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ORIGINAL ARTICLE

A new constitutively active mutant of AMP-activated protein kinase inhibits anoxia-induced apoptosis of vascular endothelial cell

Daisuke Nagata[1,2], Arihiro Kiyosue[2], Masao Takahashi[2], Hiroshi Satonaka[3], Kimie Tanaka[1,2], Masataka Sata[2], Tetsuo Nagano[4], Ryozo Nagai[2] and Yasunobu Hirata[2]

The inhibition of apoptotic changes in vascular endothelial cells is important for preventing vascular damage from hypoxia. AMP-activated protein kinase (AMPK) has recently been identified as playing a role in vascular protection. Although the chemical reagent 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) has been used to stimulate AMPK activity, AICAR has been associated with several nonspecific reactions. We therefore constructed a new constitutively active mutant of AMPKα1 (NcaAMPK), which lacks the autoinhibitory domain in AMPKα1 and in which threonine 172 has been replaced with aspartate. We investigated whether NcaAMPK has an anti-apoptotic effect in vascular endothelial cells under anoxic conditions. NcaAMPK, or green fluorescent protein (GFP) as a control, was overexpressed in human umbilical vein endothelial cells (HUVECs). After HUVECs were incubated for 40 h under normoxic or anoxic conditions, we examined cell viability, caspase 3/7 activity, and expression and phosphorylation levels of apoptosis-related proteins. Cell viabilities under anoxic conditions were improved in NcaAMPK-overexpressing cells. Anoxia increased caspase 3/7 activity, but NcaAMPK reduced this increase significantly. NcaAMPK overexpression increased protein kinase B/Akt Ser473 and endothelial nitric oxide synthase Ser1177 phosphorylation, but pretreatment with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) did not decrease the viability of NcaAMPK-overexpressing HUVECs. Furthermore, co-expression of a dominant-negative Akt reduced the improvement in cell viability and the suppression of poly (ADP-ribose) polymerase cleavage by NcaAMPK under anoxic conditions. In conclusion, NcaAMPK inhibited anoxia-induced apoptosis in vascular endothelial cells through Akt activation, suggesting that activation of AMPK might protect against ischemic vascular injury.
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Keywords: Akt; AMP-activated protein kinase; anoxia; apoptosis; vascular endothelial cell

INTRODUCTION

An important initial step in the pathogenesis of atherosclerosis is endothelial damage by various factors, such as inflammatory cytokines.[1] A large body of evidence has shown that hypoxia is a pivotal factor modulating endothelial cell function and survival.[2] Although necrosis is a major pathway for regulating ischemia- and reperfusion-induced cardiomyocyte death, apoptosis has recently been identified as another important regulator.[3] Furthermore, apoptosis of vascular endothelial cells is thought to be a pivotal regulator of vascular damage, suggesting that apoptosis of endothelial cells could be an important therapeutic target for preventing cardiovascular diseases.

AMP-activated protein kinase (AMPK) has been identified as a key regulator of cellular ATP levels.[4] AMPK was identified as a homolog of yeast sucrose non-fermenting 1 and is known as a metabolite-sensing protein kinase.[5] AMPK is a heterotrimeric serine/threonine protein kinase consisting of a catalytic α-subunit and two regulatory subunits, β and γ.[6] There are multiple isoforms of each AMPK subunit, with α1, α2, β1, β2, γ1, γ2, and γ3 forming heterotrimers[7] that differ in tissue and subcellular localization. In mammalian cells, AMPK is activated by increases in the AMP/ATP ratio,[4] which occur under conditions of hypoxia or anoxia.[8,9] When the AMP/ATP ratio increases, AMPK is partially activated by a conformational change after combining with AMP and is fully activated when phosphorylated at Thr172 by an AMPK kinase (AMPKK), which is now known to be LKB1 serine/threonine kinase.[10] Activated AMPK phosphorylates and downregulates several anabolic enzymes, including 3-hydroxy-3-methylglutaryl-CoA reductase or acetyl-CoA carboxylase, and shuts off the ATP-consuming synthetic pathway.[4] In addition to such energy-saving

[1]Department of Molecular Research for Vascular Diseases, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [2]Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [3]Department of Nephrology and Endocrinology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan and [4]Department of Chemistry and Biology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan
Correspondence: Dr D Nagata, Department of Molecular Research for Vascular Diseases, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: dskngtendo0504-tky@umin.ac.jp
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effects, AMPK is important for protecting cellular function under energy-restricted conditions, such as hypoxia/anoxia.[11]

To investigate the functions of AMPK in mammalian cells, we and other investigators have used 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), which is a chemical AMPK activator. AICAR is phosphorylated and converted to 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl-5′-monophosphate (ZMP) in mammalian cells. ZMP mimics the effect of AMP and activates AMPK.[12,13] However, AICAR increases not only ZMP but also ZTP[12] and, as a chemical rather than a specific enzyme, may have other nonspecific effects on ATP-requiring reactions. Constitutively active mutants of AMPK provide a more specific method for examining the functions of AMPK. Several kinds of constitutively active AMPK mutants have been investigated, including AMPKα1 (amino acids (aa) 1–312, Thr172→Asp: T172D),[14,15] AMPKγ2 (Arg302→Gln),[16] and AMPKγ1 (His150→Arg).[17] Although it has been reported that maximum activity of AMPK requires all three subunits,[6] AMPKα1 (1–312, T172D) lacks both the binding domain for interactions with the β-subunit and the autoinhibitory domain, which inhibits the self-kinase activity.[14] The β-subunit plays a role in modulating subcellular localization through its phosphorylation and myristoylation,[18,19] so that the ability to bind the β-subunit might be critical for the catalytic α-subunit to select its appropriate substrates. We have constructed a replication-defective adenoviral vector expressing a new constitutively active AMPKα1 mutant (NcaAMPK), which lacks the autoinhibitory domain (aa 313–392) but has the complex formation domain (aa 393–548).[14] As we found that NcaAMPK functions as a specific and continuous activator of AMPK, we investigated whether NcaAMPK overexpression could inhibit the pro-apoptotic pathway induced by anoxia in human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Chemical reagents

N^G-nitro-L-arginine methyl ester (L-NAME), wortmannin, and other chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Antibodies

AMPKα1, phospho-AMPKα (Thr172), phospho-protein kinase B/Akt (Ser473), phospho-endothelial nitric oxide synthase (eNOS) (Ser1177), and poly (ADP-ribose) polymerase (PARP) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Actin, Akt, and eNOS antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Myc-tag and hemagglutinin (HA)-tag antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA).

Cell culture and anoxic conditions

HUVECs were purchased from Kurabo (Osaka, Japan) and cultured in HuMedia EG2 (Kurabo). HUVECs were used for experiments within passages 6–8. In some experiments, HUVECs were transduced with the indicated replication-defective adenoviral vectors at a multiplicity of infection of 50 plaque-forming units (50 MOI) for 1 day. The medium was then changed to a low-serum medium, HuMedia basic medium (EB2) with 0.2% fetal bovine serum, to reduce the effects of stimulation by serum mitogens.

After incubation in the low-serum medium for 8 h, adenovirus-infected cells were incubated in a normoxic or anoxic incubator for 40 h. In the pilot experiments, we found that 24 h anoxia was too short to evaluate cell viability because cell survival rates under these conditions were >90%. Furthermore, even under normoxic conditions, >72 h incubation in the low-serum medium induced high rates of cell death >40%. We conclude that 40–48 h anoxic conditions are suitable for evaluating effects of anoxia on cell death without the cell-damaging effects of low serum. For anoxic conditions, we used the Anoxic Chamber System (Coy Laboratory Products, Grass Lake, MI, USA). To exclude the effects of reoxygenation after anoxia, every procedure (such as protein extraction) was performed under anoxic conditions in this anoxic chamber.

Construction of adenoviral AMPKα1 mutant vectors and other adenoviral vectors

Total RNA was extracted from rat vascular smooth muscle cells (rVSMCs) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was produced by reverse transcription from purified rVSMC RNA with an RNA LA PCR kit (TAKARA, Otsu, Japan) using a random 9mer primer. Synthetic oligo primers for PCR and for creating point mutations are as follows:

primer 1
GGAATTCGCCATGGAGCAGAAGCTTATCTCCGAGGAGGACCTCGGTG
GCGGCGAGAAGCAGAAGCACGACGGG
primer 2
CCGCTCGAGTTAGTACAGGCAGCTGAGGACCTC
primer 3
GCTCTAGAGTACAGGCAGCTGAGGACCTC
primer 4
GCTCTAGAAAGGCAAAGTGGCATTTGGGGATTCGAA
primer 5
GCTCTAGAGCATGCTCGAGTTACTGTGCAAGAATTTTAATTAGATTTG
CACACACATTTCA
primer 6
ATGTCAGATGGTGAATTTTTAAGAGATAGCTGTGGCTCGCCCAATTATG
primer 7
CATAATTGGGCGAGCCACAGCTATCTCTTAAAAATTCACCATCTGACAT

To make a conventional caAMPK (CcaAMPK) cDNA fragment that lacks aa 313–548, we performed PCR with KOD FX DNA polymerase (Toyobo, Osaka, Japan) using the rVSMC cDNA, primer 1 (which includes a myc-tag sequence), and primer 2. This fragment was digested with the restriction enzymes *Eco*RI and *Xho*I and ligated to pcDNA 3.1/Zeo(+) (Invitrogen) digested with *Eco*RI/*Xho*I. To make a Thr-to-Asp mutation at residue 172, we used a QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, using primers 6 and 7 to introduce the point mutation. To construct the NcaAMPK cDNA fragment, which has the complex formation domain but lacks the autoinhibitory domain, we amplified a fragment A using primer 1/primer 3 and a fragment B using primer 4/primer 5. We digested fragments A and B with *Eco*RI/*Xba*I and *Xba*I, respectively. Fragment A was then ligated to pcDNA 3.1/Zeo(+) digested with *Eco*RI/*Xba*I. Next, this vector was digested with *Xba*I and ligated with *Xba*I-digested fragment B. The direction of fragment B was confirmed by direct sequencing. To make a Thr-to-Asp mutation at residue 172, we used the QuickChange II XL Site-Directed Mutagenesis Kit, as for CcaAMPK construction. Schematics of the complete constructs are shown in Figure 1. We used an adenoviral construction kit (AdEasy adenovirus vector system; Stratagene) to make replication-defective adenoviral vectors for CcaAMPK and NcaAMPK. Briefly, pcDNA 3.1/Zeo(+) plasmid vectors for CcaAMPK and NcaAMPK were digested with *Kpn*I/*Xho*I, and the fragments were ligated to a *Kpn*I/*Xho*I-digested pAdTrack-CMV plasmid, which was a generous gift from Dr Walsh (Boston University). We followed the manufacturer's instructions after this procedure. An adenoviral vector expressing green fluorescent protein (GFP) was obtained from Qbiogene (Illkirch, France) and used as a control for infection level. The HA-tagged dominant-negative (dn) Akt adenovirus was also generous gift from Dr Walsh.

AMPK assay

The AMPK assay using SAMS peptide (HMRSAMSGLHLVKRR, Upstate Biotechnology) was performed according to the methods available at the Animal Model of Diabetic Complications Consortium (AMDCC) website (http://www.amdcc.org/shared/phenotype/showAssay.aspx?id=260).

Western blot analysis

Western blot analysis was carried out as previously described.[9] An ECL-PLUS Western Blotting Detection kit (GE Healthcare, Piscataway, NJ, USA) was used

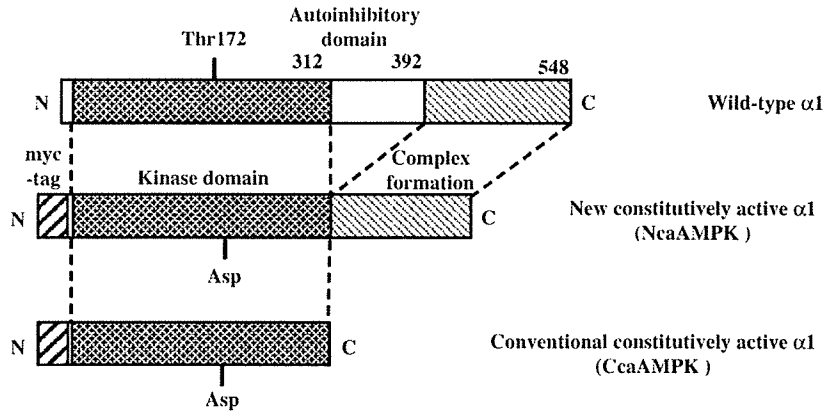

Figure 1 The construction of two AMPKα1 mutants, conventional constitutively active α1 (CcaAMPK) and new constitutively active α1 (NcaAMPK). Wild-type rat AMPKα1 contains a kinase domain, autoinhibitory domain, and the domain for complex formation. The autoinhibitory domain inhibits the AMPKα1 kinase activity. CcaAMPK lacks both the autoinhibitory domain and the domain for complex formation. At the target phosphorylation site of upstream kinases in the kinase domain of CcaAMPK, Asp was substituted for Thr172 to mimic phosphorylation. We constructed NcaAMPK by adding the domain for complex formation to the C terminus of CcaAMPK. Both CcaAMPK and NcaAMPK have a myc tag to distinguish their expression from that of endogenous AMPKα1.

for detection. The density of the bands was quantified using the Scion Image program (Scion Corporation, Frederick, MD, USA). Each experiment was repeated 3–4 times.

Intracellular protein crosslinking experiments using photoactivated amino acids

To evaluate intracellular AMPK protein complex formation in NcaAMPK-overexpressing cells, we used the photoactivated amino acid system (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, ultraviolet (UV)-sensitive L-photo-leucine and L-photo-methionine, which are incorporated in AMPK subunits or overexpressed AMPK mutants, are crosslinked to each other intracellularly after UV irradiation. Cell lysates were then extracted with cell lysis buffer, and AMPK protein complex formation was evaluated by western blotting.

WST-1 assay

To evaluate cell viability, we performed an assay using the WST-1 reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Caspase 3/7 assay

We performed a caspase 3/7 assay using a Caspase-Glo 3/7 assay kit and GloMax96 luminometer (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical analyses

Values are expressed as the mean ± s.e.m. Statistical comparisons were performed using analysis of variance with Scheffe's F procedure for *post hoc* analyses. P-value <0.05 was considered to be statistically significant.

RESULTS

NcaAMPK shows higher kinase activity and forms an AMPK complex more efficiently than CcaAMPK

To compare the AMPK activity of NcaAMPK and CcaAMPK, we performed a kinase assay by measuring the radiolabeled phosphorylation rate of SAMS peptide corrected by the amount of protein (Figure 2a). The basal levels of AMPK activity in GFP-overexpressing control cells was 1030 ± 140 c.p.m. per μg protein. Although the kinase activity of CcaAMPK-overexpressing cells (1860 ± 160 c.p.m. per μg protein) was significantly higher than that of controls ($P<0.05$), the activity of NcaAMPK-overexpressing cells (3310 ± 250 c.p.m. per μg protein) was significantly higher than that of CcaAMPK ($P<0.01$). We confirmed the expression of the CcaAMPK and NcaAMPK mutants by western blot (Figure 2b).

As NcaAMPK kinase activity is higher than CcaAMPK, we hypothesized that NcaAMPK would bind more effectively to the β-subunit than CcaAMPK and its kinase activity would be intensified. To show the formation of AMPK complexes including NcaAMPK, we used the UV light-sensitive photoactivated amino acid system. In NcaAMPK-overexpressing HUVECs, positive bands of approximately 130 kDa were found when using an anti-myc-tag or anti-AMPKβ antibody (Figure 2c). In CcaAMPK-overexpressing cells, we did not find myc-tag positive bands of approximately 110 kDa, which indicates AMPK complex formation including the CcaAMPK mutant. When using the AMPKβ antibody, we found weaker intensity bands with a molecular weight of ~135 kDa in the GFP- and CcaAMPK-overexpressing cells. These might be endogenous AMPK complexes with expected molecular weights of 137 kDa (α1β1γ1 or α2β1γ1). These results suggest that NcaAMPK, which has the complex formation domain, can form active AMPK complexes more effectively than CcaAMPK.

NcaAMPK overexpression inhibits anoxia-induced cell death

We performed a WST-1 assay in HUVECs overexpressing GFP, CcaAMPK, or NcaAMPK to compare the effect of the AMPK mutants on cell survival under anoxic conditions. Cell viability was significantly higher ($P<0.01$) in NcaAMPK-overexpressing cells than in either GFP or CcaAMPK-infected cells (Figure 3a). The lower panels in Figure 3a show phase-contrast micrographs of these cells. NcaAMPK overexpression inhibited cell death and kept cells attached to the bottom of the culture dish.

We also measured caspase 3/7 activity to investigate whether anoxia-induced cell death might be accompanied by an increase in caspase activity. Caspase 3/7 activity was inhibited by 33% in NcaAMPK cells relative to control GFP cells (Figure 3b). CcaAMPK did not inhibit caspase 3/7 compared with controls (Figure 3b).

NcaAMPK increases Akt and eNOS phosphorylation

Previous studies, including ours, suggested that AMPK upregulates the PKB/Akt signal,[9,20] which is known to be an important regulator of cell survival in endothelial cells. Consistent with these reports, we found that Akt Ser473 was more highly phosphorylated in NcaAMPK-overexpressing cells than in controls (Figure 4a). Furthermore, eNOS

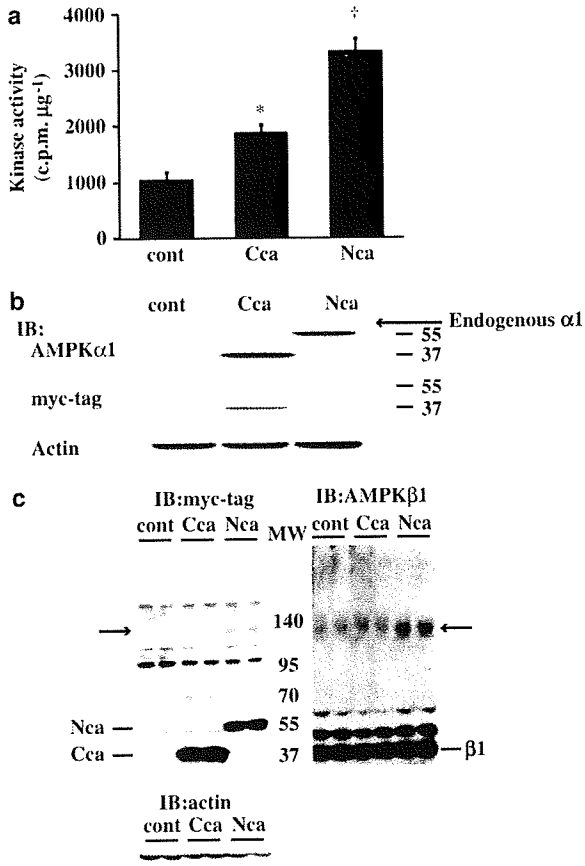

Figure 2 The kinase activity of CcaAMPK and NcaAMPK. **(a)** Cell lysates of control (cont), CcaAMPK-overexpressing (Cca), or NcaAMPK-overexpressing (Nca) HUVECs were subjected to an AMPK kinase assay using SAMS peptide and γ^{32}P-ATP. The activity of Nca was significantly higher than that of cont or Cca. $^{*}P<0.05$ *vs.* cont, $^{\dagger}P<0.01$ *vs.* Cca. **(b)** Western blots using the cell lysates from the experiment shown in panel a. Overexpression of CcaAMPK or NcaAMPK was estimated by using anti-AMPKα1 and anti-myc-tag antibodies. Actin was blotted as an internal control. **(c)** Western blots using a UV light-sensitive photoactivated amino acid system. In NcaAMPK-overexpressing cells, positive bands of ~130 kDa were found when using the anti-myc-tag and anti-AMPKβ antibodies (indicated by arrows). Myc-tag-positive bands of ~110 kDa were not found in CcaAMPK-overexpressing cells. These results suggest that NcaAMPK has higher kinase activity than CcaAMPK because it forms a tighter and more effective AMPK complex. IB, immuno blot.

Ser1177, which is known to be phosphorylated by Akt and AMPK,[21] was also phosphorylated (Figure 4a). Phosphoinositide 3 (PI3) kinase is an upstream kinase of Akt, and pretreatment with the PI3 kinase inhibitor wortmannin (1 μmol l^{-1}) inhibited Akt and eNOS phosphorylation (Figures 4a and b), suggesting that NcaAMPK phosphorylates Akt and eNOS in a PI3 kinase-dependent manner. Thus, eNOS might be phosphorylated both directly by AMPK and, at least in part, by the PI3–Akt pathway.

As we have previously shown that nitric oxide has an anti-apoptotic effect in endothelial cells,[22] we used the WST-1 assay to investigate whether the nitric oxide synthase inhibitor L-NAME could inhibit the anti-apoptotic cell survival effect of NcaAMPK. Although we used L-NAME concentrations from 1.0 to 5.0 mmol l^{-1}, L-NAME pretreatment did not inhibit cell survival (Figure 4c).

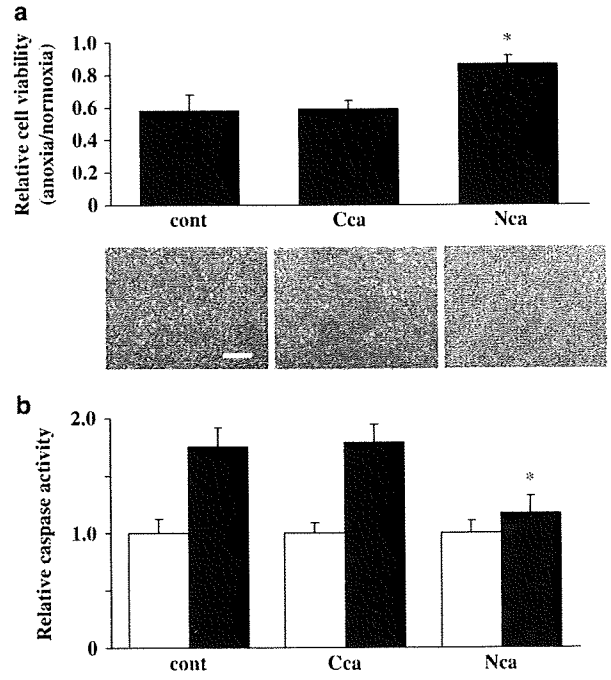

Figure 3 NcaAMPK overexpression inhibits anoxia-induced cell death and caspase 3/7 activity. **(a)** Ratio of cell viability in anoxia to that in normoxia, as determined in the WST-1 assay. NcaAMPK overexpression ameliorated the anoxia-induced cell death ratio compared with CcaAMPK or control (GFP). Lower panels show phase-contrast pictures of HUVECs. Scale bar indicates 200 μm. **(b)** In control cells, anoxia increased caspase 3/7 activity. Although CcaAMPK overexpression did not inhibit caspase 3/7 activity, NcaAMPK overexpression significantly inhibited it. Blank bars and filled bars represent normoxia and anoxia, respectively. cont, GFP; Cca, CcaAMPK; and Nca, NcaAMPK. Each bar represents the mean ± s.e.m., $N=6$. The experiments were performed three times. $^{*}P<0.01$ *vs.* cont, Cca.

NcaAMPK increases the phosphorylation of Akt but does not modulate proteins of the bcl-2 family

In agreement with the results of our previous report,[9] the phosphorylation of Akt Ser473 decreased under anoxic conditions in control cells (Figures 5a and b). However, in NcaAMPK-overexpressing cells, Akt phosphorylation was maintained at a high level even under anoxic conditions. The expression levels of two members of the Bcl-2 family, Bax and Bcl-xl, did not change in NcaAMPK-overexpressing cells or under anoxic conditions (Figure 5a). Bcl-2 was not detected by western blot analysis.

NcaAMPK suppresses anoxia-induced cell death and PARP cleavage but co-expression of dnAkt abrogates this suppression

We showed that NcaAMPK overexpression augments the resistance of HUVECs to anoxia (Figure 3). However, co-expression of dnAkt partially but significantly abrogated this anoxia resistance in NcaAMPK-overexpressing cells (Figure 6a). Next, we performed western blot analyses to investigate whether PARP cleavage was inhibited more effectively in NcaAMPK-overexpressing cells than in controls. The band intensities of cleaved PARP were higher under anoxic conditions than under normoxic conditions. The band intensity of the cleaved smaller fragment of PARP was lower in NcaAMPK-overexpressing cells than in controls under anoxic conditions (Figure 6b, lane 4). However, overexpression of dnAkt partially but

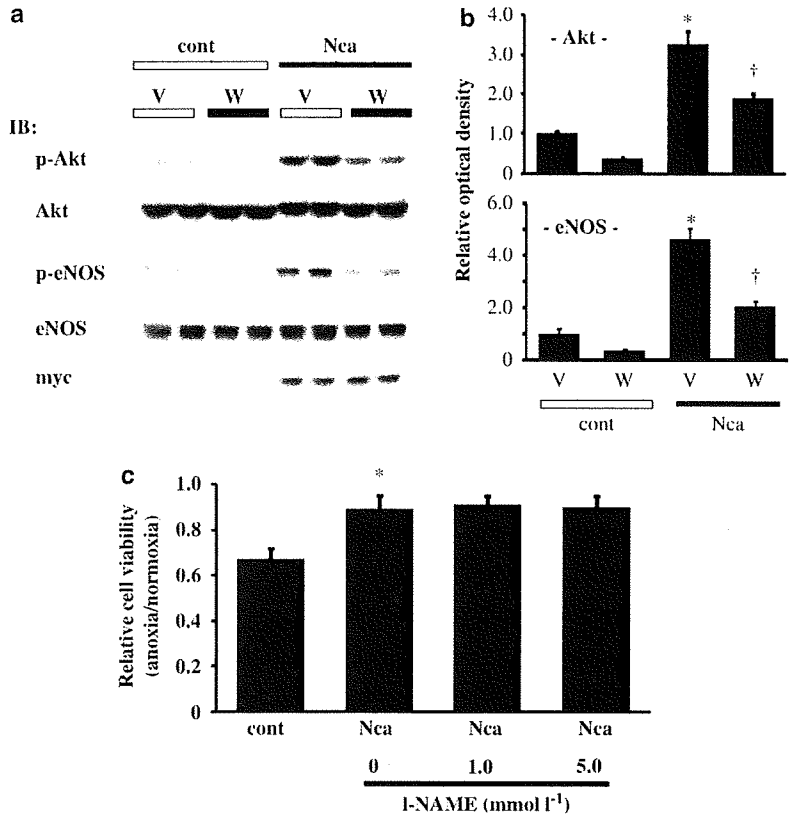

Figure 4 NcaAMPK overexpression increases Akt and eNOS phosphorylation levels. (**a**) NcaAMPK increases phosphorylation of Akt Ser473 and eNOS Ser1177. The PI3 kinase inhibitor wortmannin inhibited this phosphorylation. V, vehicle; W, wortmannin, cont: GFP, Nca: NcaAMPK. IB: immuno blot. (**b**) Density levels of Akt Ser473 and eNOS Ser1177 after normalization to the amount of total loaded protein are shown. The mean value of V/cont was fixed to 1.0. Each bar represents the mean ± s.e.m., N=4. *P<0.005 *vs.* cont. †P<0.01 *vs.* V. (**c**) Cell viability under anoxic conditions relative to that under normoxic conditions as measured using the WST-1 assay. The NOS inhibitor L-NAME did not abrogate the inhibition of anoxia-induced cell death by NcaAMPK. Each bar represents the mean ± s.e.m., N=6. *P<0.01 *vs.* cont.

significantly abrogated the inhibition of PARP cleavage in NcaAMPK-overexpressing cells under anoxic conditions (Figure 6b, lane 8).

DISCUSSION

The results of this study suggest that AMPK activation by NcaAMPK inhibits anoxia-induced apoptosis in HUVECs through the activation of Akt. Although ischemia and reperfusion have been shown to injure the cardiovascular system, AMPK activation can prevent these harmful effects of ischemia.[23] AMPK is also a downstream target of adiponectin,[24,25] the most cardiovascular-protective adipocytokine,[26] which inhibits cardiomyocyte apoptosis partially through AMPK activation.[27] Conversely, apoptosis increases in cardiomyocytes after ischemia and reperfusion when AMPK activation is impaired.[23] Thus, AMPK clearly plays an anti-apoptotic role in the cardiovascular system, although details of the mechanism remain to be elucidated, especially in the vasculature.

Some investigators[28,29] have shown that AICAR-induced AMPK activation inhibits apoptosis in vascular endothelial cells, whereas other investigators[30,31] have reported that AICAR treatment, rather than increasing cell viability, actually promotes apoptotic changes in nonendothelial cell lines. AICAR has also been reported to have nonspecific effects in addition to its ability to activate AMPK.[12,32,33] Therefore, we have been very eager to find a more specific activator of AMPK to clarify whether AMPK signaling actually inhibits anoxia-induced apoptosis in vascular endothelial cells. A constitutively active AMPK mutant containing only the α-subunit kinase domain, CcaAMPK, provided an alternative, but the kinase domain alone possesses weak kinase activity in mammalian cells, and maximum AMPK activity requires all three subunits.[6,14] We therefore constructed a replication-defective adenovirus expressing NcaAMPK, which has higher kinase activity and suppresses anoxia-induced apoptotic cell death more efficiently than CcaAMPK. Although higher dose, 100 MOI, CcaAMPK transduction in the cells increased SAMS peptide phosphorylation rate by ~35% compared with 50 MOI, the dose we used in this study, we did not find more potent inhibition of cell death in CcaAMPK-overexpressing cells with 100 MOI (data not shown). Our results suggest that the ability of NcaAMPK to bind the β-subunit might contribute to its more effective suppression of anoxia-induced apoptosis than that of CcaAMPK.

It has been previously reported that Akt is phosphorylated and activated in AMPK-activated endothelial cells,[9,20,34] and this activation is PI3-kinase dependent.[20,34] In this study we found that Akt Ser473 was also phosphorylated in NcaAMPK-overexpressing HUVECs. PI3 kinase inhibition by wortmannin suppressed this Akt phosphorylation, suggesting that NcaAMPK also upregulates Akt in a PI3 kinase-dependent manner. The anti-apoptotic effect of Akt has been proposed to be partially due tophosphorylation of transcription factors of the forkhead box gene, group O (FoxOs) and Bcl-xL/Bcl-2-associated death promoter (Bad).[35–37] As we reported previously that phosphorylation levels of Akt were gradually downregulated under hypoxic

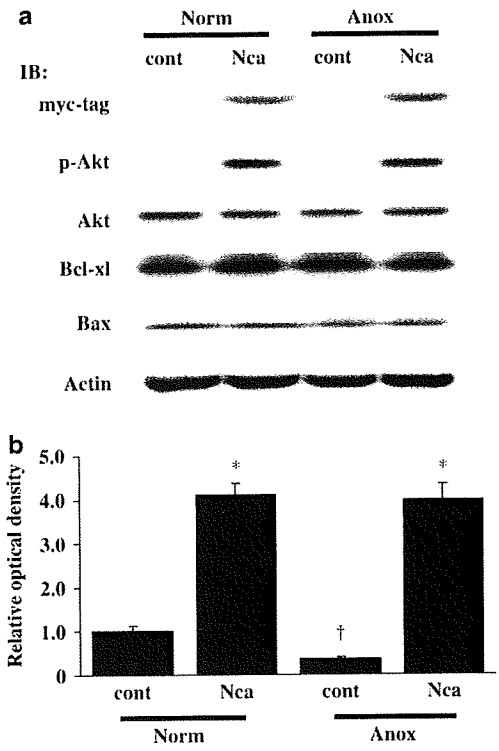

Figure 5 NcaAMPK increases phosphorylation levels of Akt under both normoxic and anoxic conditions. Basal phosphorylation levels of Akt in controls were downregulated under anoxic conditions compared with normoxic conditions. However, in NcaAMPK-overexpressing cells, Akt phosphorylation was maintained at high levels even under anoxic conditions. Neither Bcl-xl nor Bax expression levels changed under anoxic conditions. The experiments were performed four times and a representative figure is shown. IB, immunoblot. (**b**) Relative phosphorylation levels of Akt were quantified using the Scion Image program. Immunoblots were normalized to total loaded protein. The mean value of cont/Norm (normoxia) was fixed to 1.0. Each bar represents the mean ± s.e.m., $*P<0.005$ *vs.* cont, $\dagger P<0.01$ *vs.* Norm.

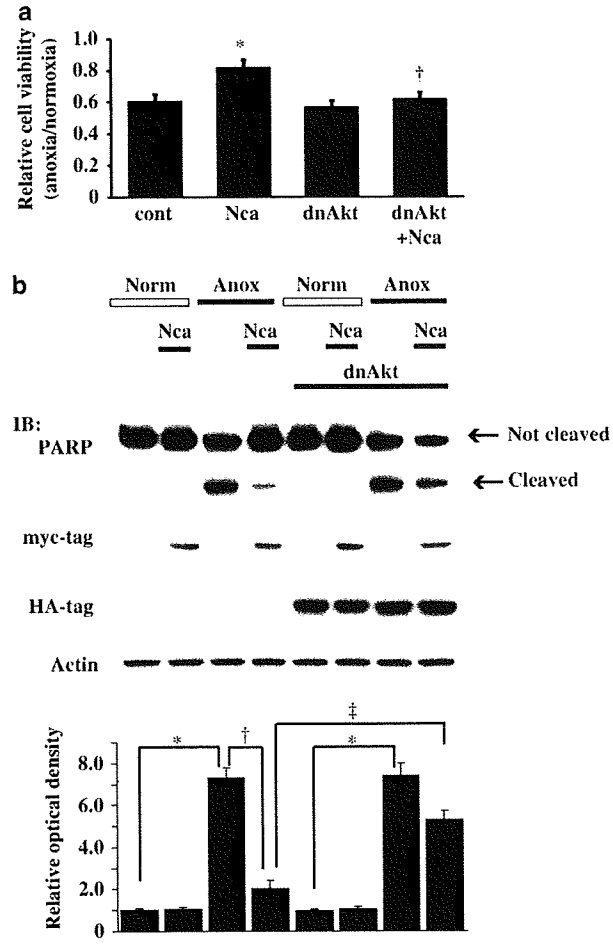

Figure 6 NcaAMPK inhibits anoxia-induced cell death and PARP cleavage but dominant-negative (dn) Akt abrogates this inhibition. (**a**) The cell viability measured in the WST-1 assay was compared under anoxic conditions among the cells expressing GFP 100 MOI (cont), NcaAMPK 50 MOI+GFP 50 MOI (Nca), GFP 50 MOI+dnAkt 50 MOI (dnAkt), and NcaAMPK 50 MOI+dnAkt 50 MOI (dnAkt+Nca). Each bar represents the mean ± s.e.m., $N=6$. $*P<0.01$ *vs.* cont, $\dagger P<0.01$ *vs.* Nca. (**b**) Anoxia-induced caspase activation increased PARP cleavage in control HUVECs, as shown with an anti-PARP antibody, and NcaAMPK overexpression inhibited PARP cleavage compared with control. However, co-overexpression of dnAkt abrogated the inhibition of PARP cleavage in NcaAMPK-overexpressing cells under anoxic conditions. Immunoblotting with myc-tag or HA tag antibodies shows the expressions of NcaAMPK and dnAkt, respectively. The experiments were performed four times and a representative figure is shown. The lower part of this figure shows a quantitative analysis of the cleaved PARP fragments. Each bar represents the mean ± s.e.m., Norm, normoxia; Anox, anoxia; Nca, NcaAMPK; IB, immunoblot. $*P<0.005$, $\dagger P<0.01$, $\ddagger P<0.05$.

conditions,[9] we also found in this study that the basal phosphorylation levels of Akt were lower under anoxic conditions than in normoxic conditions (Figure 5). However, phosphorylation was maintained at high levels in NcaAMPK-overexpressing cells even under anoxic conditions. As overexpression of dnAkt did not reverse completely the inhibition of anoxia-induced PARP cleavage in NcaAMPK-overexpressing HUVECs, we suggest that NcaAMPK inhibits apoptosis of endothelial cells not only through Akt signaling but also through other, as yet unknown, signaling pathways under anoxic conditions.

Very recently, Young[11] published a thought-provoking review on the function of AMPK in the cardiovascular system under ischemic stress. He suggested that AMPK functions as a 'major conductor of the stress signaling orchestra' in ischemic cardiovascular cells. As AMPK functions as an energy sensor and inhibits ATP-consuming reactions in endothelial cells, improvement of intracellular energy status might be a major factor for preventing cell death under anoxic/hypoxic conditions. However, we revealed in this study that AMPK activation plays an anti-apoptotic role in HUVECs, at least partly through the Akt pathway under anoxic conditions. AMPK might therefore play a beneficial role in the ischemic vasculature, although the details of the mechanism remain to be elucidated.

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ORIGINAL ARTICLE

In early-stage diabetic retinopathy, risk of cardiac events after implantation of sirolimus-eluting stent is higher than after coronary artery bypass surgery

Akihiro Masuzawa[a], Takayuki Ohno (MD)[a,*], Shinichi Takamoto (MD, FJCC)[a], Noboru Motomura (MD)[a], Minoru Ono (MD, FJCC)[a], Hideo Fujita (MD)[b], Jiro Ando (MD)[b], Toshihiro Morita (MD)[b], Yasunobu Hirata (MD)[b], Ryozo Nagai (MD, FJCC)[b], Akira Hirose (MD)[c], Takashi Shigeeda (MD)[c], Satoshi Kato (MD)[c], Makoto Araie (MD)[c]

[a] *Department of Cardiothoracic Surgery, The University of Tokyo, 7-3-1, Hongo, Bunkyo-Ku, Tokyo, 113-8655, Japan*
[b] *Department of Cardiology, The University of Tokyo, Tokyo, Japan*
[c] *Department of Ophthalmology, The University of Tokyo, Tokyo, Japan*

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Summary
Background: Patients with diabetic retinopathy (DR) have an increased risk of death from coronary heart disease and myocardial infarction. The purpose of this study was to compare the outcomes of revascularization strategies (sirolimus-eluting stent [SES] and coronary artery bypass surgery [CABG]) in patients with DR according to the stage of retinopathy: non-proliferative retinopathy (NPDR) and proliferative retinopathy (PDR).
Methods: From April 2004 until February 2007, 627 patients including 51 NPDR and 62 PDR patients underwent SES implantation. For each retinopathy group, a historical comparison group at the same stages of retinopathy undergoing CABG was selected. Cardiac events were defined as a composite of cardiac death, myocardial infarction, and repeat revascularization.

* Corresponding author. Tel.: +81 3 5800 8654; fax: +81 3 5684 3989.
E-mail addresses: takohno-tky@umin.net, takohno@hotmail.com (T. Ohno).

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Results: The average follow-up from the time of the initial revascularization was 27.7 ± 8.5 months for NPDR-SES patients, 69.6 ± 36.6 months for NPDR-CABG patients, 26.4 ± 9.7 months for PDR-SES patients, and 68.3 ± 44.2 months for PDR-CABG patients; and Kaplan–Meier estimates of the percentages of events at 24 months were 47.0%, 22.8%, 28.5%, and 26.0%. Kaplan–Meier curves for cardiac events differed significantly between the SES group and the CABG group in NPDR patients ($p = 0.04$), whereas the curves did not differ significantly between the two groups of PDR patients. The adjusted hazard ratio of SES implantation for cardiac events in the entire group of DR patients was 1.75 (95% confidence interval [CI] 1.02–3.00, $p = 0.04$).
Conclusions: SES implantation is not a suitable method of revascularization in DR patients, especially in NPDR patients. CABG may become the first-choice revascularization technique for these patients.

Introduction

Diabetic retinopathy (DR) is a frequent and early microvascular complication of diabetes mellitus, and can be easily evaluated by direct ophthalmoscopy. Epidemiological evidence indicates that the stage of DR is associated with an increased risk of death and myocardial infarction [1–4]. Previously, we have shown that the effect of treatment on survival of coronary artery bypass surgery (CABG) is more apparent among diabetic patients with retinopathy than those without [5]. After CABG, however, long-term survival is not satisfactory in advanced DR [6]. Therefore, we hypothesized that patients with early-stage DR probably have particularly strong indications for CABG.

Since the advent of the drug-eluting stent (DES), striking reductions in angiographic restenosis have often been extrapolated in support of more widespread use of percutaneous coronary intervention (PCI) in diabetic patients with coronary heart disease in the hope of improving survival. Recently, we revealed that patients with early-stage DR have an increased risk of target-vessel failure after the coronary implantation of sirolimus-eluting stents (SESs), and that SES implantation had a greater risk of adverse cardiac events than did CABG in diabetic patients with DR [7,8]. However, no data based on analysis of the different stages of diabetic retinopathy are available on the outcome after SES implantation versus CABG. The purpose of this study was, therefore, to compare the outcomes of the two-revascularization strategies (SES implantation versus CABG) in diabetic patients on the basis of the severity of retinopathy: non-proliferative retinopathy (NPDR), and proliferative retinopathy (PDR).

Methods

The procedures of revascularization; the methods of the diagnosis of DR and patient care; the details of the endpoints were the same as described previously [8].

Selection of patients

Between April 2004 and February 2007, 627 consecutive patients underwent implantation of SES (Cypher; Cordis, Johnson & Johnson, Miami Lakes, Florida) for coronary artery disease at the University of Tokyo Hospital, Tokyo, Japan. Patients were eligible for inclusion in this study if they had DR. We identified 113 such patients, and classified them into two groups: those with NPDR ($n = 51$) (retinal microaneurysm, retinal hemorrhages, soft exudates, hard exudates, intraretinal microvascular abnormalities, or venous beading) and those with PDR ($n = 62$) (the presence of new vessels, preretinal or vitreous hemorrhage, panretinal photocoagulation scars, and history of vitrectomy). For each group, a historical comparison group with the same stage of retinopathy was selected by reviewing the data of 80 consecutive patients who underwent isolated CABG at our hospital between June 1988 and December 2006.

Endpoints

Briefly, the primary end point was adverse cardiac events, defined as a composite of death from cardiac causes, myocardial infarction, stent thrombosis in cases of SES implantation, and repeat revascularization, during 2 years after the initial

Table 1 Patient backgrounds.

Variables (continuous)	Median ± SIQR		p-Value	Median ± SIQR		p-Value
	NPDR-SES (n = 51)	NPDR-CABG (n = 40)		PDR-SES (n = 62)	PDR-CABG (n = 40)	
Age	67 ± 6.0	65 ± 4.3	0.68	67.5 ± 5.9	67 ± 6.6	0.1
Body Mass Index	23.6 ± 2.1	23.3 ± 1.7	0.39	23.8 ± 2.4	23.1 ± 1.7	0.71
Serum creatinine	0.96 ± 0.12	0.84 ± 0.17	0.11	1.36 ± 2.34	0.99 ± 0.52	0.24
Estimated GFR	56.7 ± 10.3	62.4 ± 11.9	0.13	40.1 ± 26.0	49.6 ± 18.7	0.17
Left ventricular ejection fraction	59.0 ± 11.75	63.0 ± 10.0	0.26	60 ± 10.2	63 ± 10.8	0.32
Glycosylated hemoglobin	7.3 ± 1.0	6.7 ± 0.85	0.08	6.9 ± 0.7	7.0 ± 0.6	0.99
Variables (characteristics)	No. (%)		p-Value	No. (%)		p-Value
	NPDR-SES (n = 51)	NPDR-CABG (n = 40)		PDR-SES (n = 62)	PDR-CABG (n = 40)	
Follow-up angiography	41 (80.4)	36 (90)	0.25	46 (74.2)	39 (97.5)	0.002*
Age >70 years	22 (43.1)	11 (27.5)	0.13	28 (45.2)	15 (37.5)	0.54
Male sex	43 (84.3)	31 (77.5)	0.43	39 (62.9)	25 (62.5)	1
Body Mass Index >25	16 (31.4)	9 (22.5)	0.48	18 (29.5)	9 (23.1)	0.64
Glycosylated hemoglobin >7%	29 (56.9)	14 (35.9)	0.06	30 (48.4)	18 (46.2)	0.84
Treatment of diabetes	43 (84.3)	30(75)	0.3	56 (90.3)	36 (90.0)	1
Oral hypoglycemic agent	21 (41.2)	21 (52.5)	0.3	13(21)	18(45)	0.01*
Insulin	22 (43.1)	10 (25.0)	0.08	43 (69.4)	19 (47.5)	0.04*
Current smoker	14 (27.5)	11 (27.5)	1	10 (16.1)	8(20)	0.61
Ex-smoker	21 (41.2)	20(50)	0.52	24 (38.7)	12(30)	0.4
Arterial hypertension	44 (86.3)	29 (72.5)	0.12	50 (80.6)	34(85)	0.79
Dyslipidemia	41 (80.4)	28(70)	0.33	46 (74.2)	25 (62.5)	0.27
Arterial disease	11 (21.6)	11 (27.5)	0.62	15 (24.2)	14(35)	0.27
Previous myocardial infarction	12 (23.5)	10(25)	1	17 (27.4)	18(45)	0.09
Previous revascularization	15 (29.4)	3 (7.5)	0.02*	23 (37.1)	4(10)	0.003*
Stroke	9 (17.6)	9 (22.5)	0.6	9 (14.5)	9 (22.5)	0.43
Serum creatinine >1.2 mg/dl	9 (17.6)	5 (12.5)	0.57	35 (56.5)	14(35)	0.04*
Requiring dialysis	5 (9.8)	2(5)	0.46	21 (33.9)	7 (17.5)	0.11
Estimated GFR <60 ml/min	33 (64.7)	17 (42.5)	0.06	48 (77.4)	27 (67.5)	0.36
Left ventricular ejection fraction <50	17 (33.3)	6 (15.4)	0.09	19 (30.6)	10 (25.9)	0.66
Congestive heart failure	9 (17.6)	10(25)	0.44	25 (40.3)	16(40)	1
Indication for revascularization						
Stable angina pectoris	11 (21.6)	15 (37.5)	0.11	15 (24.2)	8(20)	0.81
Unstable angina pectoris	11 (21.6)	14(35)	0.17	10 (16.1)	9 (22.5)	0.44
Acute myocardial infarction	8 (15.7)	5 (12.5)	0.77	10 (16.1)	3 (7.5)	0.24
Silent myocardial ischemia	21 (41.2)	7 (17.5)	0.02*	28 (45.2)	20(50)	0.69
Multivessel disease	48 (94.1)	40(100)	0.25	56 (90.3)	39 (97.5)	0.24
3-vessel disease	31 (60.8)	31 (77.5)	0.11	44(71)	34(85)	0.15
Diseased coronary artery						
Left anterior descending	51(100)	39 (97.5)	0.44	60 (96.8)	40(100)	0.52
Left circumflex	41 (80.4)	34(85)	0.78	54 (87.1)	38(95)	0.31
Left main	12 (23.5)	18(45)	0.04*	9 (14.5)	13 (32.5)	0.047*
Right	38 (74.5)	34(85)	0.3	53 (85.5)	36(90)	0.56

Continuous data between groups were compared using Wilcoxon rank sum test. Categorical variables were compared by Fisher's exact test.

CABG, coronary-artery-bypass surgery; GFR, glomerular filtration rate; SES, sirolimus-eluting stent; SIQR, semi-inter-quartile range.

* $p < 0.05$ indicate significant differences.

Table 2 24-month outcomes.

Adverse cardiac events	NPDR-SES ($n=51$)[a]	NPDR-CABG ($n=40$)[a]	PDR-SES ($n=62$)[a]	PDR-CABG ($n=40$)[a]
In-hospital events				
Death from cardiac causes	0 (0.0)	0 (0.0)	1 (1.6)	0 (0.0)
Myocardial infarction	0 (0.0)	1 (2.5)	1 (1.6)	0 (0.0)
Repeat revascularization				
Percutaneous	1 (2.0)	3 (7.5)	0 (0.0)	5 (12.5)
Surgical	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Stent thrombosis	0 (0.0)	–	0 (0.0)	–
Out-of-hospital events				
Death from cardiac causes	1 (2.0)	0 (0.0)	2 (3.2)	0 (0.0)
Myocardial infarction	3 (5.9)	0 (0.0)	1 (1.6)	0 (0.0)
Repeat revascularization				
Percutaneous	18 (35.3)	4 (10.0)	14 (22.6)	5 (12.5)
Surgical	2 (3.9)	1 (2.5)	1 (1.6)	0 (0.0)
Stent thrombosis	2 (3.9)	–	0 (0.0)	–

A composite of deaths from cardiac causes, myocardial infarction, and repeat revascularization was termed "adverse cardiac events".
CABG, coronary-artery-bypass surgery; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; SES, sirolimus-eluting stent.
[a] No. of patients (%).

coronary revascularization. Myocardial infarction was defined as the presence of new Q waves in at least two contiguous leads and an elevated creatinine kinase MB fraction; or in the absence of pathologic Q waves, an increase in creatinine kinase level to more than twice the upper limit of the normal range plus a high level of creatinine kinase MB or troponin I. Stent thrombosis was defined as acute coronary syndromes with angiographic documentation of vessel occlusion or thrombus within the target vessel; or in the absence of angiographic confirmation, either acute myocardial infarction in the distribution of the treated vessels or death from cardiac causes within 30 days. Patients undergoing SES implantation were asked to agree to a coronary angiographic follow-up study at 6 months or earlier if anginal symptoms occurred. Patients undergoing CABG were asked to agree to a postoperative coronary angiographic study during the initial hospitalization. Repeat revascularization was considered to be driven by ischemia: angiographic stenosis by more than 50% in any target vessel or in any graft (in the case of CABG) with angina symptoms, or by more than 75% stenosis without symptoms.

Statistical analysis

Continuous data between groups were compared using Wilcoxon rank sum test. Categorical variables were compared by Fisher exact test. Survival and adverse cardiac event-free survival were analyzed by Kaplan–Meier analysis, and differences between groups were compared with the log-rank test. Univariate Cox proportional-hazards models were calculated for the entire group and the stratified groups according to various variables. A multivariate Cox model minimizing the Akaike information criterion was automatically selected. A two-sided

Table 3 Cox multivariate analysis.

Variables	Hazard ratio	(95% confidence intervals)	p-Value
SES implantation	1.75	(1.02–3.00)	0.042*
NPDR	1.63	(0.98–2.70)	0.06
Male	0.66	(0.38–1.15)	0.14
Age <70 years	1.98	(1.12–3.48)	0.019*
Stroke	0.43	(0.21–0.89)	0.022*

Variables were selected to minimize the Akaike information criterion.
NPDR, non-proliferative diabetic retinopathy; SES, sirolimus-eluting stent. * $p<0.05$ indicate significant differences.

p-value of <0.05 was considered indicative of a statistically significant difference. Statistical analyses were performed using R 2.6.0 for Windows (R Development Core Team [2007]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org).

Results

Patient characteristics

Table 1 lists the baseline and angiographic characteristics of the enrolled patients, stratified by the severity of DR and the revascularization strategy. The number of patients with silent myocardial ischemia was higher in the NPDR-SES group than in the NPDR-CABG group ($p=0.02$). Patients with increased serum creatinine (>1.2 mg/dl) and patients using insulin were more often observed in the PDR-SES group than in the PDR-CABG group ($p=0.04$). Follow-up angiography was performed in many more PDR-CABG patients than PDR-SES patients ($p=0.002$). CABG patients were more likely to have left main coronary artery stenosis in both NPDR and PDR groups ($p=0.04$ and 0.047, respectively). Previous revascularizations were more often observed in patients with SES implantation in both NPDR and PDR groups ($p=0.02$ and 0.003, respectively).

Outcome

The average follow-up from the time of the initial revascularization was 27.7±8.5 months for NPDR-SES patients, 69.6±36.6 months for NPDR-CABG patients, 26.4±9.7 months for PDR-SES patients, and 68.3±44.2 months for PDR-CABG patients. Table 2 lists adverse cardiac events observed in each group. Repeat revascularization was the most common event in all groups. Kaplan–Meier estimates of the rate of cardiac events at 24 months were 47.0% in NPDR-SES patients, 22.8% in NPDR-CABG patients, 28.5% in PDR-SES patients, and 26.0% in PDR-CABG patients. Kaplan–Meier cumulative event-free curves for cardiac events differed significantly between SES patients and CABG patients in NPDR patients ($p=0.04$), but did not differ significantly in the entire group of DR patients ($p=0.07$) and in PDR patients ($p=0.60$) (Fig. 1). There were also significant differences in univariate analyses comparing revascularization procedures in subgroups of dyslipidemia, dependence on insulin and/or oral hypoglycemic agents, age (<70 years), and male gender (Fig. 2). In Cox univariate analyses, there was no significant risk factor of adverse

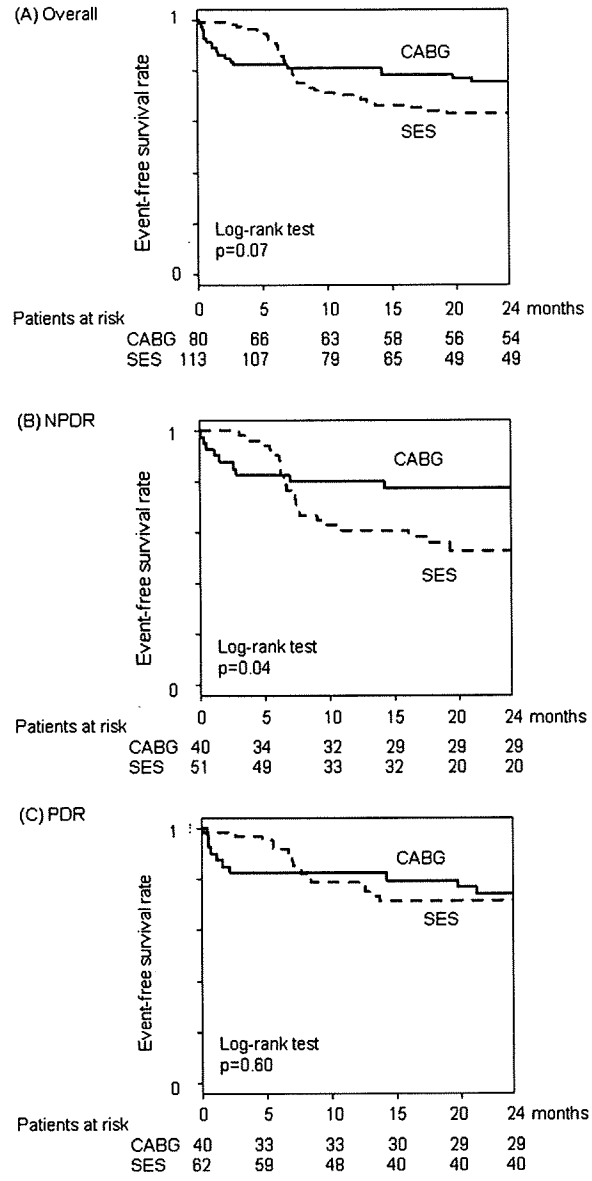
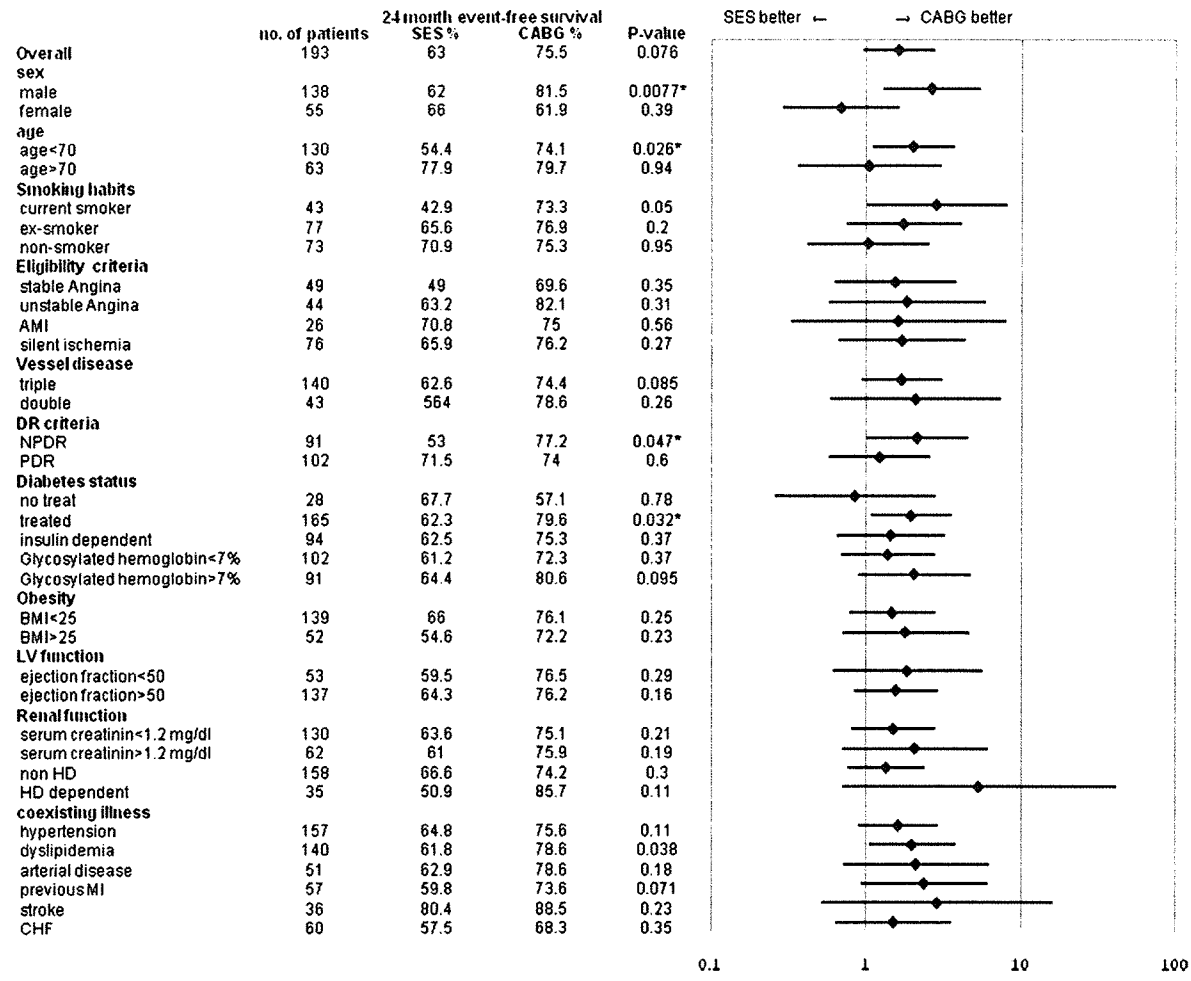


Figure 1 Kaplan–Meier cumulative event-free curves for adverse cardiac events (a composite of deaths from cardiac causes, myocardial infarction, and repeat revascularization) of diabetic patients who received sirolimus-eluting stents (SES) or coronary artery bypass surgery (CABG): (A) overall, (B) with non-proliferative retinopathy (NPDR), and (C) with proliferative retinopathy (PDR).

cardiac events in the entire group of DR patients (data not shown). In Cox multivariate analysis in the entire group of DR patients, revascularization procedure, severity of DR, stroke, sex, and age (<70 years) were selected as variables to minimize the Akaike information criterion. Of those variables, revascularization procedure, age (<70 years), and stroke were significantly associated with adverse



	no. of patients	24-month event-free survival SES %	CABG %	P-value
Overall	193	63	75.5	0.076
sex				
male	138	62	81.5	0.0077*
female	55	66	61.9	0.39
age				
age<70	130	54.4	74.1	0.026*
age>70	63	77.9	79.7	0.94
Smoking habits				
current smoker	43	42.9	73.3	0.05
ex-smoker	77	65.6	76.9	0.2
non-smoker	73	70.9	75.3	0.95
Eligibility criteria				
stable Angina	49	49	69.6	0.35
unstable Angina	44	63.2	82.1	0.31
AMI	26	70.8	75	0.56
silent ischemia	76	65.9	76.2	0.27
Vessel disease				
triple	140	62.6	74.4	0.085
double	43	564	78.6	0.26
DR criteria				
NPDR	91	53	77.2	0.047*
PDR	102	71.5	74	0.6
Diabetes status				
no treat	28	67.7	57.1	0.78
treated	165	62.3	79.6	0.032*
insulin dependent	94	62.5	75.3	0.37
Glycosylated hemoglobin<7%	102	61.2	72.3	0.37
Glycosylated hemoglobin>7%	91	64.4	80.6	0.095
Obesity				
BMI<25	139	66	76.1	0.25
BMI>25	52	54.6	72.2	0.23
LV function				
ejection fraction<50	53	59.5	76.5	0.29
ejection fraction>50	137	64.3	76.2	0.16
Renal function				
serum creatinin<1.2 mg/dl	130	63.6	75.1	0.21
serum creatinin>1.2 mg/dl	62	61	75.9	0.19
non HD	158	66.6	74.2	0.3
HD dependent	35	50.9	85.7	0.11
coexisting illness				
hypertension	157	64.8	75.6	0.11
dyslipidemia	140	61.8	78.6	0.038
arterial disease	51	62.9	78.6	0.18
previous MI	57	59.8	73.6	0.071
stroke	36	80.4	88.5	0.23
CHF	60	57.5	68.3	0.35

Figure 2 Hazard ratios for cardiac events were obtained from Cox proportional hazard models comparing sirolimus-eluting stents (SESs) versus coronary artery bypass surgery (CABG) for the entire group and within each of the specified subgroups. Horizontal lines indicate 95% confidence intervals of each hazard ratio. * $p < 0.05$ indicate significant differences.

cardiac events. Severity of DR itself was not significantly associated with adverse cardiac events. But, NPDR patients tended to have higher risk of cardiac events than PDR patients. The hazard ratio of SES for cardiac events in the entire group of DR patients was 1.75 (95% CI 1.02—3.00; $p = 0.04$) (Table 3).

Discussion

In the present study, we compared 24-month outcomes between the two revascularization strategies (SES implantation versus CABG) in patients with different stages of DR. Univariate analysis revealed that NPDR patients had a higher risk of adverse cardiac events following SES implantation than following CABG. Multivariate analysis in the entire group of DR patients demonstrated that the choice of SES implantation was an independent predictor of cardiac events following coronary revascularization. This finding suggests that SES implantation is not suitable for revascularization in patients with DR, especially with NPDR. The incidence of cardiac events in our SES patients seems higher than that reported in previous studies of SESs [9—11]. This high incidence can be attributed to the inclusion in the present study of patients with a high frequency of challenging morphology such as multivessel disease and small coronary vessels, in contrast with the simple lesions of the previous studies, and also can be attributed to the DR itself. A higher incidence of target vessel failure after SES implantation in NPDR patients, and after bare metal stent implantation in DR patients was already reported [7,12].

From the perspective of disease progression, PDR is more severe than NPDR. Our PDR patients who

underwent SES implantation were more likely to have impaired renal function, suggesting that they had more severe microvascular disease. Nevertheless, an increased risk of cardiac events was noted in NPDR patients who underwent SES implantation. This result was somewhat unexpected and perplexing. NPDR may represent a more active disease process than PDR with regard to systemic inflammation.

Epidemiologic evidence indicates that the risks of coronary artery disease, death, and myocardial infarction among NPDR patients are higher than those among diabetic patients without retinopathy [1–4]. SES implantation is targeted at existing 'culprit' lesions but not future lesions, and therefore does not reduce the risk of myocardial infarction following revascularization. In contrast, CABG bypasses most of the epicardial vessels, including those at risk for future events, which are responsible for most myocardial infarctions, and may render these events less fatal. Therefore, CABG could offer the most benefit to NPDR patients. Although congestive heart failure was not included in the adverse cardiac events because of the retrospective nature of this study, we showed previously that PDR patients are at high risk for congestive heart failure after CABG, suggesting that PDR patients have irreversible diabetic cardiomyopathy [13]. NPDR patients usually have less severe renal and cardiac functional impairment compared with PDR patients. Taken together, we believe that the indication for early CABG may become particularly strong for NPDR patients before proceeding to the stage of PDR.

There are some caveats associated with the present study. First, to approximate the stage of DR at the time of the procedure, we used ophthalmologic records collected up until the time of revascularization; therefore, the retinopathy stage at the time of the procedure may have been underestimated in some patients. In this study, according to the protocol of eye screening for DR, most NPDR patients underwent eye examination every 12 months, and PDR patients, every 6 months. Second, the clinical outcomes were retrospectively evaluated; too small a number of the patients were disabled to use propensity score matching optimal for the comparison of totally different therapeutic strategies: SES implantation and CABG. Adjustment by Cox multivariate analysis should explain covariate effect adequately. Because our clinical practice incorporates close follow-up of our patients at our hospital, the retrospective nature of the analysis should not pose a big problem.

Since the number of patients was small, and the follow-up short, we did not find any survival differences between CABG and SES implantation patients. The Bypass Angioplasty Revascularization Investigators (BARIs) revealed an increased survival risk for PCI compared to CABG in patients with treated diabetes [14]. On the other hand, a recently published meta-analysis of six randomized trials that reported outcomes in diabetic patients showed no significant survival differences between CABG and PCI [15]. Because each randomized trial had a small number of patients with diabetes of unknown severity, comparisons between outcomes of CABG and PCI in diabetic patients are still controversial. Both CABG and PCI are still undergoing improvements, such as the use of the off-pump approach for CABG, or that of a drug-eluting stent. New and ongoing randomized trials and large case-matched studies are expected to add to our understanding of the differences between CABG and PCI.

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ベリプラスト症例　心臓外科領域

講師 小野　稔 東京大学大学院医学系研究科臓器病態外科学心臓外科

先天性AT-Ⅲ欠損症患者の大動脈基部置換手術における「ベリプラスト浸漬サージセルの重層圧迫」による止血

症例

●症例	27歳・男性（体重65kg）
●既往歴	幼少期よりアトピー性皮膚炎。先天性アンチトロンビンⅢ欠損症。
●家族歴	父親：深部静脈血栓症、母親：肺梗塞のため下大静脈フィルターの留置あり。
●病歴と経過	2001年12月、急性大動脈解離（DeBakey Ⅰ型）を発症し、他院でBentall手術（InterGard 28mmおよびSJM 25mm）が施行された。術後経過は良好で、術後3週間で退院となった。2004年10月、突然右上肢のしびれと失語が出現し、他院へ入院し脳梗塞と診断された。その9日後に40℃以上の発熱があり、血液培養からメチシリン感受性黄色ブドウ球菌（MSSA）が検出され、心エコー検査で人工弁への疣贅の付着が認められた。これはアトピー性皮膚炎の悪化による皮膚感染が感染性心内膜炎の原因と考えられた。体幹CT検査で腎梗塞と脾梗塞が発見された。このとき、さらにアンチトロンビンⅢ低値が判明し、家族歴から「先天性アンチトロンビンⅢ欠損症」と診断された。感受性のある抗菌薬治療にもかかわらず、入院1カ月後の心エコー検査で疣贅の増大と人工弁周囲に膿瘍の形成が認められ、11月22日、同種大動脈基部置換の目的で当院へ転院となった。

手術時の持続的出血に対して、フィブリングルーの直接投与ではグルーが洗い流されてしまい効果は期待できない。こうした場合、筆者らは「ベリプラスト浸漬サージセルの重層圧迫」を行い確実な止血を得ている。今回、先天性アンチトロンビンⅢ（以下、AT-Ⅲ）欠損症患者への大動脈基部置換手術の際の止血に対しても、この方法が奏効したので報告する。

入院時所見

入院時、抗菌薬2剤（ABPC+ GM）が投与中であった。バイタルサインは安定していたが、37.3℃の微熱が観察された。白血球数は6,000、CRPは3.31で、肝機能と腎機能に異常は認められなかった。数回測定したAT-Ⅲの活性値は33～40％（正常値80％以上）であった。術前のため、ワーファリンを中止してヘパリンによる抗凝固療法に切り替えた。AT-Ⅲ製剤であるアンスロビンP 1,500単位を数日ごとに投与したが、活性化部分トロンボプラスチン時間を45～50秒に延長させるために、ヘパリンの1日所要量は25,000～28,000単位と高用量であった。

当院で行った心エコー検査では、LVDd / Dsが48 / 35、LVEFは60％で、左室壁運動には異常はなかった。大動脈弁位人工弁には開放制限や弁周囲逆流はなく、明らかな疣贅も見られないが、大動脈基部後方に膿瘍を疑わせる3.5×2.5 cm大のスペースが認められた（写真1）。転院後、手術待機中に失語の再発が見られ、CT検査で右側頭葉に新たな脳梗塞が指摘された。保存的治療で軽快し、2005年1月、同種大動脈弁による大動脈基部置換手術を施行した。

手術所見

全身麻酔導入後に、AT-Ⅲ製剤（アンスロビンP）1,500単位を投与して体外循環に備えた。アンスロビンP投与30分後のAT-Ⅲ活性は108％で、活性化凝固時間（ACT）は102秒であった。慎重に再胸骨正中切開を行い、心臓および大動脈を剝離した。ヘパリン20,000単位を静注後のACTは503秒であった。左大腿動脈送血、上下大静脈脱血で人工心肺を確立した。右上肺静脈から左心ベントを挿入し、28℃を目標に冷却を開始した。大動脈基部を十分に剝離した後に、上行大動脈を遮断した。心筋保護は、初回は順行性に晶質性心筋保護液（crystalloid cardioplegia）を投与して、2回目以降は冠静脈洞から逆行性に血液心筋保護液（blood cardioplegia）を20分ごとに投与した。人工血管を離断して観察したが、人

工弁には明らかな異常は認めなかった。人工弁を摘出すると、無冠尖弁輪に沿った人工血管の縫合不全があり、膿瘍腔が認められた。膿瘍腔を可及的に掻爬・洗浄して、大動脈裏面にある膿瘍腔壁を開放した(**写真2**)。

心筋虚血時間が2時間を超えてからは、逆行性blood cardioplegia投与後の20分間はポンプ血(non-cardioplegic blood)を持続灌流して心筋保護の強化を図った。東京大学組織バンクから提供された同種大動脈弁(ドナー：60歳・男性、弁輪径23mm、血液型一致)を解剖学的位置関係が一致するように吻合した。無冠尖部分は大動脈弁輪が欠損し、膿瘍腔入口部もあるために、線維三角から僧帽弁前尖弁輪にかかるように大きく吻合糸をかけた。膿瘍腔の背側は切開・開放して閉鎖腔にならないように気を付けた。ベントカニューレからポンプ血を左室内に注入・加圧して、吻合部からの出血を確認した。ベリプラストを吻合部心筋に十分に摺りこむように適用した。残りの人工血管成分を可及的に除去して、左・右の順番に冠動脈をCarrel patch法で再建した。順行性cardioplegiaを注入して冠動脈吻合部の確認を行い、同種大動脈弁の遠位吻合を完了した(**写真3**)。

大動脈の遮断解除後、1度の除細動で間もなく洞調律に復帰した。人工心肺時間414分、大動脈遮断時間は326分に及んだが、人工心肺からの離脱は中等量のカテコラミン補助下に容易に行えた。人工心肺

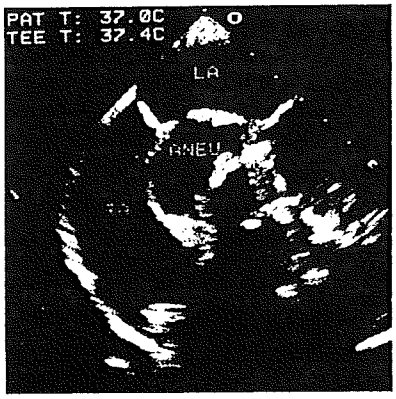

写真1　術前の心エコー像
LA：左心房、ANEU：仮性瘤、RA：右心房
術前の経食道心エコー検査で、人工弁背側に仮性瘤を認めた。

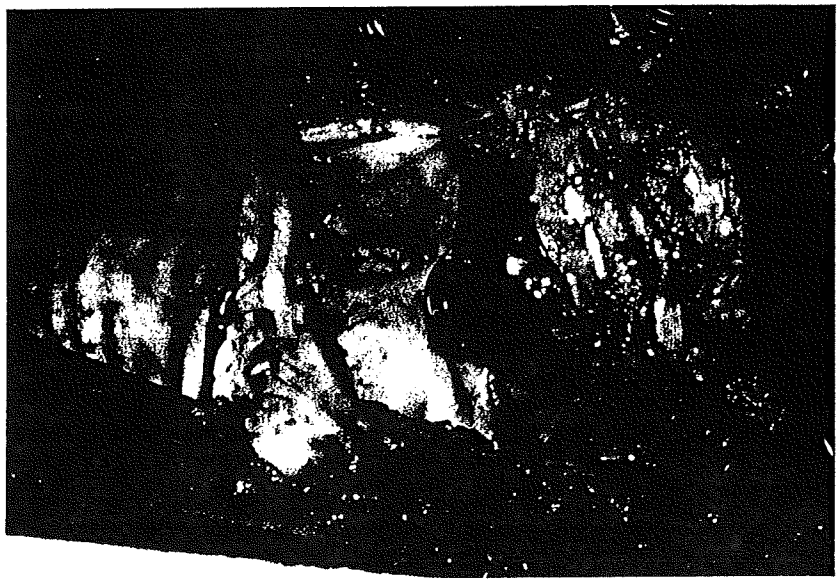

写真2　術中写真①
人工弁を一部取りはずした像。仮性瘤内を鉗子で示している(右が頭側)。

写真3　術中写真②
手術完成写真(下が頭側)。

中は1時間ごとにACTの測定を行い、人工心肺開始後5時間まではACTは480秒以上であった。6時間目の測定で448秒と短縮が見られたため、ヘパリン2,000単位を追加投与した。プロタミン中和後(250mg)にはACTは160秒に短縮し、止血はほぼ良好であった。しかし、血圧の上昇に伴い、大動脈基部吻合部からの出血が増加してきた。これに対しては、筆者らが開発した「ベリプラスト浸漬サージセルの重層圧迫」で間もなく止血を得ることができた。

術後経過は良好で、術後12時間で抜管となった。6週間の抗菌薬治療の後、退院となった。術後3年経過したが、感染の再燃はなく同種大動脈弁の機能も良好である(**写真4**)。

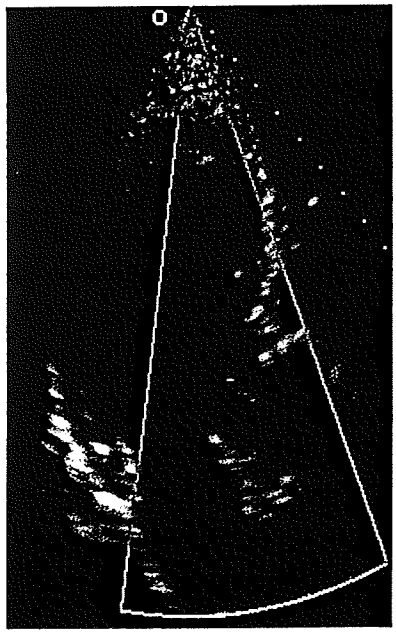

写真4　術後3年の心エコー像
同種大動脈弁の機能は良好で、逆流もほとんど見られなかった。

考察

セリンプロテアーゼ阻害作用を有するAT-Ⅲは、主にトロンビンを介して抗凝固作用を発揮するが、IXa、Xa、XIa、XIIaなどの凝固因子にも作用する。ヘパリンに結合することによって、AT-Ⅲの抗凝固作用は劇的に(10,000倍)増強する。AT-Ⅲ欠損症は遺伝性の場合と後天的に発症することがあるが、その過凝固状態のために深部静脈血栓症や肺動脈血栓塞栓症を発症しやすい。遺伝性の場合には常染色体優性遺伝の形態を取り、男女差はなく、2,000人から5,000人に1人の発症と考えられている。先天性AT-Ⅲ欠損症には、"単純に合成が低下する" Ⅰ型と、"機能を失った異常AT-Ⅲが産生される" Ⅱ型とがある。通常AT-Ⅲは、正常活性値の30～60％に低下している。ヘパリンの持続投与を受けている患者でAT-Ⅲが減少する(正常の60％以下)ことがあることが知られており、後天性AT-Ⅲ減少症と呼ばれている。

AT-Ⅲ欠損症の場合、ヘパリン投与による抗凝固作用が発揮されにくく、人工心肺症例で1,562単位/kgのヘパリン投与を必要とした症例も報告されている。しかし、大量のヘパリン投与を行うと"heparin rebound"と呼ばれる現象が起こる場合がある。これは、血中にある大量のヘパリンの一部が血管外の脂肪組織に浸透し、術後しばらくしてから血中に逆浸透してきて出血傾向が出現するというものである。AT-Ⅲ欠損症を合併した症例においてヘパリン感受性を正常化させるためには、主に2つの方法がある。

1つは新鮮凍結血漿を投与することである。しかし、投与する至適単位についてのはっきりした目安がないうえに、ウイルス肝炎などが感染するリスクを完全には排除し切れないという問題点を有する。2つ目は、本症例のように濃縮AT-Ⅲ製剤を投与する方法である。ヘパリン投与の1～2時間前にアンスロビンPなど濃縮AT-Ⅲ製剤を静注すれば、ヘパリン300単位/kgの通常量投与で十分なACTの延長が得られる。濃縮AT-Ⅲ製剤の投与量は1,500～3,000単位であると思われる。本症例のように投与後にAT-Ⅲ活性値を測定すれば、より確実である。人工心肺中は、術前AT-Ⅲ活性が正常の症例でも体外循環回路によるAT-Ⅲの消費が起こる。体外

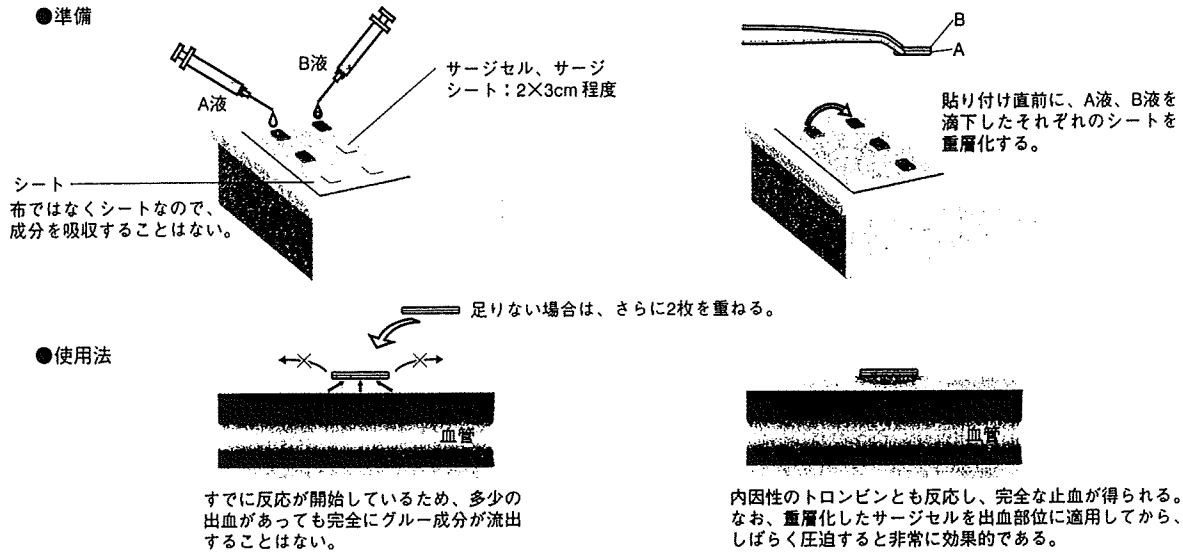

図1　ベリプラスト浸漬サージセルの重層圧迫

循環中にACTが短縮してくる場合には、濃縮AT-Ⅲ製剤の追加投与、またはヘパリンの少量追加投与で対応できる。

本症例のような再開心術や広範な組織破壊を伴った感染性心内膜炎では、どれほど丁寧で確実な剥離や吻合操作を行っても、吻合部や剥離面からの止血に難渋することがしばしばある。筆者らは、周囲組織が脆弱なために出血が危惧される吻合部や切開閉鎖部に対しては、血流や血圧の負荷がかからない大動脈遮断中にベリプラストPを好んで使用している。また、吻合部から流れ落ちないように、サージセルやタココンブで土手を作って必要な部分だけに効果的に投与することもよく行う。多くの場合には、こうした方法で十分に止血が期待できる。

しかし、特に大動脈吻合部では、自己圧の上昇に伴って人工心肺離脱前後に吻合部などからの出血が増加することがある。この場合に、筆者らがしばしば行う方法が、今回紹介した「ベリプラスト浸漬サージセルの重層圧迫」である(図1)。出血が持続的にあるとフィブリングルーを直接投与しても洗い流されてしまい効果が期待できないが、この「ベリプラスト浸漬サージセルの重層圧迫」では、貼り付け直前にサージセルAとサージセルBを重層化して反応を開始させるために、多少の出血があっても完全にグルー成分が流失することはなく、止血に貢献できる。このサージセルABの重層をさらに重ねることによって、確実な止血が得られやすいと考えている。

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Original Article: Laboratory Investigation

Near Infrared Spectroscopy study of the central nervous activity during artificial changes in bladder sensation in men

Shinya Matsumoto, Akira Ishikawa, Haruki Kume, Takumi Takeuchi and Yukio Homma

Department of Urology, The University of Tokyo Hospital, Tokyo, Japan

Objectives: To investigate the regional specificity of multi-channel near infrared spectroscopy (NIRS) on the detection of urination-related cortical activation in healthy men.

Methods: Frontal lobe blood flow was measured non-invasively in 20 resting subjects using NIRS before and after micturition. The relative concentrations of oxyhemoglobin were recorded over the frontal lobe during artificial changes in bladder sensation with a 52-channel NIRS machine. An increase in oxyhemoglobin represented locally increased blood flow. In artificially induced bladder sensation (desire to void), the lower abdomen of each participant was pressed by the examiner's hand to keep each participant feeling the desire to void for 5 s. Each participant received this series of tasks three times before and after urination. Activated concentrations calculated by contrast of subtracting the two different conditions were designed to disclose the brain areas that are involved during artificial changes in bladder sensation. The measurements were repeatedly carried out for each subject on different days to reveal the reproducibility and intra-individual variability of the results.

Results: Cerebral responses during artificial changes in bladder sensation were bilaterally associated with increased levels of oxyhemoglobin in the frontal area. Oxyhemoglobin increases during the compression maneuver were significantly larger than those during an empty bladder in several bilateral frontal channels ($P < 0.05$).

Conclusions: Frontal regions were activated during artificial changes in bladder sensation before micturition using a 52-channel NIRS machine.

Key words: bladder sensation, cerebral blood flow, near infrared spectroscopy.

Introduction

Although there are several modalities for the treatment of bladder control problems such as overactive bladder, the effect of control is not always satisfactory. Many recent studies have demonstrated that bladder control problems are caused in the lower urinary tract. Neurogenic and myogenic causes have been suggested as probable causes,[1] but no consensus has been reached. In 1995, the center of voiding was found in the periadequeductal gray (PAG) and in the pons (pontine micturition center; PMC);[2,3] however, urination itself would be controlled by supra-PAG, especially the frontal cerebral lobe, because it is necessary for us to control desires to void in many situations in our daily life.

The aim of the present study was to investigate the regional specificity of multi-channel near infrared spectroscopy (NIRS) on the detection of urination-related cortical activation in healthy men. We examined frontal cerebral lobar activity during artificial changes in bladder sensation using near infrared spectroscopy, which is a non-invasive neuroimaging method that measures changes in the oxyhemoglobin, deoxyhemoglobin and total hemoglobin concentrations in the cerebral cortex, and has not been used in urological study before. NIRS was first developed for non-invasive monitoring of cerebral oxygenation.[4,5] Its ability to measure the secondary metabolic signals accompanying neural activities was demonstrated.[6] The greatest advantage of NIRS compared with functional magnetic resonance imaging (f-MRI) and positron emission tomography (PET) is that recordings can be made without having to fix the subjects' body and brain in the apparatus. Brain imaging by PET and fMRI requires a large apparatus and subjects are not free to move around. NIRS is more suitable for recording desire to void under undisturbed and unconstrained measurement circumstances. Several studies successfully showed that NIRS was used for imaging of the sensorimotor cortex of newborn infants surprisingly under an unsedated condition.[7,8]

The present study investigates the feasibility of near infrared spectroscopy (NIRS) to measure changes in frontal lobe blood flow in response to increased bladder sensation (up to desire to void).

Correspondence: Shinya Matsumoto MD, Department of Urology, Faculty of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: shinyamatsumoto-jua@umin.ac.jp

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Methods

Subjects

Adult volunteers (22 right-handed healthy men between 23 and 45 years old; mean age 31 ± 6.5) participated in this experiment. Each subject signed written informed consent before the experimental procedures. We asked 22 right-handed healthy Japanese men to undergo a cystometry examination, but all subjects refused our request. All had no history of genitourinary manifestation and cognitive impairment in the past. Out of 22 subjects, two were excluded due to some urination disorder detected by pre-exam interview. One participant described nocturia. The other male showed an overactive bladder symptom score[9] of 3 (normal score <3). Therefore, we did not include either of these persons. The present study was approved by the institutional review board of Tokyo University Graduate School of Medicine.

Procedure

Because Japanese volunteers had not agreed to receive an examination of cystometry, we decided that 'desire to void' should be made artificially. To evaluate the effect of an artificial micturition desire, each subject received the NIRS study with the first desire to void (voiding can be delayed for at least 30 min). Visual analogue scales (VAS) (a