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PHARMACO-ECONOMICS AND PHARMACO-EPIDEMIOLOGY

A Novel Data Mining Approach to the Identification of Effective Drugs or Combinations for Targeted Endpoints—Application to Chronic Heart Failure as a New Form of Evidence-based Medicine

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Summary. Background: **Data mining is a technique for discovering useful information hidden in a database, which has recently been used by the chemical, financial, pharmaceutical, and insurance industries. It may enable us to detect the interesting and hidden data on useful drugs especially in the field of cardiovascular disease.**

Methods & Results: **We evaluated the current treatments for chronic heart failure (CHF) in our institute using a decision tree method of data mining and compared the results with those of large-scale clinical trials. We enrolled 1,100 patients with CHF (NYHA classes II–IV and EF <40%) who were hospitalized at the National Cardiovascular Center during the past 31 months. Drugs prescribed at discharge were extracted from the clinical database. Both echocardiograms and plasma BNP level at 6–12 months after discharge were determined prospectively. It was found that beta-blockers, angiotensin converting enzyme inhibitors, and angiotensin II receptor antagonists independently improve both the plasma BNP level and %fractional shortening (FS), while oral inotropic agents increased the plasma BNP level and decreased %FS. These findings agree with evidence accumulated from several large-scale trials. Interestingly, statins, histamine receptor blockers, and alpha-glucosidase inhibitors also attenuated the severity of CHF, suggesting the possibility of new treatment of CHF.**

Conclusion: **Clinical data mining using Japanese CHF patients yielded almost identical data to the results of large-scale trials, and also suggested novel and unexpected candidates for CHF therapy. Further validation of the data mining approved in the cardiovascular field is warranted.**

Key Words. **chronic heart failure, Evidence based medicine, large-scale clinical trials, data mining method, novel therapy, cardiovascular disease**

Introduction

Evidence-based medicine (EBM) is an established way of testing drugs and a straightforward and ethically sound way to evaluate each treatment. Furthermore, EBM provides a direction and rationale for clinicians to manage their patients. However, there are several issues with regard to large-scale trials. First of all, large-scale trials require immense expense, prodigious labor, and sophisticated infrastructure to accomplish, so such trials cannot be performed frequently [1]. Secondly, prediction of the outcome is required at the time of planning and optimization of all the possible factors is not easy. Thirdly, a combinational explosion problem may be encountered when all drugs are used in the actual clinical situation. Fourthly, the different backgrounds of patients can make results controversial, even when almost identical large-scale trials are carried out [1,2]. Lastly and importantly, large-scale trials cannot produce or even predict the new treatment. To compensate these defects of large-scale trials, we propose the use of a novel method of data mining to detect effective drugs or drug combinations from the medical records of large numbers of patients. However, data mining methods have never been used in the medical field to find effective drugs or combinations of drugs.

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To test the applicability and feasibility of data mining methods, we examined whether this method could identify effective drugs that have been proven to decrease mortality, and whether it could discover novel drugs to improve the pathophysiology of diseases. Accordingly, we examined patients with CHF at the National Cardiovascular Center in Japan.

Methods

Patients

We enrolled 1,100 consecutive patients with CHF who were hospitalized and discharged from the National Cardiovascular Center between July 1, 2000 and January 31, 2003. CHF was defined on the basis of cardiac symptoms (NYHA classes II–IV) and cardiac dysfunction (EF of less than 40%).

Echocardiograms

During 6-12 months after discharge echocardiograms were performed and checked by doctors who had no information of the prescription of the patients at discharge. Echocardiographical measurements were performed using the Guidelines of the American Society of Echocardiography. Left ventricular end-diastolic and end-systolic dimensions were recorded by M-mode (SSA 260A, SSH 160A(Toshiba), Sonos 2000(Hewlett Packard), SSD 870, SSD 2200(ALOKA)). Fifteen trained cardiac echocardiography technicians randomly obtained echocardiograms of all of the patients in one echo-laboratory in our institute, which then were checked by one of two specialists.

The measurements of plasma BNP levels

Blood was sampled from each patient in the sitting position in a syringe containing both EDTA (1 mg/dl) and aprotinin (103 KIU/ml). Serum was separated within 6 hours and the samples were stored at −20°C until the measurements. The concentration of BNP was measured within 1 week after the plasma sampling by an immunoradiometric assay (IRMA) method (Shionoria BNP test in SRL laboratory, Tokyo, Japan). The test is a one-step immunoradiometric assay that uses two different monoclonal antibodies that recognize the C-terminal structure and the disulfide bond-mediated ring structure of BNP 32, respectively.

Table 1. Baseline Characteristics in hospitalization

Male : Female	776 : 324	Underlying disease	
Age (years old)	63.3 ± 1.3	Primary DCM	13%
LVDd/LVDs (mm)	61.9 ± 0.3/ 51.6 ± 0.39	Secondary DCM	14%
%FS (%)	16.8 ± 0.19	Valvular disease	32%
NYHA (II/III/IV)	440/506/154	HHD	13%
		IHD	28%

HHD: hypertensive heart disease, IHD : ischemic heart disease.

Table 2. Classification of %FS, BNP, and LVDd

%FS:	abnormally low	%FS ≤ 14%
	low	14% < %FS ≤ 25%
	gray	25% < %FS ≤ 30%
	normal	30% < %FS
BNP: (pg/ml)	normal	BNP ≤ 20
	gray	20 < BNP ≤ 200
	high	200 < BNP ≤ 1000
	very high	1000 < BNP ≤ 2000
	abnormally high	2000 < BNP
LVDd: (mm)	normal	LVDd ≤ 55
	Gray	55 < LVDd ≤ 60
	high	60 < LVDd ≤ 70
	abnormally high	70 < LVDd

Abbreviations: %FS: fractional shortening, LVDd: left ventricle end-diastolic dimension.

Data mining analysis

Both echocardiograms and plasma BNP levels over 6–12 months after the discharge were collected prospectively. Drugs prescribed at discharge were extracted from the clinical database. To make the numerical data more suitable for data mining, we classified fractional shortening (%FS), the plasma BNP level, and left ventricular end-diastolic diameter (LVDd) as shown in Table 2.

A total of 158 drugs were divided into 58 groups according to their pharmacological characteristics, such as inotropic agents (Table 3), and their doses were normalized.

A decision tree (C5.0) was used to analyze the relationship between the data. The details of the decision tree have been described by Podgorelec et al. [3]. A decision tree is a reliable and effective decision making technique, and provides high accuracy for the classification with a simple representation of gathered knowledge. Namely, a decision tree is the powerful and automatic subgroup analysis using the power of computer. Both %fractional shortening (FS) and the BNP level were considered as criterion variables and drug data as explanatory variables, since both %FS and BNP are known to be intermediate endpoints for the mortality or morbidity of patients with CHF. We performed the procedure twice, once using all 52 drug-groups and again using only the 21 drug groups frequently employed for cardiovascular disease.

Study organization

The study protocol was approved by the ethics committee of National Cardiovascular Center, and written consent was obtained from each patient.

Disclosure of a role of fundings

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Table 3. The 52 drug groups used for analysis

21 drug groups	Additional 31 drug groups
Beta-blockers	Antiulcer agents
Coronary dilators	Digestive enzyme agents
Adenosine-related agents	Proton pump inhibitors (PPIs)
Ca-antagonists	Medicines for intestinal disorders
Anti-arrhythmic drugs	Gastric agents
Pressor agents	Purgatives
Angiotensin-converting enzyme inhibitors (ACEIs)	Anti-allergy drugs
Alpha-blockers	Antigout drugs
Angiotensin receptor blocker (ARB)	Medicines for prostatic hypertrophy
Cardiotonic agents	Vitamins
Antiplatelet drugs	Anti-osteoporosis drugs
Anticoagulants	Bone metabolic turnover drugs
Anti-aldosterone drugs	Potassium agents
Diuretics	Serum potassium lowering agents
Insulin	Hematinic agents
Oral diabetic medicines	Anti-tuberculous agents
Alpha-glucosidase inhibitors	Respiratory anti-inflammatory drugs
Statins	Qral anti biotics
Choleretic drugs	Respiratory mucolytic agents
Anti-thyroid drugs	Broncodilators
Thyroid hormones	Anti-inflammatory drugs, painkillers
	Parasympathetic stimulants
	Antianxiety drugs
	Antiepileptics
	Tranquilizers
	Psychopharmaceuticals
	Antidinics
	Sedatives and hypnotics
	Iodine gargles
	Troches
	Topical steroids

A total of 159 drugs were divided into 52 groups.

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Results

Patient characteristics

Of the 1,100 patients, 776 and 324 were men and women, respectively, and the average age was 63.3 years. In detail, 13% and 14% of the patients suffered from primary or secondary dilated cardiomyopathy (DCM), respectively, while 32% had a valvular disease, 13% had hypertensive heart disease, and 28% had ischemic heart disease. Since we received DCM patients as candidates for heart transplantation from all over Japan, and these patients were relatively young, the average age was rather young compared with ordinary hospitals that care for myocardial infarction or hypertension.

Digitalis, diuretics, angiotensin converting enzyme inhibitors (ACEI), angiotensin-receptor blockers (ARB), spironolactone, and beta-blockers were used to treat 51, 68, 59, 12, 26, and 49%, respectively. The values of LVDd, %FS and the plasma BNP levels at 6–12 months after discharge were 54 mm, 29%, and 218 pg/ml, respectively (Table 1). As these are data on treatment, this suggests that we received patients with severe chronic heart failure, since our institute receives many patients with moderate-severe heart failure from all over Japan.

Data mining analysis using a decision tree

Figures 1–5 show the results of our decision tree analysis, where the left side represents the roots and the right side represents the branches. The final branch contains two numbers in parentheses and classification of a criterion variable (e.g. normal, gray, etc.). The numbers indicate the support and the confidence, respectively, where the support is the number of patients belonging to the branch and the confidence is the percentage of patients who fit the rule of the branch.

%FS

Figure 1 shows the tree for %FS with 21 drug groups. Data mining method revealed that ARB, calcium-antagonists, and alpha-glucosidase inhibitors can increase %FS, while inotropic agents decreased %FS in a dose-dependent manner. Figure 2 shows the analysis for %FS with 58 drug groups. Vitamins, ARB, alpha-glucosidase inhibitors, calcium antagonists, and ACEI also increased %FS.

The plasma BNP level

Figure 3 shows the decision tree for the plasma BNP level with 21 drug groups. Beta-blockers improved the plasma BNP level in a dose-dependent way. Calcium-antagonists, statins, and antiplatelet drugs also decreased the plasma BNP level, while inotropic agents caused the plasma BNP level to increase. Figure 4 shows the analysis of the plasma BNP level with 58 drug groups. Either ACEI or statins decreased the plasma BNP level, but either magnesium oxide or inotropic agents increased the plasma BNP level. Interestingly, either anti-ulcer drugs such as the blockers of histamine receptors (famotidine) or proton pump inhibitors (PPI) exerted beneficial effects depending on the conditions.

LVDd

Figure 5 shows the tree for LVDd. No difference was observed between the analysis with 21 drug groups and 58 drug groups. Beta-blockers decreased LVDd in both cases.

Discussion

We have shown that data mining may become a unique method for exploring useful drugs or drug combinations

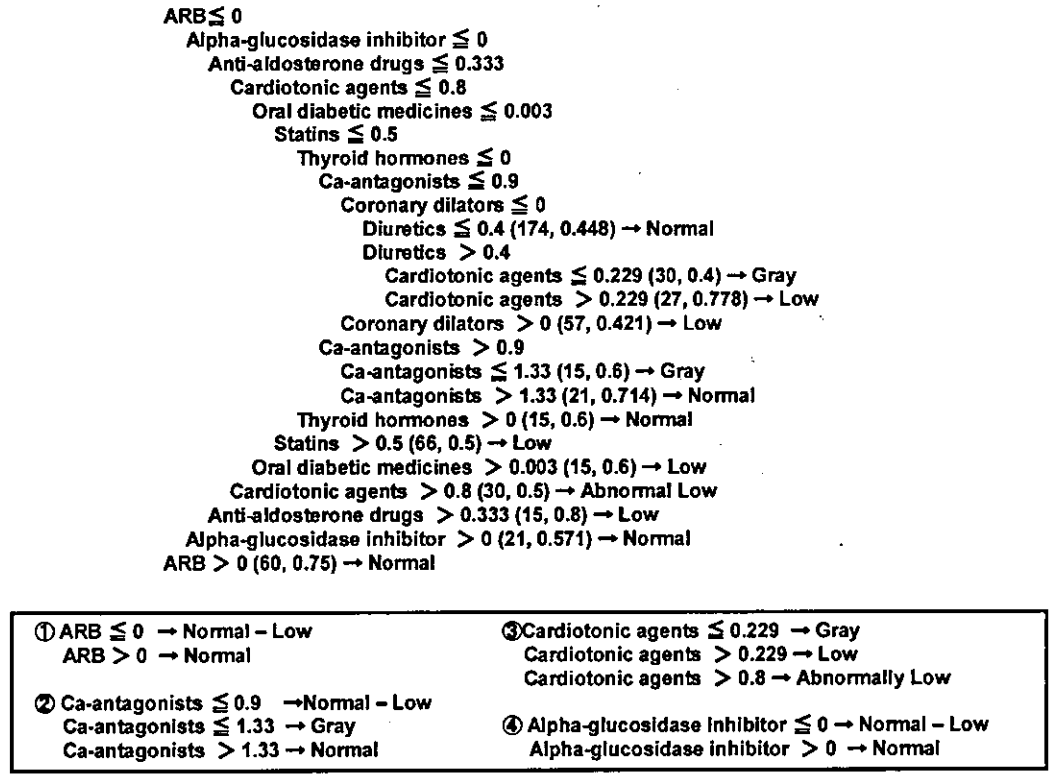

Fig. 1. Analysis of %FS with 21 drug groups. The upper panel shows the decision tree. The lower panel represents the simplified rules extracted from the tree. %FS (%): Abnormally Low: [0,14], Low(14,25), Gray: (25,30), Normal: (30,∞).

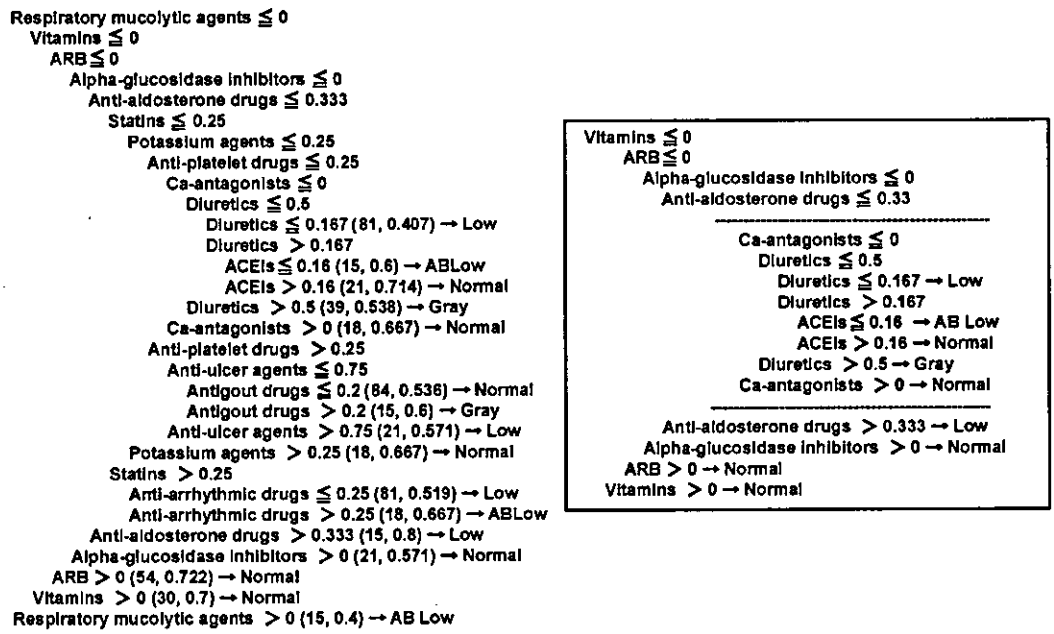

Fig. 2. Analysis of %FS with 52 drug groups. The left panel shows the decision tree. The right panel represents the simplified rules extracted from the tree. %FS (%): Abnormally Low: (0,14), Low: (14,25), Gray: (25,30), Normal: (30,∞).

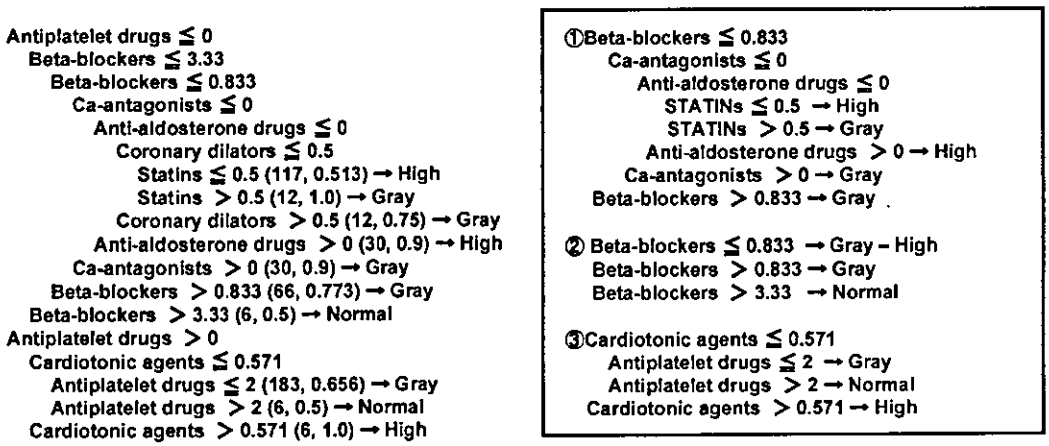

Fig. 3. Analysis of BNP with 21 drug groups. The left panel shows the decision tree. The right panel represents the simplified rules extracted from the tree. BNP(ug/ml), Normal: (0,20), Gray: (20,200), High: (200,1000), Abnormally High: (1000,2000), Very Abnormally High: (2000,∞).

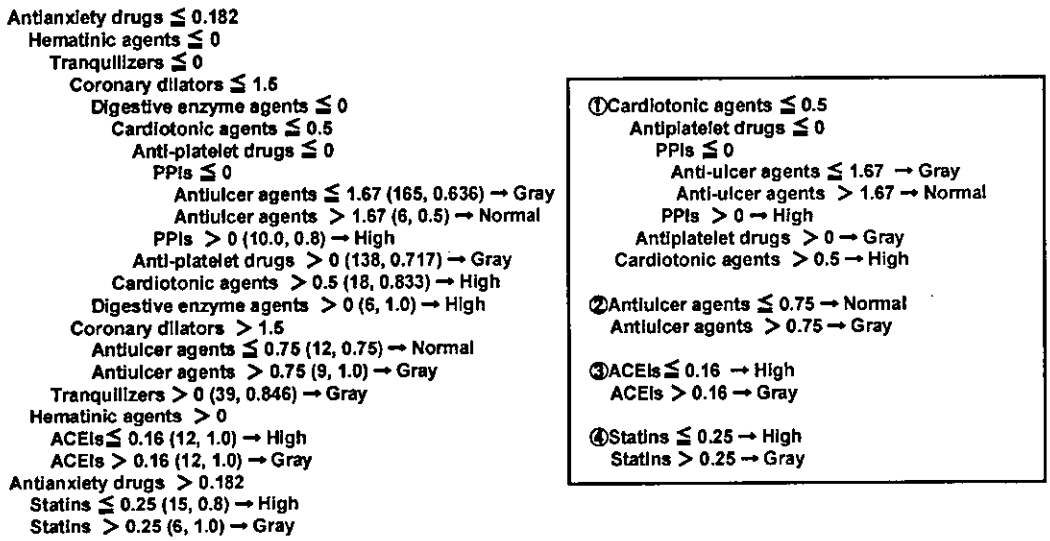

Fig. 4. Analysis of BNP with 52 drug groups. The left panel shows the decision tree. The right panel represents the simplified rules extracted from the tree. BNP(ug/ml), Normal: (0,20), Gray: (20,200), High: (200,1000), Abnormally High: (1000,2000), Very Abnormally High: (2000,∞).

from large clinical databases. Of course, the large-scale clinical trial is a very powerful method to show whether or not a certain drug is useful in a large number of patients. The analytical power of medical data mining does not seem to be less than that of the original large-scale trials. This is because the results of the original large-scale trials are also obtained by clinical data mining. We found that either beta-blockers or ACEI decreases the plasma BNP levels, and that ARB increases %FS, and that beta-blockers decrease LV dimension. On the other hand, inotropic agents decreased %FS. The original large-scale trials indicated that beta-blockers [4], ACEI [5, 6], or ARB [5,6] decreased both mortality and morbidity in patients with CHF, while inotropic agents increased mortality. Also, an increase in %FS [7], and a decrease in either plasma BNP level [8,9] or ventricular dimensions [10] are intermediate endpoints that predict the decrease in mortality and morbidity. Accordingly, medical data mining may be able to reproduce the results of large-scale clinical trials.

The present study suggests that unexpected drugs might be effective for the treatment of heart failure. Such information was not provided by large-scale trials. First, medical data mining suggested that statins might be effective for heart failure. Interestingly, we have previously reported that statins decrease cardiac

Diuretics ≦ 0.667
Antiplatelet drugs ≦ 1.75 (456, 0.572) → Normal
Antiplatelet drugs > 1.75 (15, 0.6) → High
Diuretics > 0.667
Beta-blockers ≦ 0.25 (48, 0.563) → High
Beta-blockers > 0.25 (33, 0.636) → Normal

Fig. 5. Analysis of LVDd with 21 and 52 drug groups. The decision tree is very simple. LVDd: Normal: (0,55), Gray: (55,60), High: (60,70), AB High: (70,∞).

hypertrophy and attenuate the severity of heart failure in mice with pressure overload [11], and we have recently found that statins are effective for CHF [12]. Furthermore, either anti-ulcer drugs such as famotidine or lansoprazole, antiplatelet drugs, or alpha-glucosidase inhibitors also attenuated the severity of CHF in the present study. These drugs are often used in patients with cardiovascular disease, but no large-scale trials have examined the effect of such non-cardiovascular drugs. These findings seem to be unexpected. However, histamine is reported to damage the tissues [13–15]. Aspirin is used as an antiplatelet drug and it attenuates the inflammatory process, while one of the mechanisms of CHF progression is believed to be inflammation [16,17]. In addition, alpha-glucosidase inhibitors attenuate postprandial hyperglycemia that increases oxidative stress [18,19], and oxidative stress is believed to be involved in heart failure [20]. Thus, these analyses may suggest new drugs for CHF that cannot be created by large-scale clinical trials.

However, there are several differences between medical data mining and large-scale trials. The cause and effect relationship is very tight in large-scale trials, while data mining theoretically indicates a possibility. The cause and effect relationship may be inverted; if a certain drug is used by the patients with severe heart failure, that drug may be determined as deleterious for heart failure. To avoid this possibility, we need to feedback the unexpected results to the experimental and clinical researches to determine the cause and effect relationship.

The important issue in a large-scale study, either an original large-scale trial or medial data mining, is to benefit patients in the clinical setting. Bozkurt [21] reported that spironolactone is used widely to treat HF without consideration of the NYHA class and ejection fraction, and without optimization of background treatment with ACEI and beta-blockers, which is against the RALES trial guidelines. The results of large-scale trials are sometimes difficult to transfer to the clinical field, e.g. confusion may arise in the clinical field with respect to the conflicting findings of the ALLHAT and ANBP2 trials [1,2]. Medical data mining can analyze the actual medication used for various conditions, with factors such as blood pressure, sex, or age being specified in the rules. This may make clinical translation easier and comprehensive analysis can be performed changing the criterion variables and explanatory variables, adding extra data, and using other data mining methods.

In conclusion, medical data mining provided almost identical data in Japanese CHF patients to the results obtained from large-scale CHF trials in the western countries, and also suggested novel candidates for CHF therapy. This method may be useful to evaluate or find new drugs in various filed of medicine, especially in the cardiovascular field.

Study Limitations

Examinations of echocardiograms and measurements of the plasma BNP levels were systematically performed only over 6-12 months after discharge. Because measurements of the parameters related to the severity of heart failure were only performed at one time point, we could not determine whether the drug usage after discharge directly improved either LV function or the plasma BNP level. On the other hand, we performed the BNP measurements and the echocardiographic examination in one laboratory in our institute. Our echocardiographic laboratory has 15 technicians for echocardiography who were trained by two specialists, and these technicians randomly performed echocardiography of all patients, which were then checked by either of two specialists. This system may maintain constantly high quality of echocardiography in our institute, which may make it possible to obtain meaningful results.

Although data mining is a very powerful method to find a useful hypothesis, and data mining can indicate the same results obtained from large-scale CHF trials, we should notice that this method is primitive rather than definitive. For example, we obtained the result that Ca channel blockers are effective for the heart failure, which has been defined by large-scale trials. However, recent evidence of VALUE and ACTION trials suggests that the long acting calcium channel blockers potently attenuate the incidence of acute myocardial infarction in high risk patients, and decrease the incidence of chronic heart failure in patients with stable angina pectoris [22,23]. On the other hand, we could not obtain the result that either ACE inhibitors or ARB decreased end-diastolic dimension as the results that beta-blockers did [24], although all of the drugs decrease mortality and morbidity of the patients with heart failure. These considerations suggest that we still need to perform prospective clinical trials for a definitive conclusion.

Contributions of Authors

Jiyoong Kim: the creation of the design of analysis
Takashi Washio: the data mining analysis and the interpretation of the data

Masakazu Yamagishi, Yoshio Yasumura, Satoshi Nakatani, Kazuhiko Hashimura, Akihisa Hanatani, kazuo Komamura: performance of the data collection and the interpretation of the data

Kunio Miyatake, Soichiro Ktamura, Hitonobu Tomoike: revising the manuscript critically for important intellectual content

Masafumi Kitakaze: final approval of this manuscript

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Original Article

Selective blockade of serotonin 5-HT2A receptor increases coronary blood flow via augmented cardiac nitric oxide release through 5-HT1B receptor in hypoperfused canine hearts

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Abstract

Serotonin (5-hydroxytryptamine [5-HT]), which induces vasoconstriction via 5-HT2A receptors in smooth muscle cells and vasodilation through activating nitric oxide (NO) synthase (NOS) via 5-HT1B receptors in endothelial cells, possesses divergent effects on regulating vascular tone. These facts lead us to consider that sarpogrelate, a 5-HT2A receptor blocker, may increase coronary blood flow (CBF) via either attenuation of vasoconstriction through 5-HT2A receptor blockade or augmentation of vasodilation by relative stimulation of NOS through 5-HT1B receptor and we tested this hypothesis in ischemic canine hearts. In open chest dogs, coronary perfusion pressure was reduced so that CBF was decreased to 33% of the baseline and kept constant. Thereafter, sarpogrelate was infused selectively into the left anterior descending artery with and without either an inhibitor of NOS (NG-nitro-L-arginine methyl ester (L-NAME)) or a 5-HT1B receptor antagonist (GR55562). An intracoronary administration of sarpogrelate increased CBF (34.0 ± 4.0 to 44.5 ± 4.4 ml/100 g/min, $P < 0.05$), along with the cardiac NOx release (3.2 ± 0.6 to 6.8 ± 1.2 nmol/ml, $P < 0.05$). The increases in both CBF and NOx by sarpogrelate were completely blunted by the co-administration of either L-NAME or GR55562. Interestingly, sarpogrelate increased the cardiac serotonin release (−4.8 ± 3.2 vs. 22.1 ± 1.5 ng/ml, $P < 0.05$, respectively) in the hypoperfused heart. Immunohistochemical analysis showed that sarpogrelate induced serotonin production in ischemic cardiac myocytes. These results suggest that sarpogrelate increases CBF via augmented cardiac NO production through 5-HT1B receptor activation along with the blockade of 5-HT2A receptors. The increase in cardiac release of serotonin may increase NO production in the ischemic heart.

Keywords: Serotonin (5-hydroxytryptamine, 5HT); Nitric oxide (NO); Ischemia; Coronary circulation; Sarpogrelate

1. Introduction

Serotonin (5-hydroxytryptamine, 5HT) has various subtypes of receptors, and causes both vasoconstriction and vasodilatation [1,2]. Serotonin causes vasoconstriction via activation of 5-HT2A receptors on vascular smooth muscle cells [3], and Vanhoutte [2] reported that serotonin can cause vasodilatation via a nitric oxide (NO)-dependent mechanism via 5-HT1B receptors. However, there is no clear consensus about the effects of serotonin on coronary circulation.

Sarpogrelate is a selective antagonist of 5-HT2A receptor widely used for patients with arteriosclerosis obliterans [4]. Since the blockade of 5-HT2A receptors by sarpogrelate may weaken the vasoconstriction and alternatively stimulates 5-HT1B receptors and therefore NO production, we hypothesized that sarpogrelate may increase coronary blood flow (CBF) in hypoperfused hearts. In the present study, we tested the effects of sarpogrelate on CBF in hypoperfused canine hearts using either NG-nitro-L-arginine methyl ester (L-

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NAME), an inhibitor of NO synthase (NOS), or a 5-HT1B receptor antagonist, GR55562. Then we determined whether sarpogrelate increases the differences in the plasma levels of NOx (metabolites of NO) and serotonin between coronary arterial and venous blood. Furthermore, we evaluated a potential mechanism by which sarpogrelate increased NOx release and identified the specific myocardium cell types from which serotonin was released using immunohistochemical technique.

2. Material and methods

All procedures were performed in careful conformance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1996). Experimental protocols were approved by the Osaka University Ethical Committee for Laboratory Animal Use.

3. Instrumentation

Thirty-three hybrid beagle dogs weighing 14–20 kg were anesthetized with sodium pentobarbital (30 mg/kg intravenously). The dogs were prepared as previously described [5]. Briefly, the proximal portion of the left anterior descending (LAD) coronary artery was cannulated and perfused with blood from the left carotid artery through an extracorporeal bypass tube. Either coronary perfusion pressure (CPP) or CBF was monitored at this tube.

A small, short collecting tube (diameter 1 mm, length 7 cm) was inserted into a small coronary vein near the perfused region to sample coronary venous blood in 33 dogs. Arterial samples were collected at the proximal edge of the bypass tube. Sarpogrelate hydrochloride was obtained from Mitsubishi Pharma Co.® (Japan), GR55562 from Tocris® (UK), L-NAME and a primary antibody against serotonin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4. Experimental protocols

4.1. Effects of sarpogrelate on coronary hemodynamics parameters in the ischemic myocardium (constant low CPP)

After hemodynamic stabilization, CPP was reduced so that CBF was decreased to 33% of baseline, using an occluder attached at the extracorporeal bypass tube. After CPP reduction, the occluder was adjusted to keep CPP constant for 5 min. Saline (n = 7; control group), sarpogrelate (10 μg/kg per min, n = 10; sarpo group), sarpogrelate + L-NAME (10 μg/kg per min, n = 9; sarpo + L-NAME group) or sarpogrelate + GR55562 (1 μg/kg per min, n = 7; sarpo + GR group) was infused selectively into the LAD, and measurements of all hemodynamic parameters were recorded at 5-min intervals for 20 min. Blood was sampled before and 20 min after the onset of hypoperfusion. We chose the dose of 10 μg/kg per min of sarpogrelate for an intracoronary administration so that the concentration of sarpogrelate in coronary circulation became nearly 10 μmol/l. This dose of sarpogrelate is known to abolish serotonin-induced vasoconstriction in an isolated human endothelium-denuded arterial segment of the left internal thoracic arteries [6]. The dose of 1 μg/kg per min of GR55562 was chosen for an intracoronary administration to achieve the concentration of GR55562 to the 1 μmol/l. This level of GR55562 inhibits vasorelaxation by sumatriptan, an agonist of 5-HT1B/1D receptors, in the rat isolated middle cerebral artery [7]. Preliminary experiments confirmed that L-NAME at the dose of the 10 μg/kg per min attenuates the coronary vasodilatory action of bradykinin (20 μg/kg per min, i.c.) by 85% ± 6%.

5. Biochemical analysis

We measured the metabolites of NO (nitrite and nitrate, NOx), using 2 ml of blood as described previously [8]. Additional blood was immediately placed on ice, and used for measurement for serotonin level as described previously [9]. The cardiac release of NOx and serotonin were defined as the differences in the plasma levels of NOx and serotonin between coronary arterial and venous blood, respectively.

6. Immunohistochemical analysis

After the hearts were perfused with phosphate-buffered saline, we sampled the hypoperfused hearts following a 15 min infusion of sarpogrelate. Immunohistochemical analysis was performed as described previously [10]. Briefly, tissue from the left ventricle of the excised hearts was fixed in 10% formaldehyde for several days and dehydrated with graded concentrations of alcohol for embedding in paraffin. Paraffin slices from each heart were stained with antibody against serotonin. All histopathological sections were scanned with a Olympus light microscope (BX40) equipped with a high resolution digital camera (Fujix HC 2000, Fujifilm).

7. Statistical analysis

The time course of changes in hemodynamic parameters in each group was compared by one-way repeated measures ANOVA. The time course of changes in hemodynamic parameters between groups was compared by repeated measures ANOVA. When ANOVA revealed a significant difference, modified Bonferroni's correction was applied. All values were expressed as mean ± S.E.M. A value of $P < 0.05$ was considered as significant.

8. Results

8.1. Effects of an intracoronary administration of sarpogrelate on coronary hemodynamics

As shown in Fig. 1, both baseline heart rate (HR) and CPP in each group were similar during the experiment. In the sarpo group, baseline HR and CPP averaged 152 ± 10 bpm and 104 ± 8 mmHg, respectively. When CBF was reduced to 33% of baseline, CPP of the LAD coronary artery was decreased to 42 ± 2 mmHg and kept constant thereafter. The intracoronary administration of sarpogrelate increased CBF compared with the control group (33.6 ± 6.1 vs. 44.5 ± 4.4 ml/100 g/min, $P < 0.05$) after 15 min of infusion, and this increase in CBF was completely abrogated by the co-administration of either L-NAME or GR55562 (Fig. 2).

8.2. Cardiac release of NOx in the ischemic canine heart

The infusion of sarpogrelate significantly increased the cardiac release of NOx compared with that of the control group after 20 min of hypoperfusion. Similarly, this increase was abolished by the co-administration of either L-NAME or GR55562 (Fig. 3).

8.3. Cardiac release of serotonin in the ischemic canine heart

Treatment with sarpogrelate for 15 min in a hypoperfused state significantly increased the cardiac release of serotonin (−4.8 ± 3.2 baseline vs. 22.1 ± 1.5 ng/ml, $P < 0.05$), which also reached a significant level compared with the control group after 20 min of hypoperfusion (Fig. 4).

8.4. Serotonin expression in the ischemic canine heart

Immunohistochemical analysis revealed that serotonin was weakly expressed in the non-ischemic heart and potently induced in the ischemic heart after sarpogrelate infusion (Fig. 5).

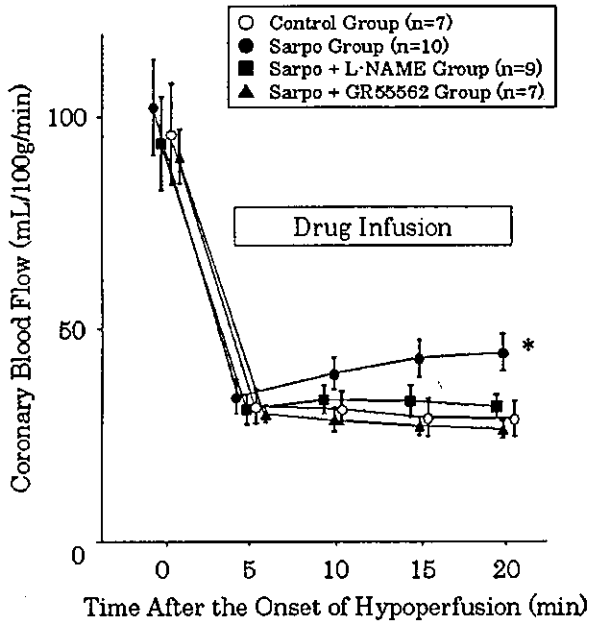

Fig. 2. Changes in CBF after infusion of sarpogrelate with and without L-NAME or GR55562. Although reduced CPP was at a constant low level, sarpogrelate increased CBF, with the effect being blunted by L-NAME or GR55562. * $P < 0.05$ versus the control group.

9. Discussion

We demonstrated here that sarpogrelate, an antagonist of 5-HT2A receptor, increased CBF via a NO-dependent mechanism through 5-HT1B receptor in hypoperfused canine hearts, along with the increase in the cardiac release of serotonin which may be produced in the ischemic myocardium.

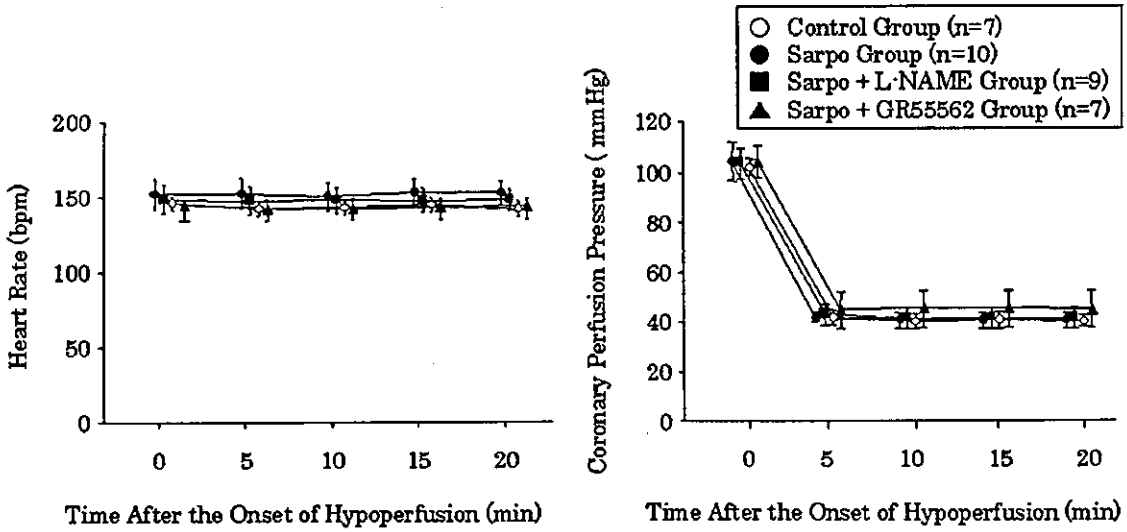

Fig. 1. Changes in HR and CPP among the control, sarpo, sarpo + L-NAME and sarpo + GR55562 groups. Both HR and CPP in each group were similar during the experiment.

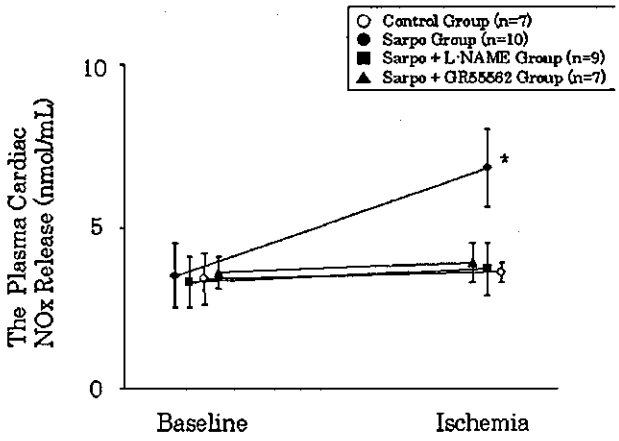

Fig. 3. Changes in the differences in the plasma levels of NOx between coronary arterial and venous blood among the control, sarpo, sarpo + L-NAME and sarpo + GR55562 groups. * $P < 0.05$ versus the control group after 20 min of hypoperfusion (ischemia).

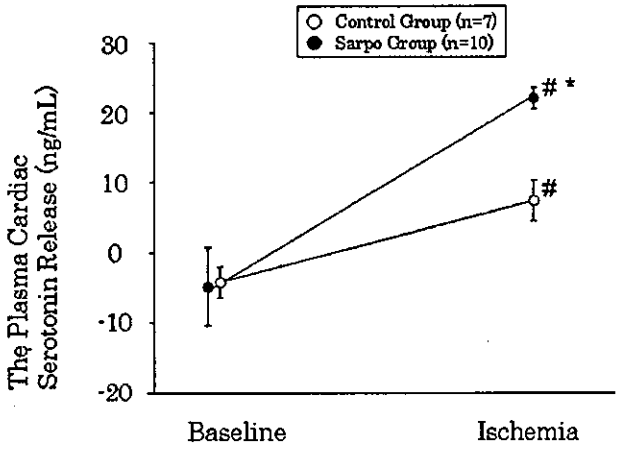

Fig. 4. Changes in the differences in the plasma levels of serotonin between coronary arterial and venous blood among the control and sarpo groups. * $P < 0.05$ versus the control group after 20 min of hypoperfusion (ischemia). # $P < 0.05$ versus before the onset of hypoperfusion (baseline) in each group.

9.1. Mechanisms by which sarpogrelate increased CBF and the cardiac release of NOx in the ischemic myocardium

In this experiment, we clearly demonstrated that sarpogrelate increased CBF in the hypoperfused hearts which may improve myocardial ischemia. Furthermore, the increase in CBF induced by sarpogrelate was abrogated by either the inhibition of NOS by L-NAME or the blockade of 5-HT1B receptor by GR55562. These results suggested that the increase in CBF induced by sarpogrelate was involved in both NOS and 5-HT1B receptors. Coincident with these findings, we confirmed that sarpogrelate increased NOx release in the coronary circulation, which was blunted by co-administration with GR55562. Thus, sarpogrelate increased CBF via a NO-dependent mechanism through 5-HT1B receptors.

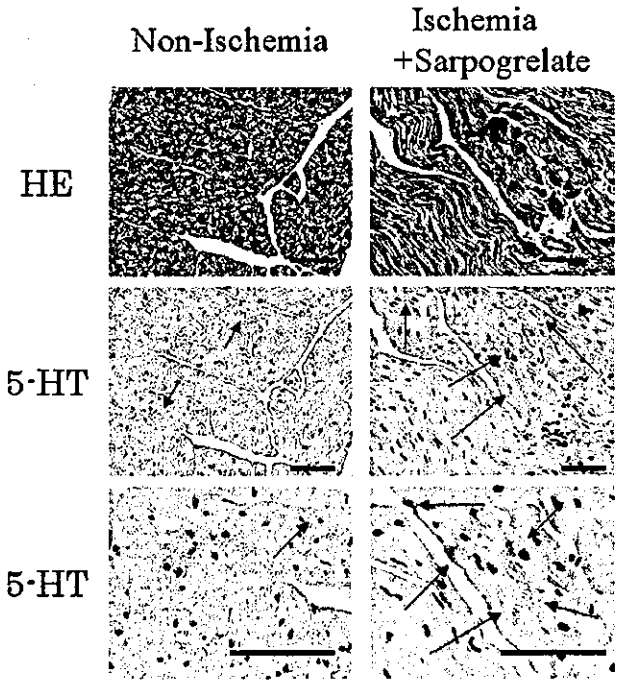

Fig. 5. Immunohistochemical analysis shows staining of serotonin in the non-ischemic myocardium and the ischemic myocardium after 20 min infusion of sarpogrelate. Upper panels show hematoxylin–eosin (HE) staining (×200). Middle (×200) and lower (×400) panels show immunohistochemical staining against 5-HT antibody. Bar indicates 1 μm.

9.2. Mechanisms by which sarpogrelate increased the cardiac release of serotonin in the ischemic myocardium

Vanhoutte [2] reported that serotonin may also enhance NO production via activation of 5-HT1B receptor in the endothelium. Furthermore, there is a report that serotonin evoked NO release in a dose dependent manner in human coronary artery endothelial cells [11]. The resulting production of NO stimulates soluble guanylate cyclase and results in vasodilation [12]. These reports suggest that increased serotonin may increase the cardiac release of NOx via 5-HT1B receptors. Our study confirmed that ischemia induced the cardiac release of serotonin, which was augmented by sarpogrelate. Thus, we suggest that sarpogrelate increased CBF via a NO-dependent pathway through the activation of 5-HT1B receptors induced by enhanced serotonin release. Serotonin is catalyzed by monoamine oxidases (MAO) [13]. A previous report has shown that ischemia decreased MAO activity in an ischemic kidney [14]. Therefore, in ischemic hearts, decreased MAO activity may increase plasma serotonin level by the accumulation of the undegraded serotonin. For the support of this idea, there is a report that the level of serotonin in the coronary effluent was elevated in ischemic isolated rat hearts, suggesting that serotonin was released from the ischemic myocardium [15]. In cardiac tissues, serotonin has been shown to be released from mast cells [16] and ganglia [17]. Although the precise mechanisms by which sarpogrelate augmented an increase in the cardiac release of

serotonin remain unclear, we demonstrated that an infusion of sarpogrelate increased serotonin staining in ischemic cardiomyocytes by immunohistochemistry. This result may partially contribute to an increase in the cardiac release of serotonin in the ischemic myocardium after infusion of sarpogrelate. Importantly, Shimizu et al. [18] showed the contradictory result that interstitial serotonin levels were increased in isolated ischemic rabbit heart that were abrogated by treatment with sarpogrelate. There might be several explanations for this discrepancy. First, we measured the plasma serotonin release in the ischemic canine heart not at the interstitial level as in the infarcted rabbit heart. In our study, we used the dogs with unimpaired endothelium on which serotonin can directly act. Second, as mentioned before, sarpogrelate might have stimulating effects on cardiac myocytes. Although this might be caused by the differences in animal species and models, further investigation is necessary to clarify serotonin released and metabolic mechanisms in the ischemic heart.

10. Clinical implications

In clinical settings, sarpogrelate is a selective 5-HT2A receptor blocker used for patients with arteriosclerotic obliterance because of its vasodilating and antiplatelet action [4]. Furthermore, sarpogrelate has been reported to be protective effects against human angina pectoris through an increase of collateral circulation [19]. We previously reported that an increase of CBF by benidipine attenuated the severity of ischemia gauged by lactate extraction rate and fractional shortening in the hypoperfused canine hearts [20]. Thus, the findings of this study may suggest another cardioprotective effect of sarpogrelate in ischemic heart disease.

11. Study limitation

Since coronary arteries in canine heart are covered with unimpaired endothelium, the beneficial effects of sarpogrelate in patients with arteriosclerosis may differ from our results. Further investigation will be needed to clarify these issues.

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Original Article

Opening of Ca^{2+}-activated K^+ channels is involved in ischemic preconditioning in canine hearts

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Abstract

Brief periods of ischemia that precede sustained ischemia can markedly reduce infarct size (IS), a phenomenon that is known as ischemic preconditioning (IP). Several investigators have shown that elevation of the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) during the antecedent brief periods of ischemia triggers the cardioprotective mechanism of IP. Since opening of Ca^{2+} activated K^+ (K_{Ca}) channels is reported to be cardioprotective, we hypothesized that these channels may be involved in the cardioprotective mechanism of IP. In anesthetized open-chest dogs, myocardial ischemia/reperfusion injury was created by occlusion of the left anterior descending coronary artery (LAD) for 90 min followed by 6 h of reperfusion. First, we showed that the treatment with NS1619, a K_{Ca} channel opener, reduced IS (IS in NS1619 group and control group, 19.8 ± 5.5% vs. 45.4 ± 3.5% of the area at risk, $P < 0.05$). Next, four cycles coronary occlusion for 5 min and reperfusion (IP) were performed before the 90-min occlusion with or without the infusion of potent K_{Ca} channel inhibitors, iberiotoxin (IbTX) and charybdotoxin (ChTX). IP markedly reduced IS (IS in the IP group was 8.2 ± 1.8%, $P < 0.01$ vs. control group). Infusion of either of K_{Ca} channel blockers during IP blunted the IS-limiting effect of IP (IS in the IP + IbTX and IP + ChTX groups was 30.7 ± 7.0% and 35.5 ± 3.7%, respectively, $P < 0.05$, vs. IP group). However, the cardioprotective effect of IP was not blunted by the treatment with ChTX when treated only during reperfusion (14.0 ± 4.1%). Thus, we conclude that the opening of K_{Ca} channel is involved in early trigger phase of the molecular mechanism of IP.

Keywords: Ischemic preconditioning; Ischemia; Reperfusion; Myocardial infarction; Ca^{2+}-activated K^+ channel

1. Introduction

Brief periods of ischemia that precede sustained ischemia can markedly decrease infarct size (IS), a phenomenon which is known as ischemic preconditioning (IP) [1–3]. This endogenous self-defense mechanism is one of the most powerful cardioprotective defenses against ischemia/reperfusion injury that has been demonstrated so far. Several investigators have previously revealed that an increase of the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) during the antecedent brief periods of ischemia triggers or mediates the cardioprotective mechanism of IP [4,5]. Among the intracellular sequelae of Ca^{2+} overload, opening of Ca^{2+}-activated K^+ channels (K_{Ca} channels) is known to occur. Indeed, we have reported that opening of K_{Ca} channels is involved in the limitation of IS by 17β-estradiol or raloxifene [6,7]. A recent report also suggested that K_{Ca} channel opening mediates cardioprotection [8], but it has not been elucidated whether cardioprotection

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} level; ChTX, charybdotoxin; IbTX, iberiotoxin; IP, ischemic preconditioning; K_{Ca} channel, Ca^{2+}-activated K^+ channel; LAD, left anterior descending coronary artery.

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due to IP is mediated through the K_{Ca} channels as well as ATP-sensitive K^+ channels.

We hypothesized that opening of the K_{Ca} channel in response to elevation of $[Ca^{2+}]_i$ may be involved in the cardioprotective mechanism of IP. We found that potent K_{Ca} channel inhibitors, charybdotoxin (ChTX) and iberiotoxin (IbTX), could abolish IP-induced cardioprotection in a canine ischemia/reperfusion model. We also found that this channel contributed to early phase of IP-induced cardioprotective machinery rather than late phase.

2. Materials and methods

All procedures were performed in accordance 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).

2.1. Instrumentation

Beagle dogs weighing 9–14 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg), intubated with a cuffed endotracheal tube, and ventilated using room air mixed with oxygen (1.5 l/min), as described previously [9]. Thoracotomy was performed through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. After an intravenous dose of heparin (500 U/kg), the proximal left anterior descending coronary artery (LAD) was cannulated and perfused with blood via an extracorporeal tube from the left carotid artery. Further heparin (100 U/kg) was administered intravenously every 3 h throughout the protocol. An occluder was attached to the bypass tube of the carotid-to-LAD shunt, and manual clamping of the tube was performed to produce myocardial ischemia. The pressure-resistant tube from the proximal portion of the cannula was connected to a multichannel recorder (Rm-6000; Nihon Kohden) to monitor arterial pressure. In addition, the left atrium was cannulated for the injection of microspheres.

2.2. Experimental protocols

2.2.1. Protocol 1: Effect of an intracoronary K_{Ca} channel opener (NS1619) on infarct size

Fig. 1 shows the details of this protocol. After hemodynamic stabilization, we injected NS1619 (11 μg/kg per min, Sigma, St. Louis, MO, USA: NS1619 group; $n = 6$) or NS1619 plus ChTX (0.3 μg/kg per min, Peptide Institute, Minoh, Osaka, Japan: NS1619 + ChTX group; $n = 6$) into the LAD through the bypass tube from 10 min before coronary occlusion until 1 h after reperfusion without a period of occlusion. We chose these doses of NS1619 and ChTX because these doses are maximal each that does not alter either systemic blood pressure or heart rate (HR), and sufficient to open and block the K_{Ca} channels, respectively. The ED_{50} of NS1619 is about 10 μM [10] and the calculated concentration

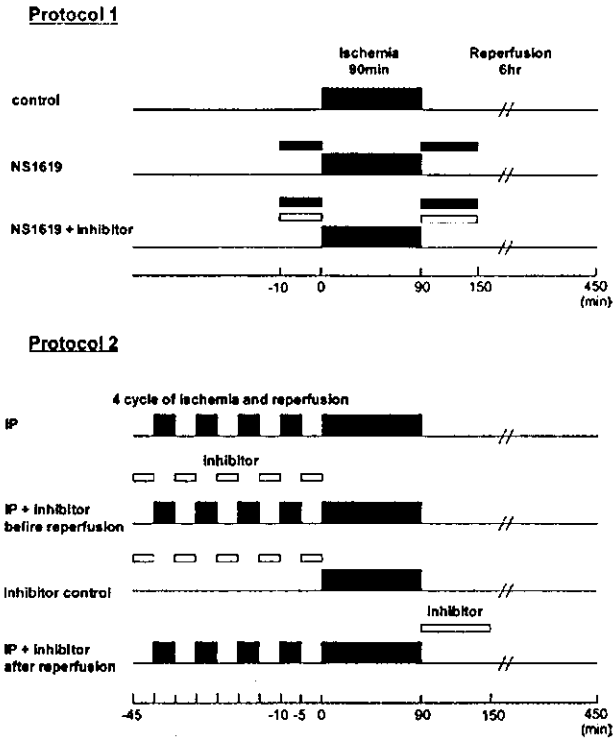

Fig. 1. All experimental protocols in this study are shown. IP: ischemic preconditioning.

of NS1619 used in this model was 40 μM. On the other hand, since K_d of ChTX is 2.1 nM [11], we need to use 10–100 times higher dose of K_d to fully block the channels. The calculated concentration of ChTX used in the present study was 100 nM, indicating that the dose of ChTX used in this protocol are sufficient to block the K_{Ca} channels.

Hemodynamic parameters were measured before the initiation of each protocol, 60 min after the onset of ischemia, and 1, 3 and 6 h after the onset of reperfusion.

2.2.2. Protocol 2: Effect of K_{Ca} channel inhibitors on IP

Fig. 1 displays the details of this protocol. After hemodynamic stabilization, four cycles of coronary occlusion for 5 min and subsequent reperfusion for 5 min (IP) were performed using the occluder with or without infusion of a K_{Ca} channel inhibitor throughout the IP procedure except during coronary occlusion. We used two different K_{Ca} channel inhibitors, IbTX (0.6 μg/kg per min, Peptide Institute) and ChTX. The following six groups were studied: Control group ($n = 6$), IP group ($n = 6$), IP + IbTX before reperfusion group ($n = 6$), IP + ChTX before reperfusion group ($n = 6$), IbTX group ($n = 6$), and ChTX group ($n = 6$). We chose the dose of IbTX because this dose is maximal and does not alter either systemic blood pressure or HR, and is sufficient to block the K_{Ca} channels. Since K_d of IbTX is 1 nM [12], we need to use 10–100 times higher dose of K_d to fully block the channels. The calculated concentrations of IbTX in this study is 200 nM, indicating that the dose of IbTX was also sufficient to block the K_{Ca} channels.

To distinguish the role of K_{Ca} channel in IP during ischemia or reperfusion period, we infused ChTX for 60 min after reperfusion in a group subjected to IP (IP + ChTX after reperfusion group, ($n = 8$)).

Hemodynamic parameters were measured at the same five times as in protocol 1.

2.3. Exclusion criteria

To ensure that all of the animals included in the analysis of IS were healthy and exposed to a similar extent of ischemia, we adopted the following criteria for exclusion of unsatisfactory dogs: (1) subendocardial collateral flow >15 ml/100 g per min, and (2) more than two consecutive attempts required to correct ventricular fibrillation with a low-energy counter pulse applied directly to the heart.

2.4. Measurement of infarct size and regional myocardial blood flow

We measured IS and regional myocardial blood flow as described previously [9]. For randomization, all measurements were done at completion of the protocol by persons without the knowledge of the treatment given to each heart.

2.5. Statistical analysis

Data are expressed as the mean ± S.E. Statistical significance was assessed with ANOVA, and if differences were found among groups, they were evaluated by Bonferroni's post-hoc test with $P < 0.05$ being considered as significant. The effect of collateral blood flow on IS was analyzed by ANCOVA, with regional collateral flow in the inner half of the left ventricular wall as covariant.

3. Results

3.1. Mortality and exclusions

We excluded 11 dogs from analysis because subendocardial collateral blood flow was greater than 15 ml/100 g per min. Ventricular fibrillation that matched the exclusion criterion occurred in seven animals during the 6-h reperfusion period and six of the seven died of ventricular fibrillation (Table 1). There were no significant differences in the number of exclusions among the groups.

3.2. Hemodynamic parameters, area at risk, and collateral blood flow

During protocols 1 and 2, the HR and mean arterial blood pressure (MAP) remained stable throughout the study (Table 2). The area at risk and the collateral blood flow were also similar among all of the groups, and there was no statistical difference (Table 3).

3.3. Infarct size

Fig. 2 shows IS in the nine groups from protocols 1 and 2. Treatment with NS1619 before and after ischemia reduced IS (19.8 ± 5.5% vs. 45.4 ± 3.5% of the area at risk, compared with the control group), while ChTX completely blocked this IS-limiting effect (42.2 ± 5.8%). IP markedly reduced IS compared with the control group (8.2 ± 1.8%). Treatment with either K_{Ca} channel inhibitor during IP blunted the IS-limiting effect (IP + IbTX group and IP + ChTX group: 30.7 ± 7.0%* and 35.5 ± 3.7%*, respectively, * $P < 0.05$ vs. the IP group). Either inhibitor alone, had no influence on IS (IbTX group and ChTX group: 37.1 ± 4.5% and 40.7 ± 6.1%, respectively). However, cardioprotective effect of IP was not influenced by treatment after reperfusion with ChTX (14.0 ± 4.1%).

ANCOVA test showed that these effects of K_{Ca} channel opener and inhibitor were independent from that of collateral blood flow (Fig. 3, and Table 4).

4. Discussion

We showed that an intracoronary administration of a K_{Ca} channel opener (NS1619) mimicked the IS-limiting effect in

Table 1
Number of dogs assigned to and excluded from each group for measurement of IS

Group	Number of dogs originally assigned	Number of dogs used for data analysis	Reason for exclusion		
			Vf (>2) during 6 h of reperfusion	Death due to Vf	High collateral flow (>15 ml/100 g per min)
Protocols 1, 2					
Control	7	6	0	1	0
NS1619	10	6	0	1	3
NS1619 + ChTX	8	6	0	0	2
IP	7	6	0	0	1
IP + IbTX before reperfusion	11	6	0	2	3
IP + ChTX before reperfusion	7	6	1	0	0
IbTX	7	6	0	1	0
ChTX	6	6	0	0	0
IP + ChTX after reperfusion	11	8	0	1	2

Vf: ventricular fibrillation, IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin.

Table 2
Hemodynamic parameters during protocol

Group	Baseline		60 min of ischemia		1 h after reperfusion		3 h after reperfusion		6 h after reperfusion	
	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)
Protocols 1, 2										
Control	99 ± 5	141 ± 7	95 ± 5	139 ± 12	98 ± 5	139 ± 9	95 ± 4	132 ± 12	92 ± 4	133 ± 9
NS1619	104 ± 4	135 ± 7	92 ± 7	134 ± 6	92 ± 5	138 ± 7	91 ± 4	146 ± 7	92 ± 4	141 ± 6
NS1619 + ChTX	106 ± 5	142 ± 3	103 ± 6	134 ± 8	100 ± 9	137 ± 9	97 ± 6	136 ± 8	93 ± 6	142 ± 8
IP	106 ± 6	134 ± 7	100 ± 7	133 ± 7	104 ± 7	134 ± 7	102 ± 6	134 ± 7	101 ± 8	132 ± 7
IP + IbTX before reperfusion	104 ± 4	135 ± 9	96 ± 7	134 ± 5	91 ± 3	134 ± 9	92 ± 7	128 ± 14	90 ± 7	133 ± 14
IP + ChTX before reperfusion	105 ± 9	143 ± 8	90 ± 10	146 ± 8	92 ± 9	129 ± 8	96 ± 7	136 ± 9	94 ± 9	138 ± 9
IbTX	101 ± 8	139 ± 8	102 ± 8	137 ± 6	104 ± 7	134 ± 7	98 ± 6	130 ± 6	92 ± 6	133 ± 6
ChTX	99 ± 3	133 ± 9	98 ± 8	136 ± 8	100 ± 7	133 ± 7	104 ± 7	130 ± 7	102 ± 8	134 ± 8
IP + ChTX after reperfusion	104 ± 2	143 ± 6	101 ± 3	135 ± 8	101 ± 3	151 ± 7	99 ± 4	142 ± 9	106 ± 4	137 ± 6

MAP: mean arterial pressure, IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin. Data were shown by mean ± S.E. There was no significant difference among each protocol.

Table 3
Collateral blood flow and area at risk among experimental groups

Group	Collateral blood flow (ml/100 g per min)	Area at risk (%)
Protocols 1, 2		
Control	7.8 ± 1.6	46 ± 4
NS1619	6.5 ± 1.3	49 ± 6
NS1619 + ChTX	7.3 ± 1.5	39 ± 2
IP	9.0 ± 1.4	41 ± 3
IP + IbTX before reperfusion	6.8 ± 1.3	51 ± 5
IP + ChTX before reperfusion	7.9 ± 1.1	42 ± 2
IbTX	7.3 ± 1.4	44 ± 4
ChTX	8.5 ± 1.1	39 ± 2
IP + ChTX after reperfusion	8.7 ± 1.0	41 ± 2

IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin. Data were shown by mean ± S.E. There was no significant difference among each protocol.

Table 4
Linear regression model test in each group

Group	Formula
Control	$y = 50.032 - 0.586x$
NS1619	$y = 30.649 - 1.667x$ *
NS1619 + ChTX	$y = 61.806 - 2.694x$
IP	$y = 15.228 - 0.786x$ *
IP + IbTX before reperfusion	$y = 49.482 - 2.792x$ *, †
IP + ChTX before reperfusion	$y = 42.162 - 0.852x$ †
IbTX	$y = 47.653 - 1.439x$
ChTX	$y = 56.948 - 1.921x$
IP + ChTX after reperfusion	$y = 37.367 - 2.703x$ *

IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin. * $P < 0.05$ vs. control group, † $P < 0.05$ vs. IP group.

a canine ischemia/reperfusion model potent, and K_{Ca} channel inhibitors (ChTX and IbTX) blocked the cardioprotective effect of IP. We also showed that the cardioprotective effect of IP was not blunted by the treatment with ChTX only during the reperfusion period. These data suggest that opening of K_{Ca} channel is involved in early phase of the molecular mechanism of IP.

Since Murry et al. [1] first demonstrated the intriguing phenomenon known as IP, numerous studies have been done to elucidate the cellular mechanisms responsible. There is considerable evidence that $[Ca^{2+}]_i$ increases transiently during ischemic episodes that produce IP and it may be the key factor in IP [4,5,13–15]. Direct measurement of $[Ca^{2+}]_i$ has shown that a brief period of ischemia increases it two to fourfold [16,17]. On the other hand, exogenous calcium triggers the IS-limiting effect, and our previous study showed that a calcium chelator abolished the cardioprotective effect of IP [5]. While $[Ca^{2+}]_i$ is increased during antecedent ischemia, it is paradoxically reduced during subsequent sustained ischemia [18,19]. Indeed, it is well recognized that ischemia/reperfusion causes intracellular Ca^{2+} overload and thus leads to the death of cardiomyocytes [20]. Generally, the K_{Ca} channel opens after elevation of $[Ca^{2+}]_i$ and causes membrane hyperpolarization, which reduces voltage-dependent Ca^{2+} influx by increasing K^+ efflux and thus prevents Ca^{2+} overload. This sequence suggests that K_{Ca} channel opening is a candidate for mediating IP.

The outward K^+ channels comprise the voltage-dependent channel (K_V channel) and the calcium-dependent channel (K_{Ca} channel). The K_{Ca} channel is separated into three subclasses (BK, IK, and SK) according to its conductance. It has been demonstrated that the K_{Ca} channel (generally BK) is present in various muscular and non-muscular tissues. In addition to its distribution in a variety of cell types, Kawakubo et al. [21] demonstrated the existence of BK channels on ventricular cardiomyocytes by using the patch-clamp technique. In addition, we have previously shown that opening of the K_{Ca} channels has a cardioprotective effect without affecting coronary blood flow. Indeed, it has been reported that the K_{Ca} channel is located on vascular smooth muscle cells (SMC), and that endothelium-derived hyperpolarizing factor causes vasodilation by activating this channel [22,23]. To exclude the possibility of an effect on coronary and collateral flow, we determined the dose of NS1619 (11 μg/kg per min) that did not increase coronary flow. In fact, there was no significant difference of collateral flow (Table 3), which implies that opening of K_{Ca} channels in cardiac tissues other than SMC may also be important.

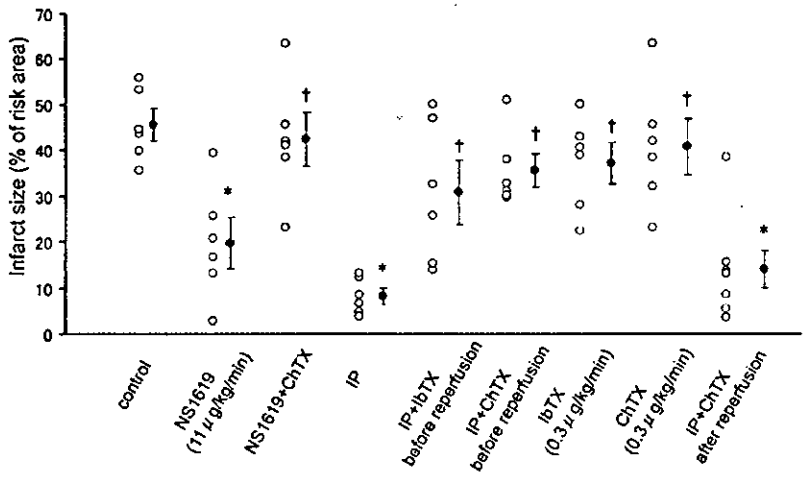

Fig. 2. IS as a percentage of the area at risk in all nine experimental groups in protocols 1 and 2. Data from individual animals and mean ± S.E. are shown. * P <0.05 vs. control group, † P <0.05 vs. IP group. IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin.

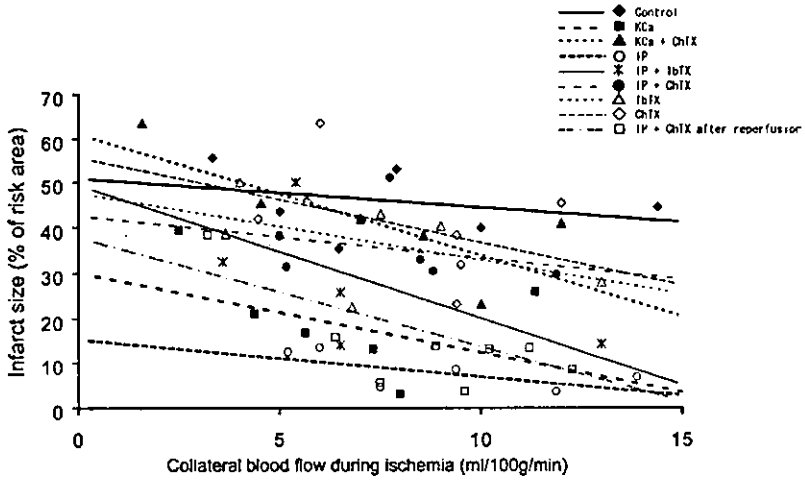

Fig. 3. IS in protocols 1 and 2 as a percentage of the risk area vs. regional collateral blood flow during ischemia. See Fig. 2 legend for explanation of each group. The results of ANCOVA test is indicated in Table 4.

Recently, it was demonstrated that infusion of NS1619 5–10 min before sustained ischemia could reduce IS through mitochondrial K_{Ca} channels in an isolated rabbit heart model [8]. It was suggested that opening of this channel improves mitochondrial ATP production and decreases both the production of reactive oxygen species and Ca^{2+} overload in the mitochondria. This mechanism may also contribute to the cardioprotective effect of the K_{Ca} channel during IP in addition to reduction of voltage-dependent Ca^{2+} influx by promotion of K^+ efflux.

Generally, some care is needed when interpreting experiments that employ pharmacological agents. ChTX mainly acts on BK channels, but it also interacts with IK1 and K_V 1.3 type channels [24]. We tested the influence of IbTX on IP as well as ChTX, since IbTX is a selective blocker of large-conductance BK type K_{Ca} channels. Our results showed that both K_{Ca} channel inhibitors abolished IP-induced cardioprotection, making it likely that this channel is involved in the mechanism of IP rather than the possibility of a nonspecific effect or the contaminating effect of other channels.

Protein kinase C (PKC) is believed to play an important role in triggering IP, and elevation of $[Ca^{2+}]_i$ activates PKC [5,25]. However, activation of PKC was reported to inhibit the K_{Ca} channel [26]. Since we have previously reported that PKA is involved in IP [27] and many authors have shown that PKA activates the K_{Ca} channel, [28–32] it seems that PKA activation following brief ischemia also has a role in opening the K_{Ca} channel as well as the elevation of $[Ca^{2+}]_i$. Adenosine has also been reported to open K_{Ca} channels, and it may be involved in the mechanism of IP [33].

In the present study, we found evidence that K_{Ca} channel opening contributes to IP, as well as the ATP-sensitive K^+ channel which is believed to play a central role in IP. Further investigations will be required to clarify the importance of the K_{Ca} channel to IP.

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