Comparison of Nitric Oxide Production in Response to Carbachol Between Macrovascular and Microvascular Cardiac Endothelial Cells

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Cardiac microvascular endothelial cells (EC) play an important role in the physiological regulation of coronary blood flow, but their function has not been rigorously examined, because suitable in vitro models have not been available. Cardiac macrovascular and microvascular EC were isolated and cultured from 14–16-week-old Sprague-Dawley rats to examine the pharmacological responses of carbachol-induced nitric oxide (NO) production using a Griess method. Carbachol-induced NO production was only detected in cardiac macrovascular EC, which suggests that endothelial production of NO differs between macrovascular and microvascular EC. Next, cardiac microvascular EC was treated with either vehicle, angiotensin-converting enzyme (ACE) inhibitor (captopril, 10 μ mol/L) or angiotensin II type 1 (AT1) receptor antagonist (CV11974, 10 μ mol/L) for 4 days. Carbachol-induced NO production was improved by captopril (136 ± 45 nmol, $p < 0.01$ vs vehicle) and CV11974 (146 ± 30 nmol, $p < 0.01$ vs vehicle). Angiotensin II concentration in the culture medium and protein expressions of endothelial nitric oxide synthase and AT1 receptor in the EC were similar among the 3 groups. Interestingly, the level of muscarinic subtype 3 (M3) receptor was significantly increased in the EC treated with captopril (214%, $p < 0.01$) and CV11974 (296%, $p < 0.01$). When cardiac microvascular EC were treated with neomycin (non-selective phospholipase C inhibitor), carbachol-induced NO production was also improved (146 ± 35 nmol, $p < 0.01$, neomycin 1 mmol/L) together with increased expression of M3 receptor ($p < 0.01$). These data suggest that the upregulation of the M3 receptor by captopril or CV11974 occurs via a phospholipase C-dependent pathway. Cardiac microvascular EC also produced NO constitutively, as did the macrovascular EC, but carbachol-induced NO production was decreased. The present data suggest that the upregulation of the M3 receptor by the ACE inhibitor and AT1 receptor antagonist is a new beneficial effect of these drugs on microvascular endothelial function. (Circ J 2002; 66: 511–515)

Key Words: ACE inhibitor; AT1 receptor antagonist; Endothelium; Muscarinic receptor; Nitric oxide

It is now established that the vascular endothelium plays a pivotal role in the regulation of blood vessel tone through the release of chemical mediators such as nitric oxide (NO).¹ Because endothelium-dependent NO-mediated vasodilation can be assessed by acetylcholine (ACh) or other muscarinic receptor agonists, a number of studies have used regional infusion of ACh and examined the resultant vasodilatation.^{2,3} On the assumption that a diminished vasodilator response to ACh reflects endothelial dysfunction, evidence for an impaired endothelium-dependent vasodilator response has been found in the microcirculation of the myocardium,⁴ leg⁵ and forearm.^{6,7} This endothelium-derived NO is not only a potent vasodilator but also an inhibitor of platelet aggregation, smooth muscle cell proliferation, monocyte adhesion and adhesion

molecule expression. Abnormalities in NO production have been demonstrated in various pathological states such as atherosclerosis,⁸ hypertension,⁹ hypercholesterolemia,¹⁰ and heart failure.¹¹ However, the detailed mechanism of impaired endothelium-derived NO production is still unclear.

The endothelium has a significantly heterogeneous structure and function among organs, and there are differences between macrovascular and microvascular endothelium cells (EC).^{12–14} A number of studies have shown that cardiac microvascular EC play important roles in physiological and pathological conditions such as hypertrophy, ischemia, and collateral development.¹⁵ In addition, endothelial dysfunction associated with hypertension and diabetes mellitus primarily occurs in cardiac microvessels. However, cardiac microvascular endothelial function has not been rigorously examined because suitable in vitro models are not available.

The purpose of this study was to examine the function of cardiac macrovascular and microvascular EC isolated from adult rat hearts.

Methods

Isolation and Culture of Adult Rat Cardiac Macrovascular and Microvascular EC¹²

Male Sprague-Dawley rats (14–16-week-old) were anes-

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thetized with sodium pentobarbital (60 mg/kg ip), and the hearts were excised and placed in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Rockville, MD, USA). The animals were handled according to the animal welfare regulations of Yamagata University, and the study protocol was approved by the Animal Subjects Committee of Yamagata University.

The hearts were immediately cannulated and retrogradely perfused with DMEM for 5 min through the ascending aorta to remove blood cells.¹² After removal of connective tissue, the atria, right ventricle and all valvular tissues, the remaining left ventricular tissue was opened by an incision along the septal wall and washed with DMEM. The outer one-quarter of the left ventricular free wall (as macrovessels or conduit vessels) and the remaining heart tissue (as microvessels or resistant vessels) were separately minced finely in 0.2% collagenase in Hank's balanced salt solution (HBSS) and incubated for 25 min at 37°C in a shaking water bath. Trypsin (0.02%) was added, and the ventricular tissue was sheared 10 times in a 10-ml pipette and incubated for another 25 min. Dissociated cells were washed with HBSS and centrifuged at 1,200×G for 5 min. Cells were resuspended in DMEM supplemented with 20% fetal bovine serum (Gibco BRL) and antibiotics (penicillin 100 IU/ml and streptomycin 100 mg/ml; Sigma Chemical Co, St Louis, MO, USA) and plated on 12-well or 100-mm culture plates.

In the preliminary study, after isolation and plating on culture plates for 4 days, confluent primary isolates were incubated with 10 µg/ml of fluorescence-conjugated acetylated low density lipoprotein (LDL) labeled with 1,1'-diiodatadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies, Stoughton, MA, USA) in DMEM with 20% fetal bovine serum overnight. Differential uptake of fluorescently labeled Ac-LDL on fluorescence microscopy indicated that the primary isolates of EC were quite homogeneous (data not shown).

After isolation and plating on culture plates, pre-confluent cardiac microvascular EC were incubated with vehicle (control), 10 µmol/L captopril (Sigma Chemical Co), 10 mmol/L CV11974 (Takeda Chemical Industries Ltd, Osaka, Japan) or 100 µmol/L neomycin (Sigma Chemical Co) in 10% CO₂ at 37°C for 4 days. Then cells were washed with Ca²⁺ 1.0 mmol/L Tyrode twice, and stimulated by 10 µmol/L carbachol for 1 h. NO in the supernatants was meas-

ured by the Griess method. In the present study, we also examined the muscarinic stimulation in cardiac macrovascular EC as a control in an identical manner to the microvascular EC.

Determination of NOx (NO₂⁻/NO₃⁻)

The cells were plated onto 12-well plates for 4 days, and washed with Ca²⁺ 1.0 mmol/L Tyrode buffer containing (mmol/L) 140 NaCl, 5.4 KCl, 1.0 MgCl₂, 1.0 Na₂HPO₄, 10 glucose, 5.0 Hepes, gassed with 10% CO₂, adjusted to pH 7.3, at 37°C twice, and then stimulated by 10 µmol/L carbachol (Sigma Chemical Co) for 1 h. NO production by carbachol was not dose-dependent over 10 µmol/L concentration (data not shown). The supernatants were used for NOx measurement. They were applied to a copper-coated cadmium reduction column, and NOx was determined by the Griess method with the use of an autoanalyzer (ENO-10, Eicom Corp, Kyoto, Japan).¹⁶

Western Blotting

When cells were 95% confluent, they were harvested by aspirating the medium and washing with ice-cold phosphate buffered saline, and were lysed by the addition of 0.5 ml of ice-cold lysis buffer containing (mmol/L) NaCl 50.0, NaF 100.0, Tris-HCl 25.0, (%) sodium deoxycholate 0.5, NP-40 2.0, sodium dodecyl sulfate (SDS) 0.2 and sodium orthovanadate 200 µmol/L at pH 7.4, along with 10 µg/ml leupeptin, 10 µg/ml aprotinin and 100 µg/ml phenylmethylsulfonyl fluoride. The cell lysates were then placed on ice for 30 min, and centrifuged at 14,000×G at 4°C for 15 min. Supernatants were either used immediately or stored at -80°C. Protein concentrations were determined using a bicinchoninic acid protein assay kit from Pierce. Equal amounts (40 µg) were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membrane (Hybond P, Amersham Pharmacia Biotech, Inc, Piscataway, NJ, USA) as previously reported.^{17,18} The membrane was blocked with 5% dried-milk in TNT solution containing 25 mmol/L Tris-HCl, 125 mmol/L NaCl, 0.2% Tween 20 at 4°C overnight and incubated with primary antibodies specific to endothelial nitric oxide synthase (eNOS),¹⁹ muscarinic subtype 3 (M₃) receptor and AT₁ receptor²⁰ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. After washing, the membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG secondary antibody (Santa Cruz Biotechnology, Inc) for 1 h at room temperature. Immunoreactive bands were visualized by

Fig 1. Comparison of NO production in response to carbachol stimulation in cardiac macrovascular and microvascular endothelial cells. In cardiac microvascular endothelial cells, treatment with captopril and CV11974 increased NO production by carbachol. Data are mean ± SD from 8 separate experiments. *p<0.01 vs basal (without carbachol) and #p<0.01 vs control (+carbachol). CCh, carbachol.

Fig 2. Effects of L-NAME on carbachol-induced NO production. Data are mean ± SD from 4 separate experiments. *p<0.01. L-NAME, N^G-nitro-L-arginine methyl ester.

Fig3. Protein levels of AT1 receptor (Left panel) and eNOS (Right panel) in cardiac microvascular endothelial cells. Data are mean $\pm$ SD from 4 separate experiments.

the enhanced chemiluminescent detection method (ECL, Amersham Pharmacia Biotech, Inc) and quantified by a densitometer with an imaging system.

Statistical Analysis

Data are expressed as mean $\pm$ SD. Statistical analysis was performed by one-way ANOVA followed by Scheffé's test. A value of $p < 0.05$ was taken as significant.

Results

Effects of ACE Inhibitor and AT1 Receptor Antagonist on NO Production by Cardiac Microvascular EC

We found that cardiac microvascular and macrovascular EC produced NO in the basal condition to a similar degree (57 ± 25 nmol vs 46 ± 18 nmol, Fig 1). However, carbachol-induced NO production was significantly decreased in the microvascular EC compared with the macrovascular EC (28 ± 12 nmol vs 197 ± 23 nmol, $p < 0.01$, Fig 1), which suggests that, in contrast to the macrovascular EC, the microvascular EC lack the capacity to generate NO in response to muscarinic stimulation. Interestingly, NO production in the microvascular EC was improved by captopril (136 ± 45 nmol, $p < 0.01$ vs vehicle) and CV11974 (146 ± 30 nmol, $p < 0.01$ vs vehicle) to the same level as the cardiac macrovascular EC. In contrast to the microvascular EC, NO production in the macrovascular EC was unchanged by either captopril (166 ± 32 nmol) or CV11974 (160 ± 21 nmol). The improvement of NO production by the microvascular EC by captopril and CV11974 was completely inhibited by N^G-nitro-L-arginine methyl ester (L-NAME, 100 mmol/L) (Fig 2), which suggests that NO is generated by NO synthase in these cells.

Angiotensin II Concentration and AT1 Receptor Expression

We then used radioimmunoassay (SRL, Tokyo, Japan) to examine the angiotensin II concentration in the cultured medium of cardiac microvascular EC and found that there were no significant differences between the groups (control: 3.38 ± 3.11 pg/ml, captopril: 5.12 ± 2.59 pg/ml, and CV11974: 2.38 ± 2.62 pg/ml).

We used Western blotting to also examine the protein expression of the AT1 receptor in the cardiac microvascular EC and as shown in Fig 3, there was no difference among the 3 groups.

Fig4. The level of M3 receptor in cardiac microvascular endothelial cells was significantly increased by captopril and CV11974. Data are mean $\pm$ SD from 3 separate experiments. * $p < 0.01$ vs control.

Fig5. Effects of neomycin (phospholipase C inhibitor) on NO production (Left) and M3 receptor expression (Right). Data are mean $\pm$ SD from 4 separate experiments. * $p < 0.01$ vs control.

Protein Expression of eNOS in Cardiac Microvascular EC

A number of previous studies have shown that the endothelial dysfunction in atherosclerosis is related to downregulation of eNOS²¹ In the present study NO production was inhibited by L-NAME in cardiac microvascular EC (Fig 2), so we used Western blotting to examine the protein expression of eNOS in these cells, but there were no differences among the 3 groups (Fig 3).

Expression of M3 Receptor and Effects of a Phospholipase C Inhibitor

In EC, vasodilation elicited by muscarinic stimulation is mediated by release of NO consequent to the activation of the M3 receptor.²² Therefore, we examined the levels of the M3 receptor protein in cardiac macrovascular and microvascular EC cultured with vehicle, captopril and CV11974. The protein level of the M3 receptor was decreased in cardiac microvascular EC given vehicle compared with the cardiac macrovascular EC (Fig 4), but was increased by the treatment with captopril and CV11974 ($p < 0.01$) to the same level as the cardiac macrovascular EC. These data suggest that the upregulation of M3 receptor is related to improvement in NO production in cardiac microvascular EC by captopril and CV11974.

To examine whether the upregulation of the M3 receptor with resultant improvement of NO production was mediated by the AT1 receptor-mediated signaling pathway, we cultured cardiac microvascular EC with a phospholipase C (PLC) inhibitor, neomycin, and as shown in Fig 5, both carbachol-induced NO production and M3 protein expres-

sion improved in a dose-dependent manner ($p < 0.01$). These data suggest that the upregulation of M₃ receptor by captopril and CV11974 occurs via a phospholipase C-dependent pathway.

Discussion

We report successful isolation of cardiac macrovascular and microvascular EC from adult rat hearts and have shown that cardiac microvascular EC express eNOS and generate NO in the basal condition, as has been reported in macrovascular EC, but that the NO production in response to carbachol is decreased. Treatment with captopril, CV11974, and neomycin improved NO production in cardiac microvascular EC associated with increased protein expression of the M₃ receptor.

Microvascular and Macrovascular Endothelial Function

The vascular endothelium plays a pivotal role in the control of vascular tone by releasing various vasoactive substances! NO is generated from the endothelium and plays a key role in many physiological and pathological conditions including the regulation of cardiovascular function, blood pressure, flow, vascular proliferation, platelet function, etc. It has been demonstrated that endothelium-dependent vasodilation is impaired in several pathological conditions including atherosclerosis and hypertension.^{23–25} The endothelium has a significantly heterogeneous structure and function among organs, and there are differences between macrovascular and microvascular endothelium.²⁶ A number of studies have shown that cardiac microvascular EC play important roles in physiological and pathological conditions such as hypertrophy, ischemia and collateral development.^{14,15} Cardiac microvascular EC release peptides and/or autacoids, which act on cardiomyocytes downstream. It has been also reported that an interaction between microvascular endothelium and cardiomyocytes is important in various pathological conditions;⁷ for example, the endothelial dysfunction associated with hypertension and diabetes mellitus occurs primarily in cardiac microvessels with a diameter of less than 250 μm . However, systematic evaluation of cardiac microvascular endothelial function has not been rigorously performed because suitable in vitro models are not available. Ando et al reported successful isolation of cardiac microvascular EC from porcine hearts and showed lower eNOS activity compared with aortic EC.¹⁴ In the present study, we compared NO production in response to carbachol between cardiac macrovascular and microvascular EC and found that carbachol-induced NO production was significantly decreased in the microvascular EC.

Effects of ACE Inhibitor and AT1 Receptor Antagonist

Carbachol-induced NO production was improved by captopril and CV11974 in the cardiac microvascular EC only. To study the mechanism of this improvement, we examined eNOS protein expression and found that the levels of eNOS did not differ among cardiac microvascular EC treated with or without captopril and CV11974. These results indicated that the decrease in carbachol-induced NO production in cardiac microvascular EC was not related to eNOS downregulation. In the present study, AT1 receptor protein expression and angiotensin II concentration were unexpectedly unchanged among the groups treated with vehicle, captopril and CV11974. We demonstrated for the

first time, to our knowledge, that the protein levels of the M₃ receptor were decreased in cardiac microvascular EC compared with macrovascular EC, but were improved by treatment with captopril, CV11974 and neomycin. Improvement in the M₃ protein levels was closely related with improved NO production in response to carbachol stimulation. These results suggest that this improvement in cardiac microvascular endothelial function is related to the inhibition of the AT1 receptor/PLC pathway. Shibata et al have suggested that carbachol increases AT2 receptor mRNA in PC12 cells and they suggest that there is crosstalk between the muscarinic and AT2 receptors, and that activation of the angiotensin II/AT2 receptor increases M₃ receptor protein expression.²⁸ Although the precise mechanisms are still unknown, clinical data showing reversed endothelial dysfunction by AT1 receptor antagonism has been reported in patients with atherosclerosis,²⁹ coronary artery disease³⁰ and hypertension.³¹ Our present data indicate that increased expression of M₃ receptor contributes, at least in part, to the improved endothelial function with this therapy.

Study Limitations

The passage of blood in the vessels generates hemodynamic forces, such as shear stress, and regulates the function of EC lining the intimal surface of the vasculature. Our in vitro study using cardiac microvascular EC was not performed under physiological conditions, so although our data indicate that increased expression of the M₃ receptor by ACE inhibitor and AT1 receptor antagonist contributes to improved endothelial function, other mechanisms may also account for this effect. We did not clarify the mechanism of improvement of carbachol-induced NO production via PLC inhibition because neomycin has some non-selective actions and we could not measure PLC activity.

Conclusion

We report successful isolation of cardiac macrovascular and microvascular EC from adult rat hearts, and primary culture of these cells may be a useful tool for investigating the pathophysiological status of cardiac microvascular endothelial function. The present data suggest that beneficial effects of ACE inhibitor and AT1 receptor antagonist on microvascular endothelial function may be related to upregulation of M₃ receptors.

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