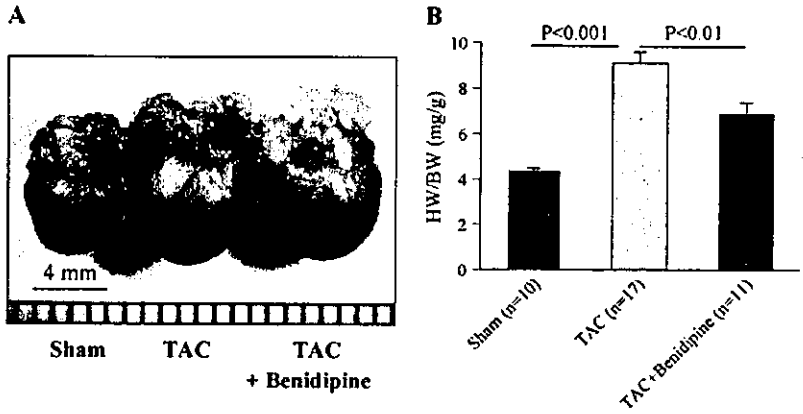

Fig. 3. Benidipine inhibits cardiac remodeling. (A) Representative pictures of whole hearts. (B) The heart to body weight ratio (HW/BW) was significantly decreased in TAC mice treated with benidipine (10 mg/kg/d) compared with untreated TAC mice.

3.3. Benidipine prevents progression from hypertrophy to heart failure

TAC induced congestive heart failure with a reduction in LVFS and increase of pulmonary congestion. LVFS measured by echocardiography was significantly higher in benidipine-treated mice than in TAC mice (Fig. 5A,B). Compared with the value for sham-operated mice, the lung weight to body weight ratio (LW/BW) was increased by about 108% in TAC mice, but only rose by 46% in benidipine-treated mice (Fig. 5C,D).

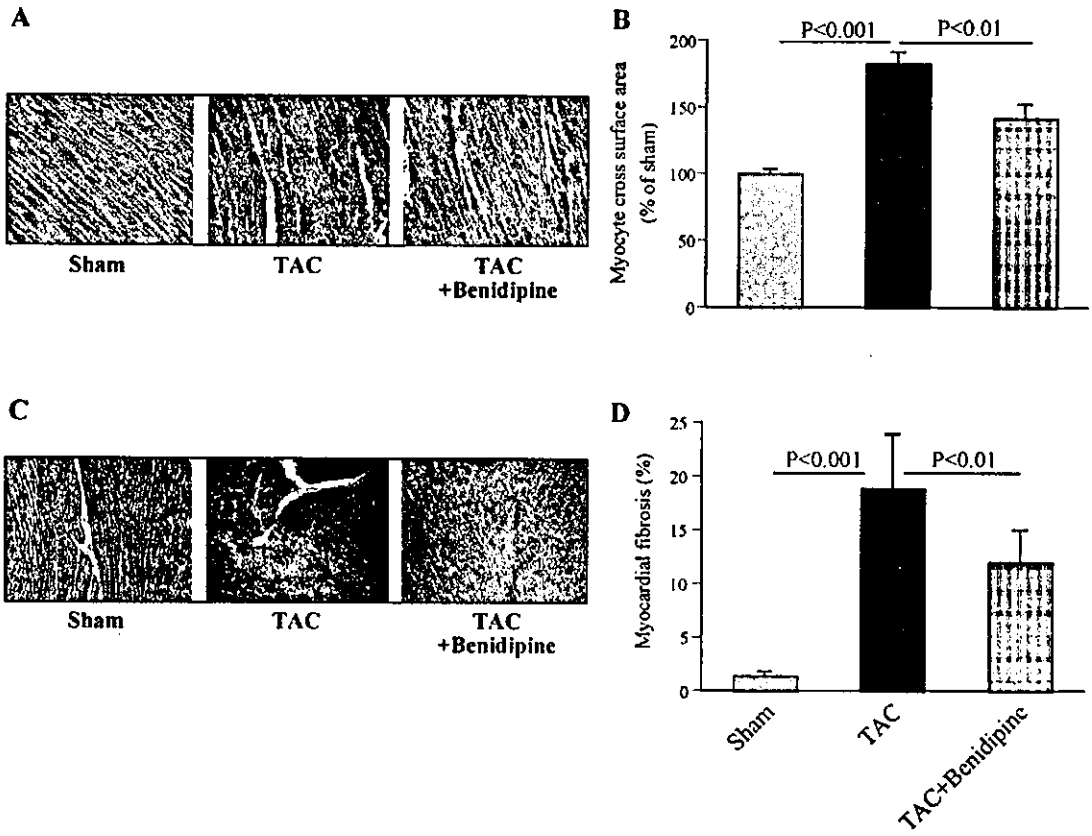

Fig. 4. Results of histological examination. (A) Representative images of the myocardium (HE stain×200). (B) The cross-sectional area of cardiac myocytes was significantly increased in TAC mice by pressure overload for 4 weeks, while treatment with benidipine blunted the enlargement of myocytes. (C) Representative pictures of myocardial fibrosis (Azan–Mallory stain×100). (D) Quantitative analysis showed that benidipine significantly inhibited myocardial fibrosis due to pressure overload for 4 weeks. Three hearts per group were used to determine the cross-sectional area of cardiac myocytes and the extent of myocardial fibrosis.

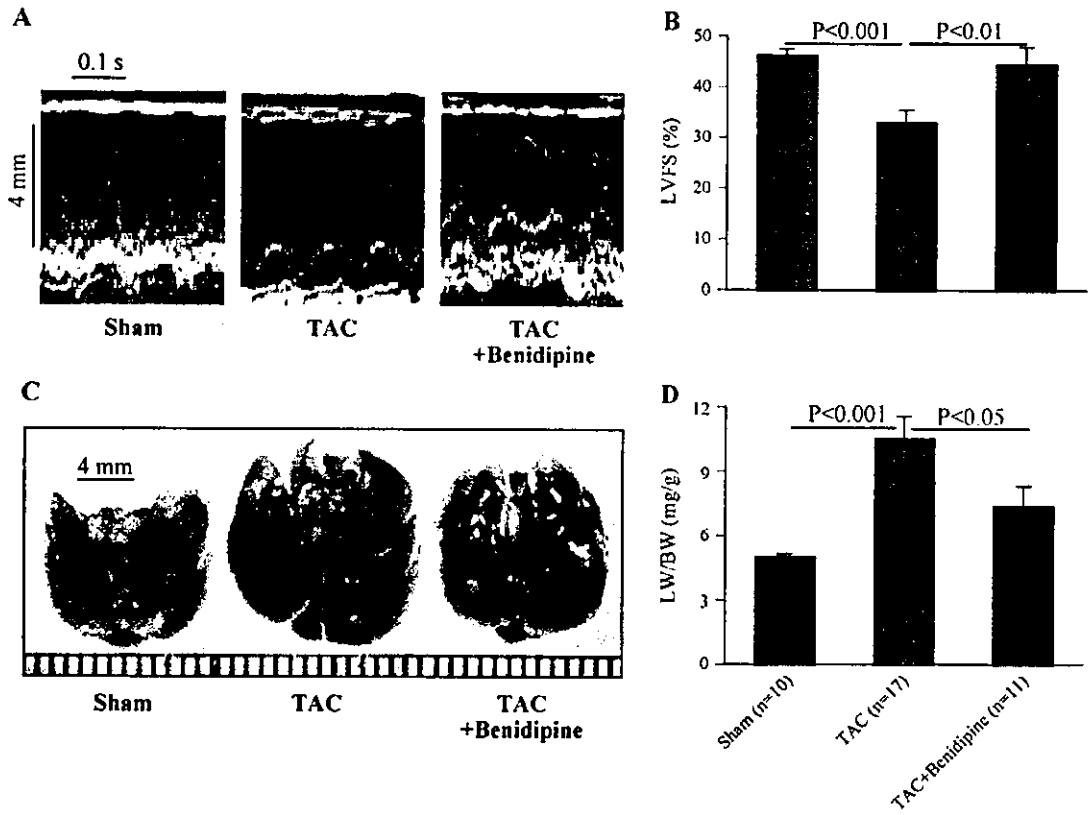

Fig. 5. Benidipine ameliorates heart failure induced by pressure overload. (A) Representative images obtained by echocardiography. (B) LV fractional shortening (LVFS) was increased by benidipine treatment. (C) Macroscopic views of lungs from each group. (D) The lung to body weight ratio (LW/BW) was significantly decreased in TAC mice treated with benidipine in comparison with untreated mice.

3.4. BNP, PIN, and procollagen IV are up-regulated in cardiac hypertrophy

Based on evidence from our laboratory and other investigators that BNP is an important molecular marker of cardiac hypertrophy or heart failure, and that both NO and fibrosis play an important role in cardiac remodeling, we assessed the expression of the BNP, PIN, and procollagen IV alpha genes in pressure-overloaded murine hearts, using microarray analysis. We found that a series of hypertrophy-related genes were up-regulated (Fig. 6A), including the BNP, PIN, and procollagen IV alpha genes, which were consistently up-regulated at both 1 and 4 weeks after TAC. Expression of calmodulin and five other procollagen genes was also increased by pressure overload (Fig. 6B).

3.5. Benidipine increases plasma NOx and down-regulates BNP, PIN, and procollagen IV alpha

As shown in Fig. 7A, the plasma level of NOx was markedly decreased in TAC mice at 4 weeks and was significantly increased in TAC mice treated with benidipine. Quantitative RT-PCR (Fig. 7B–D) demonstrated that benidipine decreased the level of BNP, a molecular marker for hypertrophy, and also down-regulated the expression of PIN and procollagen IV alpha$_1$. These changes supported our other findings in vitro and in vivo that benidipine inhibits cardiac hypertrophy and improves cardiac function partly by increasing the release of NO.

4. Discussion

4.1. Major findings

The present study is the first to evaluate the inhibitory effect of benidipine on cardiac remodeling induced by TAC in mice. The major findings of this study include the observations that (1) benidipine inhibits the increase of protein synthesis by cardiac myocyte stimulated by phenylephrine; (2) cardiac hypertrophy, myocardial fibrosis, and heart failure in pressure-overload mice were ameliorated by treatment with benidipine; and (3) an NO synthase inhibitor partially blocked the beneficial effect of benidipine on myocyte hypertrophy, while benidipine down-regulated protein inhibitor of neuronal nitric oxide synthase and increased the plasma NO level. These findings suggest that benidipine improves cardiac remodeling via an effect on the NO signaling pathway.

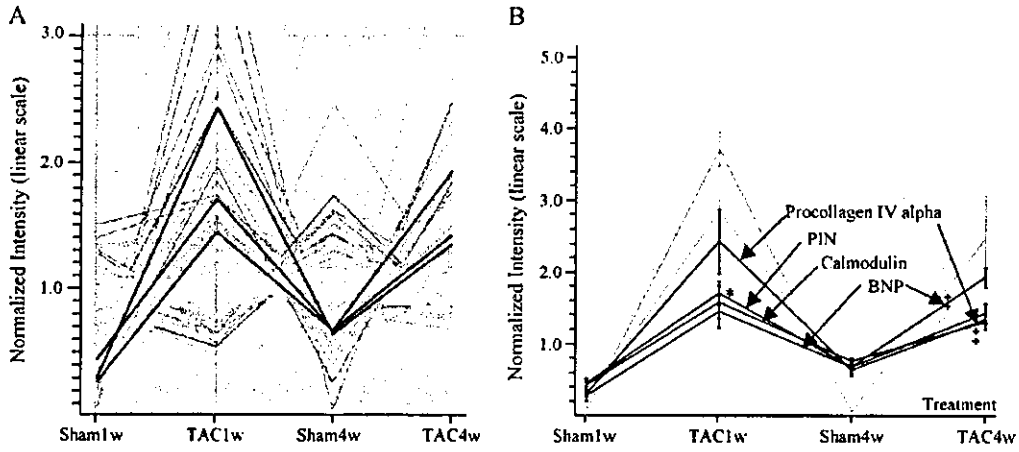

Fig. 6. cDNA microarray analysis of pressure-overload or sham-operated murine hearts. (A) From a total of 12,488 genes, three target genes were selected. These genes were functionally related to cardiac hypertrophy, heart failure, and nitric oxide signaling or fibrosis. (B) The three target genes were significantly up-regulated at 1 and 4 weeks after TAC relative to the levels in corresponding sham mice. Calmodulin and five other procollagen genes also showed up-regulation in response to pressure overload. The number of mice tested in each group was two. *$P<0.05$ vs. sham at 1w, †$P<0.05$ vs. sham at 4W (ANOVA).

4.2. Role of NO in cardiac remodeling

NO has been recognized as an important regulator of cardiac remodeling since it can influence both cardiac hypertrophy and heart failure. NO has been reported to exert an antihypertrophic effect in the hearts of spontaneously hypertensive rats without changing the blood pressure [18], which is in agreement with the results of this study. It is generally recognized that hemodynamic factors regulate cardiac myocyte hypertrophy [19], but exceptions have also been frequently reported. We previously reported that hydralazine significantly reduces the systemic blood pressure but does not have any effect on cardiac hypertrophy. In contrast, some drugs inhibit cardiac myocyte hypertrophy in the absence of a significant effect on hemodynamic, as we have reported previously [11,12,14]. Exogenous NO has

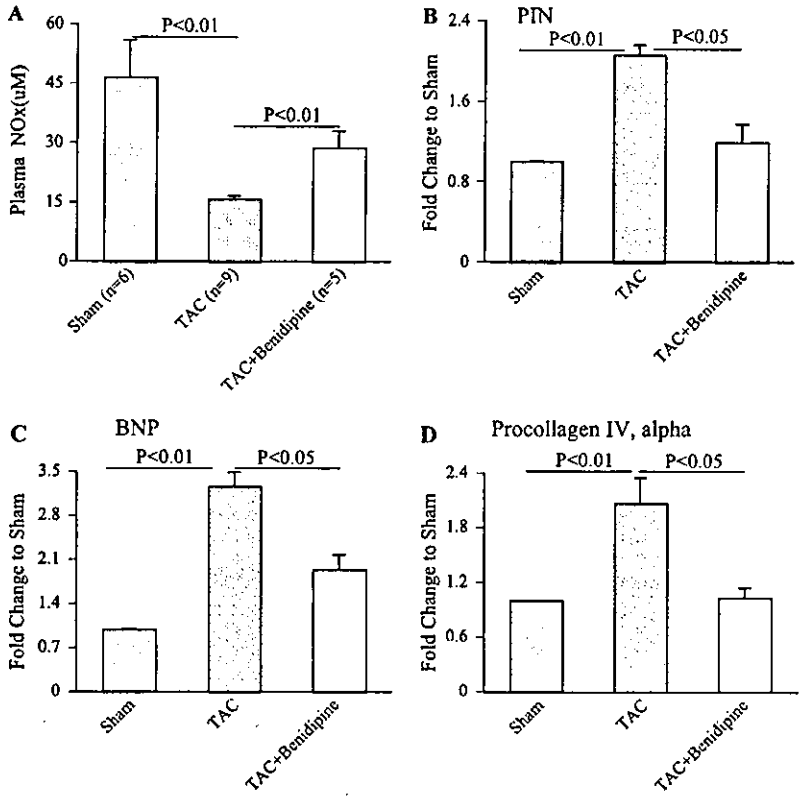

Fig. 7. Plasma nitric oxide level (A) and real-time PCR of the three target genes (B–D). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. PIN—protein inhibitor of neuronal nitric oxide synthase; BNP—natriuretic peptide precursor type B. $n=4$ per group for real-time PCR.

also been demonstrated to cause dose-dependent inhibition of alpha$_1$-adrenoceptor-stimulated protein synthesis in neonatal rat myocytes [7]. These results support our finding that benidipine caused a concentration-dependent decrease of PE (an alpha$_1$-adrenoceptor agonist)-stimulated protein synthesis by cardiac myocytes, and that this effect was blunted by NO synthase inhibitor. In addition, benidipine attenuated cardiac hypertrophy in pressure-overload mice without a significant change of blood pressure, and this antihypertrophic effect was at least partially mediated via the down-regulation of myocardial PIN. PIN has been demonstrated to regulate three types of NO synthase (NOS) [20]. Since both neural NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed in the myocardium, consistent up-regulation of PIN during the progression of cardiac hypertrophy, as noted in this study, is likely to decrease the release of NO. Interestingly, our data showed that benidipine significantly increased circulating NO levels, providing direct evidence for the abovementioned hypothesis that NO may play an important role in regulating cardiac hypertrophy. Although we did not monitor the blood concentration of benidipine, the dose that we used was effective for increasing the production of NO and consequently for attenuating cardiac hypertrophy.

We also found that benidipine could ameliorate progression from cardiac hypertrophy to heart failure, as confirmed by echocardiography, assessment of pulmonary congestion, and measurement of BNP expression. These results are partially attributable to the increase in NO production. Indeed, we have previously reported that benidipine increases coronary blood flow and reduces the severity of myocardial ischemia via an NO-dependent mechanism [5], and benidipine also improves cardiac remodeling induced by the eNOS inhibitor L-NAME [4]. Studies using genetically engineered mice have provided substantial evidence for a critical role of NO in cardiac remodeling. After myocardial infarction, LV dilation is more marked, heart function is more severely impaired, and long-term mortality is higher in eNOS-deficient mice compared with wild-type mice [8]. In contrast, congestive heart failure is less severe, and survival is increased in eNOS transgenic mice receiving coronary ligation [21]. It is worth noting that the preventive effect of benidipine on progression to heart failure may be secondary to its antihypertrophic effect. Further studies are needed to examine whether benidipine is effective in animals or humans with chronic heart failure.

4.3. Fibrosis and cardiac remodeling

Fibrosis of the myocardium plays a pivotal role in the process of cardiac remodeling. In the present study, we found that benidipine could significantly inhibit myocardial fibrosis in pressure-overload mice, a result that agrees with previous findings [9]. Although collagen type I and collagen type III produced by cardiac fibroblasts are the major components of the myocardial collagen matrix, type IV collagen is also expressed by both cardiac myocytes and fibroblasts and is a major component of the basement membrane [22,23]. Type IV collagen was reported to be increased in the hearts of diabetic rat [24] and is found in the fibrotic cardiac lesions of patients with DCM [25]. The angiotensin II-induced increase of fibronectin mRNA in the myocardium is accompanied by a similar increase of type I collagen, type IV collagen, and atrial natriuretic factor steady-state mRNA [26]. In this study, cDNA microarray analysis showed significant up-regulation of procollagen IV alpha at both 1 and 4 weeks after TAC, suggesting that this may be a potentially important gene in cardiac remodeling. Down-regulation of this gene by benidipine might have made an important contribution to the inhibition of cardiac remodeling.

4.4. Benidipine and cardiac sympathetic activity

Long-term cardiac sympathetic activation is detrimental to the heart, so one of the major aims of antihypertensive therapy is to reduce sympathetic tone. Differences in the formulations and pharmacokinetics of CCBs have various clinical influences, altering the effect of these drugs on blood pressure, heart rate, and cardiac sympathetic activity. Short-acting dihydropyridine CCBs enhance noradrenaline release from the sympathetic nerves [27]. In contrast, evidence suggests that long-acting calcium antagonists do not significantly affect sympathetic tone and may exert a more favorable clinical effect [28–30]. Our data showed that benidipine did not increase the heart rate. Moreover, benidipine prevented progression from cardiac hypertrophy to failure, suggesting that it does not enhance sympathetic tone. It is even possible that benidipine counteracts sympathetic activation in cardiac hypertrophy by increasing the release of NO because a reduced action of NO often contributes to overall sympathetic excitation in heart failure (review [31]).

4.5. Perspectives

In summary, this study provided evidence of the beneficial effect of a long-acting calcium antagonist, benidipine, on cardiac remodeling. Benidipine inhibited cardiac myocyte hypertrophy both in vitro and in vivo and also inhibited progression from cardiac hypertrophy to failure due to LV pressure overload. These effects were potentially mediated via an influence on the NO signaling pathway.

The question of whether CCB therapy increases cardiovascular events has attracted worldwide attention. Recent clinical trials have largely settled this question [29,30,32], but CCBs are still linked with a slightly increased risk of heart failure. However, the PRAISE trial revealed that amlodipine, a long-acting CCB, was not associated with increased mortality or morbidity in patients with severe

CHF [29]. Our studies and other investigations have consistently confirmed that amlodipine increases NO production [4,10,33,34]. Benidipine may also be beneficial for patients with hypertension-induced CHF, but a well-designed clinical trial is needed to investigate this point.

Acknowledgement

This work was supported by a Research Grant for Sensory and Communicative Disorders (H14-tokushitsu-38) from the Japanese Ministry of Health and Labor and Welfare.

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Amlodipine ameliorates myocardial hypertrophy by inhibiting EGFR phosphorylation

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Received 7 December 2004
Available online 29 December 2004

Abstract

The effects of long-acting calcium channel blockers on pressure overload-induced cardiac hypertrophy have been little studied in experimental animals and the underlying mechanisms are not fully understood. We previously reported that cardiomyocyte hypertrophy could be induced via phosphorylation of the epidermal growth factor receptor (EGFR). In this study, we investigated whether amlodipine attenuates cardiac hypertrophy by inhibiting EGFR phosphorylation. We found that amlodipine dose-dependently inhibited epinephrine-induced protein synthesis and EGFR phosphorylation in cultured neonatal rat cardiomyocytes. Our in vivo study revealed that amlodipine could ameliorate myocardial hypertrophy induced by transverse aortic constriction (TAC) in C57/B6 mice. One week after TAC, amlodipine treatment (3 mg/kg/day) significantly reduced the heart-to-body weight ratio (6.04 ± 0.16 mg/g vs. 6.90 ± 0.45 mg/g in untreated TAC mice, $P < 0.01$). These results indicate that amlodipine ameliorates cardiomyocyte hypertrophy via inhibition of EGFR phosphorylation.

Keywords: Calcium channel blocker; Cardiomyocyte; Hypertrophy; Epidermal growth factor; Phosphorylation; Mouse

Calcium channel blockers (CCBs) are widely used for the treatment of hypertension. Amlodipine is a long-acting dihydropyridine CCB that is effective for lowering the blood pressure, amelioration of cardiac remodeling, and reduction of mortality and morbidity [1]. However, the mechanisms underlying the beneficial effects of CCBs on cardiac remodeling are not fully understood. We have reported that stimulation of the G protein-coupled receptor (GPCR) in cardiomyocytes causes the release of heparin-binding epidermal growth factor (HB-EGF), which subsequently binds to the epidermal growth factor receptor (EGFR) and produces cardiac hypertrophy [2]. There is evidence that calcium channels play an important role in activation of the EGFR [3]. Calcium channels were reported to be involved in endothelin-1-induced activation of the EGFR [3], and calcium channels also induce tyrosine phosphorylation of this receptor to levels that can activate the mitogen-activated protein kinase signaling pathway [4]. In addition, blockade of calcium uptake and mobilization by mammary gland epithelial cells suppress EGF-induced cell proliferation [5]. Considering these findings, we hypothesized that amlodipine may ameliorate cardiomyocyte hypertrophy by inhibiting EGFR phosphorylation. In the present study, we evaluated the effect of amlodipine on EGFR phosphorylation induced by a GPCR agonist in vitro and

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doi:10.1016/j.bbrc.2004.12.112

on cardiomyocyte hypertrophy induced by left ventricular pressure overload in vivo.

Materials and methods

Cell culture. Rat neonatal ventricular myocytes were isolated as described previously [2], and were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% FBS (Equitech-Bio). The medium was changed to serum-free medium after 72 h and cells were cultured under serum-free conditions for 48 h before addition of agents. Protein synthesis by the cultured cells was evaluated through analysis of [^{3}H]leucine incorporation [2,6]. Cardiomyocytes were exposed to either epinephrine (Epi: 10^{-5} M) or HB-EGF (10^{-8} M) for 24 h in the presence or absence of amlodipine (kindly provided by Sumitomo Pharmaceuticals, Japan), and the increase of [^{3}H]leucine incorporation was examined.

EGFR phosphorylation. Cultured cardiomyocytes were exposed to 10^{-5} M Epi or 10^{-8} M HB-EGF with or without pretreatment by amlodipine (10^{-6} or 10^{-9} M) or HB-EGF neutralizing antibody #19 for 30 min. Cells were lysed by incubation for 20 min at 4 °C in a buffer (50 mM Tris–HCl, pH 7.3; 150 mM NaCl; 2 mM EDTA; 0.5% sodium fluoride; 10 mM sodium pyrophosphate; 0.5 mM Na_3VO_4; 100 μg/ml phenylmethylsulfonyl fluoride; 2 μg/ml aprotinin; protease inhibitor cocktail; and 1% Nonidet P-40). Immunoprecipitation with an antibody directed against the EGFR and immunoblotting using phosphorylation antibody (Anti-pY) were performed as described elsewhere [7].

Animal model. All procedures were performed in accordance with the institutional guidelines for animal research. Male C57BL/6 mice (8–9 weeks-old, wt 19–25 g) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg intraperitoneally). The animal model of pressure overload was created as described previously [8]. Briefly, transverse aortic constriction (TAC) was produced by tying a 7-0 suture tied twice around the aorta and a 27-gauge needle, after which the needle was gently removed to yield 60–80% constriction of the aortic arch.

To determine whether amlodipine could attenuate cardiac hypertrophy induced by TAC, we treated the mice with saline (TAC group) or oral amlodipine 3 mg/kg/day. To confirm that the extent of pressure overload was similar between the amlodipine-treated and untreated groups, we measured the pressure in the ascending aorta of 2–3 mice from each group using a 1.4 F Millar catheter on the 2nd day after TAC. The tail-cuff blood pressure and heart rate (BP-98A, Softron, Tokyo, Japan) were examined before sacrifice. One week after the induction of pressure overload, mice were killed to determine organ weights and perform morphometric analysis. The cross-sectional surface area of cardiomyocytes was measured using three hearts in each group with the method described previously [6].

Statistical analysis. Multiple comparisons were performed by one-way ANOVA with the Tukey–Kramer exact probability test. Results are reported as means ± SEM. For all analyses, $P < 0.05$ was considered statistically significant.

Results and discussion

Amlodipine attenuates the induction of cardiomyoctyte protein synthesis by epinephrine

As shown in Fig. 1A, amlodipine markedly inhibited epinephrine-induced neonatal rat cardiomyocyte protein synthesis over a concentration range of 10^{-7}–10^{-5} M. Epinephrine is one of the GPCR agonists and is well known to induce cardiomyocyte hypertrophy. Pignier et al. [9] reported that hypertrophy induced by long-term stimulation of α_1-adrenoceptors is accompanied by an increase in the expression of functional calcium channels in neonatal rat cardiomyocytes, indicating the existence of a novel α_1-mediated pathway for positive regulation of the L-type calcium current. This agrees with our finding that blockade of L-type calcium channels inhibits cardiomyocyte hypertrophy. There is substantial evidence to support the notion that calcium signaling pathways contribute to the progression of cardiac hypertrophy [10,11], so it is likely that blockade of calcium signaling would lead to the regression of hypertrophy.

Amlodipine causes concentration-dependent inhibition of EGFR phosphorylation induced by epinephrine

Based on our earlier demonstration that EGFR activation by GPCR agonists led to the development of cardiac hypertrophy [2] and the present in vitro finding that

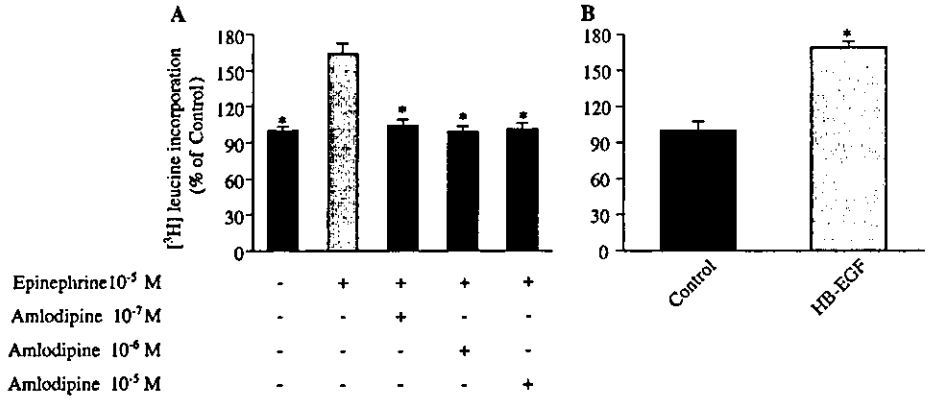

Fig. 1. Effect of amlodipine and HB-EGF on protein synthesis in rat cardiomyocytes. (A) Protein synthesis stimulated by epinephrine (10^{-5} M) was inhibited by amlodipine at concentrations ranging from 10^{-7} to 10^{-5} M. *$P < 0.01$ vs. epinephrine alone. (B) HB-EGF (10^{-8} M) significantly increased myocyte protein synthesis. *$P < 0.01$ vs. Control.

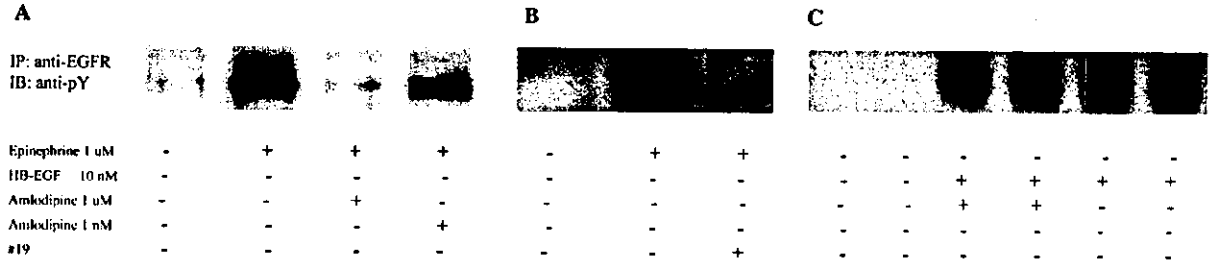

Fig. 2. EGFR phosphorylation and release of HB-EGF. (A) EGFR phosphorylation showed concentration-dependent inhibition by amlodipine. (B) HB-EGF neutralizing antibody #19 blocked epinephrine-induced EGFR phosphorylation. (C) Amlodipine did not influence EGFR phosphorylation induced by HB-EGF. Each experiment was repeated at least three times.

amlodipine inhibits cardiomyocyte protein synthesis stimulated by a GPCR agonist (epinephrine), we hypothesized that amlodipine may also inhibit cardiomyocyte hypertrophy by preventing tyrosine phosphorylation of the EGFR. In the present study, HB-EGF significantly increased protein synthesis by neonatal rat cardiomyocytes (Fig. 1B), a finding that agreed with our previous report [2]. Interestingly, we also demonstrated that amlodipine inhibits EGFR phosphorylation in cardiomyocytes in a concentration-dependent manner (Fig. 2A). In recent years, information about the mechanisms related to Ca^{2+} influx has accumulated. Zwick et al. [12] reported that calcium-dependent EGFR activation led to subsequent activation of the Ras/mitogen-activated protein pathway in neurons. In addition, Kawanabe et al. [3] have shown that Ca^{2+} influx plays an important role in endothelin-1-induced EGFR activation, and endothelin-1 is well known to stimulate cardiomyocyte growth.

Amlodipine inhibits epinephrine-induced release of HB-EGF

We previously reported that phenylephrine induces EGFR activation by increasing the release of the HB-EGF ectodomain [2]. Here we found that amlodipine could inhibit EGFR activation by reducing the epinephrine-induced release of HB-EGF. Since the extracellular level of the ectodomain of HB-EGF (soluble HB-EGF) was generally too low to be detected by Western blotting, we assessed it by an indirect method. If epinephrine induces release of the HB-EGF ectodomain, its depletion was assumed to block epinephrine-induced EGFR activation. As expected, we found that an HB-EGF neutralizing antibody #19 almost completely prevented epinephrine-induced phosphorylation of the EGFR (Fig. 2B), suggesting that epinephrine-induced EGFR activation is mediated by the release of HB-EGF, at least in newborn rat cardiac myocytes. When we investigated whether amlodipine prevents HB-EGF-induced activation of the EGFR, we found that this drug did not have any influence on HB-EGF-mediated EGFR phosphorylation (Fig. 2C), suggesting that it acts upstream of HB-EGF. Finally, we revealed that amlodipine caused marked inhibition of epinephrine-induced phosphorylation of the EGFR (Fig. 2A), a result that supported an inhibitory effect of the drug on EGFR activation by preventing the release of HB-EGF. Further studies are needed to elucidate the exact mechanism by which CCBs inhibit EGFR phosphorylation. Src kinase is reported to contribute to EGFR activation by GPCR agonists [13,14], while a link between calcium release through L-type calcium channels and Src has also been demonstrated [4,15–18], and the release of calcium seems to be necessary for activation of Src [4,18]. Thus, it is likely that amlodipine blocks the signal transduction pathway upstream of Src.

Amlodipine inhibits myocardial hypertrophy in vivo

We used a well-established mouse model of left ventricular pressure overload to further confirm the preventive effect of amlodipine on cardiac hypertrophy. An increase of GPCR agonists, such as catecholamines [6], angiotensin II, and endothelin-1, is known to occur in the myocardium of these mice. Since EGFR activation leads to cardiomyocyte hypertrophy [2] and amlodipine inhibits epinephrine-induced EGFR phosphorylation in cardiomyocytes in vitro, as shown in the present study, it would seem plausible that amlodipine also attenuates cardiac hypertrophy induced by TAC. Indeed, consistent with our in vitro results, we found that oral administration of amlodipine (3 mg/kg/day) for 1 week markedly ameliorated cardiac hypertrophy. Histological examination confirmed that myocyte hypertrophy was less severe (Figs. 3A and B) in mice treated with amlodipine. Compared with sham mice, the heart-to-body weight ratio (HW/BW) increased by about 43% in TAC mice, while the amlodipine-treated mice only showed an increase of about 25% (Fig. 3C). Cardiomyocytes cross-surface area was also significantly decreased in amlodipine-treated mice (Fig. 3D). Hemodynamic parameters are summarized in Table 1; amlodipine did not significantly affect either the tail-cuff systolic blood pressure or the heart rate. Ascending aortic pressure was similar in the TAC and amlodipine-treated TAC

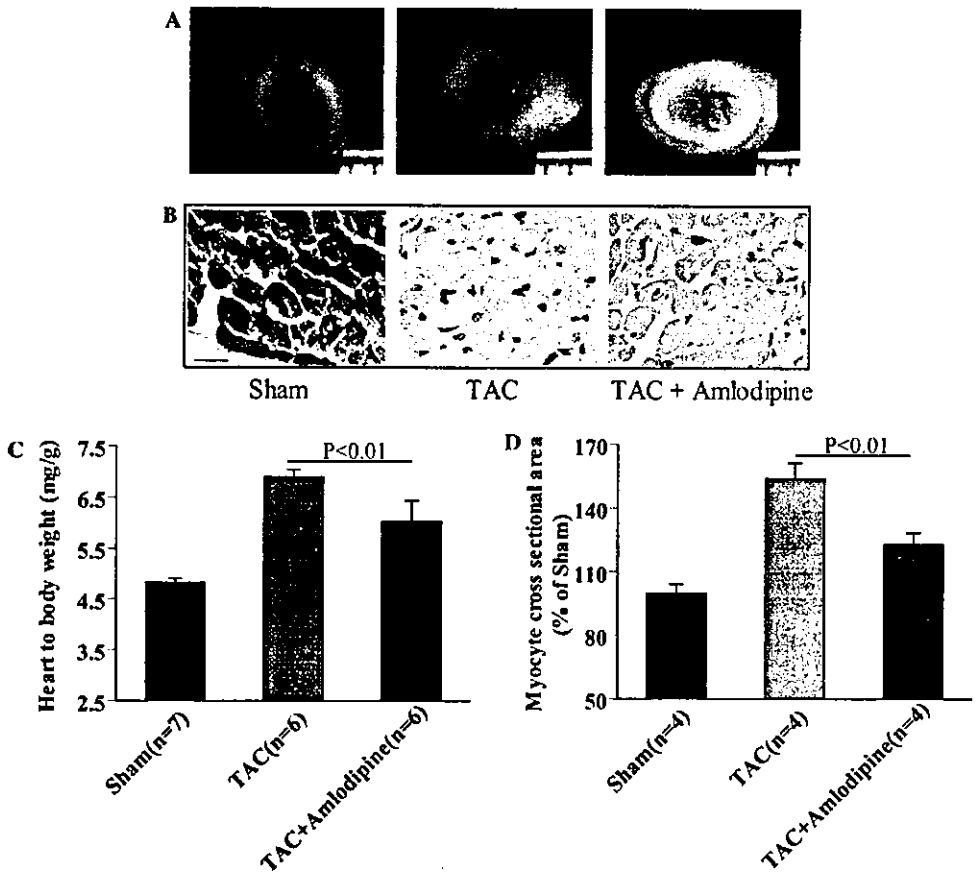

Fig. 3. Effects of amlodipine on cardiac hypertrophy induced by pressure overload in mice. (A) Representative cross-sections of hearts from the three groups. (B) Histological examination showed that cardiomyocyte hypertrophy was less severe in hearts of amlodipine-treated mice (Bar, 20 μm, HE stain). The heart-to-body weight ratio (HW/BW) (C) and cardiomyocyte cross-sectional surface area (D) were significantly lower in TAC mice treated with amlodipine (3 mg/kg/day) in comparison with untreated TAC mice.

Table 1
General characteristics in three experimental groups

Group	AASBP (mmHg)[a]	BW (g)	Tail SBP (mmHg)	HR (bpm)
Sham ($n = 7$)	101 ± 5	23 ± 0.2	112 ± 4	644 ± 26
TAC ($n = 6$)	157 ± 9^{b}	22.4 ± 0.3	100 ± 5^{b}	670 ± 24
TAC+amlodipine ($n = 6$)	161 ± 8^{b}	$20.1 \pm 0.8^{b,c}$	93 ± 3^{b}	675 ± 19

TAC, transverse aortic constriction; AASBP, ascending aortic systolic blood pressure (SBP); AASBP was measured in three mice in each group at 2nd day after TAC, while those mice were randomly selected and did not receive amlodipine treatment, because we just wanted to confirm that the pressure overload was similar between TAC and amlodipine-treated groups. BW, body weight; HR, heart rate. BW, tail SBP, and HR were measured before sacrifice.

[a] $n = 2$ in each group.
[b] $P < 0.05$ vs. Sham.
[c] $P < 0.05$ vs. TAC.

groups, indicating that there was no significant difference of the pressure load on the left ventricle.

Our data suggested that amlodipine was effective for ameliorating cardiomyocyte hypertrophy independently of any decrease in the blood pressure. This antihypertrophic effect was attributable, at least partly, to the inhibition of EGFR phosphorylation by amlodipine and this drug is also likely to exert an antihypertrophic effect through the nitric oxide signaling pathway, as indicated by previous studies [19].

Although various clinical trials have demonstrated that amlodipine is effective and safe for the treatment of hypertension and reducing cardiac events [20–22], the underlying mechanisms remain poorly understood. The present study is the first to show that amlodipine ameliorates cardiac hypertrophy by inhibiting EGFR

activation. This suggests the possibility of using the regulation of Ca^{2+} influx as a therapeutic approach for controlling cell growth and proliferation.

Acknowledgments

This work was supported by a grant for Research on Sensory and Communication Disorders (H14-tokushitsu-38) from the Health and Labor Sciences Research Grants provided by the Ministry of Health, Labor and Welfare in Japan.

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Cardiovascular Drugs and Therapy 18 483–489 2004

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

This study was supported by a Grant-in-aid for Human Genome, Tissue Engineering, and Food Biotechnology (H13-Genome-011) and a Grant-in-aid for Comprehensive Research on Aging and Health

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	Oral 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.

(H13-21seiki(seikatsu)-23) from the Health and Labor Sciences Research Grants of the Japanese Ministry of Health, Labor and Welfare.

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

```
ARB ≦ 0
  Alpha-glucosidase inhibitor ≦ 0
    Anti-aldosterone drugs ≦ 0.333
      Cardiotonic agents ≦ 0.8
        Oral diabetic medicines ≦ 0.003
          Statins ≦ 0.5
            Thyroid hormones ≦ 0
              Ca-antagonists ≦ 0.9
                Coronary dilators ≦ 0
                  Diuretics ≦ 0.4 (174, 0.448) → Normal
                  Diuretics > 0.4
                    Cardiotonic agents ≦ 0.229 (30, 0.4) → Gray
                    Cardiotonic agents > 0.229 (27, 0.778) → Low
                Coronary dilators > 0 (57, 0.421) → Low
              Ca-antagonists > 0.9
                Ca-antagonists ≦ 1.33 (15, 0.6) → Gray
                Ca-antagonists > 1.33 (21, 0.714) → Normal
            Thyroid hormones > 0 (15, 0.6) → Normal
          Statins > 0.5 (66, 0.5) → Low
        Oral diabetic medicines > 0.003 (15, 0.6) → Low
      Cardiotonic agents > 0.8 (30, 0.5) → Abnormal Low
    Anti-aldosterone drugs > 0.333 (15, 0.8) → Low
  Alpha-glucosidase inhibitor > 0 (21, 0.571) → Normal
ARB > 0 (60, 0.75) → Normal
```

① ARB ≦ 0 → Normal – Low
ARB > 0 → Normal

② Ca-antagonists ≦ 0.9 → Normal – Low
Ca-antagonists ≦ 1.33 → Gray
Ca-antagonists > 1.33 → Normal

③ Cardiotonic agents ≦ 0.229 → Gray
Cardiotonic agents > 0.229 → Low
Cardiotonic agents > 0.8 → Abnormally Low

④ Alpha-glucosidase inhibitor ≦ 0 → Normal – Low
Alpha-glucosidase inhibitor > 0 → Normal

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,∞).

```
Respiratory mucolytic agents ≦ 0
  Vitamins ≦ 0
    ARB ≦ 0
      Alpha-glucosidase inhibitors ≦ 0
        Anti-aldosterone drugs ≦ 0.333
          Statins ≦ 0.25
            Potassium agents ≦ 0.25
              Anti-platelet drugs ≦ 0.25
                Ca-antagonists ≦ 0
                  Diuretics ≦ 0.5
                    Diuretics ≦ 0.167 (81, 0.407) → Low
                    Diuretics > 0.167
                      ACEIs ≦ 0.16 (15, 0.6) → ABLow
                      ACEIs > 0.16 (21, 0.714) → Normal
                  Diuretics > 0.5 (39, 0.538) → Gray
                Ca-antagonists > 0 (18, 0.667) → Normal
              Anti-platelet drugs > 0.25
                Anti-ulcer agents ≦ 0.75
                  Antigout drugs ≦ 0.2 (84, 0.536) → Normal
                  Antigout drugs > 0.2 (15, 0.6) → Gray
                Anti-ulcer agents > 0.75 (21, 0.571) → Low
            Potassium agents > 0.25 (18, 0.667) → Normal
          Statins > 0.25
            Anti-arrhythmic drugs ≦ 0.25 (81, 0.519) → Low
            Anti-arrhythmic drugs > 0.25 (18, 0.667) → ABLow
        Anti-aldosterone drugs > 0.333 (15, 0.8) → Low
      Alpha-glucosidase inhibitors > 0 (21, 0.571) → Normal
    ARB > 0 (54, 0.722) → Normal
  Vitamins > 0 (30, 0.7) → Normal
Respiratory mucolytic agents > 0 (15, 0.4) → AB Low
```

```
Vitamins ≦ 0
  ARB ≦ 0
    Alpha-glucosidase inhibitors ≦ 0
      Anti-aldosterone drugs ≦ 0.33
      ----------
        Ca-antagonists ≦ 0
          Diuretics ≦ 0.5
            Diuretics ≦ 0.167 → Low
            Diuretics > 0.167
              ACEIs ≦ 0.16 → AB Low
              ACEIs > 0.16 → Normal
          Diuretics > 0.5 → Gray
        Ca-antagonists > 0 → Normal
      ----------
    Anti-aldosterone drugs > 0.333 → Low
  Alpha-glucosidase inhibitors > 0 → Normal
ARB > 0 → Normal
Vitamins > 0 → Normal
```

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,∞).

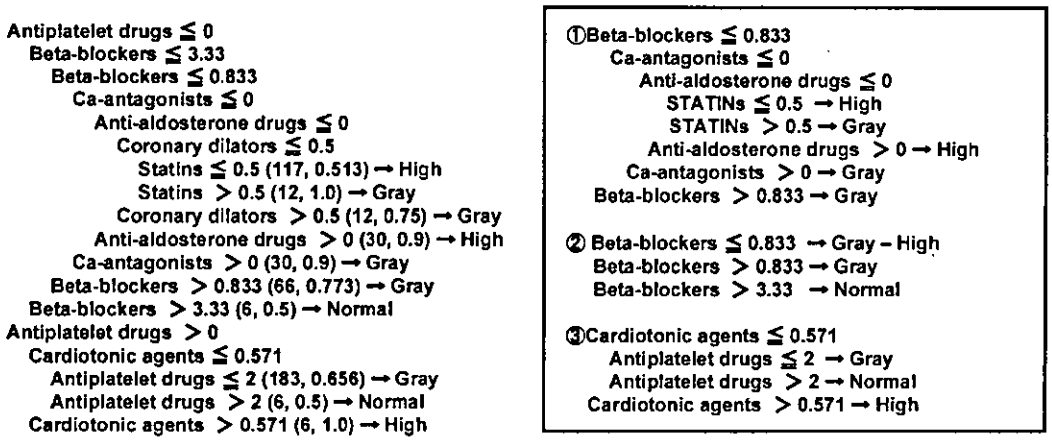

Fig. 3. Analysis of BNP with 21 drug groups. The left panel shows the decision tree. The right panel represents the simplified rules extracted from the tree. BNP(ug/ml), Normal: (0,20), Gray: (20,200), High: (200,1000), Abnormally High: (1000,2000), Very Abnormally High: (2000,∞).

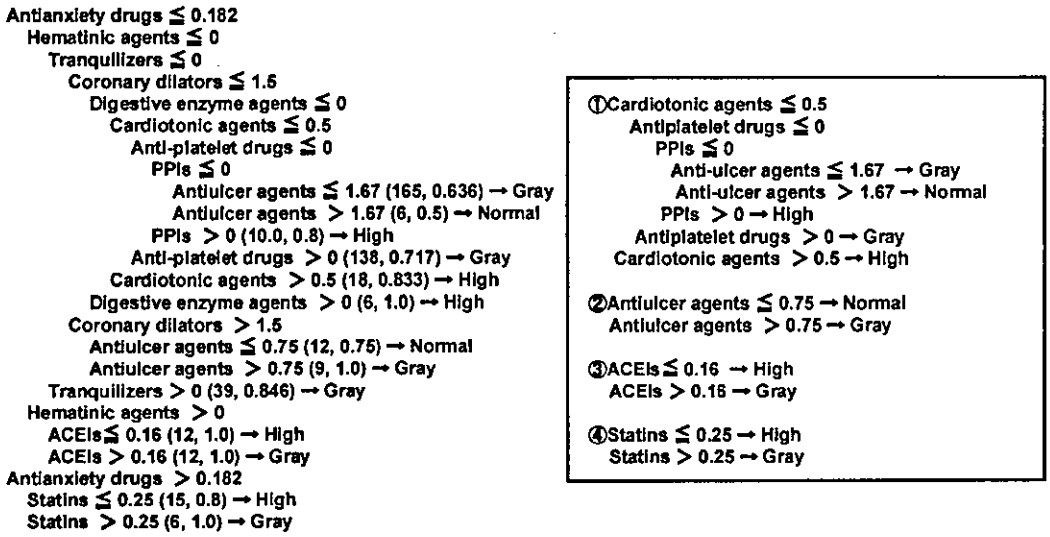

Fig. 4. Analysis of BNP with 52 drug groups. The left panel shows the decision tree. The right panel represents the simplified rules extracted from the tree. BNP(ug/ml), Normal: (0,20), Gray: (20,200), High: (200,1000), Abnormally High: (1000,2000), Very Abnormally High: (2000,∞).

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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Journal of Molecular and Cellular Cardiology 37 (2004) 1219–1223
Journal of Molecular and Cellular Cardiology
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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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Received 11 April 2004; received in revised form 22 September 2004; accepted 27 September 2004

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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0022-2828/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.yjmcc.2004.09.016

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.