

Fig. 7. Renal functional parameters of NCX1^{+/+} wild-type and NCX1^{+/-} heterozygous ARF mice, with or without KB-R7943 pretreatment. BUN (a), Per (b), Ccr (c), Uosm (d), and FENa (e). Each column and bar represents the mean ± S.E.M. *, P < 0.01, compared with wild-type ARF (-KB-R7943) mice; †, P < 0.01, compared with heterozygous ARF (-KB-R7943) mice.

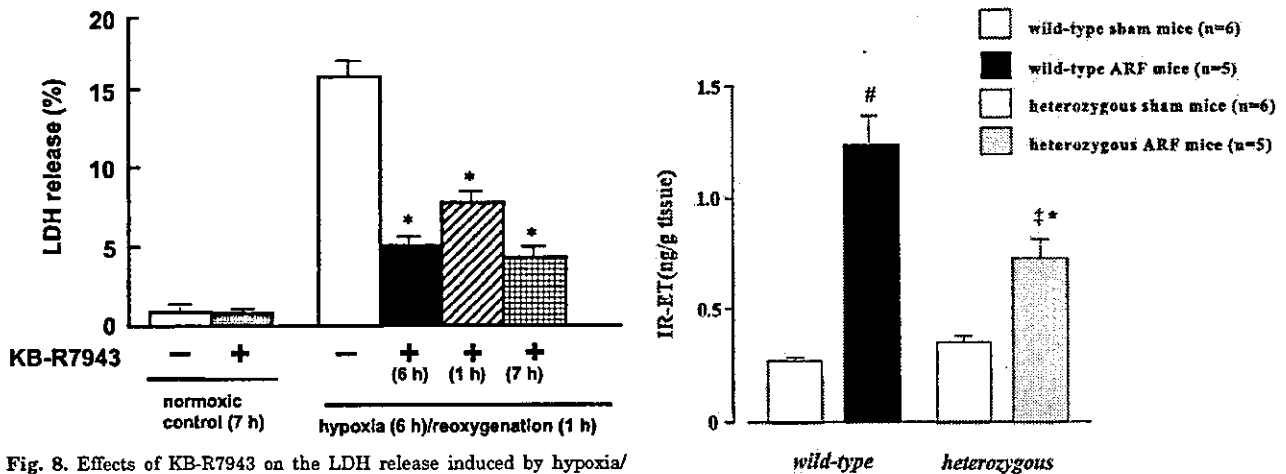


Fig. 8. Effects of KB-R7943 on the LDH release induced by hypoxia/reoxygenation in LLC-PK₁ cells. The cells were exposed to 7 h of normoxia or 6 h of hypoxia followed by 1 h of reoxygenation. KB-R7943 (10 μM) was added to the culture medium at the beginning of hypoxia and/or reoxygenation. Each column and bar represents the mean ± S.E.M. *, P < 0.01, compared with hypoxia and/or reoxygenation without KB-R7943.

Fig. 9. Endothelin-1 content in the kidney of NCX1^{+/+} wild-type and NCX1^{+/-} heterozygous mice, with or without ARF. Each column and bar represents the mean ± S.E.M. #, P < 0.01, compared with wild-type sham mice; †, P < 0.01, compared with heterozygous sham mice; *, P < 0.05, compared with wild-type ARF mice. IR-ET, immunoreactive endothelin.

differences in renal function between NCX1^{+/+} and NCX1^{+/-} sham-operated control mice (Fig. 4). Alternatively, transgene technique using kidney-specific promoter to overexpress NCX1 protein and/or a conditional knockout technique would be also useful.

There is growing evidence that ET-1 is closely related to the development of the ischemic ARF. It has been demon-

strated that ET-1 content (Shibouta et al., 1990; Matsumura et al., 2000) and ET-1 mRNA expression (Firth and Ratcliffe, 1992; Wilhelm et al., 1999) are elevated in renal tissues after ischemia/reperfusion. Our previous study has shown that daily oral administration of the ET_A-selective antagonist ABT-627, but not the ET_B-selective antagonist A-192621, had a beneficial effect on ischemia/reperfusion-induced renal

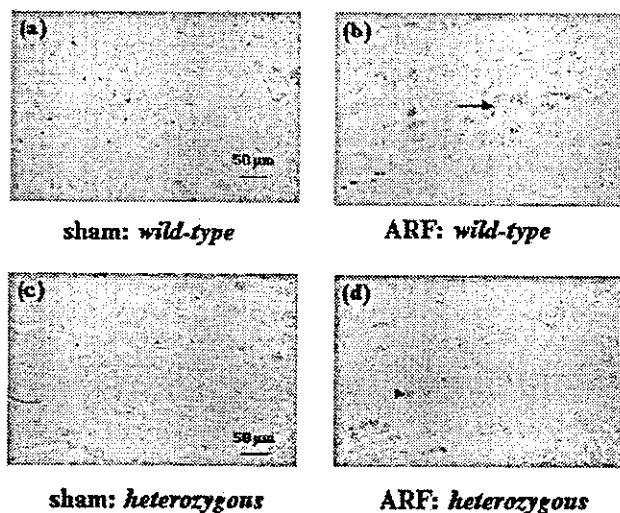


Fig. 10. Immunohistochemistry for endothelin-1 in medulla (outer zone outer stripe) of the kidney of NCX1^{+/+} wild-type and NCX1^{+/-} heterozygous mice, with or without ARF. Arrows indicate endothelin-1 peptide expression in lumens of necrotic tubular cells. The peptide expression was more intense in NCX1^{+/+} (b) than in NCX1^{+/-} mice (d).

dysfunction and tissue injury (Kuro et al., 2000). In addition, an ET-converting enzyme inhibitor, phosphoramidon (Matsumura et al., 1990a), was found to overcome ischemia/reperfusion-induced renal injury (Vemulapalli et al., 1993; Matsumura et al., 2000). Taken together, it seems likely that the up-regulation of renal ET-1 production and ET_A receptor-mediated actions are responsible for the pathogenesis of ischemic ARF. In the present study, there was an only moderate increment of ET-1 content in the postischemic kidney of NCX1^{+/-} mice, compared with the case in NCX1^{+/+} mice. In immunohistochemical study to determine the localization of ET-1 peptide in the postischemic kidney, an enhanced staining pattern was observed in necrotic tubular cells more markedly in NCX1^{+/+} mice than in NCX1^{+/-} mice. When the present study was in progress, similar localization pattern of ET-1 peptide in the kidney after the ischemia and 24 h of reperfusion was demonstrated by Wilhelm et al. (2001). Previously, Wilhelm et al. (1999) demonstrated increased ET-1 expression in the peritubular capillary network of the kidney after ischemia. Because this capillary bed is an extension of the efferent arteriole of the glomerulus and represents the primary blood supply of the tubules, it seems likely that ET-1 expressed at this site causes ongoing vasoconstriction in the peritubular capillary network and hypoxia in the neighboring tubules, and leads to tubular necrosis. Taken together, they hypothesized that ET-1 is first expressed in increased quantities in the peritubular capillary network shortly after the onset of renal ischemia and then transported across the basement membrane of the adjacent tubular epithelial cells, which are then sloughed off during the development of acute tubular necrosis. However, it remains obscure whether an overproduction of ET-1 in the ischemic kidney occurs in vascular endothelium, in tubular cells or in both, because ET-1 gene expression and the peptide production are up-regulated under the hypoxic condition, both in tubular cells and endothelial cells (Kourembanas et al., 1991; Ong et al., 1995).

One possible candidate for factors causing ET-1 overpro-

duction in the kidney exposed to the ischemia/reperfusion may be an intracellular Ca²⁺ accumulation, which is an important mediator for the pathogenesis of ischemia/reperfusion injury of the kidney (Schrier et al., 1987). An increase of calcium entry has been known to induce the expression of ET-1 gene in endothelial cells (Rubanyi and Polokoff, 1994). We demonstrated that KB-R7943 efficiently improves the renal dysfunction and tissue injury induced by the ischemia/reperfusion, accompanying the suppression of increase in ET-1 content in the kidney after the ischemia/reperfusion (Yamashita et al., 2001). Taken together, ET-1 overproduction seems to be positioned down-stream to the Ca²⁺ overload, in the cascade of ischemia/reperfusion-induced renal injury, although ET-1 overproduction may result in the further increase of intracellular Ca²⁺ level.

The medullary thick ascending limb of the loop of Henle and the proximal tubule (pars recta), both situated in the outer medulla of the kidney, are the nephron segments that are most susceptible to ischemic injury (Brady et al., 2000). Also in our study, an ischemia/reperfusion produced a marked medullary tubular necrosis, in which ET-1 peptide was abundantly observed. On the other hand, NCX1 is known to be abundant in distal tubular portion, compared with proximal portion (Yu et al., 1992; Bourdeau et al., 1993). We also observed a marked Na⁺/Ca²⁺ exchange activity in the distal tubules, compared with the case using proximal tubules. However, based on that proximal tubules are more sensitive to ischemic injury, NCX1 expressed in proximal portions may be more important in the ischemia/reperfusion-induced renal injury. It remains to be determined whether NCX1 protein is localized and functions in necrotic site of the nephron.

In separate experiments, we noted the ameliorative effect of KB-R7943 on hypoxia/reoxygenation-induced injury in LLC-PK₁ cells, which are derived from pig kidney and have characteristics of proximal tubules. Hypoxia/reoxygenation technique using LLC-PK₁ is known as in vitro model system of ischemia/reperfusion-induced renal tubular injury (Yonehana and Gemba, 1999). KB-R7943 was effective by the treatment not only during the hypoxia but also after the hypoxia, suggesting that Ca²⁺ influx via NCX1 during reoxygenation is more important in the hypoxia/reoxygenation-induced cell injury. This observation is in agreement with our previous report indicating that both pre- and postischemic treatments with KB-R7943 overcame the ischemia/reperfusion-induced renal injury (Yamashita et al., 2001).

In conclusion, NCX1^{+/-} mice exhibited an attenuated development of the ischemia/reperfusion-induced renal injury. It seems most likely that Ca²⁺ overload via the reverse mode of Na⁺/Ca²⁺ exchange, followed by renal ET-1 overproduction, plays an important role in the pathogenesis of ischemia/reperfusion-induced ARF. Taken together with the pharmacological evidence that an inhibitor of NCX1 could overcome the ischemia/reperfusion-induced renal injury in both cases of pre- and postischemic treatments, selective and potent inhibitors of NCX1 may be beneficial in the treatment of ischemic ARF in humans.

References

- Bourdeau JE, Taylor AN, and Iacopino AM (1993) Immunocytochemical localization of sodium-calcium exchanger in canine nephron. *J Am Soc Nephrol* 4:105-110.
- Brady HR, Brenner BM, Clarkson MR, and Lieberthal W (2000) Acute renal failure,

- in *Brenner and Rector's The Kidney*, 6th ed. (Brenner BN ed) pp 1201-1262, W.B. Saunders Company, Philadelphia.
- Chapman RA and Tunstall J (1987) The calcium paradox of the heart. *Prog Biophys Mol Biol* 50:67-96.
- Cross HR, Lu L, Steenbergen C, Philipson KD, and Murphy E (1998) Overexpression of the cardiac Na⁺-Ca²⁺ exchanger increases susceptibility to ischemia/reperfusion injury in male, but not female, transgenic mice. *Circ Res* 83:1215-1223.
- Cross HR, Radda GK, and Clarke K (1995) The role of Na⁺/K⁺ATPase activity during low flow ischemia in preventing myocardial injury; a 31P, 23Na and 87Rb NMR spectroscopic study. *Magn Reson Med* 34:673-685.
- Dennis SC, Coetzee WA, Cragoe EJ Jr, and Opie LH (1990) Effects of proton buffering and amiloride derivatives on reperfusion arrhythmias in isolated rat hearts. Possible evidence for an arrhythmogenic role of Na⁺/H⁺ exchange. *Circ Res* 66:1156-1159.
- Firth JD and Ratcliffe PJ (1992) Organ distribution of the three rat endothelin messenger RNAs and the effects of ischemia on renal gene expression. *J Clin Invest* 90:1023-1031.
- Fujita K, Matsumura Y, Kita S, Miyazaki Y, Hisaki K, Takaoka M, and Morimoto S (1995) Role of endothelin-1 and ET_A receptor in the maintenance of deoxycorticosterone acetate-salt-induced hypertension. *Br J Pharmacol* 114:925-930.
- Gellai M, Jugus M, Fletcher T, Nambi P, Ohlstein EH, Elliot JD, and Brooks DP (1995) Non peptide endothelin receptor antagonists V, Prevention and reversal of acute renal failure in the rat by SB 209670. *J Pharmacol Exp Ther* 275:200-206.
- Gesek FA, Wolff DW, and Strandhoy JW (1987) Improved separation method for rat proximal and distal renal tubules. *Am J Physiol* 22:F358-F365.
- Goldfarb D, Iaina A, Gavendo SS, Kapular S, and Eliahou HE (1983) Beneficial effect of verapamil in ischemic acute renal failure in the rat. *Proc Soc Exp Biol Med* 172:389-392.
- Iwamoto T, Pan Y, Nakamura TY, Wakabayashi S, and Shigekawa M (1998) Protein kinase C-dependent regulation of Na⁺/Ca²⁺ exchanger isoforms NCX1 and NCX3 does not require their direct phosphorylation. *Biochemistry* 37:17230-17238.
- Iwamoto T, Watano T, and Shigekawa M (1996) A novel isothiourea derivative selectively inhibits the reverse mode of Na⁺/Ca²⁺ exchange in cells expressing NCX 1. *J Biol Chem* 271:22391-22397.
- Kourembanas S, Marsden PA, McQuillan LP, and Faller DV (1991) Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. *J Clin Invest* 88:1054-1057.
- Kuro T, Kohnou K, Kobayashi Y, Takaoka M, Opgenorth TJ, Wessale JL, and Matsumura Y (2000) Selective antagonism of ET_A but not ET_B receptor is protective against ischemic acute renal failure in rats. *Jpn J Pharmacol* 82:307-316.
- Ladilov Y, Haffner S, Balser-Schafer C, Maxeiner H, and Piper HM (1999) Cardio-protective effects of KB-R7943; a novel inhibitor of the reverse mode of Na⁺/Ca²⁺ exchanger. *Am J Physiol* 276:H1868-H1876.
- Matsumura Y, Hisaki K, Takaoka M, and Morimoto S (1990a) Phosphoramidon, a metalloproteinase inhibitor, suppresses the hypertensive effect of big endothelin-1. *Eur J Pharmacol* 185:103-106.
- Matsumura Y, Ikegawa R, Takaoka M, and Morimoto S (1990b) Conversion of porcine big endothelin to endothelin by an extract from the porcine aortic endothelial cells. *Biochem Biophys Res Commun* 167:203-210.
- Matsumura Y, Kuro T, Kobayashi Y, Umekawa K, Ohashi N, and Takaoka M (2000) Protective effect of SM-19712, a novel and potent endothelin converting enzyme inhibitor, on ischemic acute renal failure in rats. *Jpn J Pharmacol* 84:16-24.
- Nakamura A, Harada K, Sugimoto H, Nakajima F, and Nishimura N (1998) Effects of KB-R7943, a novel Na⁺/Ca²⁺ inhibitor, on myocardial ischemia/reperfusion injury. *Folia Pharmacol Jpn* 111:105-115.
- Ong ACM, Jowett TP, Firth JD, Burton S, Karet FE, and Fine LG (1995) An endothelin-1 mediated autocrine growth loop involved in human renal tubular regeneration. *Kidney Int* 48:390-401.
- Philipson KD and Nicoll DA (2000) Sodium-calcium exchange: a molecular perspective. *Annu Rev Physiol* 62:111-133.
- Rubanyi GM and Polokoff MA (1994) Endothelins: molecular biology, biochemistry, pharmacology, physiology and pathophysiology. *Pharmacol Rev* 46:325-415.
- Scholz W, Albus U, Lang HJ, Linz W, Martorana PA, Englert HC, and Scholkens BA (1993) Hoe 694, a new Na⁺/H⁺ exchange inhibitor and its effects in cardiac ischemia. *Br J Pharmacol* 109:562-568.
- Schrier RW, Arnold PE, Van Putten VJ, and Burke TJ (1987) Cellular calcium in intravascular leukocyte accumulation in acute renal failure. *Kidney Int* 32:313-321.
- Shibouta Y, Suzuki N, Shino A, Matsumoto H, Terashita Z, Kondo K, and Nishikawa K (1990) Pathophysiological role of endothelin in acute renal failure. *Life Sci* 46:1611-1618.
- Solez K, Kramer EC, Fox JA, and Heptinstall RH (1974) Medullary plasma flow and intravascular leukocyte accumulation in acute renal failure. *Kidney Int* 6:24-37.
- Tani M and Neely JR (1989) Role of intracellular Na⁺ in Ca²⁺ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts: possible involvement of H⁺-Na⁺ and Na⁺-Ca²⁺ exchange. *Circ Res* 65:1045-1056.
- Vemulapalli S, Chiu PJ, Chintala M, and Bernardino V (1993) Attenuation of ischemic acute renal failure by phosphoramidon in rats. *Pharmacology* 47:3188-3193.
- Wakimoto K, Kobayashi K, Kuro-O M, Yao A, Iwamoto T, Yanaka N, Kita S, Nishida A, Azuma S, Toyoda Y, et al. (2000) Targeted disruption of Na⁺/Ca²⁺ exchanger gene leads to cardiomyocyte apoptosis and defects in heart beat. *J Biol Chem* 275:36991-36998.
- Watano T, Kimura J, Morita T, and Nakanishi H (1996) A novel antagonist, No. 7943, of the Na⁺-Ca²⁺ exchange current in guinea-pig cardiac ventricular cells. *Br J Pharmacol* 119:555-563.
- Wilhelm SM, Simonson MS, Robinson AV, Stowe NT, and Schulak JA (1999) Endothelin up-regulation and localization following renal ischemia and reperfusion. *Kidney Int* 55:1011-1018.
- Wilhelm SM, Stowe NT, Robinson AV, and Schulak JA (2001) The use of the endothelin receptor antagonist, tezosentan, before or after renal ischemia protects renal function. *Transplantation* 71:211-216.
- Wilson DR, Arnold PE, Burke TJ, and Schrier RW (1984) Mitochondrial calcium accumulation and respiration in ischemic acute renal failure in the rat. *Kidney Int* 25:519-526.
- Yamashita J, Itoh M, Kuro T, Kobayashi Y, Ogata M, Takaoka M, and Matsumura Y (2001) Pre-ischemic or post-ischemic treatment with a novel Na⁺/Ca²⁺ exchange inhibitor, KB-R7943, shows renal protective effects in rats with ischemic acute renal failure. *J Pharmacol Exp Ther* 296:412-419.
- Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, and Masaki T (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (Lond)* 332:411-415.
- Yonehana T and Gemba M (1999) Ameliorative effect of adenosine on hypoxia-reoxygenation injury in LLC-PK₁, a porcine kidney cell line. *Jpn J Pharmacol* 80:163-167.
- Yu AS, Hebert SC, Lee SL, Brenner BM, and Lytton J (1992) Identification and localization of renal Na⁺-Ca²⁺ exchanger by polymerase chain reaction. *Am J Physiol* 263:F680-F685.

Address correspondence to: Dr. Yasuo Matsumura, Department of Pharmacology, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan. E-mail: matumrh@gly.oups.ac.jp

IMAGES IN CARDIOLOGY.....

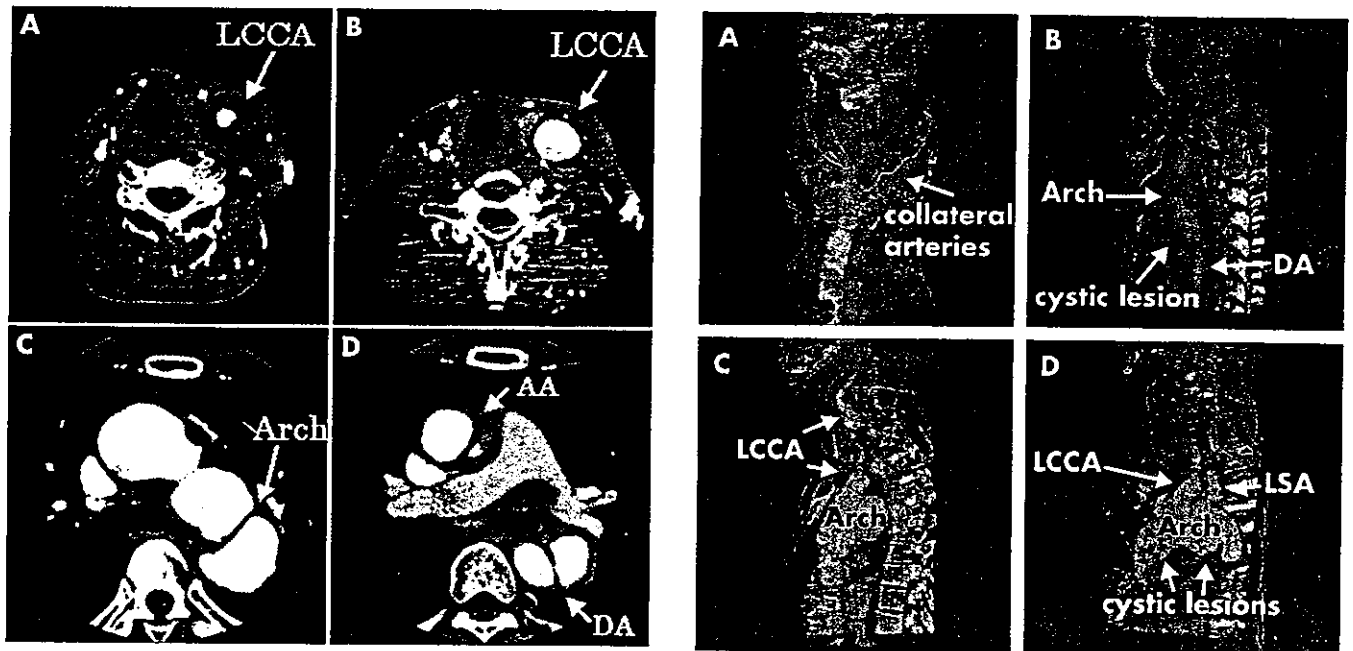
Multiple cystic aneurysms in aortitis demonstrated by three dimensional volume rendering images of multislice computed tomography

A 29 year old man presented with slight fever and pulsation at the left neck originating from a gradually expanding palpable mass. Multislice computed tomography (CT) (Aquilion, Toshiba, Tokyo, Japan) was performed with a 1 mm slice thickness, helical pitch 5.5, and 100 ml of iodinated contrast material (300 mg/ml) delivered intravenously at a rate of 3 ml/s. An aneurysm in the left common carotid artery (LCCA) with a mural thrombus was revealed. The aortic arch, proximal portion of the descending aorta (DA) and ascending aorta (AA) appeared to be separated, as if indicating dissection of the lumen. Three dimensional volume rendering images showed collateral arteries around the anterior region of the neck and a cystic lesion from the distal aortic arch to the proximal DA after which the peripheral part of the aorta heads rightward, then downward. In axial source images at this level, the lumen of the distal portion of the

aortic arch and proximal portion of the DA appears separated. Cut plane volume rendering images show distal and proximal portions of the LCCA aneurysm. Stenosis and post-stenotic dilation in the proximal portion of the left subclavian artery (LSA) are observed. Multiple cystic lesions are shown at the inferior border of the aortic arch, which in the axial images (panel D, left) appeared as aortic dissection. Thus, three dimensional volume rendering images showed the presence of multiple cystic aneurysms, but not aortic dissections. CT and blood serum studies indicated inflammation and enabled the diagnosis of aortitis, and steroid therapy was started.

N Funabashi
N Komiyama
I Komuro

komuro-ky@umin.ac.jp



Images in Cardiovascular Medicine

Coronary Artery Patency After Metallic Stent Implantation Evaluated by Multislice Computed Tomography

Nobusada Funabashi, MD; Nobuyuki Komiyama, MD; Noriyuki Yanagawa, RT;
Takafumi Mayama, MD; Katsuya Yoshida, MD; Issei Komuro, MD

A 56-year-old man presented to our hospital because of chest pain on effort. Conventional coronary angiography revealed high-grade stenosis in the proximal and distal portions of the left anterior descending branch (LAD). We therefore performed percutaneous transluminal coronary angioplasty with metallic stent implantation at the stenotic sites. Four months after the stent placement, ECG-gated enhanced multislice computed tomography (CT) (Light Speed Ultra, General Electric) was performed with a 1.25-mm slice thickness, helical pitch 3.25, to evaluate the coronary artery lumen for restenosis at the stent implantation sites. After intravenous injection of 100

mL of iodinated contrast material (350 mgI/mL), CT scanning was performed with retrospective ECG-gated reconstruction. After acquisition, volume data were extracted from end-diastole, and volume-rendering images were generated (M900 Zio). In the axial source image and cut-plane volume rendering image, the patency of the coronary arterial lumen of the proximal portion of the LAD surrounded by the metallic stent (arrows, Figure 1, arrows) was observed without any artifact from the metallic stent. The next day, conventional coronary angiography showed no significant luminal stenosis of the proximal (Figure 2, arrow) and distal portions of the LAD.

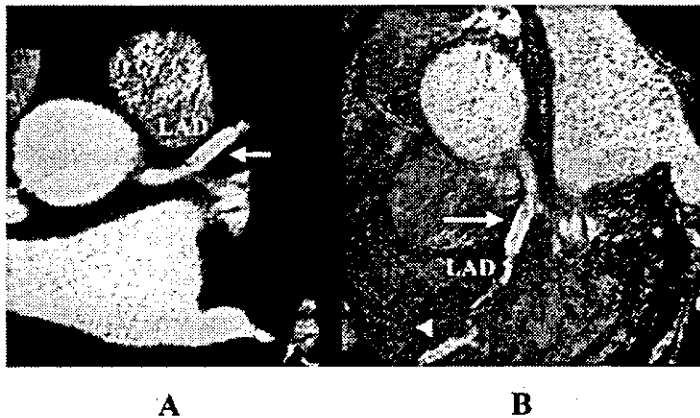


Figure 1. Axial source image (A) and volume rendering (VR) image (B) of the proximal portion of the LAD using end-diastolic data from enhanced multislice CT. Arrows show the stent located in the proximal portion of the LAD. A, The axial source image shows the patent coronary arterial lumen surrounded by the metallic stent (arrow). B, Cut-plane VR image from the superior left anterior view shows the vessel lumen surrounded by the metallic stent (arrow). The color curves were set to represent the vessel lumen filled with contrast material as yellow and the metallic stent as white. The metallic stent with CT numbers above those of the contrast-enhanced coronary artery lumen could be distinguished by VR, and the patency of the lumen surrounded by the metallic stent appeared to be maintained. The arrowhead in the VR image indicates the stent located in the distal portion of the LAD. Patency of the coronary artery lumen of the distal portion of the LAD could not be evaluated.

From the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine (N.F., T.M., K.Y., I.K.); and the Center for Cardiovascular Interventions (N.K., I.K.) and the Department of Radiology (N.Y.), Chiba University Hospital, Chiba, Japan.

Correspondence to Issei Komuro, MD, Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba City, Chiba 260-8670, Japan. E-mail komuro-ky@umin.ac.jp

The editor of Images in Cardiovascular Medicine is Hugh A. McAllister, Jr, MD, Chief, Department of Pathology, St Luke's Episcopal Hospital and Texas Heart Institute, and Clinical Professor of Pathology, University of Texas Medical School and Baylor College of Medicine.

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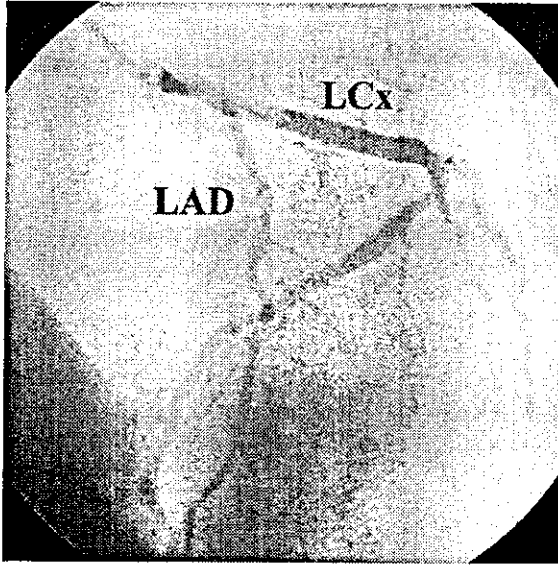


Figure 2. Left anterior oblique and cranial projection of conventional coronary angiogram showing no significant luminal stenosis of the proximal portion of the LAD where the metallic stent was located (arrow). LCx indicates left circumflex branch.

Improvement of Left Ventricular Dysfunction During Exercise by Walking in Patients With Successful Percutaneous Coronary Intervention for Acute Myocardial Infarction

Tomomi Koizumi, MD; Akira Miyazaki, MD; Nobuyuki Komiyama, MD; Kezhu Sun, MD; Takashi Nakasato, MD; Yoshiaki Masuda, MD; Issei Komuro, MD

A growing body of evidence suggests that walking reduces the incidence of coronary events, so the present study investigated whether walking influences left ventricular function in 30 patients with acute myocardial infarction (AMI) who had undergone successful percutaneous coronary intervention (PCI). The patients were randomly assigned to either a 3-month exercise training program of walking (group W, n=15) or a control group (group C, n=15). At both the beginning and end of the study, patients underwent exercise stress echocardiography to determine left ventricular ejection fraction (LVEF) at rest and during exercise. At baseline, there was no difference in LVEF at rest or during exercise between the two groups. After 3 months, LVEF during exercise was significantly improved compared with at rest in group W ($61\pm 3\%$ during exercise vs $57\pm 5\%$ at rest, $p<0.01$), whereas no difference was observed between the LVEF at rest and that during exercise in group C ($54\pm 5\%$ at rest vs $52\pm 7\%$ during exercise, NS). Walking may be beneficial for improving left ventricular function during exercise in patients with AMI. (Circ J 2003; 67: 233–237)

Key Words: Cardiac rehabilitation; Exercise; Ventricular remodeling; Walking

Walking is associated with a lower overall mortality rate in older physically capable men¹ and with the prevention of coronary heart disease in women²; but it is not known whether walking is beneficial for ventricular function. Moreover, the specific effect of walking (ie, low-intensity activity) on the remodeling process in patients who have had a myocardial infarction (MI) and who have undergone successful coronary angioplasty has not been fully elucidated.

We examined the effect of a 3-month walking program on left ventricular function in patients who have had successful percutaneous coronary intervention (PCI) for acute MI (AMI).

Methods

Patient Selection

From August 1998 to May 1999, 30 patients aged less than 80 years who had survived an AMI at Chiba University Hospital were studied. All patients had a typical history of chest pain, ST segment elevation of at least 2 mm, or ST-T abnormalities in 2 continuous precordial electrocardiographic (ECG) leads, persistent asynergy evaluated by 2-dimensional echocardiography or by left ventriculogram, and a typical pattern of elevated concentrations of serum

myocardial enzymes. Exclusion criteria were arrhythmia (ie, atrial fibrillation, bundle branch block, frequent extrasystole), failed PCI (ie, inadequate coronary reflow; residual diameter stenosis $>50\%$ in the infarct-related artery), significant valvular heart disease, contraindications for exercise, signs or symptoms of cardiac failure, rest left ventricular ejection fraction (LVEF) less than 30% on echocardiography, echocardiographic images of inadequate quality for quantitative analysis, inability to give informed consent, malignant disease or life-threatening disease at entry to the study, or an orthopedic or neurological disorder of walking. When the patients arrived at hospital within 12 h of the onset of MI, they underwent emergency PCI (9 patients), or if more than 12 h had passed after the onset, and there was no chest pain at rest, no recurrent arrhythmia because of ischemia recorded on ECG and the patient's hemodynamics were stable, elective PCI was performed after cardiac rehabilitation of approximately 1 week (21 patients). We did not perform thrombolytic therapy in all patients.

One month after PCI (ie, baseline), the patients were randomly allocated to either a 3-month training program of walking (group W: 15 patients) or a control group (group C: 15 patients). All patients underwent functional evaluation at Chiba University Hospital at both the beginning and end of the study (ie, after the 3-month training program): physical examination, heart rate, blood pressure, and a standard supine exercise stress testing on a bicycle ergometer with 2-dimensional B-mode echocardiography. Reinfarction, heart failure or angina were the clinical end-points and the criteria for withdrawing patients from the study.

Protocol of the Walking Program

We recommended the patients in group W use a pedome-

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Department of Cardiovascular Science and Medicine, Graduate School of Medicine, Chiba University, Chiba, Japan
Mailing address: Tomomi Koizumi, MD, PhD, Department of Cardiovascular Science and Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.
E-mail: tkoizumi-circ@urmin.ac.jp

Table 1 Initial Patient Data

	Group W (n=14)	Group C (n=15)
Age (years)	54±12	59±9
M/F	13/1	13/2
History, n		
Hypertension	7 (50%)	4 (27%)
Diabetes	5 (36%)	2 (13%)
Hyperlipidemia	7 (50%)	9 (60%)
Smoking habit	9 (64%)	10 (67%)
PCI (emergency/elective)	4/10	5/10
Collateral grade (+/-) in first angiography	2/12	3/12
Stent plantation in IRA, n	12 (86%)	13 (87%)
IRA: LAD, n	7 (50%)	7 (47%)
Patency of IRA, n	14 (100%)	15 (100%)
Peak creatine kinase (IU/L)	1,481±1,776	1,393±1,744
HR (beats/min)	74±11	69±5
Systolic BP (mmHg)	132±16	127±17
Rate-pressure products at rest (HR×BP)	9,773±2,175	8,766±1,446
EDVI at rest (ml/m ²)	99±16	92±12
ESVI at rest (ml/m ²)	46±11	43±7
EF at rest (%)	56±5	54±5

Values are mean±SD or number (%) of patients.

PCI, percutaneous coronary intervention; IRA, infarct related artery; LAD, left anterior descending artery; BP, blood pressure; HR, heart rate; EDVI, end diastolic volume index; ESVI, end systolic volume index; EF, ejection fraction.

Differences between groups are not statistically significant except history of diabetes: $p < 0.05$.

Table 2 Medications Taken During Study Period

	Group W (n=14)	Group C (n=15)
Calcium antagonists	7 (50%)	6 (40%)
ACE inhibitors	5 (36%)	4 (27%)
β -blockers	1 (0.1%)	0 (0%)
Lipid-lowering drugs	7 (50%)	9 (60%)
Ticlopidine hydrochloride	12 (86%)	13 (87%)
Aspirin (81 mg)	14 (100%)	15 (100%)
Isosorbide mononitrate	14 (100%)	15 (100%)
Others	3 (21%)	5 (33%)

Differences between groups are not statistically significant.

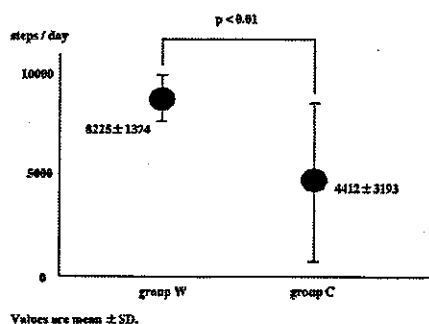


Fig 1. Average number of daily steps after the study period was significantly greater in group W than that of group C ($p < 0.01$).

ter and walk for more than 30 min every day.³ We did not specify an exact walking speed, but advised participants to walk at a moderate speed that did not feel too easy or leave them feeling exhausted after the walk. Patients in group C were given educational support without a formal exercise program, including walking more than 30 min, and they were encouraged to continue their usual lifestyle and avoid strenuous physical activity. We asked all patients in both groups to record the number of daily steps on the walking calendars that we provided.

Exercise Stress Echocardiography

The bicycle ergometric test was performed in the left supine position with an angle of 15°. The protocol began with a workload of 25 W, with a 25 W increase every 2 min. A 12-lead ECG was monitored continuously, and recorded on paper from all leads every minute. Sphygmomanometric blood pressure and heart rate were measured every 2 min. The maximum exercise stress time was measured in each study. Each patient underwent a complete B-mode echocardiographic study during the stress test, with adequate visualization of the apical cardiac chambers and ventricular walls, using a commercially available LOGIQ500MR (GE Yokogawa Medical Systems, Tokyo). Echocardiographic images were recorded with a 2.5- or 3.0-MHz probe on 1.25-cm S-VHS videotape. Left ventricular (LV) function and wall motion were assessed using a Freeland System (Tomtec Imaging System, Boulder, CO, USA). The LV end-diastolic volume index (EDVI: ml/m²) and end-systolic volume index (ESVI: ml/m²) were determined, and the LVEF was calculated by the centerline method. These parameters were measured at rest and 4 min after the start of stress testing. All measurements were derived in blinded fashion by a single experienced operator, from 3 consecutive cardiac cycles, and mean values were considered for analysis.

Statistical Analysis

Baseline characteristics of the 2 groups were compared

Table 3 Hemodynamic Variables at Rest

	Group W			Group C		
	Beginning	end	p value	Beginning	end	p value
Maximal exercise time (min)	5.7±1.6	7.4±1.7	<0.01	5.7±1.6	5.8±1.7	NS
HR (beats/min)	74±11	69±10	<0.05	69±5	70±4	NS
Systolic BP (mmHg)	132±16	128±15	NS	127±17	133±16	<0.05
Rate pressure product (HR×systolic BP)	9,773±2,175	8,851±1,973	<0.05	8,766±1,446	9,246±1,270	<0.05
Ejection fraction (%)	56±5	57±5	NS	54±5	54±5	NS
EDVI (ml/m ²)	99±16	96±12	NS	92±12	92±15	NS
ESVI (ml/m ²)	46±11	43±8	NS	43±7	42±7	NS

Values are mean±SD.

EDVI, end-diastolic volume index; ESVI, end-systolic volume index; HR, heart rate; BP, blood pressure.

Table 4 Hemodynamics During Exercise

	Group W			Group C		
	Rest	During exercise	p value	Rest	During exercise	p value
EDVI (ml/m ²)						
Beginning	99±16	96±16	NS	92±12	85±12	<0.05
End	99±12	110±15	<0.01	92±15	88±12	NS
ESVI (ml/m ²)						
Beginning	46±11	48±14	NS	43±7	42±8	NS
End	43±8	38±6	NS	42±7	43±7	NS
Ejection fraction (%)						
Beginning	56±5	52±10	NS	54±5	52±7	NS
End	57±5	61±3	<0.01	54±5	52±7	NS

Values are mean±SD.

EDVI, end-diastolic volume index; ESVI, end-systolic volume index.

using the unpaired t-test for discrete variables. All data were analyzed by comparing the initial value (at the beginning) with that at the end of the study. StatView (Abacus concept, Calabosus, CA, USA) was used to perform ANOVA procedures comparing the initial value with that at the end of assessment within a group. Differences were considered significant at $p < 0.05$. Results are expressed as mean values±SD. Pearson's product moment correlation and regression analysis were used for assessment of the relationship between the mean number of daily steps and the change of LVEF from at rest to during exercise [(LVEF during exercise)–(LVEF at rest)] in all study patients.

Results

One patient, diagnosed as having prostate cancer during the study period, was withdrawn, so 29 patients (57±11 years, 26 male, 3 female) completed the study (ie, they were evaluated at both the beginning and end of the study); there were 14 in group W and 15 in group C.

The baseline clinical characteristics of the 29 patients who completed the study are shown in Table 1. The initial data were similar for age, heart rate (HR), systolic blood pressure (SBP), rate–pressure product, peak creatine kinase, EDVI, ESVI and LVEF. Medications taken during study period are shown in Table 2; 19 patients discontinued angiotensin-converting enzyme inhibitors because of side effects (ie, dry cough etc). There were no cases of reinfarction, heart failure, sudden cardiac death or angina during

the study period.

The average number of daily steps in group W was significantly greater than that in group C (group W: 8,225±1,374 vs group C: 4,412±3,193, $p < 0.01$; Fig 1).

Hemodynamic variables at rest are shown in Table 3. After 3 months, the HR was significantly decreased compared with that at the beginning in group W, whereas there was no difference between the beginning and end evaluations of HR in group C. After 3 months, SBP was significantly increased compared with that at the beginning in group C, while SBP at the beginning was similar to that at the end of evaluation in group W. The rate-pressure product at the end of the study was significantly decreased compared with that at the beginning in group W, whereas it was significantly increased in group C. At the end of evaluation at rest, both groups showed no change in EDVI, ESVI and LVEF compared with the respective values at the beginning. At the beginning, the maximum exercise stress time was similar in both groups, but by the end of the evaluation, it was significantly increased in group W and remained unchanged in group C.

The hemodynamics at rest and during exercise are shown in Table 4. At the beginning, comparing the values at rest with those recorded during exercise, EDVI, ESVI and LVEF were unchanged in each group except for EDVI, which was significantly decreased during exercise in group C. At the end of the evaluation, again comparing the values at rest with those during exercise, EDVI was significantly increased, ESVI was unchanged and LVEF was significant-

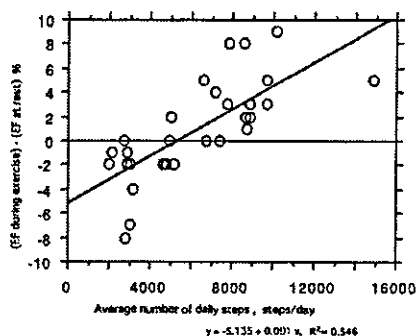


Fig 2. Relationship between the change in the LVEF from at rest to during exercise at the end of the study and the average number of daily steps. There was a significant positive correlation. LVEF, left ventricular ejection fraction.

ly increased in group W, whereas none of these parameters changed in group C. A strong correlation was found between the mean number of daily steps and the change in LVEF from at rest to during exercise at the end of evaluation ($r=0.74$, $p<0.0001$, $y=-5.135+0.001x$, $R^2=0.546$; Fig 2). Walking more than 8,000 steps daily increased the LVEF from at rest to during exercise (Fig 2).

We did not observe new or exercise-induced wall motion abnormalities during exercise either at the beginning or the end of the evaluation in any study patients. Ischemic ST-T changes or exercise-induced arrhythmia were not recorded on the ECG, and chest symptoms were absent during exercise stress-testing at both the beginning and end of the evaluation in the 2 groups.

Discussion

Physical training promotes exercise tolerance and improves cardiac perfusion in patients who have had a MI.⁴ Epidemiologically, physical activity is associated with a decrease in the risk of coronary heart disease,⁵ but the effect of physical training on LV dysfunction in the early phase of the recovery process following AMI has not been sufficiently investigated, especially in patients who have undergone successful coronary angioplasty. In fact, there have been conflicting reports^{7,10} of the effects of exercise training on ventricular function or remodeling after MI and it has been unclear about the involvement of coronary angioplasty or residual ischemia. Although most studies suggest that exercise training improves work capacity, Cobb et al demonstrated that improved exercise performance was not accompanied by improved ventricular function at rest or exercise in 11 patients.⁶ Those patients may have had residual ischemia in the myocardium, because they experienced chest pain or ST-T segment changes consistent with myocardial ischemia during the bicycle exercise. Jugdutt et al reported that exercise-training might be injurious in patients with an extensive transmural infarct that has not healed completely and low level exercise training did not have an adverse effect on LV function and topography.⁷ However, they did not state whether the patients had undergone coronary angioplasty. In contrast, Giannuzzi et al reported that training did not appear to have any effect on the remodeling process, and actually lessened LV dilatation and regional dysfunction.^{8,9} Dubach et al¹⁰ showed that high-intensity residential cardiac rehabilitation had no dele-

terious effects on LV volume, function or wall thickness, regardless of infarct area, in patients with MI. Those patients had 1- to 3-vessel coronary artery disease or a lesion in the left main coronary artery and had undergone PCI or bypass surgery.

It is noteworthy that the study by Jugdutt et al differs from that of Dubach et al in 2 respects; that is, whether coronary angioplasty was performed, and whether the patients were free of residual ischemia in the non-infarct zones of the ventricles. If patients with MI have residual stenosis of the infarct-related artery, exercise-induced ischemia may be harmful to the incompletely healed infarcted zone and to the non-infarcted zone with regard to ventricular function or remodeling. If exercise-induced ischemia of the non-infarcted myocardium endures, we could not expect an improvement of ventricular function during exercise. Indeed, previous reports about ventricular function at rest and during exercise have shown that in patients with coronary artery disease, the LVEF decreases or does not change, and regional wall motion abnormalities appear during exercise!^{11,12} It is possible that such patients have residual stenosis in the infarct-related arteries and that exercise-induced ischemia is still present. In contrast, the most common response to exercise in normal volunteers is an increased LVEF!¹³

Our results indicate that walking is associated with substantial amelioration of LV dysfunction during exercise in patients who have undergone successful coronary angioplasty for AMI. It is possible that the slightly increased EDVI during exercise in group W permitted greater stroke output to be achieved during walking, by virtue of the Starling effect. Increasing ventricular volume during exercise may not be LV diastolic dysfunction, because the EDVI at rest was not different after training. These findings suggest an improvement in LV diastolic function during exercise.

The role of exercise training in the prevention of ventricular remodeling has not been clearly documented in patients with MI who have undergone coronary angioplasty. Ventricular remodeling may initially serve as an adaptive process, but can only partially compensate for muscle loss; eventually, if the infarct is progressing because of residual ischemia, ventricular topographic changes become severe and decompensation occurs. The most important factor affecting ventricular remodeling is considered to be persistent myocardial ischemia. Several studies in humans have supported the concept of opening an infarcted artery (even relatively late) to improve post-infarct survival and LV function; results suggested that infarct vessel patency predicted survival and lower incidence of late LV dilatation!^{14,15}

In the present study, all the patients underwent successful coronary angioplasty for the residual stenosis in infarct-related arteries and there was no exercise-induced ischemia in the non-infarcted myocardium. Because exercise-induced wall motion abnormalities were not observed and the non-infarcted zone exhibited good contraction during exercise with echocardiographic evaluation, exercise stress may have beneficial effects on the non-infarcted myocardium (ie, viable tissue). It is interesting that exercise stress affects the salvaged myocardium after escape from ischemia.

The explanatory mechanisms are probably multifactorial for the positive effect on ventricular function during exercise of the walking program in the present study. An experimental study in a rat model¹⁶ showed that the improvement in myocardial perfusion and oxidative metabolism by

exercise training after transient myocardial ischemia might have facilitated functional recovery of dysfunctional, but still viable myocardium. The findings that fatty acid metabolism and the concentration of free fatty acids returned to normal after exercise training, lead a hypothesis that exercise training can positively influence myocardial lipid oxidation. Adachi et al¹⁷ reported that 2 weeks of physical training after coronary artery bypass graft surgery improves ventilatory response to exercise and increases cardiac output during exercise, an effect that they considered to be related closely to the patency of blood vessels. Vasoactive substances, including nitric oxide, regulate vascular tone and exercise training can attenuate nitric oxide and improve β -adrenoceptor activity!¹⁷ One of the reasons why the LVEF improved during exercise in the present group W may have been an improvement of oxidative metabolism or neurohumoral factors in the non-infarcted myocardium, including salvaged myocardium after escape from ischemia (central effect), as well as peripheral adaptation.

Study Limitations

In the present study, walking (low intensity activity) was found to improve LV dysfunction because the amount of infarcted myocardium in this study was relatively small (maximum creatine kinase: $1,617 \pm 1,732$ IU/L). The effects of low intensity activity on extensive myocardial infarction complicated with severe ventricular dysfunction were not demonstrated in this study. Because maximal oxygen uptake capacity (VO_2 max) was not measured, we cannot directly evaluate the peripheral effect on ventricular function. In the present study, the HR and rate-pressure products were decreased and blood pressure did not change after training in group W, whereas the blood pressure and rate-pressure products increased and HR did not change after training in group C. These findings suggest that walking induces an increase in stroke volume and may be a result of peripheral adaptations, as well as cardiac adaptation (ie, central effect). Unfortunately, observational studies have a limited capacity to describe the relationship between physical activity and risk of disease, because of difficulties in quantifying highly variable behavioral patterns from self-reported information and individual recall. Although the number of steps was assessed by a self-administered questionnaire, the correlations between physical activity as reported on the questionnaire and as recorded in 4 1-week diaries, or recalled after 1 week, were reasonably high ($r=0.62$, and $r=0.79$, respectively)¹⁸

Conclusion

The prospective data from this study indicate that walking is associated with substantial amelioration of LV dysfunction during exercise in patients with AMI who have undergone successful coronary angioplasty for the residual stenosis in infarct-related arteries. Walking more than 8,000 steps daily is therefore recommended for these patients.

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References

- Hakim AA, Petrovitch H, Burchfiel CM, Ross GW, Rodriguez BL, White LR, et al. Effects of walking on mortality among nonsmoking retired men. *N Engl J Med* 1998; 338: 94–99.
- Manson JE, Hu FB, Rich-Edwards JW, Colditz GA, Stampfer MJ, Willett WC, et al. A prospective study of walking as compared with vigorous exercise in the prevention of coronary heart disease in women. *N Engl J Med* 1999; 341: 650–658.
- Pate RR, Pratt M, Blair SN, Haskell WL, Macera CA, Bouchard C, et al. Physical activity and public health: A recommendation from the Center for Disease Control and Prevention and the American College of Sports Medicine. *JAMA* 1995; 273: 402–407.
- Linxue L, Nohara R, Makita S, Hosokawa R, Hata T, Okuda K, et al. Effect of long-term exercise training on regional myocardial perfusion changes in patients with coronary artery disease. *Jpn Circ J* 1999; 63: 73–78.
- Berlin JA, Colditz GA. A meta-analysis of physical activity in the prevention of coronary heart disease. *Am J Epidemiol* 1990; 132: 612–628.
- Cobb FR, Williams RS, McEwan P, Jones RH, Coleman RE, Wallace AG. Effects of exercise training on ventricular function in patients with recent myocardial infarction. *Circulation* 1982; 66: 100–108.
- Jugdutt BI, Michorowski BL, Kappagoda CT. Exercise training after Q wave myocardial infarction: Importance of regional left ventricular function and topography. *J Am Coll Cardiol* 1988; 12: 362–372.
- Giannuzzi P, Tavazzi L, Temporelli PL, Corra U, Imparato A, Gattone M, et al. Long-term physical training and left ventricular remodeling after anterior myocardial infarction: Results of the exercise in anterior myocardial infarction (EAMI) trial. *J Am Coll Cardiol* 1993; 22: 1821–1829.
- Giannuzzi P, Temporelli PL, Corra U, Gattone M, Giordano A, Tavazzi L. Attenuation of unfavorable remodeling by exercise training in postinfarction patients with left ventricular dysfunction: Results of the exercise in left ventricular dysfunction (ELVD) Trial. *Circulation* 1997; 96: 1790–1797.
- Dubach P, Myers J, Dziekan G, Goebels U, Reinhart W, Vogt P, et al. Effect of exercise training on myocardial remodeling in patients with reduced left ventricular function after myocardial infarction: Application of magnetic resonance imaging. *Circulation* 1997; 95: 2060–2067.
- Borer JS, Bacharach SL, Green MV, Kent KM, Epstein SE, Johnston GS. Real-time radionuclide cineangiography in the noninvasive evaluation of global and regional left ventricular function at rest and during exercise in patients with coronary artery disease. *N Engl J Med* 1977; 296: 839–844.
- Jones RH, McEwan P, Newman GE, Port S, Rerych SK, Scholz PM, et al. Accuracy of diagnosis of coronary artery disease by radionuclide measurement of left ventricular function during rest and exercise. *Circulation* 1981; 64: 586–601.
- Upton MT, Rerych SK, Newman GE, Bounous EP Jr, Jones RH. The reproducibility of radionuclide angiographic measurements of left ventricular function in normal subjects at rest and during exercise. *Circulation* 1980; 62: 126–132.
- White HD, Norris RM, Brown MA, Brandt PW, Whitlock RM, Wild CJ. Left ventricular end-systolic volume as the major determinant of survival after recovery from myocardial infarction. *Circulation* 1987; 76: 44–51.
- Pfeffer MA, Lamas GA, Vaughan DE, Parisi AF, Brownwald E. Effect of captopril on progressive ventricular dilatation after anterior myocardial infarction. *N Engl J Med* 1988; 319: 80–86.
- Chen L, Nohara R, Hirai T, Li X, Kataoka K, Hosokawa R, et al. Effects of exercise training on myocardial fatty acid metabolism in rats with depressed cardiac function induced by transient ischemia. *Jpn Circ J* 2001; 65: 550–555.
- Adachi H, Ito H, Sakurai S, Takahashi T, Toyama T, Niato S, et al. Short-term physical training improves ventilatory response to exercise after coronary artery bypass surgery. *Jpn Circ J* 2001; 65: 419–423.
- Wolf AM, Hunter DJ, Colditz GA, Manson JE, Stampfer MJ, Corsano KA, et al. Reproducibility and validity of a self-administered physical activity questionnaire. *Int J Epidemiol* 1994; 23: 991–999.

Characteristic Effects of α_1 - $\beta_{1,2}$ -Adrenergic Blocking Agent, Carvedilol, on $[Ca^{2+}]_i$ in Ventricular Myocytes Compared With Those of Timolol and Atenolol

Atsushi Yao, MD; Osami Kohmoto, MD*; Tomomi Oyama, MD; Yasuyuki Sugishita, MD; Tatsuya Shimizu, MD; Kazumasa Harada, MD; Hiroshi Matsui, MD; Issei Komuro, MD; Ryozo Nagai, MD; Hiroshi Matsuo, MD*; Takashi Serizawa, MD*; Takashi Maruyama, MD**; Toshiyuki Takahashi, MD

Beta-adrenergic stimulation and the resultant Ca^{2+} load both seem to be associated with progression of heart failure as well as hypertrophy. Because the α_1 -, $\beta_{1,2}$ -blocker, carvedilol, has been shown to be outstandingly beneficial in the treatment of heart failure, its direct effects on intracellular calcium ion concentration ($[Ca^{2+}]_i$), including antagonism to isoproterenol, in ventricular myocytes were investigated and then compared with a selective β_1 -blocker, atenolol, and a non-selective $\beta_{1,2}$ -blocker, timolol. At 1–300 nmol/L, carvedilol decreased the amplitude of $[Ca^{2+}]_i$ by ~20% independently of its concentration, which was a similar effect to timolol. All the β -blockers at 10 nmol/L decreased the amount of cAMP, but atenolol had the least effect. Carvedilol in the μ mol/L order further diminished the amplitude of $[Ca^{2+}]_i$ transients, and at 10 μ mol/L increased the voltage threshold for pacing myocytes. These effects were not observed with timolol or atenolol. L-type Ca^{2+} currents (I_{Ca}) were decreased by carvedilol in the μ mol/L order in a concentration dependent manner. As for the β -antagonizing effect, the concentrations of carvedilol, timolol, and atenolol needed to prevent the effect of isoproterenol by 50% (IC_{50}) were 1.32, 2.01, and 612 nmol/L, respectively. Furthermore, the antagonizing effect of carvedilol was dramatically sustained even after removal of the drug from the perfusate. Carvedilol exerts negative effects on $[Ca^{2+}]_i$, including inhibition of the intrinsic β -activity, reduction of I_{Ca} in the μ mol/L order, and an increase in the threshold for pacing at $\geq 10 \mu$ mol/L. Data on the IC_{50} for the isoproterenol effect suggest that carvedilol could effectively inhibit the $[Ca^{2+}]_i$ load induced by catecholamines under clinical conditions. (Circ J 2003; 67: 83–90)

Key Words: β -blocker; Carvedilol; Calcium; Ion channels; Myocytes

Carvedilol is an α_1 and $\beta_{1,2}$ -adrenergic blocking agent with other effects, such as a scavenger for oxidation and an inhibitor of apoptosis,^{1,2} and has been shown to improve the prognosis of patients with congestive heart failure.^{1,3} Although the mechanism is not fully elucidated, some other β -blocking agents have been reported to have beneficial effects on heart failure,^{4–10} suggesting that continuous β -adrenergic stimulation progresses heart failure. Carvedilol is also known to directly inhibit L-type calcium current (I_{Ca}) similar to Ca^{2+} antagonists, and diminishes the influx of Ca^{2+} in ventricular myocytes¹¹ as well as vascular smooth muscle cells in concentrations of the μ mol/L order.¹² However, most calcium antagonists have failed to decrease the mortality of patients with heart failure.^{13,14} In addition, the physiological blood concentra-

tion of carvedilol has been reported to be 100–600 nmol/L after a single oral dose or daily doses of 12.5, 25, or 50 mg.¹⁵ Because most (~98%) of carvedilol in the blood is thought to bind to albumin,¹⁷ the inhibitory effect of carvedilol on I_{Ca} may not be of clinical significance. The beneficial effects of carvedilol on heart failure, therefore, are most probably exerted through antagonizing β -agonists independently of blocking of the L-type Ca^{2+} channel, although other effects of carvedilol such as antioxidation and anti-apoptosis might be involved.

In ventricular myocytes, $\beta_{1,2}$ -receptors couple with the Gs protein complex, which is a positive modulator of adenylyl cyclase, and directly increases I_{Ca} . Once β -agonists are bound to $\beta_{1,2}$ -receptors, adenylyl cyclase is activated to produce more cAMP, leading to the activation of protein kinase A (PKA). Activated PKA subsequently phosphorylates the L-type Ca^{2+} channel and phospholamban, resulting in the augmentation of I_{Ca} and the Ca^{2+} uptake rate of the sarcoplasmic reticulum (SR) Ca-ATPase, respectively, both of which cooperatively contribute to an increase in the SR Ca^{2+} content and enhancement of Ca^{2+} induced Ca^{2+} release from the SR (CICR). In this way, β -agonists augment the amplitude of intracellular calcium ion concentration ($[Ca^{2+}]_i$) transients, mostly through the PKA cascade as well as through direct interaction of the Gs protein with the L-type Ca^{2+} channel. Recently we have found that a β -agonist, isoproterenol, induces hypertrophic

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Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, Tokyo and *Second Department of Internal Medicine and **Department of Pharmacology, Saitama Medical University, Saitama, Japan

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Mailing address: Atsushi Yao, MD, Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: atsushiyao@aol.com

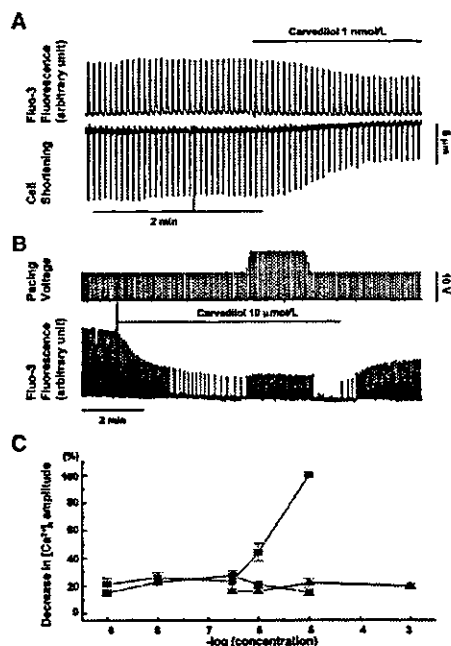


Fig 1. Effects of carvedilol on $[Ca^{2+}]_i$ in ventricular myocytes. (A) Carvedilol of 1 nmol/L decreased $[Ca^{2+}]_i$ amplitudes and cell shortenings. (B) In a 10- μ mol/L carvedilol solution, myocytes lost their responsiveness to field stimulation, which was rescued to some extent by increasing the pacing voltage or by wash-out of carvedilol. (C) Carvedilol (\blacksquare), timolol (\bullet) and atenolol (\blacktriangle) all decreased $[Ca^{2+}]_i$ amplitudes at tested concentrations, but carvedilol at $\geq 1 \mu$ mol/L further decreased $[Ca^{2+}]_i$ amplitudes, which was divergent from the effects of the other two drugs. The effect of 10 μ mol/L carvedilol was 100%, indicating the loss of responsiveness to field stimulation. Each data point is expressed as mean \pm SEM, consisting of 4–6 recordings.

responses, such as the activation of MAP kinase and calcineurin, through a Ca^{2+} -dependent pathway in cultured neonatal rat ventricular myocytes¹⁸. Therefore, the catecholamine-induced Ca^{2+} load could be associated with hypertrophy, and in this respect, the Ca^{2+} load induced by catecholamines could be regarded as a Ca^{2+} overload-condition such as ischemia–reperfusion,¹⁹ metabolic inhibition,²⁰ cardiac glycoside treatment²¹ and so on. On the other hand, Marx et al have recently reported that hyperphosphorylation of FKBP12.6 by PKA is pathophysiologically important for aspects of heart failure such as decreased ventricular function and arrhythmia²². In this respect, we propose the importance of the catecholamine-induced $[Ca^{2+}]_i$ load and PKA activation for hypertrophic responses and the pathophysiology of heart failure, respectively, although it is not clear that the transition from hypertrophy to heart failure is associated with Ca^{2+} load. $[Ca^{2+}]_i$ transients are not only direct parameters for $[Ca^{2+}]_i$, but also can be used as a very sensitive indicator for the activity of PKA. Thus, it is suitable to utilize $[Ca^{2+}]_i$ transients as a parameter in order to evaluate the effects of β -blockers.

The purposes of the present study were to elucidate whether carvedilol directly influenced $[Ca^{2+}]_i$ cycling in beating ventricular myocytes by itself, and how much carvedilol antagonized a β -agonist, isoproterenol, in terms of $[Ca^{2+}]_i$, as well as evaluating the clinical importance of these effects of carvedilol. These effects of carvedilol were also compared with those of a selective β_1 -blocker, atenolol,

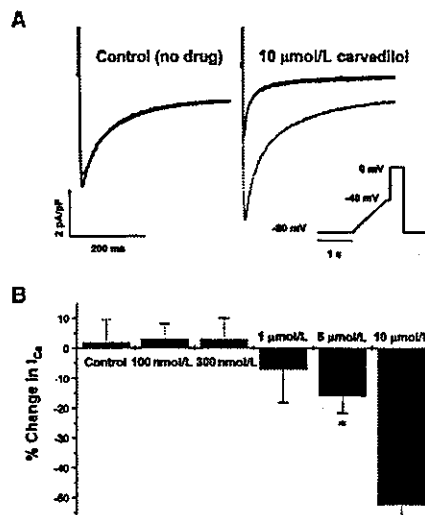


Fig 2. Direct effects of carvedilol on I_{Ca} . (A) Run-down phenomenon was not observed (thick line in the left panel) 10 min after starting the measurement of I_{Ca} (control value, thin line). On the other hand, 10 min treatment with 10 μ mol/L carvedilol markedly decreased I_{Ca} (thick line in the right panel), compared with the control recordings (thin line). The insert shows the protocol of the voltage clamp steps. (B) Summary of the mean values of I_{Ca} in myocytes treated with carvedilol at various concentrations. Each data point is mean \pm SEM of 4–10 recordings normalized to the control values.

and a non-selective $\beta_{1,2}$ -blocker, timolol.

Methods

Ventricular Myocyte Isolation

The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). We isolated ventricular myocytes from hearts of male guinea pigs (300–500 g) as described previously²³. In brief, hearts were dissected from anesthetized animals and rapidly attached to a Langendorff perfusion system. All perfusion solutions were maintained at 37°C and pH 7.4. After perfusion with 0 mmol/L Ca^{2+} solution consisting of (mmol/L) 126 NaCl, 4.4 KCl, 1.0 $MgCl_2$, 13 NaOH, 24 HEPES, 2.5 g/L taurine, 0.65 g/L creatine monophosphate, 0.55 g/L sodium pyruvate, 0.14 g/L NaH_2PO_4 , and 2 g/L glucose, the hearts were subsequently digested with 100 μ mol/L Ca^{2+} solution containing 100 mg/dl of type II collagenase (Worthington Biochemicals, Freehold, NJ, USA) and 10 mg/dl of protease (Sigma Chemical Co, St Louis, MO, USA,) for 8–12 min. Then, after the hearts were washed with 100 μ mol/L Ca^{2+} solution without the enzymes, both ventricles were excised, minced and shaken gently in 100 μ mol/L Ca^{2+} solution. The cell suspension was filtered through a fine metallic tea filter. Isolated myocytes were kept in 1 mmol/L Ca^{2+} solution at room temperature, and were used within 6 h after the isolation procedure.

Measurements of $[Ca^{2+}]_i$ Transients

$[Ca^{2+}]_i$ was measured with a method described previously²³. Briefly, isolated myocytes were incubated for 30 min in 3–4 μ mol/L fluo-3 AM containing normal HEPES, which consists of (mmol/L) 126 NaCl, 4.4 KCl, 1.0 $MgCl_2$, 1.08

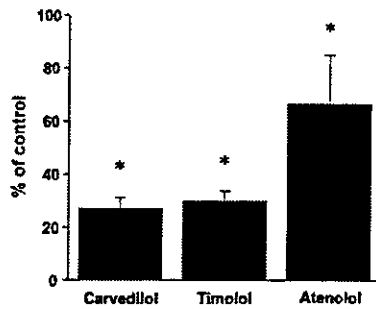


Fig 3. Decreases in the amount of cAMP by β -blockers. Treatment with one of carvedilol, timolol and atenolol at 10 nmol/L significantly decreased the amount of cAMP by $72.9 \pm 4.2\%$, $70.1 \pm 3.8\%$ and $33.3 \pm 18.4\%$, respectively. The bar graphs represent mean \pm SEM after normalizing to the control values, obtained from 3 series of experiments (* $p < 0.0001$).

CaCl₂, 13 NaOH, 11 glucose, 24 HEPES (pH 7.4 at 25°C). After washing in dye-free normal HEPES solution for more than 15 min, a single cell was isolated in the field to be excited by 485-nm light, and the emission of 530 nm was collected. The intensity of 530-nm fluorescence increases as $[Ca^{2+}]_i$ increases. The flow rate of the perfusate was ≈ 2.0 ml/min and the volume of the solution in the chamber was kept constant ($\approx 1-1.5$ ml) during the experiments. Measurements were performed at room temperature (24–26°C).

Cell Shortening Measurement

Ventricular myocytes were electrically stimulated by a platinum electrode with a $\times 1.5$ threshold voltage of 4-ms duration every 4 s (0.25 Hz). Both longitudinal edges of the myocytes were continuously monitored with a video motion detector, and cell shortening was measured as described previously.²³ In some experiments, $[Ca^{2+}]_i$ and cell shortening were simultaneously recorded.

I_{Ca} Measurement

I_{Ca} was recorded with a whole cell voltage clamp method. Ventricular myocytes were dialyzed with a microelectrode (1.5–2.5 M Ω) filled with a pipette solution containing (mmol/L) 130 CsCl, 10 NaCl, 0.5 MgCl₂, 5 K₂ATP, 5.5 glucose, 10 HEPES, and 10 EGTA, which was titrated to pH 7.2 with KOH. I_{Ca} was activated with a voltage step to 0 mV for 400 ms from -40 mV of 100-ms duration following linear ramp step for 1 s from a holding potential of -80 mV (Fig 2A). Measurements of I_{Ca} were performed in normal HEPES solution at room temperature every 2 min after an initial 5-min dialysis of the cytosol with the pipette solution. To prevent the run-down phenomenon, myocytes were voltage-clamped at -80 mV between the measurements. The flow rate of the perfusate was ≈ 0.5 ml/min, and the volume of the solution in the chamber was kept constant ($\approx 1-1.5$ ml) during the experiments.

Assessment of β -Blocking Effects

Because carvedilol is lipophilic and its effect is difficult to wash out (as shown in Fig 5B), we first defined the expected amplitude of $[Ca^{2+}]_i$ transients stimulated by 1 μ mol/L isoproterenol with 10 records. Briefly, a fluo-3-loaded myocyte was paced for 2 min until stabilized, and then the $[Ca^{2+}]_i$ transient was recorded as control data.

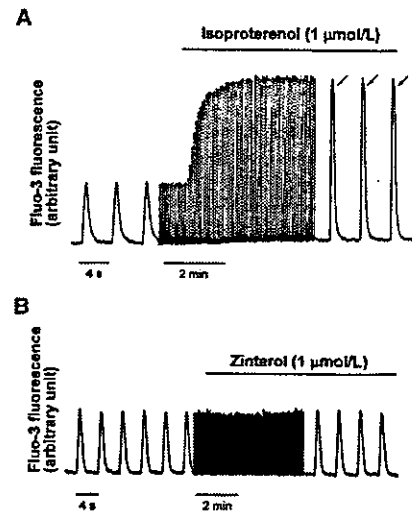


Fig 4. Effects of isoproterenol and zinterol on $[Ca^{2+}]_i$ in ventricular myocytes. (A) Isoproterenol (1 μ mol/L) markedly increased $[Ca^{2+}]_i$ amplitudes, and provoked an after-peak hump (arrow). (B) A β_2 -stimulator, zinterol (1 μ mol/L), did not affect $[Ca^{2+}]_i$ transients.

Consecutively, the myocyte was treated with isoproterenol for 5 min, and then the $[Ca^{2+}]_i$ transient was recorded. The amplitude of the $[Ca^{2+}]_i$ transient treated with isoproterenol was expressed as normalized to that of the control. The mean value of the isoproterenol-treated $[Ca^{2+}]_i$ transients was $263 \pm 27\%$ ($n=10$), which was defined as the expected amplitude. Assuming that without the β -blocking effect the amplitude of the $[Ca^{2+}]_i$ transients should reach 263% of the control amplitudes, we then defined the formula to calculate the inhibition rate:

$$\% \text{ inhibition} = 100 \times (263 - b) / (263 - a)$$

where a and b are, respectively, the amplitude when a myocyte is pretreated with a β -blocker and that when the myocyte is consecutively treated with the β -blocker and 1 μ mol/L isoproterenol. Each $[Ca^{2+}]_i$ amplitude in this formula was expressed normalized to the control $[Ca^{2+}]_i$ amplitude.

Cyclic AMP (cAMP) Measurements

After the myocytes were isolated, an equal amount of cell suspension in normal HEPES solution was poured into laminin-coated dishes of 60 mm diameter. After the cells in the dishes were treated with drugs according to the protocols, the solution was vacuumed and cells were then harvested with 500 μ l of the cell lysis buffer 1B included in the cAMP assay kit (BIOTRACK™, Amersham Pharmacia Biotech UK Ltd). The amount of cAMP was measured according to the manufacturer's protocol. The amount of protein was also measured with a modified Bradford method using the dye reagent from the BIO-RAD Protein Assay (BIO-RAD Laboratories, CA, USA). The relative amount of cAMP was calculated by dividing the concentration of cAMP by that of protein, and normalizing it to the control value in each series of the experiment. Experiments were performed at room temperature.

Drug Preparation

If a drug could not be dissolved in water, it was first

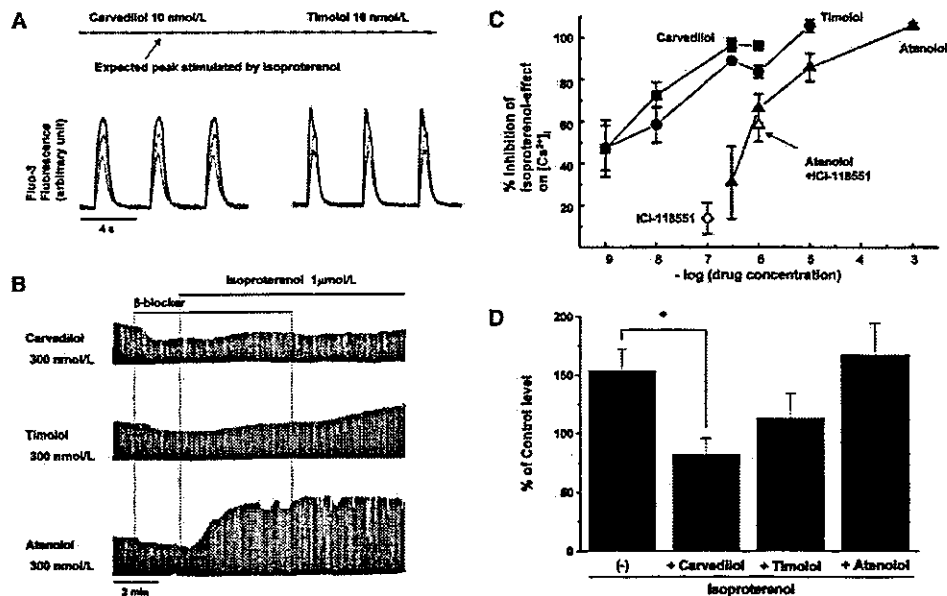


Fig 5. Comparison of carvedilol, timolol, and atenolol on the inhibition of isoproterenol. (A) Both carvedilol and timolol at 10 nmol/L decreased $[Ca^{2+}]_i$ amplitudes (thin line) from the control level (dotted line). Addition of 1 μ mol/L isoproterenol still increased $[Ca^{2+}]_i$ amplitudes in the carvedilol-treated (left thick line) and the timolol-treated (right thick line) myocytes, although these β -blockers well inhibited the effects of isoproterenol. Note that the after-peak hump is observed only in the timolol-treated myocyte. (B) At 300 nmol/L, both carvedilol and timolol almost completely suppressed the effects of isoproterenol on $[Ca^{2+}]_i$. In contrast, atenolol showed little antagonism to isoproterenol. Note that the antagonizing effect of carvedilol is sustained long after its removal from the perfusate, which contrasted with that of timolol. (C) Mean values of percent inhibition of isoproterenol effects by carvedilol (■), timolol (●) and atenolol (▲), plotted as a function of their concentrations. Their IC_{50} values were calculated by a curve-fitting technique to be 1.32, 2.01, and 612 nmol/L, respectively. The inhibitory effects of the combination (Δ) of 1 μ mol/L atenolol and 1 μ mol/L ICI-118551, a β_2 -selective blocker, were similar to that of atenolol (1 μ mol/L) alone. ICI-118551 at 100 nmol/L by itself had a slight inhibitory effect. Data are expressed as the mean \pm SEM of 4–6 recordings. (D) Treatment with isoproterenol (1 μ mol/L) for 30 min significantly increased the amount of cAMP by 54.2 \pm 17.8% (vs control, $n=6$, $p<0.05$). After 15-min pretreatment with each β -blocker at 300 nmol/L, myocytes were treated with isoproterenol for 30 min in the presence of the β -blocker. Carvedilol significantly prevented the increase in the amount of cAMP by isoproterenol ($n=6$, $*p<0.01$). Data are expressed as the mean \pm SEM of 6 records normalized to the control in each series of experiment.

dissolved in a suitable solvent at a concentration 10,000 times higher than that for use. Then the drug in the solvent was diluted with the perfusate. The solvent was dimethyl sulfoxide for carvedilol and forskolin, methanol for atenolol, and water for the others.

Data Acquisition and Statistics

Voltage data converted from original current signals, fluorescence signals, cell shortening and pacing signals were digitized with a 12-bit A/D converter (Digidata 1200) and then stored on personal computers using the software of P-clamp 6.0 or Axo scope 1.1 (Axon Instruments, Burlingame, CA, USA), which was also used to analyze the data. Comparison was performed with unpaired t-test, or paired t-test if data were obtained from the same cell. $p<0.05$ was considered statistically significant.

Results

Direct Effects of Carvedilol on $[Ca^{2+}]_i$

We first examined the direct effects of carvedilol on $[Ca^{2+}]_i$ in ventricular myocytes and found that the amplitude of $[Ca^{2+}]_i$ was attenuated by carvedilol of 1 nmol/L, which was accompanied by decreased cell shortening (Fig 1A). Furthermore, in a 10 μ mol/L carvedilol solution,

myocytes reversibly lost their responsiveness to field stimulation (Fig 1B) and this loss of responsiveness to field stimulation was overcome by increased voltage for field stimulation. These results suggest that carvedilol has a direct negative inotropic effect on ventricular myocytes by lowering peak $[Ca^{2+}]_i$. The mean data on the $[Ca^{2+}]_i$ lowering effects of carvedilol, timolol, and atenolol are summarized in Fig 1C, showing that all of the β -blockers can be thought to exert negative inotropic action by diminishing the peak of $[Ca^{2+}]_i$ transients. At a concentration ≥ 1 μ mol/L, carvedilol further diminished the $[Ca^{2+}]_i$ amplitude, which contrasted with the effects of timolol and atenolol. Timolol and atenolol showed neither apparent concentration-dependent attenuation of the $[Ca^{2+}]_i$ amplitude nor induced pacing failure in myocytes at tested concentrations.

Effects on I_{Ca}

The results for $[Ca^{2+}]_i$ just described implied that carvedilol at concentrations of the μ mol/L order may directly decrease I_{Ca} , facilitating the decrease in $[Ca^{2+}]_i$ amplitude, so we assessed the direct effect of carvedilol on I_{Ca} . We first confirmed that I_{Ca} was stable for 5–15 min after starting the dialysis of the cytosol with the pipette solution and as shown in Fig 2A, carvedilol at 10 μ mol/L markedly decreased I_{Ca} . Carvedilol at ≥ 5 μ mol/L directly and signifi-

cantly decreased I_{Ca} in a concentration-dependent manner (Fig 2B). From these data, it can be deduced that the $[Ca^{2+}]_i$ -lowering effect of carvedilol at ≤ 300 nmol/L is not the result of a reduction of I_{Ca} . Because $10 \mu\text{mol/L}$ carvedilol suppressed the I_{Ca} by approximately 52%, the pacing failure in a $10 \mu\text{mol/L}$ carvedilol solution can not be fully explained by the reduced I_{Ca} .

Alteration of Intrinsic β -Activity by Carvedilol

To further investigate the mechanism of the direct negative inotropism by carvedilol, as well as that of timolol and atenolol, we performed an additional experiment. Earlier reports^{24,25} have suggested that some β -receptors transmit to the active form spontaneously and in a self-acting way, and that some β -blockers inhibit this transmission, resulting in decreased production of cAMP (inverse agonism). In this regard, we measured the amount of cAMP in myocytes treated with each β -blocker for 30 min. As shown in Fig 3, the amount of cAMP was significantly decreased by 10 nmol/L of carvedilol, timolol, and atenolol, although the effect of atenolol was relatively weak. Therefore, occupation of the β -receptors by the blockers seems to lead to attenuation of the intrinsic PKA activity, which consequently decreases the $[Ca^{2+}]_i$ amplitude. These results suggest that the direct negative inotropic action by carvedilol at ≤ 300 nmol/L is at least in part associated with a decrease in the amount of cAMP, which is probably a result of a decrease in the intrinsic β -activity.

Beta-Antagonism of Carvedilol

We then examined the antagonism of carvedilol to isoproterenol and compared its effect with that of timolol and atenolol. First, we examined by how much $1 \mu\text{mol/L}$ isoproterenol increased the amplitude of $[Ca^{2+}]_i$ transients and found that it markedly increased the amplitude, with after-peak humps seen in most cases (Fig 4A). On the other hand, a selective β_2 -stimulator, zinterol, did not appear to affect the $[Ca^{2+}]_i$ transients (Fig 4B), suggesting that the effect of isoproterenol is likely β_1 -dependent. Next, after the $[Ca^{2+}]_i$ transients were stabilized, myocytes were pretreated with one of the β -blockers for 2 min and subsequently treated with $1 \mu\text{mol/L}$ isoproterenol for 5 min in the presence of the β -blocker. We found that 10 nmol/L carvedilol effectively inhibited the $[Ca^{2+}]_i$ rise, and always prevented the after-peak hump, which was observed frequently with 10 nmol/L timolol (Fig 5A). Among the tested concentrations, the minimal concentration to always eliminate the after-peak hump was 10 nmol/L, 300 nmol/L, and $10 \mu\text{mol/L}$ for carvedilol, timolol, and atenolol, respectively. Because the after-peak hump was observed more frequently as the β -stimulation increased, the inhibitory effect of carvedilol seemed to be stronger than that of timolol. At the concentration of 300 nmol/L, both carvedilol and timolol almost completely suppressed the isoproterenol-induced $[Ca^{2+}]_i$ rise (Fig 5B). In contrast, atenolol appeared to have much less inhibitory effects. In addition, the time course of washing out the effects of carvedilol was much slower than timolol, implying that the binding affinity of carvedilol might be higher. The inhibitory effect of carvedilol was stronger than that of timolol as well as that of atenolol, as indicated by the IC_{50} (1.32 , 2.01 , and 612 nmol/L, respectively) (Fig 5C).

We also examined the inhibitory effects of these β -blockers on isoproterenol-induced increases in the amount of cAMP and found that 300 nmol/L carvedilol significant-

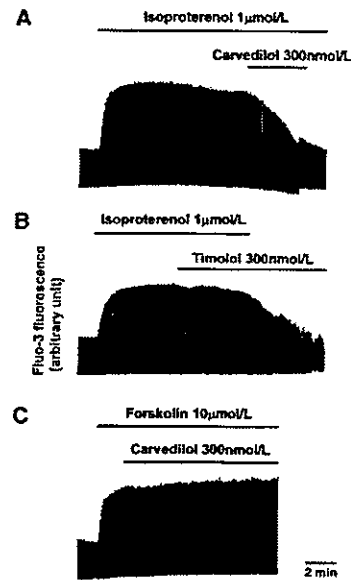


Fig 6. Direct interaction between the β -blockers and post-receptor activation pathway. After pretreatment with isoproterenol ($1 \mu\text{mol/L}$), the addition of 300 nmol/L of carvedilol dramatically diminished $[Ca^{2+}]_i$ amplitudes (Panel A). In contrast, timolol (300 nmol/L) had no apparent effect on $[Ca^{2+}]_i$ under similar conditions, although subsequent removal of isoproterenol accelerated the attenuation of $[Ca^{2+}]_i$ amplitudes (Panel B). A direct activator of adenylyl cyclase, forskolin ($10 \mu\text{mol/L}$), increased $[Ca^{2+}]_i$ amplitudes in a similar manner to isoproterenol, but the addition of carvedilol (300 nmol/L) did not affect the $[Ca^{2+}]_i$ amplitudes (Panel C). These data were confirmed to be reproducible in several independent experiments.

ly prevented the isoproterenol-induced increase in the amount of cAMP, to a greater degree than timolol (Fig 5D). Atenolol did not show any apparent inhibition. In these serial experiments, atenolol seemed to be a comparatively and distinctively weak β -antagonist, but it was much more β_1 -selective than the other two. Therefore, it seems that the β_2 -activation by isoproterenol may compensate for the β_1 -blocking by atenolol, resulting in apparently less inhibition of isoproterenol-induced $[Ca^{2+}]_i$ enhancement. To rule out this possibility, 2 more experiments were performed. Myocytes were exposed to isoproterenol in the presence of atenolol and $1 \mu\text{mol/L}$ ICI-118551, a selective β_2 -blocker, after pretreatment with them. There was no obvious difference in the $[Ca^{2+}]_i$ amplitudes between the atenolol alone and the atenolol with ICI-118551 treatment groups (Fig 5C). Also, 100 nmol/L ICI-118551 did not significantly inhibit the effects of isoproterenol. Taken together with the data on the effect of zinterol (Fig 4B), the effects of isoproterenol on $[Ca^{2+}]_i$ amplitudes were exerted almost exclusively via β_1 -dependent pathway, and the β_2 -activation by isoproterenol seems not to be the reason for the weak antagonizing ability of atenolol in guinea pig ventricular myocytes.

To examine the affinity of carvedilol for β_1 -receptor, myocytes pretreated with isoproterenol ($1 \mu\text{mol/L}$) were subsequently and additionally exposed to 300 nmol/L of carvedilol or timolol. Carvedilol rapidly diminished the amplitude of $[Ca^{2+}]_i$ close to the control level, and the effect was sustained after carvedilol was washed out (Fig 6A). On the other hand, timolol showed only slight effects on

[Ca²⁺]_i for 5 min, although the [Ca²⁺]_i amplitude quickly diminished after isoproterenol was washed out (Fig 6B). Because the pretreatment with timolol showed nearly the same inhibitory effect as carvedilol, these data further imply the prominent affinity of carvedilol for the β₁-receptors. However, there is the possibility of direct interaction of carvedilol with the post-receptor activation pathway, including interaction with PKA pathway. To examine this possibility, myocytes were perfused with a solution containing 300 nmol/L carvedilol and 10 μmol/L forskolin after the [Ca²⁺]_i transients were well enhanced and stabilized in a solution containing 10 μmol/L forskolin. Unlike isoproterenol, carvedilol did not affect the [Ca²⁺]_i amplitudes enhanced by forskolin (Fig 6C). Therefore, the downstream PKA pathway may not be directly modulated by carvedilol.

Among the 3 β-blockers, carvedilol possesses the strongest antagonistic actions to isoproterenol, and its binding affinity for the β₁-receptors is distinctive

Discussion

In the present study, we have shown the direct [Ca²⁺]_i-lowering effects of carvedilol in guinea pig ventricular myocytes: the decrease in the intrinsic β-activity (shown as a decrease in the amount of cAMP), the significant reduction of the L-type Ca²⁺ current at concentrations ≥5 μmol/L, and the increase in the voltage threshold for pacing at concentrations ≥10 μmol/L.

The ability of carvedilol to prevent the isoproterenol-induced enhancement of [Ca²⁺]_i amplitudes was slightly stronger than timolol, and much more so than atenolol, as indicated by IC₅₀ of 1.32, 2.01, and 612 nmol/L, respectively. In addition, carvedilol seems to have a relatively strong affinity for β₁-receptors.

Negative Modulation of [Ca²⁺]_i by Carvedilol

Our data for [Ca²⁺]_i demonstrate that carvedilol exerted a negative inotropic action even at very low concentrations (1 nmol/L) by lowering [Ca²⁺]_i. Furthermore, carvedilol significantly decreased the I_{Ca} in a concentration-dependent manner at ≥5 μmol/L. Therefore, the negative inotropic effect of carvedilol at ≤300 nmol/L is not necessarily because of the reduction of the I_{Ca}. As described^{24,25} the number of β-receptors is an important factor in maintaining the intrinsic β-activity of ventricular myocytes, even in the absence of catecholamines. Some β-blockers appear to prevent β-receptors from spontaneously transmitting to the activated form, resulting in decreased production of cAMP (inverse agonism). In the present study, carvedilol, timolol, and atenolol negatively modulated the [Ca²⁺]_i amplitudes and all three induced a decrease in the amount of cAMP in ventricular myocytes. A decrease in the amount of cAMP is supposed to lead to a decrease in the PKA activity, so the mechanism for the negative modulation of [Ca²⁺]_i amplitudes by the β-blockers is at least and in part associated with a reduced I_{Ca} and/or SR Ca-ATPase function because of the decrease in the amount of intracellular cAMP. From this result, one might wonder why the measured I_{Ca} was not influenced by the decrease in the amount of cAMP induced by carvedilol at ≤1 μmol/L. One possible reason is that the voltage-clamped myocytes were dialyzed with a cAMP-free pipette solution, and therefore the intracellular cAMP concentration might be already very diluted. This is widely recognized as a reason for the run-down phenomenon of I_{Ca} and hence the effects of carvedilol on the amount of cAMP

were no longer reflected in the size of I_{Ca}, although the activation of adenylyl cyclase might locally increase cAMP and activate PKA to increase the I_{Ca}. Carvedilol at ≥5 μmol/L seems to directly reduce the I_{Ca}, but not through the cAMP-dependent pathway, to further decrease [Ca²⁺]_i amplitudes. In a 10-μmol/L carvedilol solution, ventricular myocytes lost their responsiveness to field stimulation, which was regained when the voltage for field stimulation was increased. Because the other two β-blockers did not mimic this phenomenon, and 10 μmol/L carvedilol did not inhibit the I_{Ca} enough to eliminate [Ca²⁺]_i transients, the pacing failure is not attributable to the alteration of the intrinsic β-activity or the inhibition of I_{Ca}. This result indicates that the L-type Ca²⁺ channel was not opened by pacing, suggesting that the depolarization by pacing was not enough to induce an action potential, otherwise a [Ca²⁺]_i increase would be observed. The 0 phase of an action potential in a ventricular myocyte is composed of Na⁺ and Ca²⁺ currents. A Na⁺ channel blocker, tetrodotoxin (10 μmol/L), did not mimic the phenomenon, but slightly decreased the [Ca²⁺]_i amplitudes (data not shown). From this result, the membrane potential depolarized by pacing probably did not reach the threshold of the Na⁺ channel, which is known to be approximately -60 mV. Presumably this carvedilol-induced pacing failure was caused by decreased membrane excitability in response to electrical stimulation, which may be caused by changes in the membrane characteristics by carvedilol. Further examination is required to elucidate the mechanism underlining this intriguing finding.

Beta-Antagonism of Carvedilol

In terms of the [Ca²⁺]_i, we assessed the antagonism of carvedilol to β-stimulation. Carvedilol antagonized the isoproterenol-induced β-stimulation most strongly among the 3 β-blockers we tested (Fig 5C) and the data for cAMP (Fig 5D) have clearly shown that carvedilol most effectively prevented the production of cAMP induced by isoproterenol, again suggesting that carvedilol is the strongest β-blocker. In addition, these inhibitory effects of the β-blockers on the production of cAMP by isoproterenol appear to be comparable to the data for [Ca²⁺]_i shown in Fig 5D. In these experiments, the antagonizing effects of a β₁-selective blocker, atenolol, were distinctively weak, implying the functional contribution of residual β₂-activation by isoproterenol. However, a specific β₂-stimulator, zinterol (1 μmol/L), did not affect the [Ca²⁺]_i amplitudes, and a specific β₂-blocker, ICI-118551 (100 nmol/L), exerted only a slight inhibitory effect on the isoproterenol-induced enhancement of [Ca²⁺]_i. Furthermore the combination of atenolol and ICI-118551 had no additional antagonizing effect, suggesting that the β₂-activation by isoproterenol is not significantly involved in [Ca²⁺]_i regulation in guinea pig ventricular myocytes. In rat and mouse ventricular myocytes, a β₂-subtype has been reported in ventricular myocardium, but is almost devoid of coupling with positive inotropic action and/or increase in [Ca²⁺]_i amplitudes²⁶⁻²⁸ Because the β₂-subtype has been shown to exist in guinea pig ventricular myocytes as well²⁸ our present results are very similar to those for rat and mouse. Regarding the uncoupling of β₂-receptor with positive inotropism, Xiao et al reviewed earlier reports and proposed 3 possible mechanisms²⁹ First, the β₂-receptor signaling pathway is extremely localized so that its activation leading to the production of cAMP can not be transmitted to the phosphorylation of the L-type Ca²⁺ channel, phospholamban, and so

on. Second, the β_2 -receptor is coupled with Gi. Third, intrinsic and/or additional PKA activation switches the coupling of the β_2 -receptor from Gs to Gi. In the present study, zinterol did not affect $[Ca^{2+}]_i$ amplitudes, suggesting that the latter two possibilities are unlikely explanations for our results, although the first mechanism could still explain our results on the uncoupling of the β_2 -receptor with $[Ca^{2+}]_i$ regulation. Very recently Zaccolo et al have shown that the localization of cAMP is limited by phosphodiesterases, and that the specificity of the subsequent events was tightly localized³⁰ which further supports the idea that the local production of cAMP via β_1 -receptor is specifically coupled with $[Ca^{2+}]_i$ regulation.

In addition, the α_1 -blocking effect of carvedilol may have influenced our results for $[Ca^{2+}]_i$ and cAMP. In guinea pig ventricular myocytes, α_1 -stimulation has been reported to shorten action potential duration by increasing the delayed rectifier K^+ current³¹ and decreasing cAMP³² both of which contribute to a decrease in the peak of Ca^{2+} transients. This hypothesis suggests that the α_1 -blocking effect of carvedilol may result in an increase in the amplitude of $[Ca^{2+}]_i$ transients, but that seems to contradict our results of carvedilol-induced decreases in the peak of $[Ca^{2+}]_i$ transients and the amount of cAMP. Therefore, the α_1 -blockade by carvedilol may have minor additional effects in the absence of α_1 -agonists under our experimental conditions, although further experiments are necessary to prove it.

Under our experimental conditions isoproterenol increased $[Ca^{2+}]_i$ amplitudes mainly via the β_1 -receptor pathway, and carvedilol possessed the strongest β_1 -antagonizing effects among the 3 β -blockers, although the functional significance of β_2 -receptors and the effect of α_1 -blockade by carvedilol remain to be elucidated.

In Vivo Kinetics of Carvedilol

From the data on drug kinetics previously reported³³ the maximum blood concentration of atenolol could be nearly $2\mu\text{mol/L}$ after a 50-mg oral dose, and most (97%) of atenolol is supposed to be free in human plasma³⁴. On the other hand, the blood concentration of carvedilol in clinical use after a single or daily oral dose of 12.5–50 mg has been reported to reach 100–600 nmol/L at maximum^{15,16} and ~98% is bound to albumin in blood¹⁷. The calculated concentration of free carvedilol is approximately 2–12 nmol/L, although neither the distribution nor the local concentration in various tissues is clear. Applying these calculations to our present results, carvedilol of 2–12 nmol/L and atenolol of ~2 $\mu\text{mol/L}$ antagonize β_1 -stimulation in ventricular myocytes to a similar degree (Fig 5C). In addition, our data on the wash-out of (Fig 5D) and additional application of (Fig 6A) carvedilol in the presence of isoproterenol imply that the binding of carvedilol to β_1 -receptors is tight as compared with isoproterenol and timolol. The washing out of carvedilol might be related to its characteristic lipophilic structure, which when dissolved in the plasma membrane may re-surface through a diffusion theory after carvedilol was washed out from the extracellular fluid. Given that diffusion is restricted on the plasma membrane³⁵ carvedilol diffusing from the membrane may be retained on the surface of the membrane, which not only accumulates carvedilol, but also consequently slows the diffusion of carvedilol from the membrane. These local kinetics of carvedilol would favor an accentuation of its effects on the surface membrane.

Carvedilol at clinical concentrations seems to effectively

inhibit β_1 -activation and the $[Ca^{2+}]_i$ -load induced by catecholamines. The characteristics of carvedilol that include a prominent β_1 -receptor affinity and/or a lipophilic structure may contribute to its efficacy.

In conclusion, carvedilol possesses various types of direct $[Ca^{2+}]_i$ -lowering effects in ventricular myocytes, which include decreasing the intrinsic β -activity, antagonizing β -stimulation at concentrations of the nmol/L order supposedly equivalent to its clinical concentration, reducing the I_{Ca} at $\geq 5\mu\text{mol/L}$, and increasing the threshold for pacing at $\geq 10\mu\text{mol/L}$. Supposing that one possible mechanism associated with the progression of hypertrophy and heart failure is catecholamine-induced $[Ca^{2+}]_i$ load and/or PKA activation, it could be partially but effectively prevented by β -blockers. In this respect, carvedilol could be regarded as one of the β -blockers potent enough to prevent $[Ca^{2+}]_i$ load and PKA activation in the clinical setting.

Acknowledgments

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References

1. Frishman WH. Carvedilol. *N Engl J Med* 1998; 339: 1759–1765.
2. Rössig L, Haendeler J, Mallat Z, Hugel B, Freyssinet JM, Tedgui A, et al. Congestive heart failure induces endothelial cell apoptosis: Protective role of carvedilol. *J Am Coll Cardiol* 2000; 36: 2081–2089.
3. Packer M, Bristow MR, Cohn JN, Colucci WS, Fowler MB, Gilbert EM, et al, for the US Carvedilol Heart Failure Study Group. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. *N Engl J Med* 1996; 334: 1349–1355.
4. Teerlink JR, Massie BM. Beta-adrenergic blocker mortality trials in congestive heart failure. *Am J Cardiol* 1999; 84: 94R–102R.
5. Heidenreich PA, Lee TT, Massie BM. Effect of beta-blockade on mortality in patients with heart failure: A meta-analysis of randomized clinical trials. *J Am Coll Cardiol* 1997; 30: 27–34.
6. Hjalmarson A. Cardioprotection with beta-adrenoceptor blockers: Does lipophilicity matter? *Basic Res Cardiol* 2000; 95(Suppl 1): I/41–I/45.
7. Böhm M, Maack C. Treatment of heart failure with beta-blockers: Mechanisms and results. *Basic Res Cardiol* 2000; 95(Suppl 1): I/15–I/24.
8. Bonet S, Agustí A, Arnau JM, Vidal X, Diogène E, Galve E, et al. Beta-adrenergic blocking agents in heart failure. *Arch Intern Med* 2000; 160: 621–627.
9. Packer M, Antonopoulos GV, Berlin JA, Chittams J, Konstam MA, Udelson JE. Comparative effects of carvedilol and metoprolol on left ventricular ejection fraction in heart failure: Results of a meta-analysis. *Clin Invest* 2001; 141: 899–907.
10. Hirooka K, Yasumura Y, Ishida Y, Hanatani A, Nakatani S, Komamura K, et al. Comparative left ventricular functional and neurohumoral effects of chronic treatment with carvedilol versus metoprolol in patients with dilated cardiomyopathy. *Jpn Circ J* 2001; 65: 931–936.
11. Cheng J, Niwa R, Kamiya K, Toyama J, Kodama I. Carvedilol blocks the repolarizing K^+ currents and the L-type Ca^{2+} current in rabbit ventricular myocytes. *Eur J Pharmacol* 1999; 376: 189–201.
12. Nichols AJ, Sulpizio AC, Ashton DJ, Hieble JP, Ruffolo RR Jr. In vitro pharmacologic profile of the novel β -adrenoreceptor antagonist and vasodilator, carvedilol. *Pharmacology* 1989; 39: 327–336.
13. Anonymous. Calcium antagonist caution. *Lancet* 1991; 337: 885–886.
14. Packer M, O'Connor CM, Ghali JK. Effect of amlodipine on morbidity and mortality in severe chronic heart failure. *N Engl J Med* 1996; 335: 1107–1114.
15. McPhillips JJ, Schwemer GT, Scott DI, Zinny M, Patterson D. Effects of carvedilol on blood pressure in patients with mild to moderate hypertension. *Drugs* 1988; 36(Suppl 6): 82–91.
16. Morgan TO, Anderson A, Cripps J, Adam W. Pharmacokinetics of carvedilol in older and younger patients. *J Hum Hypertens* 1990; 4: 709–715.

17. Morgan T. Clinical pharmacokinetics and pharmacodynamics of carvedilol. *Clin Pharmacokinet* 1994; 26: 335–346.
18. Zou Y, Yao A, Zhu W, Kudoh S, Hiroi Y, Shimoyama M, et al. Iso-proterenol activates extracellular signal-regulated protein kinases in cardiomyocytes through calcineurin. *Circulation* 2001; 104: 102–108.
19. Ikeda Y, Gohra H, Hamano K, Zempo N, Ueyama T, Ohkusa T, et al. Effects of cardioplegic arrest and reperfusion on rabbit cardiac ryanodine receptors. *Jpn Circ J* 2001; 65: 330–334.
20. Yao A, Takahashi T, Aoyagi T, Kinugawa K, Kohmoto O, Sugiura S, et al. Immediate-early gene induction and MAP kinase activation during recovery from metabolic inhibition in cultured cardiac myocytes. *J Clin Invest* 1995; 96: 69–77.
21. Allen DG, Eisner DA, Pirolo JS, Smith GL. The relationship between intracellular calcium and contraction in calcium-overloaded ferret papillary muscles. *J Physiol* 1985; 364: 169–182.
22. Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Roseblit N, et al. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): Defective regulation in failing hearts. *Cell* 2000; 101: 365–376.
23. Zaniboni M, Yao A, Barry WH, Musso E, Spitzer KW. Complication associated with rapid caffeine application to cardiac myocytes that are not voltage clamped. *J Mol Cell Cardiol* 1998; 30: 2229–2235.
24. Samama P, Pei G, Costa T, Cotecchia S, Lefkowitz RJ. Negative antagonists promote an inactive conformation of the β_2 -adrenergic receptor. *Mol Pharmacol* 1994; 45: 390–394.
25. Schutz W, Freissmuth M. Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends Pharmacol Sci* 1992; 13: 376–380.
26. Laflamme MA, Becker P. Do β_2 -adrenergic receptors modulate Ca^{2+} in adult rat ventricular myocytes? *Am J Physiol* 1998; 274: H1308–H1314.
27. Rohrer DK, Desai KH, Jasper JR, Stevens ME, Regula DPJr, Barsh GS, et al. Targeted disruption of the mouse β_1 -adrenergic receptor gene: Developmental and cardiovascular effects. *Proc Natl Acad Sci USA* 1996; 93: 7375–7380.
28. Brodde OE. β_1 - and β_2 -adrenoceptors in human heart: Properties, function, and alterations in chronic heart failure. *Pharmacol Rev* 1991; 43: 203–243.
29. Xiao RP, Cheng H, Zhou YY, Kuschel M, Lakatta EG. Recent advance in cardiac β_2 -adrenergic signal transduction. *Circ Res* 1999; 85: 1092–1100.
30. Zaccolo M, Pozzan T. Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science* 2002; 295: 1711–1715.
31. Dirksen RT, Sheu SS. Modulation of ventricular action potential by alpha 1-adrenoceptors and protein kinase C. *Am J Physiol* 1990; 258: H907–H911.
32. Buxton IL, Brunton LL. Action of the cardiac alpha 1-adrenergic receptor: Activation of cyclic AMP degradation. *J Biol Chem* 1985; 260: 6733–6737.
33. Ishizaki T, Oyama Y. Atenolol dose-finding studies. *Drugs* 1983; 25(Suppl 2): 42–49.
34. Barber HE, Hawksworth GM, Kitteringham NR, Swann JM. Protein binding of atenolol and propranolol to human serum albumin and in human plasma. *Br J Clin Pharmacol* 1978; 6: 446.
35. Yao A, Spitzer KW, Ito N, Zaniboni M, Lorell BH, Barry WH. The restriction of diffusion of cations at the external surface of cardiac myocytes varies between species. *Cell Calcium* 1997; 22: 431–438.

Beating is necessary for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes¹

YOSHIHIRO IJIMA, TOSHIO NAGAI, MIHO MIZUKAMI, KATSUHISA MATSUURA, TAKEHIKO OGURA,* HIROSHI WADA, HARUHIKO TOKO, HIROSHI AKAZAWA, HIROYUKI TAKANO, HARUAKI NAKAYA,* AND ISSEI KOMURO²

Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan; and *Department of Pharmacology, Chiba University Graduate School of Medicine, Chiba, Japan

SPECIFIC AIMS

Skeletal myoblasts are thought to be a good cell source for autologous transplantation to myocardium, but the safety and efficacy of their transplantation are still controversial. Recent studies have revealed that skeletal muscle possesses a stem cell population that is distinct from myoblasts. The present study was performed to elucidate whether skeletal muscle stem cells can transdifferentiate into cardiomyocytes and develop electro-mechanical coupling with neighboring cardiomyocytes.

PRINCIPAL FINDINGS

1. Skeletal muscle-derived cells expressed cardiac TnT and ANP in coculture with cardiomyocytes

Skeletal muscle cells were prepared from green fluorescent protein (GFP) -expressed transgenic mice and cultured with cardiac myocytes of neonatal rats. Expression of cardiac-specific proteins such as cardiac troponin T (TnT) and atrial natriuretic peptide (ANP) was examined by immunostaining. Although the majority of GFP-positive cells did not express cardiac TnT and ANP, a few GFP-positive cells (~0.02%) expressed cardiac TnT and ANP. Expression of cardiac TnT was first observed at day 2 from the start of the coculture. At day 4, GFP-positive cells expressing cardiac TnT showed a fine striated pattern, indicating sarcomere formation (Fig. 1A, B). ANP staining was observed at perinuclear region at day 2 (Fig. 1C). Complete colocalization of GFP signal and cardiac TnT was confirmed by merged picture of confocal image (Fig. 1D). The expression of these two cardiac-specific proteins in GFP-positive cells suggests that skeletal muscle-derived cells transdifferentiate into cardiomyocytes.

2. Skeletal muscle-derived cells expressed Nkx2E and GATA4 in coculture with cardiomyocytes before expression of cardiac TnT and ANP

We next examined the expression of cardiac transcription factors Nkx2E (formerly named as Csx/Nkx2.5)

and GATA4 in skeletal muscle-derived cells using the coculture system. Expression of Nkx2E was examined by double staining with anti-Nkx2E antibody and anti-cardiac TnT antibody. Expression of GATA4 was examined by double staining with anti-GATA4 antibody and anti-ANP antibody. At day 1 from starting the coculture, expression of Nkx2E and GATA4 was observed in GFP-positive cells. On day 2 some skeletal muscle-derived cells expressed both Nkx2E and cardiac TnT, and GATA4 and ANP. These results suggest that cardiac transcription factors Nkx2E and GATA4 were expressed in skeletal muscle-derived cells 1 day earlier than cardiac TnT and ANP.

3. Skeletal muscle-derived cells expressed connexin 43 and cadherin at the junction of neighboring cardiomyocytes

To investigate whether transdifferentiated skeletal muscle-derived cells contact with cardiomyocytes via gap and adherence junctions, expression of connexin 43 and cadherin was examined. Cells were double stained by anti-connexin 43 antibody and cardiac TnT or anti-pan-cadherin antibody and anti-ANP antibody. Expression of connexin 43 was observed at the junction of cardiac TnT-expressing skeletal muscle-derived cells and neighboring cardiomyocytes. Expression of cadherin was observed at the junction of ANP-expressing, skeletal muscle-derived cells and neighboring cardiomyocytes. Skeletal myoblasts and skeletal myotubes expressed little connexin43 and cadherin at the junction of neighboring cardiomyocytes as reported previously. These results suggest that skeletal muscle-derived cells contact with cardiomyocytes through gap junctions and adherence junctions after transdifferentiation into cardiomyocytes.

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² Correspondence: Department of Cardiovascular Science and Medicine, Chiba University School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670 Japan. E-mail: komuro-ky@umin.ac.jp