimuli, including tumor necrosis factor (TNF)-α, interferon (INF)-γ, IL-1β [5] and thrombin [6,7]. Although studies have clearly demonstrated that NF-κB is essential for ICAM-1 expression [8,9], other studies have reported that TNF-α upregulates ICAM-1 expression via activation of the c-Jun N-terminal kinase (JNK). Further, Gorgolis et al. reported that p53-mediated induction of ICAM-1 after DNA damage occurs via a NF-κB-independent mechanism [10–12]. Finally, JNK is a member of the MAP kinase family that is activated by a variety of stimuli, including cell stress and cytokines. Thrombin also activates JNK in various cell types, and thus may play a role in the inflammatory response in vascular endothelial cells [13,14].

The concordance of these data suggests that JNK may mediate thrombin-induced ICAM-1 expression. Thus, the goal of the present study was to determine whether JNK and other MAP kinase family members mediate thrombin-induced ICAM-1 expression in vascular endothelial cells and to characterize the mechanism(s) by which this regulation may occur in concert with other molecules, particularly NF-κB.

2. Materials and methods

2.1. Materials

Human thrombin and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against ICAM-1 (G-5) (1:500), JNK1 (FL) and β-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An antibody against IκB-α was purchased from Cell Signaling (Beverly, MA). An antibody against phospho-JNK was purchased from Promega (Madison, WI). An antibody against phospho-Src (pY418) was purchased from Biosource International (Camarillo, CA). An antibody against Src (clone 327) was purchased from Oncogene Research Products (San Diego, CA). PP2, PD98059, SP600125, JNKI1, SB203580, and BAY11-7082 were purchased from Calbiochem (San Diego, CA). YM-254890 was provided by Yamanouchi Pharmaceutical Co. (Ibaraki, Japan).

2.2. Plasmids

FLAG-tagged JNK was provided by Dr. R. J. Davis (University of Massachusetts Medical School, MA). Constitutively active Ras (Ras V14) was generated by site-directed point mutagenesis. Constitutively active $G\alpha_q$ ($G\alpha_q$ RC) and $G\alpha_{13}$ ($G\alpha_{13}$ QL) were provided by Dr. M. I. Simon (California Institute of Technology, CA). The β-adrenergic receptor kinase C-terminus (βARK-ct) was provided by Dr. R. J. Lefkowitz (Duke University Medical Center, NC). cDNA for Csk was provided by Dr. J. A. Cooper (Fred Hutchinson Cancer Research Center, WA) and was cloned into pcDNA3.1. ICAM-LUC containing 1393 bp of the 5′ regulatory region of the ICAM-1 promoter-linked firefly luciferase reporter gene was provided by Dr. C. Stratowa (Boehringer Ingelheim, Vienna, Austria). IgκB-LUC containing the κB site-integrated luciferase reporter was provided by Dr. A. Takaori (Kyoto University).

2.3. Cell culture and transfection

Bovine aortic endothelial cells (BAEC; purchased from Clonetec, Palo Alto, CA) and human umbilical vein endothelial cells (HUVEC; purchased from Cell Applications, Inc., San Diego, CA) were cultured in endothelial basal medium containing the specific growth supplements recommended by the manufacturer. Cells at passages 4 to 8 were plated on dishes coated with 1% gelatin. For transfection experiments, BAEC at 50% confluence were transfected using the FuGENE6 (Roche, Basel, Switzerland) method, as previously described [15]. In brief, cells were incubated for 8 h in medium containing the FuGENE reagent along with the gene constructs of interest. After transfection, cells were placed in serum-free medium overnight. Next, the cells were challenged with thrombin for the indicated periods of time and then harvested.

2.4. Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western blot analysis were performed as described previously [16,17]. In brief, cells were lysed in gentle lysis buffer (25 mM Tris [pH 7.5], 50 mM NaF, 10 mM sodium pyrophosphate, 137 mM NaCl, 10% glycerol, 1% NP-40, 5 mM EDTA, 1 mM Na_3VO_4, 10 μg/mL leupeptin, and 1 mM PMSF), and centrifuged. Lysates containing equal amounts of soluble proteins were precleared and incubated with antibodies overnight at 4 °C. Antibody complexes were collected by incubation with protein G-agarose for monoclonal antibody. Precipitates were washed in lysis buffer and then resuspended in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated with a primary antibody and were visualized using the ECL system (Amersham-Pharmacia Co., Buckinghamshire, UK). Images were captured using

Adobe Photoshop, and the band intensities were quantified using NIH Image 1.61.

2.5. Luciferase assay

BAEC were seeded in 12-well plates at 50% confluence for transfection experiments. Cells were allowed to incubate with gene constructs (ICAM-LUC or IgκB-LUC), the Renilla luciferase vector and FuGENE6 reagent for 8 h followed by incubation in serum-free medium overnight. Next, cells were treated with thrombin (3 U/mL) for 8 h and then harvested. Luciferase activity was measured using the Dual-Luciferase Assay Kit (Promega). Firefly luciferase activity was normalized to the Renilla luciferase activity and expressed as a fold increase, in which the control value in the absence of thrombin was defined as 1.0 (control). Repetitive experiments were performed in triplicate.

2.6. Assays for JNK activity

JNK kinase assay was performed as described previously [18]. Briefly, approximately 250 μg of cell lysate protein was incubated with 3 μg GST-c-Jun (1-169) coupled to glutathione agarose at 4 °C for 4 h under constant rotation. Agarose beads were washed three times with buffer B (12.5 mM MOPS [pH 7.2], 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM $MgCl_2$, 1 mM DTT, and 0.1% Triton X-100) containing 0.25 M NaCl. Beads were then incubated in modified buffer B (standard preparation plus 10 mM $MgCl_2$, 1 mM $MnCl_2$, 50 μM ATP, and 15 μCi of [γ-^{32}P] ATP) for 10 min at 30 °C. For cotransfection experiments, exogenous FLAG-tagged JNK1 was immunoprecipitated with FLAG antibody, and the kinase reaction was performed in modified buffer B with 2 μg GST-c-Jun. The reaction was terminated with sample buffer, and relative protein concentrations were determined via SDS-PAGE and autoradiography. Otherwise, JNK activity was determined by Western blot analysis using anti-phospho-JNK antibody.

2.7. Ras activation assay

A Ras Activation Assay Kit, which included GST-fusion proteins containing Raf binding domain (GST-Raf RBD), was purchased from Upstate (Lake Placid, NY). The assay was performed as described previously [19]. Briefly, HUVEC were lysed in MLB (25 mM HEPES [pH 7.5], 150 mM NaCl, 25 mM NaF, 10% glycerol, 0.25% Na deoxycholate, 10 mM $MgCl_2$, 1% Triton X-100, 1 mM EDTA, 1% NP-40, 1 mM Na vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). After centrifugation, lysates were incubated with 10 μg of the fusion protein bound to beads, and the mixture was placed on a rocker plate at 4 °C for 45 min. Bound proteins were collected by centrifugation, and beads were washed 3 times with MLB and then resuspended in SDS sample buffer. Bound Ras was determined by Western blot analysis with anti-Ras (1 : 1000) antibodies.

2.8. ICAM-1 mRNA analysis

Total RNA was isolated from 1×10^6 HUVEC, which had either been left untreated or been stimulated with thrombin (3 U/ml, 6 h)±actinomycin D (10 μg/ml, 1 h), using TRIZOL Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After isolation, 1 μg of RNA was reverse-transcribed into cDNA with an oligo (dT)^{20} primer using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) as described in the manufacturer's instructions. The mRNA levels were analyzed by the LightCycler quantitative RT-PCR strategy (Roche, Basel, Switzerland). Specific primers for ICAM-1 and β-actin were obtained by Search-LC (Heidelberg, Germany). The ICAM-1 mRNA expression was normalized to housekeeping β-actin expression.

2.9. Statistical analysis

Statistical comparisons were made using two-tailed Student's t test. Experimental values were reported as mean±S.D. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Thrombin induces ICAM-1 expression in endothelial cells

Thrombin (3 U/ml) induced an increase in ICAM-1 protein expression in HUVEC in a time-dependent manner (Fig. 1A). Increases in ICAM-1 expression were observed beginning at 4 h after thrombin treatment with a peak between 12 and 24 h. To examine whether this increase was due to increases in ICAM-1 transcription, the ICAM-1 promoter activity was analyzed by luciferase assay using BAEC transfected with ICAM-LUC (Fig. 1B). Thrombin treatment resulted in increases in ICAM-1 promoter activity to 10.3±2.1-fold of basal levels at the 8-h time point. Similarly, ICAM-1 mRNA levels increased in response to thrombin with a peak at 6 h in HUVEC (data not shown). This induction of ICAM-1 mRNA was completely inhibited by the transcriptional inhibitor, actinomycin D (Fig. 1C). These data indicate that thrombin-induced increase in ICAM-1 expression occurs secondary to increases in transcription.

3.2. JNK regulates thrombin-induced ICAM-1 expression in endothelial cells

To determine the role of extracellular signal-regulated kinase (ERK1/2), JNK and p38 MAP kinase in ICAM-1

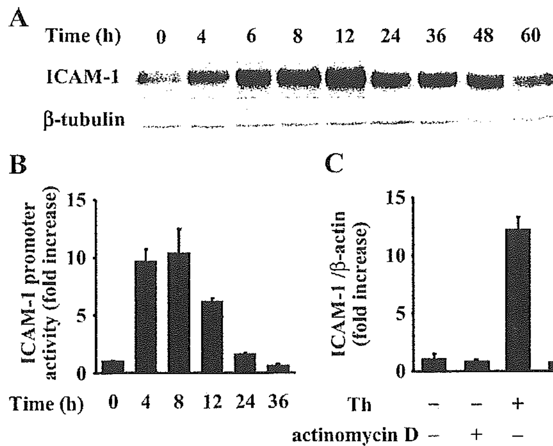

Fig. 1. Thrombin-induced ICAM-1 expression in endothelial cells. (A) Confluent HUVEC were serum-starved and challenged with thrombin (3 U/ml) for the indicated periods. Western blotting was performed with ICAM-1 antibody as described in Materials and methods. ICAM-1 protein level was normalized to that of β-tubulin. (B) BAEC, transfected with a plasmid encoding the ICAM-1 promoter linked with the luciferase reporter gene (ICAM-LUC), were placed in serum-free medium for 16 h. Cells were treated with thrombin (3 U/mL) for the indicated periods and then harvested. Luciferase activity was measured via luminometer. ICAM-1 promoter activity was normalized to Renilla luciferase activity and expressed as a fold increase. (C) HUVEC were incubated with actinomycin D (10 μg/ml) for 1 h before treatment with thrombin (th, 3 U/mL) for 6 h. ICAM-1 mRNA levels, quantitated by real time RT-PCR, were normalized to β-actin mRNA levels. In (B) and (C), values are the mean±S.D. for three separate experiments.

expression, Western blot analysis was performed to measure the change in thrombin-induced ICAM-1 expression following treatment with inhibitors of the MAP kinase family. Treatment of cells with the JNK specific inhibitors, SP600125 (3–30 μM) or a JNK inhibitory peptide (JNKI1) (3–30 μM), resulted in significant attenuation of thrombin-induced ICAM-1 protein expression in a concentration-dependent manner (67±3% and 72±7% inhibition at 30 μM, respectively; Fig. 2A). In contrast, treatment of cells with the MEK inhibitor, PD98059, or the p38 inhibitor, SB203580, had minimal effect on thrombin-induced ICAM-1 expression. Similarly, thrombin-induced ICAM-1 promoter activity was also inhibited by SP600125 or by JNKI1, while PD98059 and SB203580 had only slight inhibitory effect (Fig. 2B). These data indicate that thrombin-induced ICAM-1 expression is mediated predominantly by JNK activity in comparison to the relatively small effect of other members of the MAP kinase family in endothelial cells.

3.3. *JNK mediates thrombin-induced ICAM-1 expression independent of the NF-κB pathway*

To determine the relative contributions of the JNK and NF-κB pathways on thrombin-induced ICAM-1 expression, Western blot analysis of thrombin-induced ICAM-1 expression was performed after treatment of cells with the JNK inhibitor, SP600125, or with the NF-κB inhibitor, BAY11-7082. Treatment of cells with either SP600125 or BAY11-

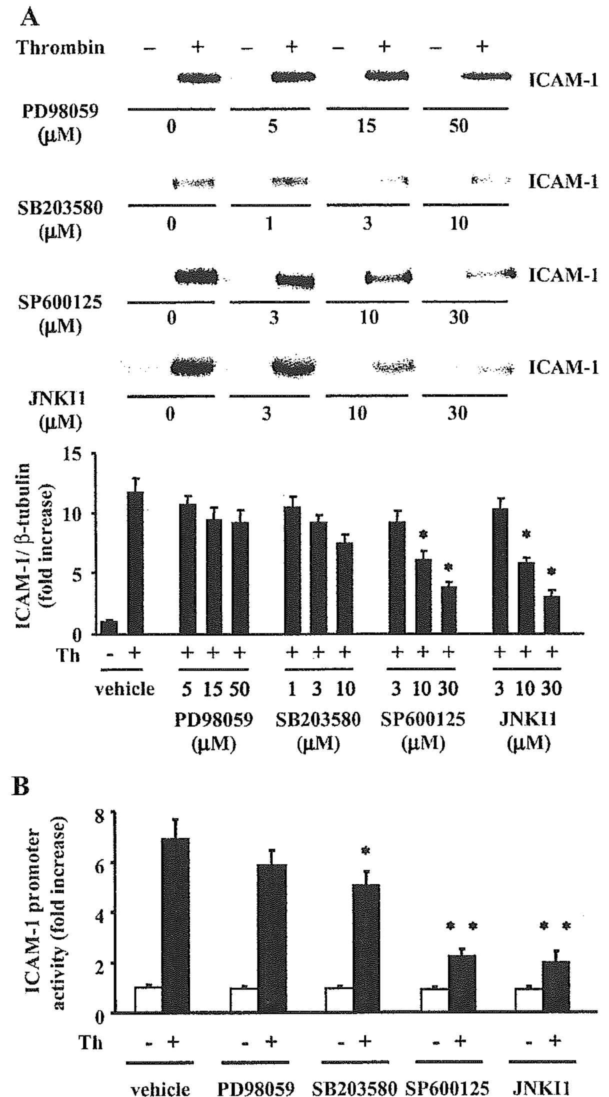

Fig. 2. Effects of MAP kinase family inhibitors on thrombin-induced ICAM-1 expression. (A) Growth-arrested HUVEC were treated with various concentrations of PD98059, SB203580, SP600125, or JNKI1 for 1 h prior to challenge with thrombin (th; 3 U/ml) for 12 h. Cell lysates were prepared and assayed for ICAM-1 and β-tubulin expression by Western blot analysis. Representative immunoblots and the corresponding bar graph are shown. In the graph, the expression of ICAM-1 was normalized to that of β-tubulin and expressed as a fold increase, in which the control ratio without thrombin was defined as 1.0 (control). (B) BAEC, transfected with ICAM-LUC, were treated for 1 h with PD98059 (50 μM), SB203580 (10 μM), SP600125 (30 μM), or JNKI1 (30 μM) prior to challenge with (filled bar) or without (blank bar) 3 U/mL thrombin for 8 h. Normalized luciferase activity was expressed as a fold increase, in which the control value without thrombin was defined as 1.0 (control). In all experiments, values are the mean±S.D. for three separate experiments. *, $p<0.05$ versus control with thrombin; **, $p<0.01$ versus control with thrombin.

7082 resulted in significant inhibition of thrombin-induced ICAM-1 expression (56±7 and 67±3% inhibition, respectively, Fig. 3A). However, treatment of cells with both agents in combination resulted in near complete inhibition (88±3% inhibition) of thrombin-induced ICAM-1 expression.

Further experiments were conducted to examine whether MAP kinases modulate thrombin-induced NF-κB activation. Phosphorylation and consequent degradation of IκB-α lead to activation of NF-κB pathway. Thrombin induced IκB-α degradation with a peak at 1 h, and this effect was not attenuated in the presence of PD98059, SP600125 or SB203580 (Fig. 3B). The MAP kinase family inhibitors did not inhibit IκB-α phosphorylation, which peaked at 10 min after thrombin stimulation (data not shown). These data indicate that JNK mediates thrombin-induced ICAM-1 expression independent of the action of the NF-κB pathway.

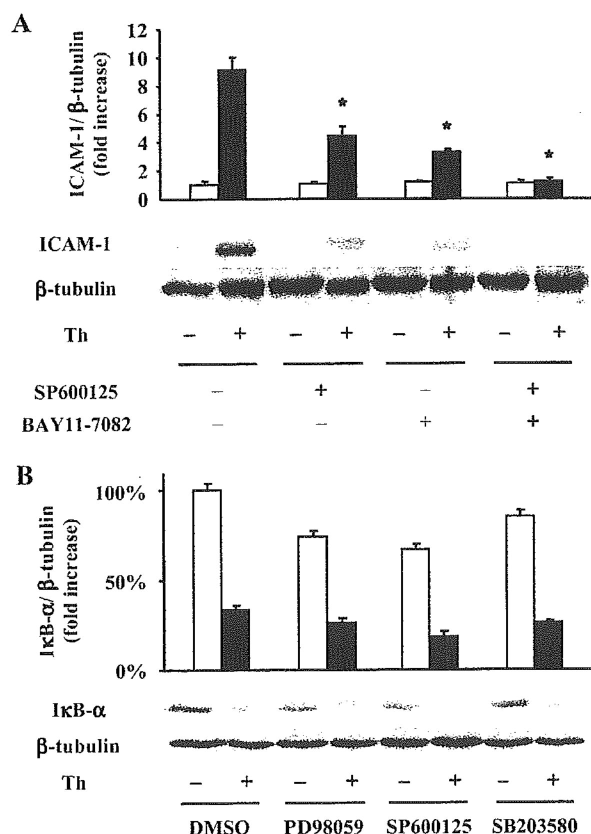

Fig. 3. Effects of JNK and NF-κB on thrombin-induced ICAM-1 expression. (A) HUVEC were treated with SP600125 (30 μM), BAY11-7082 (10 μM), or the combination of the two compounds prior to challenge with 3 U/mL thrombin (th) for 12 h. Western blot analysis was performed with anti-ICAM-1 and β-tubulin antibody. ICAM-1 expression was normalized and presented as a fold increase in the bar graph. (B) IκB-α degradation assay. HUVEC were treated with PD98059 (50 μM), SP600125 (30 μM), or SB203580 (10 μM) for 1 h before thrombin stimulation. Western blot analysis was performed with anti-IκB-α antibody. IκB-α expression was normalized to that of β-tubulin in the bar graph, in which the control ratio without thrombin was defined as 100%. Representative immunoblots and the corresponding bar graphs are shown. Values are the mean±S.D. for three separate experiments. *, $p<0.05$ versus control with thrombin.

3.4. Thrombin receptor-induced JNK activation and ICAM-1 expression is mediated by the $G\alpha_q$ and $G\beta\gamma$ subunits

To characterize the mechanisms by which JNK mediates thrombin-induced ICAM-1 expression, thrombin-induced JNK activity was assayed following modulation of various signal transduction elements. Thrombin (3 U/mL) induced rapid activation of JNK, with a peak at 10–20 min (Fig. 4A), and JNK activation was still detectable at 60 min.

Thrombin binds to PAR-1, which couples to $G\alpha_q$, $G\alpha_{13}$ and $G\alpha_i$ [1]. To determine whether $G\alpha_q$ mediates thrombin-induced JNK activation and ICAM-1 expression, the $G\alpha_q$-selective inhibitor, YM-254890 was utilized [20]. As shown in Fig. 4B and C, YM-254890 potently suppressed thrombin-induced JNK activation and ICAM-1 protein expression. Further, thrombin-induced ICAM-1 promoter activity was markedly inhibited by YM-254890, while overexpression of constitutively active $G\alpha_q$ resulted in induction of ICAM-1 promoter activity to levels 2.4±0.5-fold above baseline levels (Fig. 4D).

To determine the role of the released Gβγ subunit of the heterotrimeric G protein, which is also capable of signal transduction, JNK activity and ICAM-1 expression were assessed following treatment of cells with a specific scavenger for Gβγ subunit, βARK1ct. Transfection of βARK1ct resulted in a decreased level of thrombin-induced JNK activation and ICAM-1 promoter activity when compared to transfection of vector alone (61±6% and 68±2% inhibition, respectively; Fig. 4E, F). These data indicate that $G\alpha_q$ and Gβγ subunits play a significant role in JNK-mediated ICAM-1 expression induced by thrombin.

3.5. Ras and Rac1 mediate thrombin-induced JNK activation and ICAM-1 expression

Thrombin activates various small G proteins, such as Ras, Rac1 and Rho [1]. As shown in Fig. 5A, overexpression of constitutively active Ras (Ras V14) or Rac1 (Rac1 QL) resulted in a significant increase in JNK activity, while overexpression of constitutively active Cdc42 (Cdc42 V12) or Rho (Rho V14) had only minimal or no effect. In contrast, overexpression of dominant-negative Ras (Ras N19) or Rac1 (Rac1 N17) inhibited thrombin-induced JNK activity (Fig. 5B), while dominant-negative Cdc42 or Rho had no effect (data not shown). In addition, dominant-negative Ras or Rac1 inhibited thrombin-induced ICAM-1 promoter activity by 59±14% and 43±2%, respectively, whereas dominant-negative Rho had no effect on ICAM-1 promoter activity (Fig. 5C). Similarly, dominant-negative Ras and Rac1 inhibited ICAM-1 promoter activation induced by constitutively active $G\alpha_q$ (Fig. 5D). Furthermore, thrombin-induced Ras activation was potently

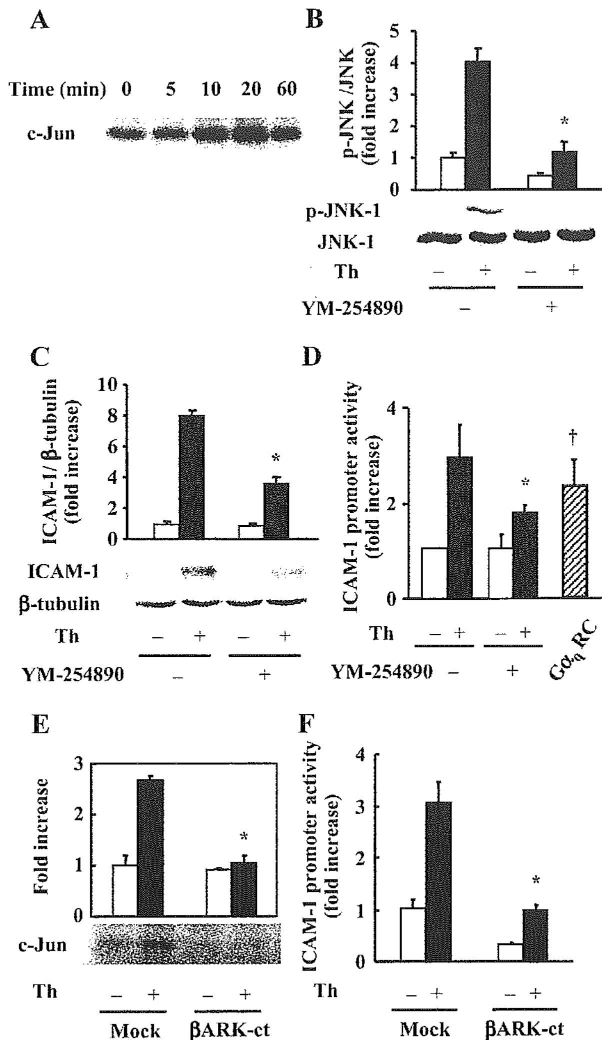

Fig. 4. Effects of $G\alpha_q$ and $G\beta\gamma$ subunits on thrombin-induced JNK activation and ICAM-1 expression. (A) Time course for thrombin-induced JNK activation. BAEC were stimulated with thrombin (3 U/mL) for indicated times. JNK activity was analyzed by a JNK kinase assay with c-*jun* as a substrate. (B) (C) Western blot analysis. HUVEC were treated with 1 μM YM-254890 for 1 h and then challenged with thrombin (th) for 10 min (B) or 1 h (C), respectively. (D) Luciferase assay. BAEC expressing ICAM-LUC were pretreated for 1 h with YM-254890 (1 μM) prior to challenge with thrombin for 8 h. BAEC cotransfected with ICAM-LUC and constitutively active $G\alpha_q$ construct were allowed to grow for 20 h. Normalized luciferase activity was expressed as a fold increase. (E) JNK kinase assay. BAEC were cotransfected with FLAG-tagged JNK and βARK-ct expression vector. Cells were stimulated with thrombin for 20 min. Exogenous JNK was immuno-precipitated with anti-FLAG antibody, and JNK activity was expressed as a fold increase, in which JNK activity in cells transfected with mock vector was defined as 1.0. (F) Luciferase assay. BAEC, cotransfected with ICAM-LUC and βARK-ct, were challenged with thrombin for 8 h. In all experiments, values are the mean±S.D. for three separate experiments. * †, $p<0.05$ versus control in the presence and absence of thrombin, respectively.

inhibited by YM-254890 (Fig. 5E), suggesting that Ras is involved in thrombin-induced JNK activation and ICAM-1 expression at a point downstream of $G\alpha_q$.

To determine whether small G proteins mediate thrombin-induced activation of NF-κB pathway, NF-κB reporter assays were performed (Fig. 5F). Preliminary data showed that NF-κB promoter activity peaked at 8 h after thrombin stimulation (7.3±0.8 fold above baseline levels, data not shown). Dominant-negative Ras, Rac and Rho had minimal or no effect on thrombin-induced NF-κB promoter. These

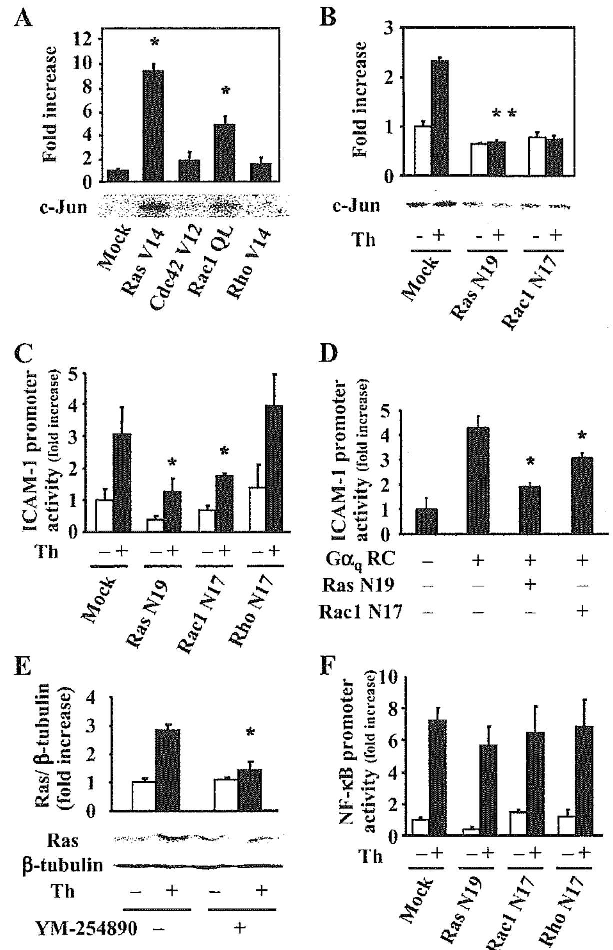

Fig. 5. Effect of small G proteins on thrombin-induced JNK activation and ICAM-1 expression. (A) (B) JNK kinase assay. In (A), BAEC were cotransfected with FLAG-tagged JNK and constitutively active small G proteins. In (B), BAEC, expressing dominant-negative small G proteins, were stimulated with thrombin (3 U/mL) for 20 min. The bar graphs represent quantified levels of JNK activity, expressed as a fold increase. (C) BAEC were cotransfected with ICAM-LUC and dominant-negative small G proteins and challenged with thrombin for 8 h. (D) BAEC were cotransfected with ICAM-LUC and constitutively active $G\alpha_q$ along with dominant-negative small G protein mutants. Cells were harvested at 36 h after transfection. (E) Ras activation assay. HUVEC were pretreated with YM-245890 (1 μM) prior to challenge with thrombin for 5 min. Ras binding activity was normalized to β-tubulin expression and expressed as a fold increase. (F) BAEC were cotransfected with IgκB-LUC and dominant-negative small G proteins and stimulated with thrombin for 8 h. Normalized luciferase activity was expressed as a fold increase. In all experiments, values are the mean±S.D. for three separate experiments. *, $p<0.05$ versus control with thrombin.

data suggest that Ras and Rac1 mediate thrombin-induced JNK activation and ICAM-1 expression in endothelial cells.

3.6. Thrombin-induced JNK activation and ICAM-1 expression is mediated by the Src kinase family

G-protein coupled receptors, including PAR1, activate the Src kinase family [16,17,21]. Treatment of cells with the Src kinase family inhibitor, PP2, attenuated thrombin-induced JNK activation in a concentration-dependent manner (Fig. 6A). Similarly, expression of an endogenous inactivator of the Src family kinases, Csk, inhibited thrombin-induced JNK activation and ICAM-1 promoter activity by 50±5% and 62±7%, respectively (Fig. 6B, C). Consistently, PP2 treatment resulted in decreased thrombin-induced ICAM-1 protein expression (Fig. 6D).

To examine the role of $G\alpha_q$ in thrombin-induced activation of c-Src, HUVEC were treated with YM-254890. YM-254890 significantly attenuated thrombin-induced activation of c-Src by 38±15% (Fig. 6E). Furthermore, to elucidate the relationship between c-Src and Ras, we examined the effect of PP2 on Ras activity. As a result, PP2 potently attenuated thrombin-induced Ras by 67±7% (Fig. 6F). These data, in combination with those shown in Fig. 5E, suggest that c-Src mediates the signal to JNK and ICAM-1 expression in response to thrombin at a point downstream of $G\alpha_q$ and upstream of Ras.

PP2 did not inhibit thrombin-induced degradation of IκB-α, suggesting that the Src kinase family does not affect activation of NF-κB (Fig. 6G). Consistent with these data, overexpression of Csk or dominant-negative Src and Fyn did not affect thrombin-induced NF-κB promoter activity (data not shown).

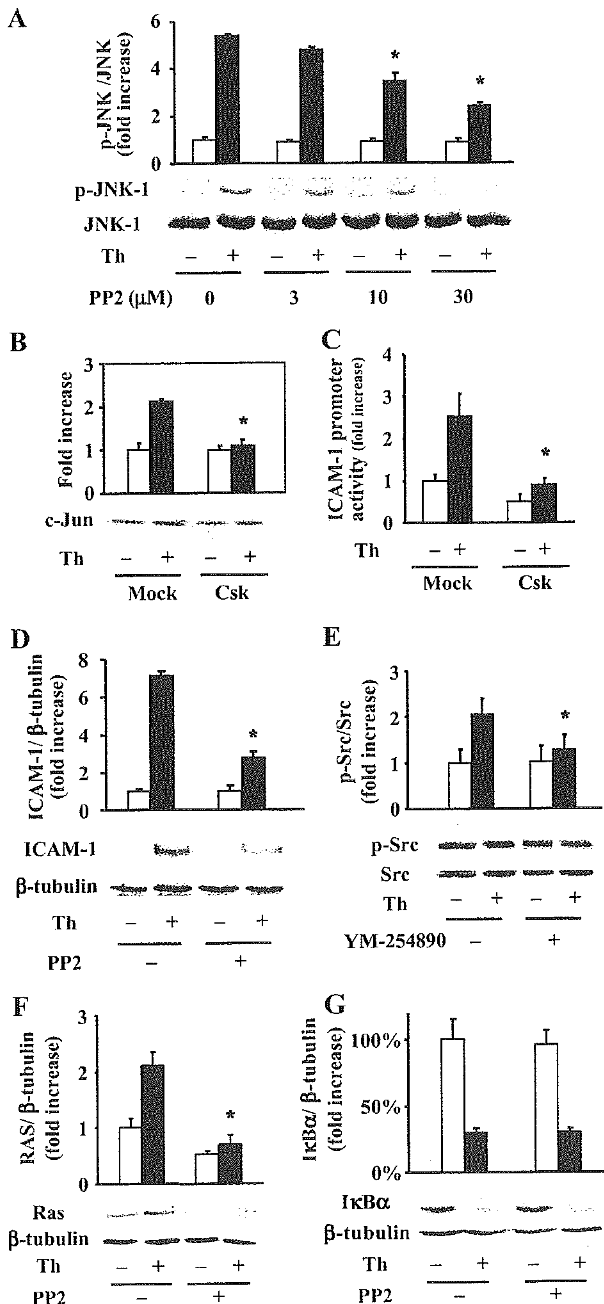

Fig. 6. Effects of Src kinase family on thrombin-induced JNK activation and ICAM-1 expression. (A) HUVEC were pretreated with PP2 (0–30 μM) for 30 min prior to challenge with thrombin (th) for 10 min. Phosphorylated JNK expression was detected by Western blot analysis and normalized to that of JNK in the bar graph. (B) JNK kinase assay. BAEC coexpressing FLAG-tagged JNK and Csk were stimulated with thrombin for 20 min. The bar graphs represent quantified levels of JNK activity as a fold increase. (C) Luciferase assay. BAEC, cotransfected with ICAM-LUC and Csk, were challenged with thrombin for 8 h. Normalized luciferase activity was expressed as a fold increase. HUVEC were pretreated with PP2 (30 μM) for 30 min prior to challenge with thrombin for 12 h (D) or 1 h (G), respectively. Expression of ICAM-1 (D) and IκB-α (G) were detected by Western blot analysis. (E) HUVEC were pretreated with YM-254890 (1 μM) for 1 h and stimulated with thrombin for 30 s. Phosphorylated Src and Src were detected by Western blot analysis. (F) Ras activation assay. HUVEC were pretreated with PP2 (30 μM) prior to challenge with thrombin for 5 min. Ras binding activity was normalized to β-tubulin expression and expressed as a fold increase. In all experiments, values are the mean±S.D. for three separate experiments. *, $p<0.05$ versus control with thrombin.

4. Discussion

The present study demonstrated that JNK is involved in thrombin-induced ICAM-1 expression in vascular endothelial cells. This study also presented the novel finding that the signal transduction cascade leading to JNK-dependent ICAM-1 expression involves $G\alpha_q$, $G\beta\gamma$, Ras, Rac1 and the Src kinase family. Further, inhibition of ERK and p38 MAP kinase had only slight effect on thrombin-induced ICAM-1 expression or ICAM-1 promoter activity, indicating that these kinases play only minor roles in this pathway. By contrast, Rahman et al. reported that the p38 MAP kinase inhibitor, SB203580, significantly inhibited thrombin-induced ICAM-1 expression [22]. The cause of this

discrepancy is unknown, but it is possible that the difference in HUVEC sources could result in a differential signaling mechanism for thrombin-induced ICAM-1 expression. De Cesaris et al. have demonstrated that SB203580 did not inhibit ICAM-1 expression in another setting [22,23], which is consistent with findings from the present study.

ERK and JNK are independently regulated by Ras and Rac1/Cdc42, respectively, in many cell types [24]. However, in the present study, thrombin-induced JNK activation and ICAM-1 promoter activity was inhibited by dominant-negative Ras as well as by dominant-negative Rac1. Several investigators have reported that Ras mediates JNK activation by various stimuli, including the response to v-Src and shear stress [25,26]. While the mechanism by which Ras activates JNK is largely unknown, Ras may activate Rac-guanine nucleotide exchange factors (GEF), such as Vav and Sos-1, via PI3K in several cell types [27]. It is unlikely that Ras and Rac1 independently regulate ICAM-1 expression via ERK and JNK, respectively, because inhibition of ERK by PD98059 had no effect on ICAM-1 expression in the present study. Alternatively, Ras may converge with the JNK pathway at a point upstream of Rac-GEFs.

PAR1 is the main thrombin receptor expressed in endothelial cells and is coupled to $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_i$ [1]. The present study focused on $G\alpha_q$ using the selective inhibitor, YM-254890, which does not inhibit $G\alpha_{12/13}$ or $G\alpha_i$ [20]. YM-254890 significantly inhibited JNK activation and ICAM-1 expression. In addition, the expression of constitutively active $G\alpha_q$ resulted in an increase in ICAM-1 promoter activity. These data indicate that $G\alpha_q$ plays a crucial role in thrombin-induced JNK activation and ICAM-1 expression. Further, these data are supported by a study by Nagao et al., which demonstrated that $G\alpha_{q/11}$ stimulates JNK activity through the Src family kinase [28]. It is possible that another Gα member, such as $G\alpha_{12/13}$, is also involved in thrombin-induced JNK activation and ICAM-1 expression. Indeed, constitutively active $G\alpha_{13}$ increased ICAM-1 promoter activity in endothelial cells (unpublished data), and Collins et al. demonstrated that the expression of constitutively active $G\alpha_{12}$ increased JNK activity [29]. In addition, Rahman et al. showed that the expression of constitutively active $G\alpha_q$ increased NF-κB activity [30]. Thus, $G\alpha_q$ may be involved in thrombin-induced JNK and NF-κB activation, both of which mediate ICAM-1 expression.

The βγ dimers of heterotrimeric proteins regulate the activity of many signaling molecules [31]. In the present study, expression of the carboxyl terminus of the β-adrenergic receptor kinase, which acts as a Gβγ scavenger, inhibited thrombin-induced JNK activation and ICAM-1 promoter activity. Several studies have reported involvement of βγ subunits in GPCR-induced activation of JNK [32,33] via Ras and Rac1. Further, Kiyono et al. showed that free βγ dimers activate Ras-GRFs, which serve as Ras GEF, as well as induce guanine-nucleotide exchange on Ras and Rac1 [34]. These data indicate that βγ subunits, in coordination with Gα subunits, mediate thrombin-induced JNK activation, possibly through Ras and Rac1.

Several investigators have demonstrated involvement of c-Src and other Src kinase family members in GPCR signaling [21], and we previously demonstrated that angiotensin II-induced c-Src activation regulated ERK activation and cytoskeletal reorganization in vascular smooth muscle cells [16,17,35]. In the present study, inhibition of the Src kinase family by expression of Csk resulted in marked suppression of JNK and ICAM-1 promoter activity, and YM-254890 significantly inhibited c-Src activation by thrombin stimulation. Furthermore, thrombin-induced Ras activation was inhibited by YM-254890 and by PP2. These data suggest that the thrombin-induced JNK activation and ICAM-1 expression are mediated, at least in part, via the $G\alpha_q$-Src-Ras axis, possibly in concert with Gβγ. Indeed, Kiyono et al. showed that the GEF activity of Ras-GRF1 toward Ras and Rac1 is dependent on phosphorylation by Src [36].

The present study did not elucidate the transcription mechanism by which JNK mediates thrombin-induced ICAM-1 expression. JNK phosphorylates c-Jun and ATF-2 and increases their ability to activate transcription, leading to c-*jun* induction and subsequent AP-1 activation [37,38]. ICAM-1 gene expression is also modulated by multiple *cis*-acting elements, including binding sites for AP-1, NF-κB and SP-1 [39], and thrombin stimulates AP-1 DNA binding and AP-1 mediated transactivation [40]. These data are consistent with those reported by Kobuchi et al., which showed that PMA and TNF-α induced ICAM-1 expression via the activation of the JNK pathway and AP-1 [12], and suggest that JNK-dependent ICAM-1 expression is mediated by AP-1.

Although previous studies have reported that NF-κB mediates thrombin-induced ICAM-1 expression [9,22], the present data suggest that the JNK pathway also plays a significant role in the signaling cascade leading to induction of ICAM-1 expression. NF-κB inhibition resulted in a significant, but not complete attenuation of thrombin-induced ICAM-1 expression. Further, inhibition of both JNK and NF-κB resulted in an additive inhibitory effect on ICAM-1 expression. Neither thrombin-induced IκB-α degradation nor NF-κB promoter activity was affected by inhibition of JNK, small G proteins or the Src kinase family, which suggests that the thrombin-activated JNK pathway and the NF-κB pathway are largely separate. De Cesaris et al. showed that TNF-α up-regulates ICAM-1 expression via activation of JNK, and Gorgoulis et al. reported that p53-mediated induction of ICAM-1 after DNA damage occurs via an NF-κB-independent mechanism [10,11], which is consistent with data from the present study.

In order to characterize which signaling molecules were involved in thrombin-induced ICAM-1 expression, we performed experiments with Western blot analysis and reporter assays with dominant-negative mutants. The data from both sets of experiments were comparable, except in

the case of Ras and Rac1, in which experiments could not be conducted because selective inhibitors for these molecules are not available. Thus, future studies to confirm the involvement of Ras and Rac1 in thrombin-induced increases in ICAM-1 expression are warranted.

In summary, the present study demonstrated that thrombin-induced ICAM-1 expression is mediated by JNK in vascular endothelial cells, and that this pathway likely involves $G\alpha_q$, $G\beta\gamma$, Ras, Rac1 and Src family kinases. These data support the potential clinical utility of therapeutic targeting of JNK for prevention of atherosclerosis.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Heart Foundation, and the Takeda Science Foundation and Tsuchiya Memorial Foundation. We thank Dr. Oren Traub for reading this manuscript.

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The Nicorandil-Induced Vasodilation in Humans Is Inhibited by Miconazole

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Abstract: Nicorandil, N-(2-hydroxyethyl)-nicotinamide nitrate, exerts its vasodilatory effects by opening ATP-sensitive potassium (K-ATP) channels and by acting as the exogenous nitric oxide (NO). It is not clear, however, whether the actions of other endothelium-dependent vasodilators, such as NO, endothelium-derived hyperpolarizing factor (EDHF), and prostaglandins, contribute to nicorandil-induced vasodilation in the vasculature in humans. We evaluated forearm blood flow (FBF) response to intraarterial infusion of nicorandil alone and in the presence of glibenclamide, a K-ATP channel inhibitor, N^G-monomethyl-L-arginine, an NO synthase inhibitor, indomethacin, a cyclooxygenase inhibitor, or miconazol, a cytochrome P-450 inhibitor, in 24 healthy male subjects. FBF was measured using strain-gauge plethysmography. Infusion of nicorandil significantly increased the FBF response in a dose-dependent manner. Intraarterial infusion of glibenclamide attenuated nicorandil-induced vasodilation (160.9 ± 21.2% versus 90.2 ± 19.4%, $P < 0.01$), and miconazole also attenuated the FBF response to nicorandil (160.9 ± 21.2% versus 66.1 ± 9.2%, $P < 0.001$). N^G-monomethyl-L-arginine or indomethacin did not alter the FBF response to nicorandil. These findings suggest that nicorandil causes vasodilation in forearm circulation in humans, at least in part through a pathway that is dependent on K-ATP channels and cytochrome P-450, but not on endogenous NO and prostaglandins. EDHF may contribute to nicorandil-induced vasodilation in humans.

Key Words: nicorandil, ATP-sensitive potassium channels, endothelium-derived hyperpolarizing factor, nitric oxide

(*J Cardiovasc Pharmacol*™ 2005;45:290–294)

Received for publication September 30, 2004; accepted December 17, 2004.
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This study was supported in part by grants-in-aide for Scientific Research (1559075100 and KOSEI151201) from the Ministry of Education, Science and Culture of Japan.
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Nicorandil, N-(2-hydroxyethyl)-nicotinamide nitrate, has been one of the most frequently used drugs in the treatment of angina pectoris and has a dual mechanism of action. In experimental models in vitro and in vivo, its distinctive pharmacological effect is opening ATP-sensitive potassium (K-ATP) channels, thereby dilating peripheral and coronary resistance arterioles, but it also possess a nitrate moiety that dilates systemic veins and epicardial coronary arteries.[1,2] The Impact of Nicorandil in Angina randomized trial (IONA) study demonstrated that nicorandil therapy decreased the composite endpoint of death from coronary heart disease in patients with stable angina.[3] In this study, nicorandil was given in addition to other standard antianginal therapy, including β-blockers, calcium-channel blockers, or long-acting nitrates. Therefore, these findings suggest that its clinical benefit is elicited by activation of K-ATP channels, which lead to the phenomenon of ischemic preconditioning and might represent an endogenous cardioprotective mechanism.[4,5]

K-ATP channels exist in vascular smooth muscle cells[6] and play an important role in the vascular responses to a variety of pharmacological and endogenous vasodilators, such as prostacyclin (PGI_2), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF). The nature of EDHF has remained controversial since the first report of its existence.[7,8] EDHF candidates include epoxyeicosatrienoic acids (EETs),[9,10] which are products of cytochrome P-450 (CYP450)-dependent arachidonate metabolism, K^+,[11] gap junctions,[12,13] and hydrogen peroxide.[14,15] It is known that K-ATP channels are activated in response to NO or PGI_2.[6,16,17] In general, the hyperpolarization mechanism of EDHF is thought to be mediated by Ca^{2+}-sensitive K^+ channels on vascular smooth muscle.[6,18–20] Some reports have suggested that EDHF may activate K-ATP channels.[21,22]

However, there is little information on the role of endogenous vasodilators, such as NO, PGI_2, and EDHF, in nicorandil-induced vasodilation in humans. Therefore, the purpose of this study was to determine the mechanisms by which nicorandil causes vasodilation in humans.

METHODS

Subjects

We studied 24 healthy Japanese men (mean age 25 ± 5 years) who had no history of cardiovascular disease,

hypertension, or other diseases. They had no history of smoking and took no medications for at least 4 weeks before the study. The study protocol was approved by the ethics committee of Hiroshima University Graduate School of Biomedical Sciences. Informed consent for participation in the study was obtained from all subjects.

Study Protocol

The study began at 8:30 AM. Subjects fasted the previous night for at least 12 hours. They were kept in the supine position in a quiet, dark, air-conditioned room (constant temperature 22–25°C) throughout the study. A 23-gauge polyethylene catheter (Hakkow Co, Okayama, Japan) was inserted under local anesthesia (1% lidocaine) into the left brachial artery for the infusion of drugs and for the recording of arterial pressure using an AP-641G pressure transducer (Nihon Koden Kogyo, Tokyo, Japan). After 30 minutes in the supine position, basal forearm blood flow (FBF), heart rate, and arterial blood pressure were measured. Then nicorandil (0.1, 0.3, 1.0 μg/min/kg) was administered intraarterially for 5 minutes at each dose. In a preliminary study, we confirmed that nicorandil at 0.1 to 3.0 μg/min/kg increased FBF without altering systemic hemodynamics. And then, the dose–response curve to intraarterial nicorandil was repeated during coadministration of indomethacin, miconazole, or glibenclamide alone. These drugs were dissolved in 0.9% saline, and indomethacin was infused at a dose of 50 μg/min, started 10 minutes before nicorandil and continued throughout. Miconazole (0.25 mg/min) and glibenclamide (125 μg/min) were similarly coadministered. Finally, N^G-monomethyl-L-arginine (L-NMMA, CLINALFA Co), an NO synthase inhibitor, was infused intraarterially at a dose of 8 μmol/min for 5 minutes, and nicorandil (0.1, 0.3, 1.0 μg/min/kg) was administered. Each study was carried out in a randomized fashion and proceeded after FBF had returned to baseline level.

Measurement of FBF

The FBF was measured with a mercury-filled Silastic strain-gauge plethysmograph (EC-5R, D. E. Hokanson, Inc, Issaquah, WA) as previously described.[23,24] Briefly, a strain-gauge was attached to the upper part of the left arm and connected to a plethysmography device, and was supported above the right atrium. A wrist cuff was inflated to a pressure of 50 mm Hg above the systolic blood pressure to exclude the hand circulation from the measurements 1 minute before each measurement and throughout the measurement of FBF. The upper arm congesting cuff was inflated to 40 mm Hg for 7 seconds in each 15-second cycle to occlude venous outflow from the arm using a rapid cuff inflator (EC-20, D. E. Hokanson, Inc). The FBF output signal was transmitted to a recorder (U-228, Advance Co.). FBF was expressed as milliliters per minute per 100 mL of forearm tissue volume. Four plethysmographic measurements were averaged for analysis of FBF at baseline and during administration of drugs. FBF was calculated from the linear portions of plethysmographic recordings by 2 independent observers blinded to the study protocol. The intraobserver coefficient of variation was 3.4%. We confirmed the reproducibility of FBF response to nicorandil in 7 healthy men (mean age 25±2 years) on 2 separate occasions. The coefficients of variation were 7.6%.

Analytic Methods

Routine chemical methods were used to determine serum concentrations of total cholesterol, high-density lipoprotein cholesterol, triglyceride, creatinine, glucose, and electrolytes. Serum concentration of low-density lipoprotein was determined using the Friendewald method.

Statistical Analysis

Results are presented as the mean ± SD. Values of $P < 0.05$ were considered significant. Comparisons of time curves of parameters during the infusion of nicorandil alone or with other agents were analyzed by 2-way ANOVA for repeated measures with Bonferroni correction.

RESULTS

Clinical Characteristics

Baseline clinical characteristics in all subjects are summarized in Table 1.

FBF Response to Nicorandil

Nicorandil significantly increased FBF in a dose-dependent manner (Fig. 1). Intraarterial infusion of miconazole significantly attenuated nicorandil-induced vasodilation (percentage change from basal FBF 178.8 ± 18.7% vs 66.1 ± 9.1%, $P < 0.001$), and glibenclamide also attenuated the FBF responses to nicorandil (percentage change from basal FBF 178.8 ± 18.7% vs 90.2 ± 19.4%, $P < 0.01$) (Fig. 2). Neither miconazole nor glibenclamide altered basal FBF. The administration of glibenclamide did not cause hypoglycemia in any of subjects.

Intraarterial infusion of L-NMMA significantly decreased basal FBF in all subjects (4.8 ± 1.8 to 3.5 ± 1.4 mL/min per 100 mL, $P < 0.01$). Indomethacin did not alter basal FBF. The dose–response curves with nicorandil were not changed by coadministration of indomethacin or L-NMMA (Fig. 3).

TABLE 1. Clinical Characteristics of Study Subjects (n = 24)

Variables	
Age (yr)	25 ± 5
Body mass index (kg/m^2)	22.0 ± 2.3
Systolic blood pressure (mm Hg)	121.2 ± 11.9
Diastolic blood pressure (mm Hg)	61.7 ± 9.0
Pulse rate (bpm)	61.9 ± 9.1
Total cholesterol (mmol/L)	4.29 ± 0.58
Triglyceride (mmol/L)	1.08 ± 0.29
HDL cholesterol (mmol/L)	1.19 ± 0.24
LDL cholesterol (mmol/L)	2.52 ± 0.54
Serum glucose (mmol/L)	4.9 ± 1.0
Serum insulin (pmol/L)	150.5 ± 38.9
FBF (mL/min per 100 mL tissue)	4.8 ± 1.8

All results are presented as the mean ± SD.

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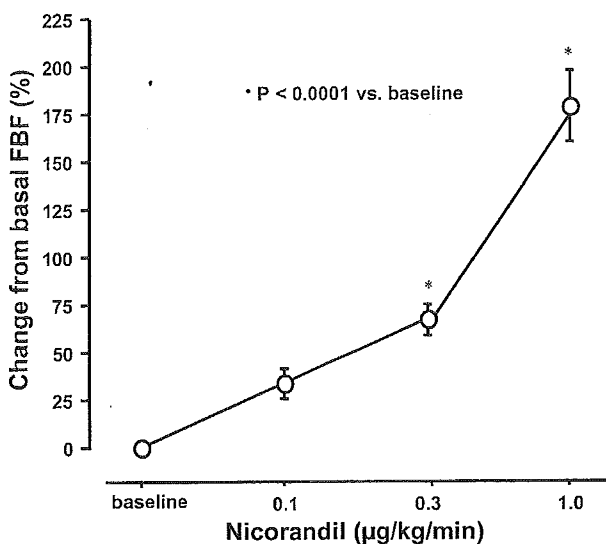

FIGURE 1. Forearm blood flow responses to intraarterial administration of nicorandil alone (○).

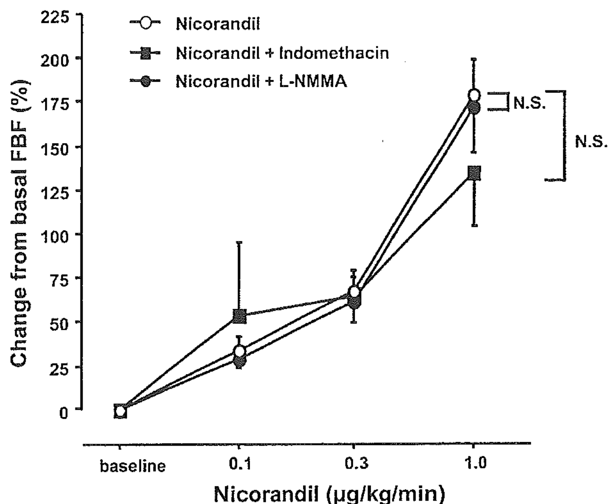

FIGURE 3. Forearm blood flow responses to intraarterial administration of nicorandil alone (○), with indomethacin (■), and with L-NMMA (●).

No significant change was observed in arterial blood pressure or heart rate during infusion of nicorandil or other agents.

DISCUSSION

The present study demonstrated that (1) intra–brachial artery infusion of nicorandil significantly increased FBF in a dose-dependent manner in healthy young men, (2) glibenclamide attenuated nicorandil-induced vasodilation, (3) miconazole attenuated nicorandil-induced vasodilation, (4) indomethacin did not alter the FBF response to nicorandil, and (5) L-NMMA did not alter the FBF response to nicorandil.

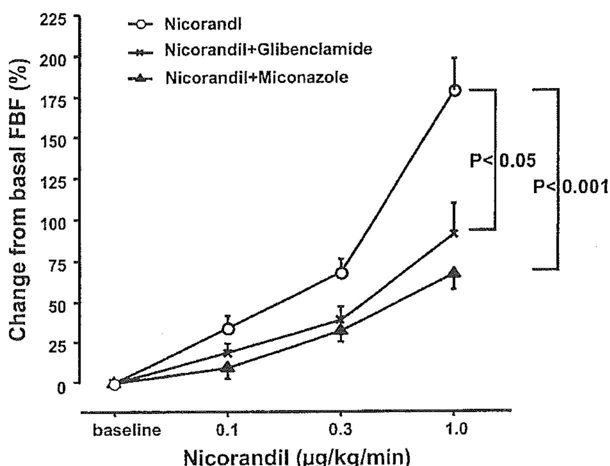

FIGURE 2. Forearm blood flow responses to intraarterial administration of nicorandil alone (○), with glibenclamide (×), and with miconazole (▲).

To our knowledge, this is the first report on the mechanism of nicorandil-induced vasodilation in humans. In the present study, to avoid factors, including aging, smoking, abnormal glucose metabolism, lipid profiles, and menstrual cycle, that alter vascular function, we recruited healthy young men. We demonstrated that miconazole and glibenclamide inhibited nicorandil-induced vasodilation by approximately 60% and 50%, respectively, in forearm circulation in the healthy male subjects. It has been certified in animal studies that nicorandil has a dual action mechanism: it opens K-ATP channels on the membranes of vascular smooth muscle cells, and it acts as exogenous NO. Glibenclamide has been used widely in animal and human studies to examine the role of K-ATP channels in blood flow regulation. The doses of glibenclamide we used were chosen to achieve peak blood concentrations that occur after oral administration of 5 mg to diabetic patients.[25–27] It has been reported that glibenclamide inhibits the forearm vasodilatory response to the selective K-ATP channel opener diazoxide but not that to the direct smooth muscle relaxant nitroprusside.[28] It is well established that glibenclamide is a specific inhibitor of K-ATP channels. Our results are consistent with results of previous studies showing that nicorandil-induced vasodilation is, at least in part, mediated by opening of K-ATP channels.[1,2]

K-ATP channels are thought to play an important role as mediators of the responses of vascular smooth muscle cells to a variety of pharmacological and endogenous vasodilators as well as to changes in metabolic activity that can directly influence blood flow in various tissues.[6] Endogenous NO produced by the L-arginine–NO synthase pathway relaxes vascular smooth muscle by several mechanisms, one of which is through activation of K^+ channels. Many previous studies have shown that endogenous NO activates K-ATP channels.[6,16]

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Therefore, it is thought that nicorandil causes vasodilation through an increase in endogenous NO in forearm vasculature. In the present study, to determine the role of NO in nicorandil-induced forearm vasodilation, we studied the effects of the NO synthase inhibitor L-NMMA. L-NMMA did not alter FBF response to nicorandil, thus suggesting that endogenous NO per se is not involved in nicorandil-induced vasodilation in humans. PGI_2 produced from arachidonic acid by the cyclooxygenase pathway is also an endothelial factor that appears to hyperpolarize and relax vascular smooth muscle, primarily via activation of K-ATP channels.[6,17] In the present study, indomethacin did not alter the FBF response to nicorandil. It is unlikely that PGI_2 contributes to nicorandil-induced vasodilation in forearm circulation in humans. Another endothelial factor that may activate K-ATP channels is the so-called EDHF. In general, its hyperpolarizing mechanism is thought to be by opening Ca^{2+}-sensitive K^+ channels on vascular smooth muscle cells.[6,18–20] In some vascular beds, EDHF seems to activate K-ATP channels.[21,22]

The nature of EDHF remains controversial.[7,8] EDHF candidates include EETs,[9,10] which are products of CYP-450-dependent arachidonate metabolism, K^+,[11] gap junctions,[12,13] and hydrogen peroxide.[14,15] Recent evidence suggests that EDHF cannot be attributed to 1 factor alone but may be a different entity depending on the vascular bed.[29,30] Most of the evidence in favor of EDHF being a short-lived EET has been obtained using bovine, porcine, canine, and human coronary arteries.[9,31–34] EETs are generated by endothelial cells and cause hyperpolarization of vascular smooth muscle cells, mediated by the opening of Ca^{2+}-sensitive K^+ channels.[9,31,35] To evaluate the role of EETs in nicorandil-induced vasodilation, we used miconazole, a nonspecific inhibitor of CYP-450. In the present study, miconazole had no effect on resting FBF, suggesting that the CYP-450 products do not influence basal microvascular tone in forearm circulation in healthy men. On the other hand, FBF response to nicorandil was suppressed by miconazole, indicating that the CYP-450 products contribute to nicorandil-induced vasodilation. Indeed, miconazole markedly attenuates EDHF-induced vasodilation. However, miconazole does not distinguish between CYP isoforms and is therefore not able to inhibit the production of all EETs or proformed EETs. In addition, we can not deny the possibility that miconazole directly inhibits Ca^{2+}-sensitive K^+ channels, and thus inhibition of nicorandil-induced vasodilation by miconazole may not necessarily be indicative of EETs as EDHF. The use of the CYP2C9 inhibitor sulfaphenazole would allow us to determine more precisely the mechanisms responsible for nicorandil-induced vasodilation in forearm circulation in humans. Unfortunately, there is a lack of specific inhibitors of CYP-450 for human use.

In conclusion, in the present study, miconazole and glibenclamide, but not L-NMMA and indomethacin, inhibited nicorandil-mediated vasodilation. These findings suggest that EDHF, but not endogenous NO and PGI_2, may contribute to nicorandil-induced vasodilation in human forearm circulation. In a future study, it would be interesting to determine whether long-term treatment of nicorandil augments vascular function in humans, including patients with heart failure.

ACKNOWLEDGMENT

The authors thank Izumi Yamashita for her secretarial assistance.

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