type with the exercise capacity or training effects in athletes or healthy persons, this is the first study to examine the association between them in patients after AMI. In particular, because CR obviously provides improved exercise tolerance and quality of life and decreased mortality in patients with CHF or after AMI, it is important to clarify whether it is genetic factors, such as the ACE genotype, that are causing the training effects of CR.

The ACE genotype affects both serum and tissue ACE levels and many studies have investigated the associations with various cardiovascular diseases. As ACE is involved in the metabolism of substances that affect vascular and cardiac remodeling, it may account for the cardiopulmonary fitness of individuals and for the differences among individuals in response to physical training. Recent studies have shown higher frequencies of the ACE I allele among endurance athletes compared with non-athlete controls[9,10] Moreover, Hangberg et al reported an association between the ACE I allele and higher $P\dot{V}O_2$ levels in postmenopausal women![11] However, there also have been conflicting reports about this association. Some studies failed to find an association between the ACE I allele and exercise capacity and did not support the hypothesis that ACE I/D polymorphism plays a major role in cardiopulmonary endurance![6] Others reported that the ACE DD allele is associated with higher levels of $P\dot{V}O_2$ and that a greater strength gain in cardiac and skeletal muscles in response to resistance training program is found in the D allele carriers![17,18]

The present study demonstrated that in post-AMI patients, the ACE genotype did not affect either the baseline exercise capacity or the training effects of a 3-month CR program, and there are several possible explanations. The subjects investigated were not healthy and the intensity of the exercise training was much less than that of endurance training in athletes. It is also well known that ethnic differences can affect genetic associations. However, at present, a physiological explanation for any association between the cardiorespiratory phenotype and ACE polymorphism has not been found and requires further investigation.

Recently, Abraham et al reported an association of the ACE DD genotype with decreased exercise tolerance 57 patients with CHF![12] They observed that those with the ACE DD genotype had more restrictive pulmonary changes and a reduced lung diffusing capacity, and they attributed this to the poorer exercise capacity in the patients with CHF. Huwang et al reported that the ACE DD genotype might be a marker of a more severe condition in Chinese Han patients with CHF![19] However, it remains controversial whether ACE polymorphism is associated with CHF?[20] In a subanalysis of the present study, we demonstrated no impact of ACE I/D polymorphism on exercise capacity in patients with LV dysfunction after AMI. As we did not assess the pulmonary function, we could not confirm Abraham's observations precisely in the current study. Also, we defined LV dysfunction as LVEF <45% and Abraham et al used LVEF <35%, which might explain the different results between the 2 studies. Exercise tolerance is a multifactorial phenotype and training benefits may be attributed to adaptations in cardiac and pulmonary performances, as well as those in the peripheral circulation and skeletal muscles. Although ACE I/D polymorphism does not appear to bean important modulator of the exercise capacity of patients with LV dysfunction after AMI, further studies are necessary to clarify the contribution of other important genetic factors in the decreased exercise capacity of CHF/LV dysfunction patients.

Study Limitations

We did not assess the effect of genotype on circulating markers of the renin–angiotensin system. However, because the association of the ACE D allele with increased plasma ACE activity has been consistently demonstrated in many previous studies, our observation suggests there is no relationship between plasma ACE activity and exercise capacity. In addition, the analysis and findings of this study are limited by the retrospective design. Finally, the study population was relatively small, especially in the subanalysis of patients with LV dysfunction. Any negative finding could thus be caused by a low statistical power. A larger study will be required if associations of the ACE genotype are to be investigated further.

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Circ J 2005; **69:** 1029–1034

Validation of the Association Between the Gene Encoding 5-Lipoxygenase-Activating Protein and Myocardial Infarction in a Japanese Population

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Background Recently, the 5-lipoxygenase activating protein gene (*ALOX5AP*) was reported to confer a risk of myocardial infarction (MI) and stroke, independent of conventional risk factors. The purpose of the present study was to validate those findings in a Japanese population.

Methods and Results The study population consisted of 1,875 subjects (males 871, females 1,004) recruited from the Suita study (control group) and 353 subjects (males 306, females 47) with MI. The promoter, all of the exons, and 3'UTR regions of *ALOX5AP* were sequenced in 96 subjects, and 8 polymorphisms were found. There were significant differences in the frequencies of the haplotypes constructed from the 2 SNPs (A162C and T8733A) between the control and MI groups. Multiple logistic analysis indicated that the homozygous genotype of the (CA) haplotype was significantly associated with a reduced risk for MI.

Conclusion The hypothesis that *ALOX5AP* contributes to susceptibility for MI was validated in a Japanese population. (*Circ J* 2005; **69:** 1029–1034)

Key Words: *ALOX5AP*; Haplotype; Myocardial infarction

Myocardial infarction (MI) is a multifactorial disease caused by environmental and genetic factors. There are an increasing number of studies that identify genes contributing to MI[1–5] for personalized prevention from the disease. Recently, the 5-lipoxygenase activating protein gene (*ALOX5AP*) was reported to confer a risk of MI and stroke, independent of conventional risk factors[6] This possibility was based on findings in genome-wide scans and subsequent case–control studies. A haplotype, HapA, defined by 4 single nucleotide polymorphisms (SNPs) and which spanned *ALOX5AP*, was shown to be associated with MI in an Icelandic population[6] and subsequently, another SNP-based haplotype within *ALOX5AP*, HapB, showed a significant association with MI in British cohorts from Leicester and Sheffield[6]

The *ALOX5AP* gene encodes the membrane-associated 5-lipoxygenase (LO)-activating protein, an important mediator of the activity of 5-lipoxygenase, a key enzyme in the biosynthesis of leukotrienes[7] Leukotrienes are not only smooth muscle constrictors but also proinflammatory mediators that are produced predominantly by inflammatory cells[8,9] Studies have indicated that inflammatory processes play an important role in the progression of atherosclerotic disease[10,11] The 5-LO pathway could be an important contributor to the pathophysiology of atherosclerosis through the formation of leukotriene (LT) B4 via an increase in vascular permeability[12] Antagonists of LTB4 have been reported to attenuate the development of atherosclerosis in apoE-deficient and LDLR-deficient mice[13] Furthermore, 5-LO has been localized to macrophages, dendritic cells, foam cells, mast cells, and neutrophilic granulocytes, and the number of cells that expressed 5-LO was markedly greater in advanced lesions[14] Leukocytes that were positive for 5-LO accumulated at distinct sites that are most prone to rupture[15] Taken together, these findings suggest that upregulation of the leukotriene pathway may contribute to the progression of atherosclerotic progression and plaque stability. However, the precise role of leukotrienes in the pathogenesis of atherothrombotic diseases awaits further investigation.

Thus, it is likely that *ALOX5AP* contributes to MI. However, we are now recognizing that the contribution of common alleles is less than expected, and any single study that considers a few thousand subjects may not be large enough to support concrete conclusions and should be viewed as providing only tentative results. The purpose of the present study was to validate the findings of DeCode genetics[6] in a Japanese population and to evaluate the possible importance of *ALOX5AP* in the pathogenesis of MI.

Methods

Study Population

The selection criteria and design of the Suita Study have been described previously[16–18] The genotypes were determined in 1,875 subjects recruited from the Suita Study between April 2002 and February 2003. The MI group consisted of 353 (males 306, females 47) randomly selected inpatients and outpatients with documented MI who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003[19]

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Table 1 Characteristics of the Study Population

	Control	MI	p-value
n	1,875	353	
Sex (% male)	46.5	86.69	<0.0001
Age (years)	64.5±0.25	58.8±0.59	<0.0001
BMI (kg/m²)	22.8±0.07	23.9±0.16	<0.0001
C-SM (%)	44.3	61.2	<0.0001
HT (%)	40.6	53.7	<0.0001
HLP (%)	27.8	51.7	<0.0001
HDL-C (mg/dl)	59.9±0.35	44.0±0.93	<0.0001
DM (%)	6.1	39.1	<0.0001

Data are mean±standard error. Differences between the 2 groups (control vs myocardial infarction (MI)) were calculated by t-test or χ^2 analysis. BMI, body mass index; C-SM, current smoking habit; HT, hypertension; HLP, hyperlipidemia; HDL-C, high-density lipoprotein cholesterol; DM, diabetes mellitus.

Table 2 Primers for Sequence Analysis

Probe	Sequence (5'-3')
P1S	GATATCAGCTGTCCCTCCCCACTG
E1S	CTCAGGGAAGTTTCCCATGACAAGG
E2S	CAGTAGAGAGCAGCTGCTGAGTACG
E3S	CCAAGTCTCCCTTACGCATCACCG
E4S	CTTGGGTCTTTTCCTGAAGTGC
E5S	GGAGCATTGTTGAGTCCAGGGAGC
P1A	GCACAACCTGCCCTGTACAGGAAG
E1A	CAAAACCTTCAAGTTGCAGCCCTG
E2A	CACAAAGCCTCTCTGGTGAAGTCC
E3A	GCTCTCACCTCTCCAGGGCTTCAC
E4A	GCTCAGGGAAAGAAGAATCAGAGGTC
E5A	GGATTACAGGTATGAGCCACCACACC

Primers used for sequence analysis of a promoter region and all of the 5 exon regions including noncoding regions in ALOX5AP.

All the subjects enrolled in the present study provided written informed consent. The present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center. The characteristics of the study population are shown in Table 1. Subjects with systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, and/or taking antihypertensive medication were categorized as having hypertension (HT). Subjects with fasting blood glucose ≥126 mg/dl, hemoglobinA1c ≥6.5%, and/or treatment for diabetes mellitus (DM) were categorized as having DM. Subjects with total cholesterol ≥220 mg/dl, triglycerides ≥150 mg/dl, and/or antihyperlipidemic medication were categorized as having hyperlipidemia (HLP).

DNA Studies

A promoter region and all of the 5 exon regions, including noncoding regions, were sequenced in 48 healthy subjects and 48 subjects with MI. Primers for sequencing are shown in Table 2. Eight polymorphisms were found and all the genotypes were determined by the TaqMan system in 1,875 control subjects and 353 subjects with MI. The probes and primers are shown in Table 3. HapA and HapB reported by DeCode,[6] which have been reported to be associated with MI and stroke, each consisted of 4 SNPs that were outside the area of our sequencing. To validate the possible importance of these haplotypes, the genotypes of 7 additional SNPs were also determined by the TaqMan system (Table 3).

Table 3 TaqMan Probes and Primers for *ALOX5AP* Genotyping

	Probe		Primer	
	VIC	FAM	F	R
A(−607)G	TTTTCTTGGAATTCAAAAA	TTTCTTGGGATTCAAAAA	AAACCTTATGTGGCTGCTACTTACC	GTGCCCCAAATACTGTCCTTCT
T(−515)C	TGTGCGTGTGTGTGTGT	TGCGTGCGTGTGTGT	TGGAATTCAAAAAAAGAAGGACAGTA	GGGCAACAGAGCAAGACTGTCT
A(162)C	CCCTTCACTCAGGG	CCCTTCAATCAGGG	CAGCGTGGTCCAGAATGGTA	TCCAAACAACCATCAAAGAAAATC
A(8640)C	AGTCCTAACCCTGATGTT	AGTCCTAAACCTGATGTTG	GCCTTTGAGCGGGTCTACACT	TTAAGGGTGGTCATCACTCCTAGAA
T(8733)C	AGCCAAGTTTCTGAGCG	TGAGCCAAGCTTCTGA	ACCACCCTTAATACCACATGTCTGT	AAGCCTCTCTGGTGAAGTCCAA
G(20616)C	CCTTCCCCCTCCCCAC	CCTTCCCGCTCCCA	AGGGAAAGAAGAATCAGAGGTCCTA	CAGGAAGAGTGACAATTCAAAACAGTA
C(28506)G	CTTCTTTTTCGGAAGTGA	CTTCTTTTTGGAAGTGACT	GTCCGTTGCTGGCATATTCA	TGGAGATCGTCTTTATGTAGTTTTCAA
A(28794)G	CTATTCCCATGCATTTT	CTATTCCCGTGCATTT	GAACAAAATGATGTCATGTCAGCTC	TGGTCACAAAACATCTTCAGAGAAC
HapA1	CCACTGTTGCCCAGTGG	AGCCACTGTTACCCAGTG	ATGATTCTTCTGACAGCATCAGCT	CATGTTGCTGTGTCCATACATAGC
HapA2, HapB2	TGCAATTCTATTTAACCTCAA	TGCAATTCTAATTAACCTCAA	TCACAAGATCCAGATGTATGTCCAA	ACTCTTAAGGTAGGTCTATGGTTGCAA
HapA3	AGAGCGCGTGTGATAA	CAGAGCGCATGTGATAA	TCGGAGGCCGTGTTTCAG	CCAGGGAGCAAGCATTAGCA
HapA4	AATTGCTCGATGAGATCCT	AATTGCTCGATGAGATCC	TGCTTTAGTTCTTGACCTCACCAA	ACCATCTGGGTTCAAGAGAGAAAT
HapB1	CTGCCTCGGCCTC	TGCCTCAGCCTCCCA	ACATCACGTAGTGTGTTGTGTAAGAAA	ACTGTCTTGAACTCCTGACCTCAG
HapB3	AACTGAGAGTAAAGATTC	AACTGAGGGTAAAGATT	TCTTTAACACCCTGTCTCCAAATACA	TGGTCCCTTCCAAAATTCATATG
HapB4	TTTTAAAAACCGAAAGGACCAC	TTTTTAAAAACTGAAAGGACCAC	TGCACCCCCACAAATACCTACAA	ATCCTGATGGCCTGGCATT

TaqMan probes and primers used to determine the genotypes of SNPs in ALOX5AP. The nucleotides of polymorphisms are underlined. HapA and HapB are defined as follows: HapA1, SG13S376; HapA2 and HapB, SG13S114; HapA3, SG13S89; HapA4, SG13S32; HapB1, SG13S377; HapB3, SG13S41, HapB4, SG13S35 (see Reference 1).

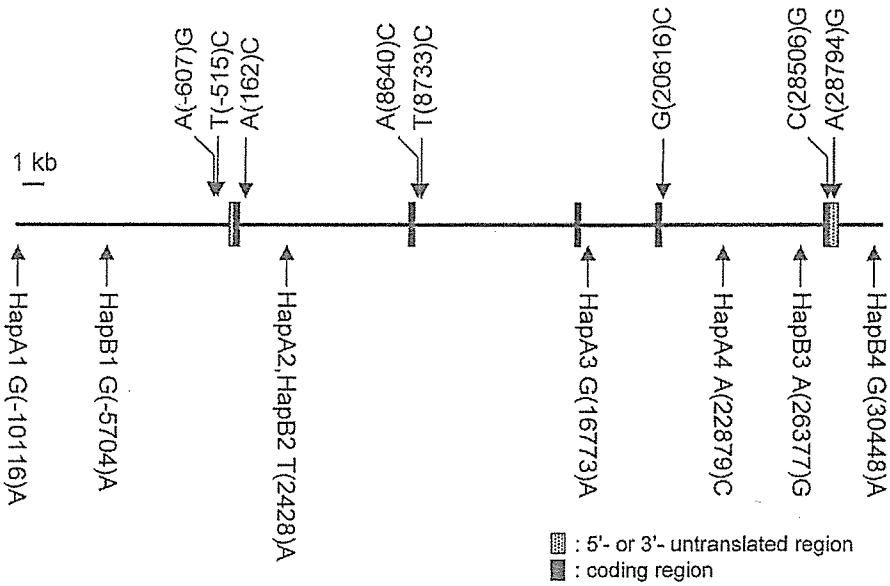

Fig 1. Schematic of the *ALOX5AP* gene and the positions of the determined polymorphisms. Gray boxes indicate the 5'- or 3'-untranslated regions, and black boxes indicate coding regions.

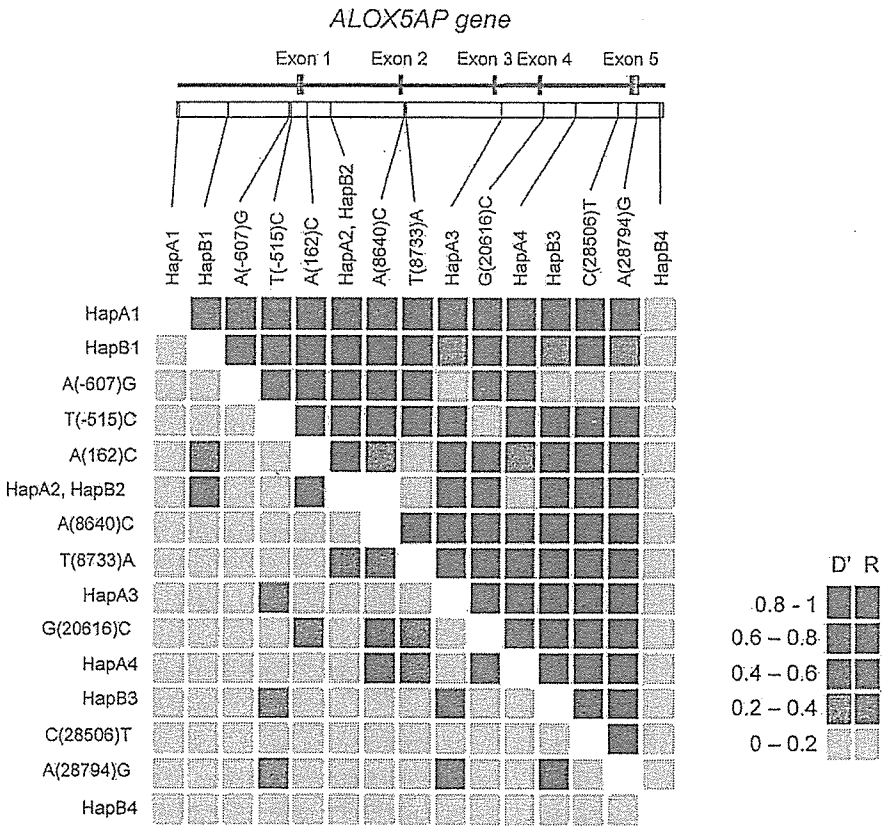

Fig 2. Linkage disequilibrium among the SNPs in the *ALOX5AP* gene. Two measures of LD are shown: D'-values in the upper right triangle and R-square values in the lower left triangle. Color-coded scales for D'-values or R-square values (measures of LD strength) are provided on the right.

Statistical Analysis

Values are expressed as mean ± standard error of the mean. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc, Cary, NC, USA) unless otherwise stated. Multiple logistic analysis was performed to obtain predictors for MI. R-square values between polymorphisms and haplotype frequencies in the control and MI groups were analyzed using the SNPAlyze Pro

Table 4 Genotype Frequencies of Each Polymorphism in *ALOX5AP* in the Total Group and in Males

Polymorphism	Control			MI			p1 value	p2 value	Control males			MI males			p1 value	p2 value
	Major	Hetero	Miner	Major	Hetero	Miner			Major	Hetero	Miner	Major	Hetero	Miner		
A(−607)G	1,843	31	1	342	11	0	0.1893	0.4430	854	17	0	295	11	0	0.1201	0.1167
T(−515)C	1,859	15	0	346	2	0	0.6451	0.5695	863	8	0	299	2	0	0.6713	0.5876
A(162)C	491	939	445	105	179	68	0.1294	0.2706	240	428	203	93	150	63	0.4989	0.4470
A(8640)C	800	833	237	146	167	39	0.5250	0.5009	371	381	118	124	146	35	0.4035	0.4553
T(8733)C	605	903	364	113	179	61	0.5697	0.3391	294	413	163	97	157	52	0.5061	0.3207
G(20616)C	1,122	651	102	210	127	16	0.7331	0.8127	510	313	48	181	111	14	0.8151	0.7954
C(28506)G	1,862	12	0	353	0	0	0.0415	0.7168	866	5	0	306	0	0	0.0823	0.8010
A(28794)G	1,847	26	1	344	7	0	0.5980	0.7270	859	11	1	299	6	0	0.5133	0.5663
HapA1 (G(−1011 6)A)	1,873	1	0	352	0	0	0.5573	0.9375	871	0	0	305	0	0		
HapA2, HapB2 (T(2428)A)	792	844	239	138	173	39	0.2976	0.1480	370	384	117	123	145	35	0.4675	0.4325
HapA3 (G(16773)A)	1,848	26	1	346	7	0	0.6031	0.7313	859	11	1	300	6	0	0.5156	0.5703
HapA4 (A(22879)C)	800	832	242	145	168	39	0.4285	0.3791	373	382	116	123	146	36	0.4620	0.4838
HapB1 (G(−5704)A)	1,249	561	64	221	121	10	0.2426	0.1736	573	266	32	200	96	9	0.8119	0.8653
HapB3 (A(26377)G)	1,847	27	1	343	9	0	0.3032	0.7148	858	12	1	298	7	0	0.4260	0.5632
HapB4 (G(30448)A)	1,875	0	0	353	0	0			871	0	0	306	0	0		

Major, Hetero and Minor indicate major genotype, heterozygous genotype, and minor genotype, respectively. For example, in the case of polymorphism A(−607)G, major, hetero, and minor refer to the AA, AG, and GG genotypes, respectively. The numbers of each genotype of each SNP are shown. P1 values were calculated by χ^2 analysis and P2 values were calculated by multiple logistic analysis including age, sex, BMI, HT, HLP, DM, and C-SM as independent variables (See Table 1 for abbreviations).

statistical package (version 3.2, Dynacom Inc). Diplotypes were also estimated by the SNPAlyze Pro statistical package.

Results

Sequence analyses in 96 subjects revealed the existence of 8 polymorphisms in Japanese. The genotypes of these 8 polymorphisms were determined by the TaqMan system in 1,875 control subjects and 353 MI subjects. Seven additional genotypes were also determined by the TaqMan system to validate the possible importance of HapA and HapB, as reported by DeCode[6] A schematic of *ALOX5AP* and its polymorphisms are shown in Fig 1. The LD values calculated by D'- or R-square values among these SNPs are shown in Fig 2.

Genotype frequencies in the control and MI groups are shown in Table 4. P1 values were calculated by chi-square analysis and P2 values were calculated by multiple logistic analysis including age, sex, body mass index (BMI), HT, HLP, DM, and current smoking (C-SM) as independent variables.

The allele frequencies of the SNPs comprising HapA and HapB were significantly different between the Icelandic and Japanese populations. The allele frequencies of HapA1, HapA3, HapB3, and HapB4 were significantly less in Japanese, and some of the HapA and HapB haplotype frequencies were too small for conducting meaningful association studies in Japanese. Thus, we conducted haplotype analyses based on the polymorphisms found in our study population.

The allele frequencies of the A162C, A8640C, T8733A, and G20616C polymorphisms exceeded 0.15 (Table 4). The polymorphisms A8640C, T8733A, and G20616C are in tight LD (Fig 2). Therefore, we constructed haplotypes with A162C and one of the polymorphisms from A8640C, T8733A, and G20616C, and compared haplotype frequencies between the control and MI groups. The most significant difference in haplotype frequency ($p<0.0001$ [1,000 permutations]) was observed in the haplotype constructed by the A162C and T8733A polymorphisms (Table 5). The haplotype (AA) was less frequent in the control than in the MI group (20.0% vs 25.8%, $p=0.003$ [1,000 permutations] The haplotype (CA) was more frequent in the control than in the MI group (23.6% vs 16.9%, $p=0.001$ [1,000 permutations]. Similar trend was also observed in male subjects only (Table 5).

Next, diplotypes of the study population were estimated and the characteristics of the subjects with the homozygous genotype of the (CA) haplotype and the others are shown in Table 6. The influence of the haplotypes on susceptibility to MI was assessed by multiple logistic analysis in which age, sex, C-SM, BMI, HT, HLP, and DM were included as independent variables. The homozygous genotype of the (CA) haplotype was significantly associated with reduced risk for MI ($p=0.0431$, odds ratio=0.4436, 95% confidence interval=0.189–0.926) over other genotypes. However, multiple logistic analysis did not conclude that the homozygous genotype of the (AA) haplotype was associated with increased risk for MI ($p=0.2901$).

Discussion

The purpose of the present study was to validate in a Japanese population the association between *ALOX5AP*

Table 5 Frequencies of the Haplotype Constructed by the Polymorphisms A162C and T8733A in the Total Group and in Males

Haplotype	Overall (%)	Control (%)	MI (%)	Permutation p-value	Overall (%)	Control males (%)	MI males (%)	Permutation p-value
A162C/T8733A				<0.0001				0.043
AT	31.0	31.3	29.4	0.388	31.4	32.0	29.8	0.361
CT	25.6	25.2	27.8	0.196	26.1	25.5	27.5	0.421
AA	20.9	20.0	25.8	0.003	21.4	20.1	25.1	0.035
CA	22.6	23.6	16.9	0.001	21.1	22.3	17.6	0.03

The percentage of the haplotype constructed by the polymorphisms A162C and T8733A is indicated. Permutation p-values were calculated by 1,000 iterations of the permutation test using the SNPAlyze Pro statistical package.
The haplotypes [AT], [CT], [AA], and [CA] mean as follows: AT, A162 and T8733; CT, C162 and T8733; AA, A162 and A8733; CA, C162 and A8733, respectively.

variants and MI that has been recently reported in Caucasian populations.

Because the genetic contribution of a single gene to common disease susceptibility seems to be very low, as observed in the I/D polymorphism of the ACE gene in cardiovascular diseases,[20,21] validation studies in other populations are very important. However, some of the allele frequencies of the HapA and HapB haplotypes were too low in our study population and so we could not replicate the previous studies by DeCode.[6] However, the haplotype that was newly identified in our study population was revealed to be significantly associated with MI. Thus, the hypothesis that *ALOX5AP* contributes to the susceptibility for MI is validated and strengthened by the present study.

The precise mechanism of how variants of *ALOX5AP* confer susceptibility for atherothrombotic diseases remains to be determined. The biological significance of the haplotype defined by A162C and T8733A remains to be solved, because these 2 polymorphisms reside in intron regions. Future studies will be needed to investigate whether the haplotype influences the production of leukotrienes by neutrophils.

Identification of *ALOX5AP* as one of the genes contributing to MI may have clinical implications. Leukotriene antagonists are currently used to treat asthma and various allergic diseases.[22] It would be interesting to determine whether these clinically useful antagonists could be helpful for the secondary prevention of MI in selected subjects defined by haplotype.

Table 6 Characteristics of Subjects With the Homozygous Genotype of the [CA] Haplotype and the Others

Genotype	[CA][CA] homozygous	Others	p-value
n	113	2,115	
Sex (% male)	45.1	53.3	0.0920
Age (years)	62.3±1.06	63.7±0.24	0.2218
BMI (kg/m^2)	23.1±0.29	22.9±0.07	0.5411
C-SM (%)	44.3	47.2	0.5453
HT (%)	37.2	43.0	0.2192
HLP (%)	28.3	31.8	0.4353
DM (%)	8.9	11.4	0.3846
MI (%)	8.0	16.3	0.0107

Data are mean±standard error. Differences between the 2 groups ([CA] [CA] homozygous vs the others) were calculated by t-test or χ^2 analysis. BMI, body mass index; C-SM, current smoking habit; HT, hypertension; HLP, hyperlipidemia; HDL-C, high-density lipoprotein cholesterol; DM, diabetes mellitus.

Acknowledgments

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Circ J 2005; **69:** 1192–1195

Assessment of MEF2A Mutations in Myocardial Infarction in Japanese Patients

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Background Recently, a mutation in the human MEF2A gene was reported to be responsible for an autosomal dominant form of coronary artery disease, so the purpose of the present study was to assess the significance of MEF2A mutations in Japanese subjects with myocardial infarction (MI).

Methods and Results The study population consisted of 589 control subjects recruited from the Suita study and 379 subjects with MI. The promoter, all the exons, and 3'-UTR regions of MEF2A were sequenced in 190 subjects with myocardial infarction. We found 2 amino acid length polymorphisms, a 7-amino acid deletion polymorphism, and a nonsense mutation (R447X) in exon 12. The length and deletion polymorphisms did not confer susceptibility to MI. Although the nonsense mutation was detected in 1 subject with MI, and in none of the control subjects, the impact of this mutation does not appear to be great; the subject had the MI while in his 70s, had 2 major risk factors, and no family history of ischemic heart disease.

Conclusion MEF2A polymorphism does not contribute appreciably to MI in the Japanese population. (*Circ J* 2005; **69:** 1192–1195)

Key Words: MEF2A; Myocardial infarction; Polymorphisms

Myocardial infarction (MI) is a multifactorial disease caused by environmental and genetic factors. There is an increasing number of studies that have identified the genes contributing to ischemic heart diseases (IHD)[1–8] and recently, a mutation in the human MEF2A gene was reported as responsible for an autosomal dominant form of coronary artery disease (CAD)[9] The 7-amino acid deletion disrupts the nuclear localization of the mature protein and reduces MEF2A-induced transcriptional activation[9] The same authors have reported MEF2A missense mutations in 4 of 207 sporadic CAD cases and estimated that MEF2A mutations contribute to approximately 2% of CAD![10] On the other hand, Weng et al recently reported a lack of MEF2A mutations in 300 CAD cases;[11] so the purpose of the present study was to assess the significance of MEF2A mutations in Japanese subjects with MI.

Methods

Study Population

The control group consisted of 589 subjects recruited from the Suita study who were at least 60 years of age with no cardiovascular disease and no family history IHD. The selection criteria and design of the Suita Study have been described previously![12–14] We excluded young subjects from the control group, because they might develop MI in their 50s and 60s. The MI group consisted of 379 randomly selected inpatients and outpatients with documented MI who were admitted to the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003 (MI group)![15] The characteristics of the study population are shown in Table 1. All subjects gave written informed consent and the present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center.

DNA Studies

A promoter region and all of the 12 exon regions were sequenced in 190 of the subjects with MI (Fig 1) and we found variations that altered the amino acid sequences in exon 12 only. Next, we sequenced exon 12 in 589 control subjects and the remaining 189 subjects with MI. The variations in exon 12 were all determined by sequencing.

Statistical Analysis

All statistical analyses were performed with the JMP

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Table 1 Characteristics of the Study Population

	Control	*MI*	*p-value*
n	*584*	*379*	
Gender (% male)	*49.3*	*85.5*	*<0.0001*
Age (years)	*70.6±0.3*	*58.0±0.4*	*<0.0001*
BMI (kg/m²)	*22.67±0.12*	*23.83±0.115*	*<0.0001*
TC (mg/dl)	*207.7±1.4*	*199.7±2.3 (n=224)*	*0.0030*
HDL-C (mg/dl)	*58.8±0.6*	*43.0±1.1 (n=194)*	*<0.0001*
Smoking			*<0.0001*
Current	*93*	*228*	
Past	*163*	*74*	
Never	*328*	*77*	
DM (%)	*7.7*	*38.7*	*<0.0001*
HT (%)	*35.5*	*52.9*	*<0.0001*

Data are mean±standard error. Differences between 2 groups (Control vs myocardial infarction (MI)) were calculated by t-test or χ^2 analysis.
BMI, body mass index; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; DM, diabetes mellitus; HT, hypertension.

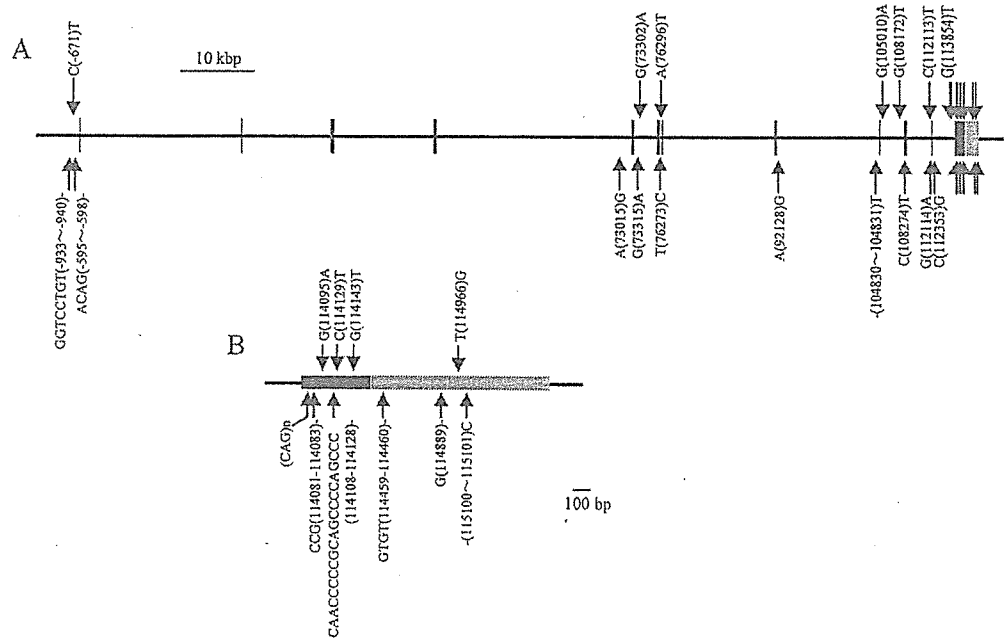

Fig 1. Scheme of the MEF2A gene. (A) Promoter region and all of the 12 exon regions are shown. (B) Expanded region of exon 12. The 5'- and 3'-UTR regions are indicated by gray boxes, and coding regions are indicated by black boxes. The 27 polymorphisms that were found are indicated by arrows.

statistical package (SAS Institute Inc, Cary, NC, USA) unless otherwise stated. Chi-squared analysis was performed to compare haplotype frequencies between the control and MI groups.

Results

We found 27 variations in MEF2A (Table 2), and 4 variations in exon 12 that altered the amino acid sequence of the MEF2A protein (Fig 2). The number of polyglutamine tandem repeats (region A) varied between 4 and 15 (genotype 1), and the number of proline tandem repeats (region B) varied between 4 and 5 (genotype 2). The 21-bp deletion (7-amino acid deletion), which was originally implicated in an autosomal dominant form of CAD[9] was also observed in region C (genotype 3) (Fig 2). We found one nonsense mutation (R447X) in exon 12 in a MI subject, and it was localized just downstream of the 21-bp deletion site (Fig 3).

The haplotype frequencies defined by the 3 genotypes are shown in Table 3: there were no significant differences between the control and MI groups.

Discussion

Wang et al reported that a mutation in the human MEF2A gene was responsible for an autosomal dominant form of CAD[9] but Weng et al could not find any MEF2A mutations in 300 cases of CAD![11] Thus, the association between mutations of MEF2A and CAD is controversial[16,17] and our results favors a lack of association.

Our results indicate that length polymorphisms in MEF2A do not contribute appreciably to MI in the Japanese population. Furthermore, the 21-bp deletion in MEF2A, which was originally implicated[9] did not seem to be associated with MI. The nonsense mutation (R447X) may affect susceptibility to MI, but the particular patient with this mutation had the MI in his 70s, and had no family history of IHD. He also had 2 risk factors: diabetes mellitus and smoking. Thus, the impact of this mutation does not appear to be great.

We sequenced all the coding regions of MEF2A in 190 subjects with MI and found neither missense nor nonsense mutations, except for R447X. Taking all our results together, MEF2A polymorphism does not appear to contribute appreciably to MI in the Japanese population.

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Table 2 The 27 Variations Found in the MEF2A Gene

Region	Polymorphisms	Sequence around polymorphisms	Amino acid mutation
Promoter	GGTCCTGT(–933～–940)–	AGACATTTTAATTTCACCTC[GGTCCTGT/–] GGTCCTGTGGTCCTGTGATC	
	C(–671)T	TGGCCGACGCTTAAGGAAAA[C/T] AGAAAAGAACCTACATGAAT	
	ACAG(–595～–598)–	TTTATTAATGTGAATCATAG[ACAG/–] CCATCAGTCTTCTATTCATT	
Intron 4	A(73015)G	CCTGTAAGTACTTTTACTTT[A/G] CCTCTACTTTTTATTTGTTG	
Intron 5	G(73302)A	GGTGATGACAACAATAAGTA[G/A] AAGGGAAGAAATGCATTTTA	
	G(73315)A	ATAAGTAGAAGGGAAGAAAT[G/A] CATTTTATTAGTATTTTTTA	
Intron 6	T(76273)C	CTTTGATGAGCAAGAATCAC[T/C] GATACATAATTGGCCTCATT	
	A(76296)T	TACATAATTGGCCTCATTTT[A/T] AAGTATTTTTTTGAAAATCA	
Intron 8	A(92128)G	GTATTTGAAGAGACAAGTCT[A/G] TTTCAAATACACAAAAATCA	
	–(104830～104831)T	TAGTGTCCAAATATGTTTTT[T/–] CTAAAGAAATATTTTGTTTG	
Intron 9	G(105010)A	CGTGTTGTTATCTAACCAAT[G/A] TTCCCTTTGTTACACAAATT	
	G(108172)T	CAGTGCTTCAGAAAATGACA[T/G] TCATATGAAACTGTGAAAAA	
Exon 10	C(108274)T	TCTTTTTTGATCTCACAGAA[T/C] ACCCAGAGGATCAGTAGTTC	N297N
Intron 10	C(112113)T	AGGAACTTTTGCAGTAGCTA[C/T] GTAAAAAATAGATTCCGTA	
	G(112114)A	GGAACTTTTGCAGTAGCTAC[G/A] TAAAAAATAGATTCCGTATG	
Intron 11	C(112353)G	TTACTCCTGGCCTACACACT[C/G] TCTTTTCCTATCAGTGACAG	
	G(113854)T	CTCTGGGCCCTTTTCCATCA[G/T] GCAGTGTCTCTACTGTATCA	
Exon 12	(CAG)n(n=4–15) (114048–114080)	GTATGACCCCATCGGGCTTC[(CAG)n] CCGCCGCCACCACCGCAGCC	(Q)n (n=4–15)
	CCG(114081–114083)–	AGCAGCAGCAGCAGCAGCAG[CCG/–] CCGCCACCACCGCAGCCCA	(P)n (n=4 or 5)
	G(114095)A	CAGCAGCCGCCGCCACCACC[G/A] CAGCCCCAGCCACAACCCCC	P435P
	CAACCCCCGCAGCCCCAGC CC(114108–114128)–	CACCACCGCAGCCCCAGCCA[CAACCCCCGCA GCCCCAGCCC/–]CGACAGGAAATGGGGCGCTC	7 amino acid deletion
Exon 12	C(114129)T	AACCCCCGCAGCCCCAGCCC[C/T] GACAGGAAATGGGGCGCTCC	R447X
	G(114143)T	CAGCCCCGACAGGAAATGGG[G/T] CGCTCCCCTGTGGACAGTCT	G451G
3'-UTR	GTGT(114459–114460)-	ATATATGTATGTGGGTGTGA[-/GTGT] GTGTGTATGTGTGGGTGTGT	
	G(114889)-	AAAACAAACAAAACAAAAAA[G/-] CCCCACACATAACTGTTTTG	
	T(114966)G	TAGCTAATAAAGAAAGAGAA[T/G] AGAAAACACGCATGAGATAT	
	-(115100～115101)C	TCATATCTTAAAAATTAAAG[-/C] AAACTGATTTTAGCTCATGT	

In the polymorphisms found in the exons, mutations of the amino acid sequence are also indicated.

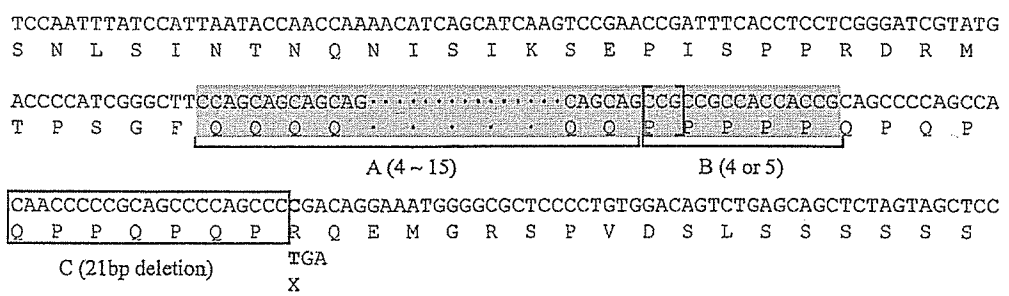

Fig 2. Nucleotide and amino acid sequences of the region containing the 4 variations in exon 12 of MEF2A. The gray box marks the polyglutamine and proline tandem repeats: the number of polyglutamine tandem repeats varies between 4 and 15, the number of proline tandem repeats varies between 4 and 5. The 21-bp deletion site is indicated by a box. The nonsense mutation (R447X) site is localized just downstream to the 21-bp deletion site.

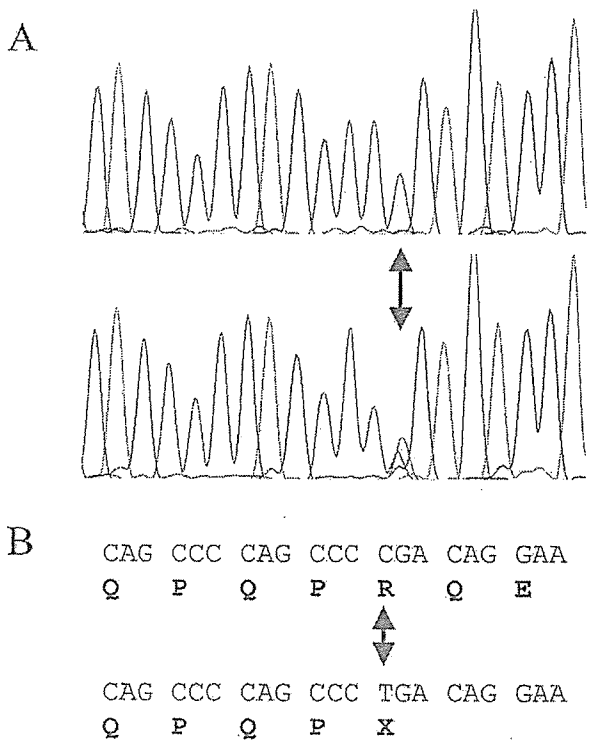

Fig 3. MEF2A nonsense mutation R447X in exon 12 in the subject with myocardial infarction (MI). (A) Sequence analysis of the control and MI subject indicated a C to T substitution at codon 447 in exon 12 of MEF2A. This mutation changes the amino acid residue arginine to stop codon (B).

Table 3 Frequencies of Haplotypes Defined by the 3 Genotypes

Haplotype	*C (numbers)*	*% (row)*	*MI (numbers)*	*% (row)*
A4B5C+	*26*	*2.23*	*10*	*1.32*
A5B5C+	*2*	*0.17*	*5*	*0.66*
A5B4C+	*1*	*0.09*	*0*	*0.00*
A6B5C+	*2*	*0.17*	*2*	*0.26*
A7B5C+	*5*	*0.43*	*2*	*0.26*
A8B5C+	*3*	*0.26*	*8*	*1.05*
A9B5C+	*396*	*33.90*	*263*	*34.61*
A9B5C–	*3*	*0.26*	*3*	*0.39*
A9B4C+	*78*	*6.68*	*50*	*6.58*
A10B5C+	*140*	*11.99*	*96*	*12.63*
A10B4C+	*0*	*0.00*	*2*	*0.26*
A11B5C+	*475*	*40.67*	*298*	*39.21*
A11B4C+	*6*	*0.51*	*1*	*0.13*
A12B5C+	*5*	*0.43*	*6*	*0.79*
A12B4C+	*0*	*0.00*	*1*	*0.13*
A14B5C+	*22*	*1.88*	*11*	*1.45*
A15B5C+	*4*	*0.34*	*2*	*0.26*
	1,168		*760*	

The frequencies of haplotypes defined by genotypes 1–3 in the control (C) or myocardial infarction (MI) groups are shown. The haplotypes are defined as follows: A represents the number of polyglutamine tandem repeats between 4 and 15 (region A in Fig 2); B represents the number of proline tandem repeats between 4 and 5 (region B); and C+ or – represents the existence or deletion of the 21-bp nucleotide (region C).

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Myocardial interstitial choline and glutamate levels during acute myocardial ischaemia and local ouabain administration

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Abstract

Aim: Noradrenaline (NA) uptake transporters are known to reverse their action during acute myocardial ischaemia and to contribute to ischaemia-induced myocardial interstitial NA release. By contrast, functional roles of choline and glutamate transporters during acute myocardial ischaemia remain to be investigated. Because both transporters are driven by the normal Na^+ gradient across the plasma membrane in a similar manner to NA transporters, the loss of Na^+ gradient would affect the transporter function, which would in turn alter myocardial interstitial choline and glutamate levels. The aim of the present study was to examine the effects of acute myocardial ischaemia and the inhibition of Na^+,K^+-ATPase on myocardial interstitial glutamate and choline levels.

Methods: In anaesthetized cats, we measured myocardial interstitial glutamate and choline levels while inducing acute myocardial ischaemia or inhibiting Na^+,K^+-ATPase by local administration of ouabain.

Results: The choline level was not changed significantly by ischaemia (from 0.93 ± 0.06 to 0.82 ± 0.13 μM, mean ± SE, $n = 6$) and was decreased slightly by ouabain (from 1.30 ± 0.06 to 1.05 ± 0.07 μM, $P < 0.05$, $n = 6$). The glutamate level was significantly increased from 9.5 ± 1.9 to 34.7 ± 6.1 μM by ischaemia ($P < 0.01$, $n = 6$) and from 8.9 ± 1.0 to 15.9 ± 2.3 μM by ouabain ($P < 0.05$, $n = 6$). Inhibition of glutamate transport by *trans*-L-pyrrolidine-2,4-dicarboxylate (*t*-PDC) suppressed ischaemia- and ouabain-induced glutamate release.

Conclusion: Myocardial interstitial choline level was not increased by acute myocardial ischaemia or by Na^+,K^+-ATPase inhibition. By contrast, myocardial interstitial glutamate level was increased by both interventions. The glutamate transporter contributed to glutamate release via retrograde transport.

Keywords acetylcholine, cardiac microdialysis, cats, coronary artery occlusion, myocardium, noradrenaline.

Acute myocardial ischaemia causes oxygen depletion and loss of ATP in the ischaemic region (Hearse 1979). Blockade of H^+-ATPase leads to noradrenaline (NA) leakage from storage vesicles and axoplasmic NA accumulation (Schömig *et al.* 1988). Intracellular acidosis causes Na^+ influx via Na^+/H^+ exchange. Inhibition of Na^+,K^+-ATPase activity reduces the Na^+ gradient across the plasma membrane. Because NA uptake transporters are driven by the normal Na^+ electrochemical gradient across the plasma membrane,

axoplasmic NA accumulation and reduction of the Na^+ gradient cause reverse transport of NA from the intracellular space to the extracellular space (Schwartz 2000). Acute myocardial ischaemia evokes the myocardial interstitial NA release in the ischaemic region via retrograde NA transport, independently of efferent sympathetic nerve activity (Schömig *et al.* 1984, Yamazaki *et al.* 1996, Akiyama & Yamazaki 1999, Kawada *et al.* 2001a).

Similar to NA, choline and glutamate are taken up into cells by plasma membrane transporters driven by the Na^+ gradient (Schwartz 2000). We hypothesized that the loss of Na^+ gradient under ischaemic conditions would interfere with the transporter function, which would in turn alter myocardial interstitial choline and glutamate levels. Choline release has been suggested as an index of ischaemic degradation of the myocardial phospholipid bilayer in isolated, Tyrode solution-perfused rat hearts (Brühl *et al.* 2004). Glutamate can be a preferred myocardial fuel during ischaemia and may have protective effects on ischaemic myocardium (Arsenian 1998). Measuring myocardial interstitial levels of these molecules *in vivo* would contribute to understanding the pathophysiology of acute myocardial ischaemia. To test the hypothesis, we employed an *in vivo* cardiac microdialysis technique and measured myocardial interstitial choline and glutamate levels in anaesthetized cats (Akiyama *et al.* 1991, 1994, Yamazaki *et al.* 1997, Kawada *et al.* 2001b). Acute myocardial ischaemia inevitably affects systemic haemodynamics and perfusion of the heart. To minimize such haemodynamic effects, we also examined the effects of Na^+,K^+-ATPase inhibition on the myocardial interstitial choline and glutamate levels by locally administering ouabain through a dialysis probe (Yamazaki *et al.* 1999, Kawada *et al.* 2002). The results of the present study indicated that the myocardial interstitial choline level was not increased by acute myocardial ischaemia or by Na^+,K^+-ATPase inhibition. By contrast, the myocardial interstitial glutamate level was increased by both interventions. The glutamate transporter contributed to glutamate release via retrograde transport.

Materials and methods

Surgical preparation

Animal care was conducted in strict accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences* approved by the Physiological Society of Japan. Adult cats weighing 2.0–4.8 kg were anaesthetized via an intraperitoneal injection of pentobarbital sodium (30–35 mg kg^{-1}) and ventilated mechanically with room air mixed with oxygen. The depth of anaesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg kg^{-1} h^{-1}) through a catheter inserted via the right femoral vein. Mean systemic arterial pressure was monitored from a catheter inserted via the right femoral artery.

With the animal in the lateral position, the left fifth and sixth ribs were resected to expose the heart. When a coronary occlusion was necessary, a 3-0 silk suture was prepared around the left anterior descending coronary artery (LAD) just distal to the first diagonal branch. With a fine guiding needle, a dialysis probe was implanted into the left ventricular free wall perfused by the LAD. Heparin sodium (100 U kg^{-1} bolus injection followed by a maintenance dose of 50 U kg^{-1} h^{-1}) was administered intravenously to prevent blood coagulation. At the end of the experiment the experimental animals were killed by an overdose of pentobarbital sodium. We confirmed that the dialysis probe had been implanted within the left ventricular myocardium.

Dialysis technique

We designed a transverse dialysis probe (Akiyama *et al.* 1991, 1994). For measurements of small molecular compounds including ACh, choline, and glutamate, we used a dialysis fibre of 50 000 molecular weight cutoff (13 mm length, 310 μm OD, 200 μm ID; PAN-1200, Asahi Chemical, Osaka, Japan) with both ends glued to polyethylene tubes (20 cm length, 500 μm OD, 200 μm ID). The dialysis probe was perfused at a rate of 2 μL min^{-1} with Ringer solution. Each sample was collected in a microtube containing 3 μL of phosphate buffer (100 mM, pH 3.5). A cholinesterase inhibitor eserine (100 μM) was added to the perfusate to measure ACh. A preliminary examination indicated that whether the perfusate-contained eserine did not affect myocardial interstitial choline levels significantly. Dead space volume between the dialysis fibre and the sample microtube was identical for ACh, choline, and glutamate measurements, and the sampling was performed taking into account the time for dialysate to traverse the dead space volume.

The dialysate ACh and choline levels were measured directly by high-performance liquid chromatography with electrochemical detection. The absolute detection limits of ACh and choline, determined with a signal-to-noise ratio of 3, were 10 and 5 fmol per injection, respectively. The dialysate glutamate level was measured by kinetic enzymatic analysis with CMA 600. The absolute detection limit of glutamate was 1 μM per injection.

Protocols

All protocols were started from 2 h after implanting the dialysis probe. To examine changes in myocardial

interstitial ACh and choline levels during acute myocardial ischaemia ($n = 6$), after collecting a 15-min baseline dialysate sample, we occluded the LAD for 60 min and obtained four consecutive 15-min dialysate samples. The full-length of the implanted dialysis fibre was located within the ischaemic area judged by discolouration of myocardium during the LAD occlusion. We then released the occlusion and collected a 15-min dialysate sample during reperfusion. To examine changes in myocardial ACh and choline levels in response to local ouabain administration ($n = 6$), after collecting a 15-min baseline dialysate sample, we replaced the perfusate with Ringer solution containing 100 μM ouabain and collected four consecutive 15-min dialysate samples.

In different groups of animals, myocardial interstitial glutamate levels were measured during acute myocardial ischaemia ($n = 6$) and during local administration of ouabain ($n = 6$). To elucidate the role of the glutamate transporter, we also examined the effects of glutamate transport inhibition by *trans*-L-pyrrolidine-2,4-dicarboxylate (*t*-PDC, 10 mM) on myocardial interstitial glutamate levels during acute myocardial ischaemia ($n = 7$) and local administration of ouabain ($n = 7$). *t*-PDC was locally administered through the dialysis probe to avoid systemic effects.

Statistical analysis

All data are presented as mean ± SE values. In each protocol, the effects of myocardial ischaemia or local ouabain administration were examined using one-way analysis of variance followed by Dunnett's test against the corresponding baseline level (Glantz 2002). The baseline as well as maximum glutamate levels with and without glutamate transport inhibition were compared by an unpaired *t*-test during acute myocardial ischaemia or during local ouabain administration (Glantz 2002). Differences were considered to be significant when $P < 0.05$.

Results

Figure 1a shows myocardial interstitial ACh level during acute myocardial ischaemia. The ACh level was increased by LAD occlusion, becoming approximately 15 times higher than the baseline level at 30–45 and 45–60 min of ischaemia. The ACh level decreased towards the baseline level upon reperfusion. Figure 1b illustrates myocardial interstitial choline level during acute myocardial ischaemia. The choline level did not change significantly throughout the ischaemic and reperfusion periods.

Figure 2a shows changes in myocardial interstitial ACh level during local administration of ouabain. The ACh level was increased by the inhibition of

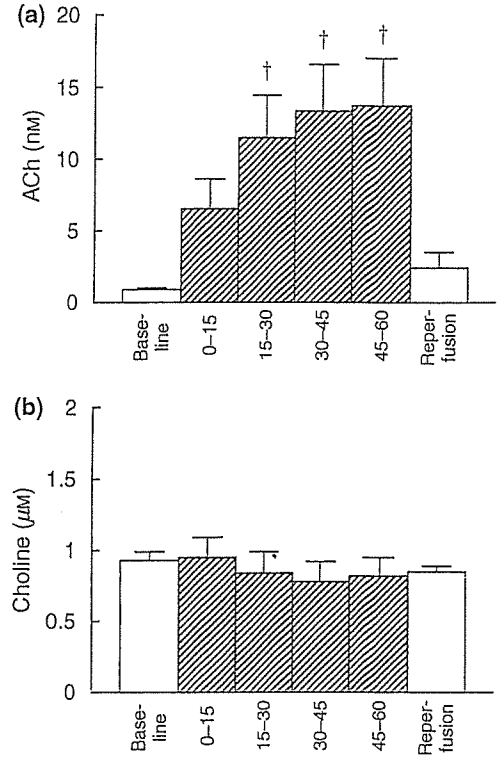

Figure 1 Changes in myocardial interstitial acetylcholine (ACh) level (a) and choline level (b) during coronary artery occlusion and reperfusion. Myocardial interstitial ACh level was significantly increased by acute myocardial ischaemia, while myocardial interstitial choline level was not changed. Data are mean ± SE. †$P < 0.01$ from baseline.

Na^+,K^+-ATPase, becoming approximately nine times higher than the baseline level at 15–30 min. The ACh level then decreased but remained significantly higher than the baseline level. Figure 2b illustrates the myocardial interstitial choline level during local administration of ouabain. The choline level was significantly lower at 0–15 and 45–60 min when compared with the baseline level.

Figure 3a shows changes in myocardial interstitial glutamate level during acute myocardial ischaemia. LAD occlusion increased the glutamate level to approximately 3.5 times higher than the baseline level at 0–15 min. Thereafter, the glutamate level was significantly higher than the baseline level throughout the ischaemic and reperfusion periods. Figure 3b illustrates the effects of glutamate transport inhibition on the ischaemia-induced glutamate release. The baseline glutamate level was significantly decreased by glutamate transport inhibition ($P < 0.05$). Although acute myocardial ischaemia and reperfusion significantly increased the glutamate level relative to the baseline level, the maximum glutamate level was attenuated to approximately one-fifth compared with that observed without glutamate transport inhibition ($P < 0.05$).

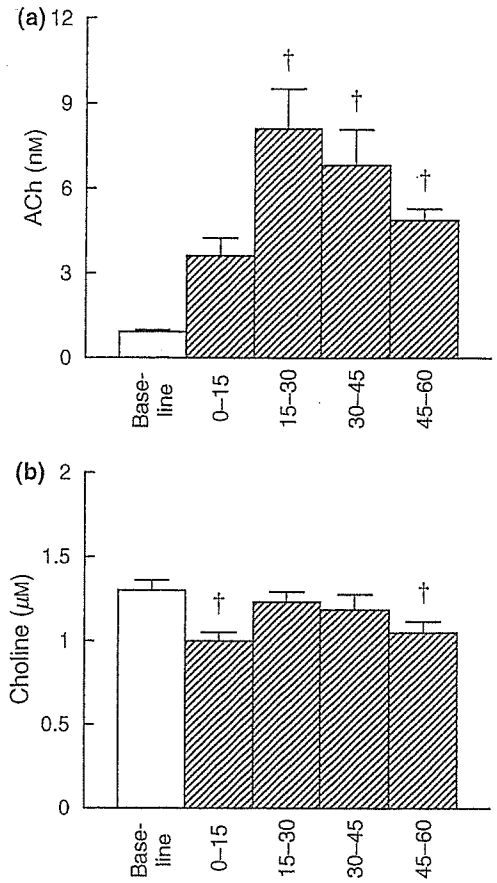

Figure 2 Changes in myocardial interstitial acetylcholine (ACh) level (a) and choline level (b) in response to the local administration of ouabain. Myocardial interstitial ACh level was significantly increased by ouabain. In contrast, myocardial interstitial choline level was decreased by ouabain. Data are mean ± SE. †$P < 0.01$ from baseline.

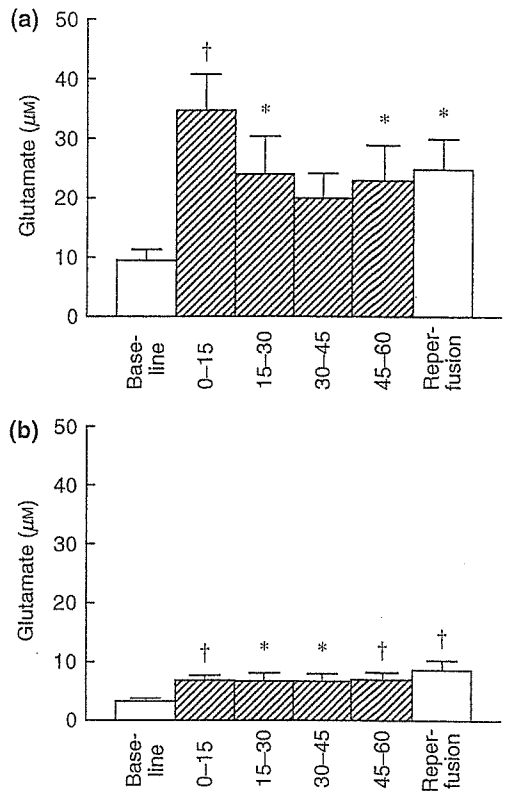

Figure 3 Changes in myocardial interstitial glutamate level during coronary artery occlusion and reperfusion without (a) and with (b) the inhibition of glutamate transporter. The glutamate level was significantly increased by acute myocardial ischaemia. The ischaemia-induced glutamate release was suppressed by the inhibition of glutamate transporter. Data are mean ± SE. †$P < 0.01$ and *$P < 0.05$ from baseline.

Figure 4a shows changes in myocardial interstitial glutamate level during the local administration of ouabain. Ouabain administration did not change the glutamate level at 0–15 min but increased the glutamate level thereafter. The glutamate level became approximately 1.8 times higher than the baseline level at 30–45 min. Figure 4b illustrates the effects of glutamate transport inhibition on ouabain-induced glutamate release. The baseline glutamate level was significantly decreased by the inhibition of glutamate transport ($P < 0.05$). Although ouabain administration increased the glutamate level relative to the baseline level, the maximum glutamate level was suppressed to approximately one-third of that observed without glutamate transport inhibition ($P < 0.05$).

Discussion

We have shown that acute myocardial ischaemia and local inhibition of Na^+,K^+-ATPase increased myocardial interstitial glutamate level but not choline level. Despite the similar Na^+ gradient dependency of corresponding transporters, myocardial interstitial glutamate and choline levels showed differential responses to the two interventions.

Changes in myocardial interstitial choline level

In the vagal nerve endings, ACh is hydrolysed to acetate and choline by acetylcholinesterase (Nicholls 1994). Choline is then taken up into the vagal nerve endings by the choline transporter driven by the Na^+ gradient. We hypothesized that loss of Na^+ gradient during acute myocardial ischaemia or local ouabain administration would increase the myocardial interstitial choline level by the interruption of choline uptake. Contrary to our hypothesis, acute myocardial ischaemia did not change myocardial interstitial choline level in the ischaemic region (Fig. 1b). Ouabain administration decreased the myocardial interstitial choline level at 0–15 and 45–60 min (Fig. 2b).

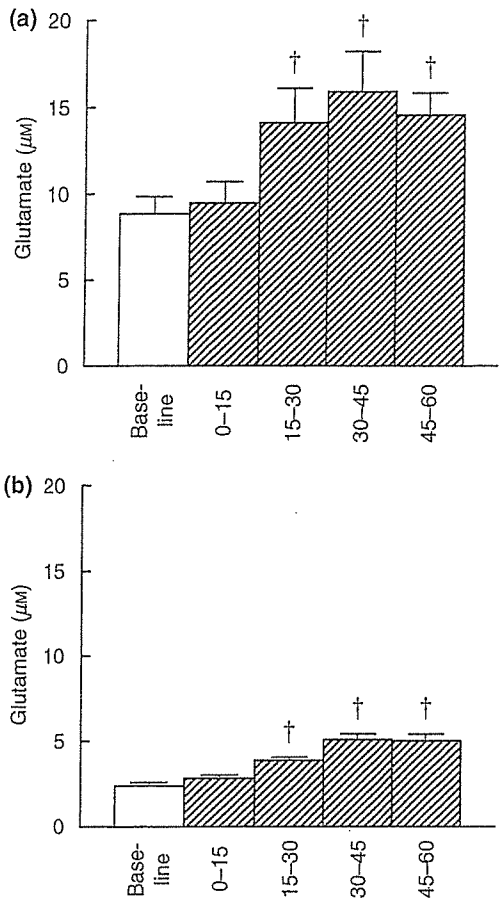

Figure 4 Changes in myocardial interstitial glutamate level in response to the local administration of ouabain without (a) and with (b) the inhibition of glutamate transporter. The glutamate level was significantly increased by ouabain administration. The ouabain-induced glutamate release was suppressed by the inhibition of glutamate transporter. Data are mean ± SE. †$P < 0.01$ from baseline.

Possible explanations for the absence of ischaemia- or ouabain-induced choline release are as follows. First, choline uptake is the rate-limiting step for ACh synthesis (Lockman & Allen 2002). Because choline in the intracellular space is rapidly consumed for ACh synthesis, the axoplasmic choline concentration might have been too low to evoke reverse transport by the choline transporter. Second, plasma choline concentration is stabilized by *de novo* choline synthesis from the catabolism of phosphatidylcholine found in cell membranes (Lockman & Allen 2002). Potential choline release may have been counterbalanced by the local stabilization mechanisms. Taking into account the recovery rate of the dialysis probe (approximately 30%), the myocardial interstitial choline concentration was 3–5 μM. Although the estimated concentration was lower than the highly regulated plasma choline concentration of approximately 10 μM, it was much higher than the ischaemia-induced maximum choline release (approximately 0.6 μM) in isolated rat hearts reported by Brühl *et al.* (2004). The present results suggest that myocardial interstitial choline level may not serve as an indicator of myocardial ischaemia in blood-perfused *in vivo* feline hearts.

By contrast with myocardial interstitial choline level, myocardial interstitial ACh level was increased both by acute myocardial ischaemia and by local administration of ouabain. Because ischaemia-induced ACh release was observed after vagal nerve transection in a previous study (Kawada *et al.* 2000), a Ca^{2+} channel-independent, regional release mechanism appears to be involved. Several reports have suggested that ouabain or ischaemia-induced intracellular Na^+ accumulation could elevate intracellular Ca^{2+} level via Na^+/Ca^{2+} exchange (Mochizuki & Jiang 1998, Li *et al.* 2000). The elevation of intracellular Ca^{2+} level may be associated with ACh release. Our previous study indicated that intracellular Ca^{2+} overload due to Ca^{2+} mobilization is responsible for the ACh release evoked by ischaemia (Kawada *et al.* 2000).

Changes in myocardial interstitial glutamate levels

Although the glutamate transporter family differs from the NA transporter family in that it requires counter-transport of K^+ instead of cotransport of Cl^-, its primary driving force is the Na^+ gradient across the plasma membrane (Schwartz 2000). Therefore, interventions that reduce the Na^+ gradient are likely to cause reverse transport of glutamate, in a similar manner to the reverse transport of NA. Acute myocardial ischaemia increased the myocardial interstitial glutamate level (Fig. 3a) as consistent with previous reports (Kennergren *et al.* 1997, 1999, Bäckström *et al.* 2003, Song *et al.* 1996). Inhibition of Na^+,K^+-ATPase also induced myocardial interstitial glutamate release (Fig. 4a). Glutamate release during acute myocardial ischaemia and local ouabain administration was significantly attenuated by the inhibition of glutamate transport (Figs 3b and 4b), suggesting the involvement of reverse transport by the glutamate transporter. Glutamate plays a vital role in keeping nitrogen balance in cells as a common amino acid in transamination reactions. The high intra-to-extracellular concentration ratio of glutamate would contribute to the retrograde transport by the glutamate transporter during the loss of normal Na^+ gradient.

In the case of myocardial interstitial NA levels, local blockade of NA uptake increased baseline NA levels, suggesting the accumulation of NA spontaneously released into the synaptic cleft (Akiyama & Yamazaki 1999). We therefore predicted that the inhibition of glutamate transport would increase the baseline gluta-

mate level. However, the inhibition of glutamate transport actually decreased the baseline glutamate level (Figs 3 and 4), suggesting that spontaneous glutamate release rather than glutamate uptake had occurred under baseline conditions. The insertion of a dialysis probe inevitably damages the myocardium. Although we waited for 2 h after implantation of the dialysis probe and the glutamate level declined with time, glutamate release from damaged myocardium may have continued. Notwithstanding this limitation, we were able to detect glutamate release in response to acute myocardial ischaemia and inhibition of Na^+,K^+-ATPase. Therefore, our interpretation that glutamate release was dependent on the reverse transport of glutamate transporter may be reasonable.

Supplementing the heart with glutamate has been shown to have beneficial effect on the recovery of contractile function in post-surgical patients (Arsenian 1998). The myocardial interstitial glutamate level remained increased during 15-min reperfusion whereas the myocardial interstitial ACh level returned towards the baseline level. Although the reason for different responses upon reperfusion was unanswered in the present study, the sustained increase in the glutamate level may have therapeutic effect on its own.

In conclusion, acute myocardial ischaemia and inhibition of Na^+,K^+-ATPase did not increase myocardial interstitial choline level despite a significant increase in myocardial interstitial ACh level. By contrast, both interventions significantly increased the myocardial interstitial glutamate level. The glutamate transporter contributed to myocardial interstitial glutamate release via retrograde transport.

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Microdialysis separately monitors myocardial interstitial myoglobin during ischemia and reperfusion

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Kitagawa, Hirotoshi, Toji Yamazaki, Tsuyoshi Akiyama, Masaru Sugimachi, Kenji Sunagawa, and Hidezo Mori. Microdialysis separately monitors myocardial interstitial myoglobin during ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 289: H924–H930, 2005. First published April 15, 2005; doi:10.1152/ajpheart.01207.2004.—Direct monitoring of myoglobin efflux during ischemia and reperfusion has been limited because of inherent sample collection problems in the ischemic region. Recently, the cardiac dialysis technique has offered a powerful method for monitoring myocardial interstitial levels of low-molecular-weight compounds in the cardiac ischemic region. In the present study, we extended the molecular target to high-molecular-weight compounds by use of microdialysis probes with a high-molecular-mass cutoff and monitored myocardial interstitial myoglobin levels. A dialysis probe was implanted in the left ventricular free wall in anesthetized rabbits. The main coronary artery was occluded for 60 or 120 min. We examined the effects of myocardial ischemia and reperfusion on myocardial interstitial myoglobin levels. Interstitial myoglobin increased within 15 min of ischemia and continued to increase during 120 min of ischemia, whereas blood myoglobin increased at 45 min of ischemia. Lactate and myoglobin in the interstitial space increased during the same period. At 60 min of ischemia, reperfusion markedly accelerated interstitial myoglobin release. The interstitial myoglobin level was fivefold higher at 0–15 min of reperfusion than at 60–75 min of coronary occlusion. The dialysis technique permits earlier detection of myoglobin release and separately monitors myoglobin release during ischemia and reperfusion. Myocardial interstitial myoglobin levels can serve as an index of myocardial injury evoked by ischemia or reperfusion.

infarction; interstitial space; membrane permeability

IT IS WELL KNOWN that certain proteins, including myoglobin, called serum cardiac markers, are released into the bloodstream in large quantities from necrotic cardiac muscle cells after myocardial infarction (20, 26, 43). However, because direct samples from the ischemic region are not readily obtainable, in situ studies on efflux of these proteins in the cardiac ischemic region have been limited (22). This problem of sample collection from the ischemic region remains unresolved. First, it is uncertain exactly when cardiac markers appear from injured myocardium. The appearance of cardiac markers indicates the turning point from reversible injury to irreversible damage (43). However, the first appearance of cardiac markers in the bloodstream is influenced by the slow transport of cardiac markers from the interstitial space into the bloodstream (20). Thus the detection of this appearance is of great value in understanding the pathophysiological events induced by myocardial ischemia. Second, recent experimental and clinical findings suggest that reperfusion itself seems to accelerate the release of cardiac markers (18, 37, 38). However, the extent to which reperfusion contributes to relative changes in their release is unclear. To determine myocardial injury evoked by reperfusion, more information is needed about the extent to which ischemia and reperfusion affect changes in the release of cardiac markers. Third, present methods used to measure infarct size require tissue analysis several hours after the ischemic event (8). Furthermore, histochemical analysis depends on the times of ischemia and reperfusion (23, 33). Concise, dissociated assessments of ischemia and reperfusion injury have been a frequent object of research.

In general, mobilization of cardiac markers from ischemic myocardium to the bloodstream has been divided into two different sequences: release from the myocardial cell to the interstitial space and transport from the interstitial space into the bloodstream (20). Therefore, if we examine the first process in in situ myocardium, we can discuss the pathophysiological changes during development of ischemic myocardial necrosis. However, little information is available on interstitial protein kinetics in the ischemic region (15). Examination of protein kinetics in the ischemic region has been limited to assessment of protein kinetics in the isolated Langendorff-perfused heart (28, 39). Recently, a cardiac dialysis technique has provided a powerful method for monitoring myocardial interstitial levels of low-molecular-weight compounds in the cardiac ischemic region (2, 6, 14, 31). Furthermore, this method is suitable for distinguishing between ischemia and reperfusion responses (32). By improving the microdialysis probes with a high-molecular-mass cutoff membrane, we have extended the molecular target to high-molecular-weight peptides and proteins and monitored myocardial interstitial protein levels.

In the present study, we chose myoglobin as one of the earliest biochemical markers in myocardial injury (4, 34). We applied the dialysis technique to the heart of anesthetized rabbits and investigated myocardial interstitial myoglobin levels during coronary occlusion and reperfusion. To address the above-mentioned issues, we compared the first appearance of myocardial interstitial myoglobin levels with that of low-molecular-weight metabolites (lactate and glycerol). Further-

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