obtained from Calbiochem (La Jolla, Calif).[15] Bovine serum albumin-conjugated aldosterone was purchased from Steraloids Inc. (Newport, RI). The primary antibody and the positive control against PKCα were commercially obtained from BD Biosciences (San Jose, Calif). Aldosterone was initially dissolved in 99.5% ethanol and diluted in saline.

Instrumentation

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.

Fifty-nine hybrid beagle dogs weighing 14 to 22 kg were anesthetized with pentobarbital sodium (30 mg/kg intravenously). The dogs were prepared as previously described.[16] Briefly, the trachea was intubated and the dog was ventilated with room air mixed with oxygen. The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. After heparinization (500 U/kg), the proximal portion of the left anterior descending coronary artery (LAD) was cannulated and perfused with blood via the carotid artery through an extracorporeal bypass tube. Either coronary perfusion pressure (CPP) or coronary blood flow (CBF) was monitored at this tube. A small collecting tube was inserted into a small coronary vein near the perfused area to sample coronary venous blood. The drained venous blood was collected in a reservoir placed at the level of the left atrium. A pair of ultrasonic crystal probes was placed in the center of the perfused area to allow the measurement of myocardial segment length with an ultrasonic dimension gauge (5 MHz; Schuessler, Cardiff by the Sea, Calif). End-diastolic length was determined at the R wave of the ECG, and end-systolic length was determined at the minimal dP/dt. Fractional shortening (FS) was calculated by the formula [(end-diastolic length)−(end-systolic length)]/(end-diastolic length), and served as an index of myocardial contractility of the perfused area.

Experimental Protocols

Protocol I: Effects of an Intracoronary Administration of Aldosterone on Systemic and Coronary Hemodynamics in the Nonischemic Hearts

First of all, to clarify dose-dependent effects of aldosterone on CBF, 20 dogs were used in this protocol. Vehicle and 3 different doses (0.05, 0.1, and 0.2 nmol/L; n=5 each) of aldosterone were randomly and selectively administered into the LAD through the extracorporeal bypass tube. We continuously infused 60 ng aldosterone in 10 mL saline into the LAD so that the final concentration of this infused aldosterone in coronary circulation became 0.1 nmol/L in the nonischemic hearts for 60 minutes. Hemodynamic parameters including heart rate, CPP, and CBF were measured 5, 10, 20, 30, 45, and 60 minutes after drug infusion.

Protocol II: Effects of an Intracoronary Administration Aldosterone on Coronary Hemodynamic and Metabolic Parameters in the Ischemic Hearts (Constant Low CPP Model)

After hemodynamic stabilization, CPP was reduced so that CBF was decreased to 33% of the control CBF using an occluder attached at the extracorporeal bypass tube. After a low level of CPP was obtained, the occluder was manually adjusted to keep CPP constant. All of the hemodynamic parameters were measured 5 minutes after the onset of hypoperfusion. Both coronary arterial and venous blood were sampled for metabolic analysis. Then, we administrated aldosterone (0.1 nmol/L, n=7) into the LAD through the extracorporeal bypass tube. The dose of 0.1 nmol/L of aldosterone was chosen because this dose of aldosterone was the minimal dose to induce the maximal coronary vasoconstriction in protocol I. In other dogs, to test the involvement of PKC in regulating CBF, we infused aldosterone with either a PKC inhibitor, GF109203X (300 ng/kg per minute; n=5), or a MR antagonist spironolactone (10 μg/kg per minute, n=5) in the ischemic hearts. An intracoronary infusion of GF109203X at this dose was reported to inhibit PKC activation without changing the coronary hemodynamic and metabolic parameters.[17]

Protocol III: Effects of Aldosterone on the Activation of PKC of Coronary Artery With and Without Ischemia

To check effects of aldosterone on PKC activation in coronary arteries, we used 4 dogs in this protocol. After the 15-minute intracoronary infusion of vehicle or aldosterone (0.1 nmol/L) with and without ischemia, the hearts were excised and the vascular segments from the LAD were modestly separated and quickly placed into liquid nitrogen (LN_2) and stored at −80°C. Then, the vascular segments obtained were separated into membrane and cytosolic fractions and the activity of PKC was checked by Western blot analysis as previously described.[18]

Biochemical Analysis

Lactate concentration was assessed by an enzymatic assay.[19] Lactate extraction ratio (LER) was calculated by multiplying the coronary arteriovenous difference in the lactate concentration by 100 and dividing it by the arterial lactate concentration.

Statistical Analysis

The time course of changes in hemodynamic parameters in each group was compared by 1-way repeated measures ANOVA, followed by the Fisher test. The time course of changes in hemodynamic parameters between groups was compared by repeated measures ANOVA, followed by the Fisher test. All values are expressed as mean±SEM, and $P<0.05$ was considered significant.

Results

Effects of an Intracoronary Administration of Aldosterone on Systemic and Coronary Hemodynamics in the Nonischemic Hearts

In the nonischemic hearts, either heart rate or CPP was not significantly changed during the infusion of aldosterone (Figure 1A and 1B). The infusion of vehicle did not change CBF throughout 60 minutes. Aldosterone at the dose of 0.1 nmol/L gradually decreased CBF from 5 minutes and reached the maximal decrease of CBF 30 minutes after the onset of hypoperfusion and did not further change CBF. Aldosterone at the dose of either 0.1 nmol/L or 0.2 nmol/L caused comparable decrease in CBF, but aldosterone at the dose of 0.05 nmol/L decreased CBF to a lesser extent than did 0.1 nmol/L (Figure 1C).

Effects of an Intracoronary Administration of Aldosterone on Coronary Hemodynamics and Cardiac Functions in the Ischemic Hearts

Before and during coronary hypoperfusion, both heart rate and CPP were unchanged with or without pharmacological interventions. There were no significant differences in baseline hemodynamics among all groups. The infusion of aldosterone (0.1 nmol/L) decreased CBF gradually from 5 minutes and reached maximal decrease at 30 minutes (Figure 2A). In the ischemic hearts, both FS (23.7±1.5% to 8.4±0.7%) and LER (41.4±3.0% to -31.7±2.9%) 30 minutes after the onset of hypoperfusion were decreased ($P<0.05$) compared with the baseline. Furthermore, the intracoronary infusion of aldosterone further decreased both FS (5.4±0.4%) and LER (−41.4±3.7%) in the ischemic hearts. Co-administration of GF109203X completely blunted the aldosterone-induced decrease in CBF (38.1±2.9 mL/100g

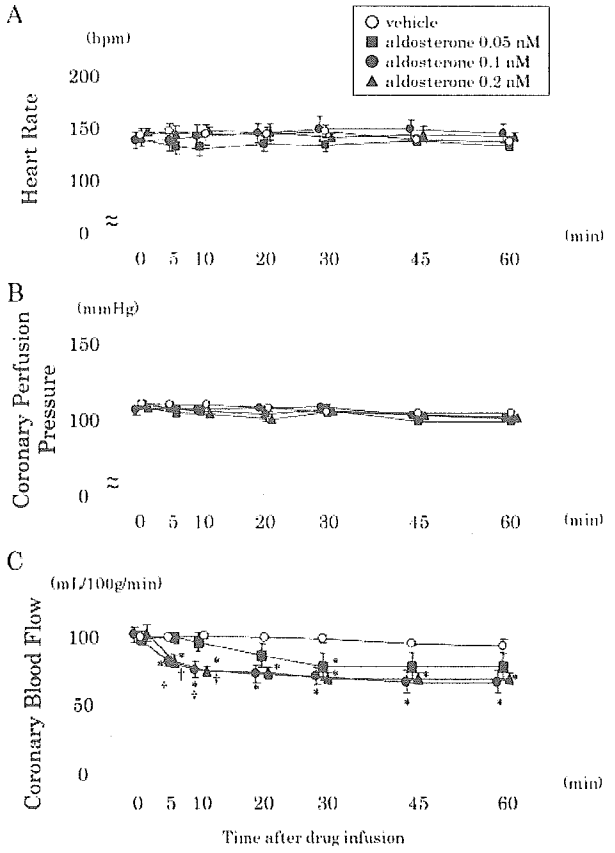

Figure 1. Nongenomic effects of aldosterone on coronary hemodynamics under nonischemic hearts. Plots of changes in heart rate (upper panel, A), coronary perfusion pressure (middle panel, B), and coronary blood flow (lower panel, C) in the nonischemic hearts during intracoronary administration of vehicle or aldosterone at the 3 doses (0.05, 0.1, and 0.2 nmol/L). *$P<0.05$ vs vehicle, †$P<0.05$ vs at the corresponding time. Data are presented as mean±SEM (n=5, each).

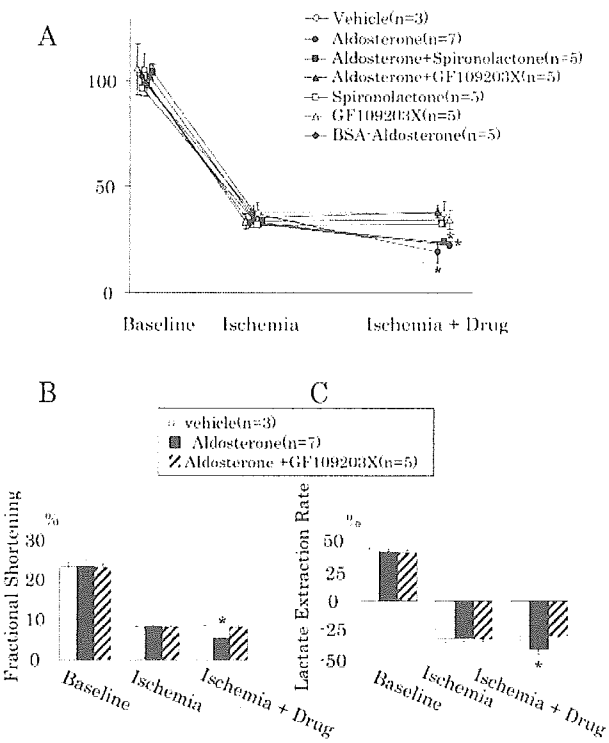

Figure 2. Nongenomic effects of aldosterone on coronary hemodynamics and myocardial contractile and metabolic functions under ischemic hearts. A, Plots of changes in coronary blood flow in the ischemic hearts with intracoronary administration of aldosterone (0.1 nmol/L) with and without either GF109203X or spironolactone, GF109203X alone, spironolactone alone, and bovine serum albumin-conjugated aldosterone. *$P<0.05$ vs vehicle. B and C, Changes in fractional shortening (left panel, B) and lactate extraction ratio (right panel, C) during the intracoronary administration of vehicle and aldosterone (0.1 nmol/L) with and without GF109203X. *$P<0.05$ vs vehicle.

per minute) (Figure 2A). This agent also blunted the aldosterone-induced decreased in both FS (8.3±0.7%) and LER (−30.2±1.3%) in the ischemic hearts (Figure 2B and 2C). The infusion of GF109203X alone (n=5) did not change CBF (33.8±3.7 to 34.5±4.3 mL/100 g per minute), FS (8.4±1.0 to 8.2±1.0%), or LER (−32.4±3.2 to −33.6±2.8%) in the ischemic hearts. Co-administration of spironolactone (n=5) did not prevent the aldosterone-induced decrease in CBF (24.0±0.5 mL/100 g per minute). The infusion of bovine serum albumin-conjugated aldosterone decreased CBF gradually from 5 minutes and reached maximal decrease at 30 minutes (22.5±0.9 mL/100 g per minute).

Effects of Aldosterone on the Activation of PKC With and Without Ischemia

As shown in Figure 3, in the nonischemic condition, aldosterone induced the translocation of PKCα from cytosolic to membrane fraction in the vascular segments of the LAD. Moreover, the ischemic insult itself induced the translocation of PKCα from cytosolic to membrane fraction and aldosterone further augmented the translocation of PKCα in the vascular segments under ischemia.

Discussion

We demonstrated here that the intracoronary administration of aldosterone rapidly decreased CBF in the ischemic as well as the nonischemic hearts in vivo. Moreover, aldosterone further worsened the contractile and metabolic functions gauged by FS and LER, respectively, in the ischemic hearts. These decreases in CBF, FS, and LER in the ischemic hearts were blunted by a PKC inhibitor but not an MR antagonist. In addition, bovine serum albumin-conjugated aldosterone reduced CBF under ischemic conditions, suggesting that the

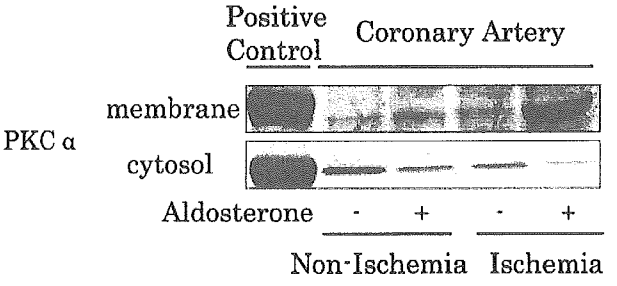

Figure 3. Effects of aldosterone on PKCα activation in coronary arteries. The representative cases in Western blotting against PKCα in the vascular tissues from the LAD.

reduction in CBF by aldosterone was mediated through possible membrane receptors, not intracellular MR. These results indicate that aldosterone nongenomically induces vasoconstriction via PKC-dependent pathways possibly through membrane receptors, which leads to the worsening of the cardiac contractile and metabolic function in the ischemic hearts.

Rapid Aldosterone-Induced Coronary Vasoconstriction in the Nonischemic and Ischemic Hearts

In this study, in the nonischemic hearts, the intracoronary administration of aldosterone decreased CBF within 30 minutes, suggesting that aldosterone nongenomically reduces CBF. Moreover, in the ischemic hearts, we observed the rapid coronary vasoconstriction induced by aldosterone along with the decrease in FS and LER, both of which indicated the contractile and metabolic deterioration, respectively. These findings suggest that the rapid decrease in CBF induced by aldosterone may cause the worsening of ischemia in the in vivo hypoperfused hearts.

Involvement of PKC in the Aldosterone-Induced Coronary Vasoconstriction

Aldosterone is reported to activate PKC in distal colon cells and cultured kidney cells, and to decrease its activity stimulated by phorbol-12-myristate-13-acetate in rat neonatal cardiomyocytes.[12–14] In our study, this nongenomic effect of aldosterone on CBF was completely blunted by the PKC inhibitor, GF109203X, confirming the involvement of the PKC activation. The dose of aldosterone at 0.1 nmol/L was reported to increase intracellular Ca^{2+} in cultured rat and rabbit vascular smooth muscle cells.[3] Consistent with this report, we showed that aldosterone activated Ca^{2+}-dependent PKCα in the vascular segments of the ischemic heart. There are some reports that endothelium nitric oxide synthase is a PKC substrate and PKC-mediated phosphorylation inhibits endothelium nitric oxide synthase activity.[20,21] Because nitric oxide is widely known to be a vasodilative agent,[22] decreased nitric oxide activity could attenuate the vascular tone, leading the decrease in CBF. Thus, there is a possibility that aldosterone induced vasoconstriction because of decreased endothelium nitric oxide synthase activity by PKC activation. Because we could not obtain antibodies that react with canine Ca^{2+} -independent subtypes of PKC, possible involvement of other subtypes of PKC was not investigated in the present study.

The Possibility of Transmembrane Receptors of Aldosterone in Canine Hearts

We demonstrated that spironolactone, a classical antagonist of intracellular MR, did not prevent aldosterone-induced vasoconstriction. In addition, bovine serum albumin-conjugated aldosterone induced vasoconstriction. Because bovine serum albumin–conjugated aldosterone would not permeate into the cytoplasm, the effects of bovine serum albumin–conjugated aldosterone on vascular tone were not mediated through intracellular MR, but rather possible membrane receptors. Arima et al suggested that aldosterone caused vasoconstriction in renal microcirculation mediated via membrane-bound receptors.[23] Although further investigation to identify the transmembrane receptors directly will be needed, these findings might support the possibility of the presence of the transmembrane receptors. These coronary vasoconstriction effects of aldosterone were categorized into AII-b according to Mannheim classifications indicating direct steroid action via nonclassical receptors.[24]

Perspectives

Recent large clinical trials resolutely established the beneficial effects of chronic blockade of aldosterone receptor using for patients with chronic heart failure after myocardial infarction.[25,26] In this study, we showed that in the ischemic hearts the nongenomic effect of aldosterone deteriorated ischemia and that this effect was blunted by the inhibition of PKC, not a MR antagonist. Our data suggest that elevated levels of aldosterone may worsen myocardial ischemia via nongenomic as well as genomic pathways in the ischemic hearts. Thus, we believe that this report throws a light on the novel clinical drug development to target nongenomic effects of aldosterone in the ischemic hearts, as well as the chronic inhibition of genomic effects of aldosterone using an antagonist against intracellular MR.

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Exacerbation of heart failure in adiponectin-deficient mice due to impaired regulation of AMPK and glucose metabolism

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Abstract

Objective: Insulin resistance (IR) was reported to be associated with chronic heart failure (CHF). Adiponectin, an insulin-sensitizing hormone with anti-inflammatory activity, improves energy metabolism via AMP-activated protein kinase (AMPK). AMPK deficiency is associated with depressed cardiac function under stress conditions. However, it is not clear whether adiponectin plays an important role in CHF. We hypothesize that deficiency of adiponectin might result in deterioration of heart failure.
Methods: Using adiponectin null mice and their littermates, we examined the effects of adiponectin on LV pressure overload-induced cardiac hypertrophy and failure, and investigated the mechanisms involved.
Results: Three weeks after transverse aortic constriction (TAC), cardiac hypertrophy (evaluated from the heart-to-body weight ratio: 7.62±0.27 in wild-type (WT) mice, 9.97±1.13 in knockout (KO) mice, $P<0.05$) and pulmonary congestion (lung-to-body weight ratio: 9.05±1.49 in WT mice, 14.95±2.36 in KO mice, $P<0.05$) were significantly greater in adiponectin KO mice than WT mice. LV dimensions were also increased in KO mice. Compared with WT TAC mice, expression of AMPKα protein was lower, while IR was higher in KO TAC mice.
Conclusion: These findings indicate that adiponectin deficiency leads to progressive cardiac remodeling in pressure overloaded condition mediated via lowing AMPK signaling and impaired glucose metabolism.

Keywords: Adiponectin; Heart failure; Myocardial hypertrophy; Metabolic syndrome

1. Introduction

The metabolic syndrome (MetS) has been identified as a constellation of important risk factors for cardiovascular disease (CVD) [1,2]. The Adult Treatment Panel III report (ATP III)[3] identified insulin resistance (IR)±glucose intolerance as an important component of MetS that is related to CVD. Clinical evidence suggests that LV hypertrophy is associated with either impaired glucose tolerance (IGT) or an increase in IR [4]. An increase in IR is also common in CHF patients with either ischemic heart disease or idiopathic dilated cardiomyopathy [5–7]. These findings lead to the concept that a strategy targeting improvement of IGT or IR should be beneficial for cardiac remodeling.

To date, there is compelling evidence that an impaired myocardial energy metabolism strongly influences cardiac remodeling [8–11]. The important role of the AMP-activated protein kinase (AMPK) in cardiac hypertrophy and failure seems to be deserving of more attention. AMPK

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activity and protein expression were both reported to be increased by pressure overload hypertrophy [8], which should be considered a compensatory mechanism for cardiac remodeling, because the overexpression of mutations of this enzyme leads to deterioration of post-ischemic cardiac dysfunction [10] or experimental glycogen storage cardiomyopathy [11]. Accordingly, we considered that AMPK might play an important role in limiting cardiac remodeling and that an increase of AMPK in the heart might inhibit remodeling by regulation of cellular metabolism to maintain energy homeostasis under stress conditions. Intriguingly, adiponectin, an endogenous adipocyte-derived insulin-sensitizing hormone, has been shown to attenuate inflammation, regulate glucose and lipid metabolism. In addition, adiponectin is able to stimulate glucose utilization and fatty acid oxidation through the activation of AMPK [12]. Furthermore, administration of adiponectin reverses IR in mice with lipoatrophy and diabetes [13,14]. The importance of adiponectin has also been demonstrated by other evidence that it may directly influence the development of cardiovascular disease [15–17]. A recent clinical investigation demonstrated that a high plasma adiponectin concentration was associated with a lower risk of myocardial infarction in men [17]. These lines of evidence strongly suggest that adiponectin might play an important role in the inhibition of cardiac remodeling via its beneficial effects on MetS. Interestingly, a recent experimental study shows that 1 week pressure overload in adiponectin-deficient mice resulted in enhanced concentric cardiac hypertrophy with an increased mortality [18]. However, to our knowledge, no previous study has evaluated the role of AMPK or adiponectin on chronic heart failure (CHF). Therefore, we aimed to test the hypothesis that adiponectin might act as an endogenous protective modulator of chronic cardiac remodeling via regulation of AMPK.

In this study, we evaluated the role of adiponectin in the progression of cardiac hypertrophy and heart failure in a model of LV pressure overload using adiponectin knockout mice, and explored the potential mechanisms involved.

2. Methods

2.1. Adiponectin knockout (KO) mice

Adiponectin KO mice were generated as described previously [19]. Wild-type (WT) littermates served as the control.

2.2. TAC model

All procedures were performed in accordance with our institutional guidelines for animal research and comply with the Declaration of Helsinki and the NIH Guide. Mice (male, 9–10 weeks old, wt 25–29 g) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg, i.p.), and transverse aortic constriction (TAC) was created as we described previously. In order to confirm that pressure overload was similar between the wild-type and the KO mice, three mice in each group were selected for measurement of the ascending aortic pressure using a 1.4 F Millar pressure catheter on the second day after TAC. The other mice were killed after 3 weeks for morphological analysis. Mice were divided into four groups: WT sham ($n=5$), WT TAC ($n=24$), KO sham ($n=5$), and KO TAC ($n=24$).

2.3. Histology

Hearts were fixed with 10% formalin. The cardiac myocyte cross-sectional surface area was measured using three hearts in each group after images were captured from HE-stained sections as described elsewhere [20]. One hundred myocytes per heart were counted, and the average area was determined. Myocardial and perivascular fibrosis were stained with Azan [21].

2.4. Echocardiography

Transthoracic echocardiography was performed with a Sonos 4500 and a 15–6 L MHz transducer (Philips, the Netherlands). Mice were fixed while conscious and good two-dimensional short-axis LV views were obtained for guided M-mode measurements of the LV posterior wall thickness (LVPW), LV end-diastolic diameter (LVEDd), LV end-systolic diameter (LVESd), LV fractional shortening (LVFS), and LV ejection fraction (EF). LVFS=(LVEDd−LVESd)/LVEDd*100, LVEF=[(LVEDd)^3−(LVESd)^3]/(LVEDd)^3*100.

2.5. Measurement of glucose and insulin

Fasting plasma glucose was measured using a blood glucose test meter (Glutestace GT-1640, Arkray Company, Japan). After 14 h withdrawal of food from the cages, whole blood sample (3 μl) was taken from mouse tails with a glucose sensor inserted in Glutestace, and the result of plasma glucose concentration was read-out 30 s later. Serum insulin levels were measured according to the protocols of the manufacturers (EIA-3440 ELISA kit, DRG, German). IR was assessed with the homeostasis model: HOMA-IR=fasting glucose level (mg/dl)×fasting insulin level (ng/ml)÷22.5.

2.6. Western blot analysis

SDS-PAGE was performed with 50 μg of protein extracted from mouse hearts. Blots were incubated with a mouse monoclonal antibody directed against anti-AMPKα_1, anti-AMPKα_2 antibodies (upstate). Signals obtained by Western blotting were quantified using Scion Image software.

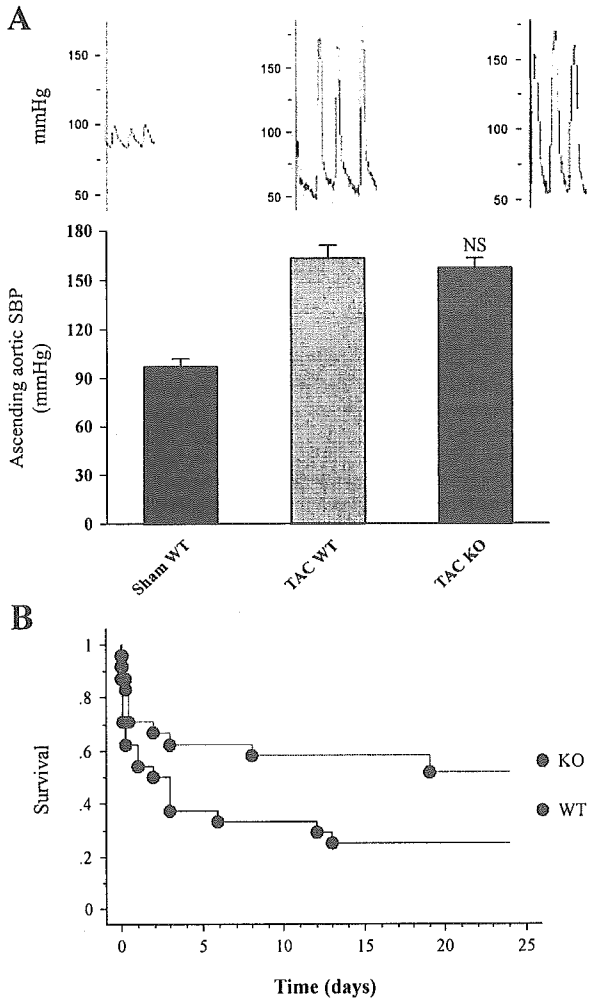

Fig. 1. Left ventricular pressure overload and survival. A) The ascending aortic systolic pressure measured with a 1.4 F catheter was similar in adiponectin KO and WT mice. NS: not significant vs. TAC WT. B) Kaplan–Meier survival analysis showed a significant higher mortality in adiponectin KO mice after TAC (Mantel–Cox test: $P=0.031$, $n=24$ in both WT and KO groups).

2.7. Statistical analysis

For all statistical tests, multiple comparisons were performed by one-way ANOVA with the Tukey–Kramer exact probability test. Survival analysis was performed using the Kaplan–Meier method. Variables with skewed distribution were transformed to logarithmic data. Results are reported as the mean±SEM and $P<0.05$ was considered statistically significant.

3. Results

3.1. LV pressure overload and survival

To evaluate the role of adiponectin in cardiac remodeling, we used mice lacking the adiponectin/*CRP30* gene. During development up to 16 weeks of age, there were no differences in growth rate and food intake between WT mice and KO (homozygous) mice [19]. The results showed that LV pressure overload was similar in WT and KO mice (Fig. 1A). The mortality after TAC was significantly higher in KO mice than WT mice (Fig. 1B). We found that acute or subacute heart failure was the main cause of death confirmed by postmortem examination (pulmonary edema or hemorrhage was noted in most of the dead mice. Lung-to-body weight ration was 13.1±2.3 mg/g for dead mice in adiponectin KO mice, 11.4±1.9 mg/g for dead mice in WT group). Body weight (BW) and blood pressure (determined by tail cuff measurement) were similar before TAC (BW: 27.1±0.4 g in KO, 27.7±0.4 g in WT) and 3 weeks after TAC (BW: 24.5±1.4 g in KO, 25.5±0.7 g in WT).

3.2. Earlier transition from hypertrophy to heart failure in KO mice

Serial echocardiographic examinations showed that the heart function evaluated by LVEF and LVFS progressively

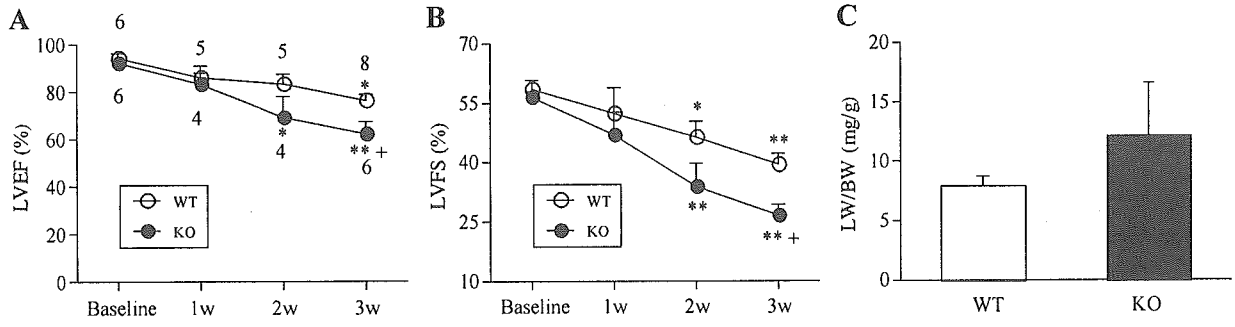

Fig. 2. The transition from hypertrophy to heart failure. A) Left ventricular ejection fraction (LVEF) and B) left ventricular fractional shortening (LVFS) were progressively depressed in adiponectin KO mice after 1 week of TAC, and the transition to heart failure occurred at 2 weeks after TAC in KO mice, which was confirmed by sacrifice to show an significant increase of lung-to-body weight ratio (C, $n=4$ for both WT and TAC mice). The number of mice in each time point for echocardiographic examination is indicated above or under the data points. *$P<0.05$, **$P<0.01$ vs. baseline, †$P<0.05$ vs. WT mice.

depressed in both adiponectin KO and WT mice over the course of 3 weeks (Fig. 2A, B). Two weeks after TAC, a significant reduction of LVEF and LVFS was noted in KO mice, indicating a proceeded transition to heart failure. To confirm the occurrence of heart failure, we sacrificed four mice in both KO and WT groups at 2 weeks after TAC and found a marked pulmonary congestion in KO mice (Fig. 2C).

3.3. Greater cardiac hypertrophy in KO mice

Three weeks after TAC, mice were sacrificed after echocardiographic examination. The wet heart-to-body weight ratio (HW/BW) was increased by 53% in TAC WT mice compared with sham WT mice, whereas HW/BW was dramatically increased by 110% in adiponectin TAC KO mice vs. sham KO mice. There was a significant difference of HW/BW between WT and KO TAC mice (Fig. 3A–C, E). The cross-sectional surface area of cardiac myocytes was significantly larger in KO mice than WT mice (Fig. 3F). There were no significant differences of HW/BW and cardiac myocyte cross-sectional surface area between WT and KO sham mice. These findings indicate that cardiac hypertrophy was far more extensive in adiponectin KO mice. We also examined myocardial and perivascular fibrosis and did not find significant difference between WT and KO TAC mice (Fig. 3D).

3.4. Worse pulmonary congestion in KO mice

We confirmed in previous studies that pulmonary edema is a reliable index of cardiac function in this model [22–24]. Severe pulmonary congestion was found in adiponectin KO mice. Compared with sham mice, the lung-to-body weight ratio (LW/BW) was increased by 170% in KO TAC mice,

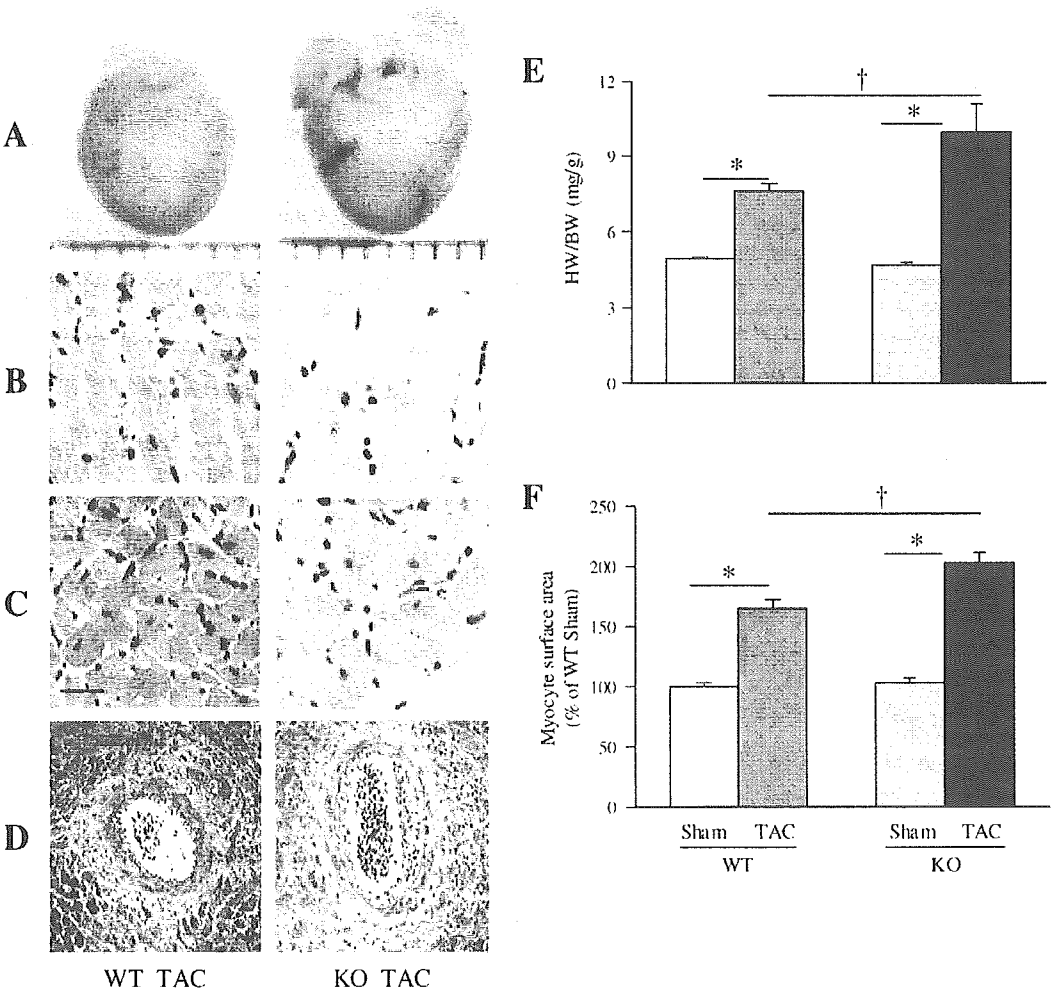

Fig. 3. Cardiac remodeling was more severe in KO mice. A) Representative pictures of cardiac hypertrophy in WT and KO mice at 3 weeks after TAC. B and C) Represent long-axis and cross-sectional views of cardiac myocytes with HE staining. D) Represents cardiac fibrosis with Azan staining (×100 magnification). HW/BW (E, $n=5$ in both sham groups, $n=8$ in WT TAC group, and $n=6$ in KO TAC group) and the cardiac myocyte cross-sectional surface area (F, $n=2$ in each sham group and $n=3$ in each TAC group) were increased significantly in KO mice compared with their wild-type (WT) littermates. *$P<0.01$, †$P<0.05$. Bar=20 μm for B and C.

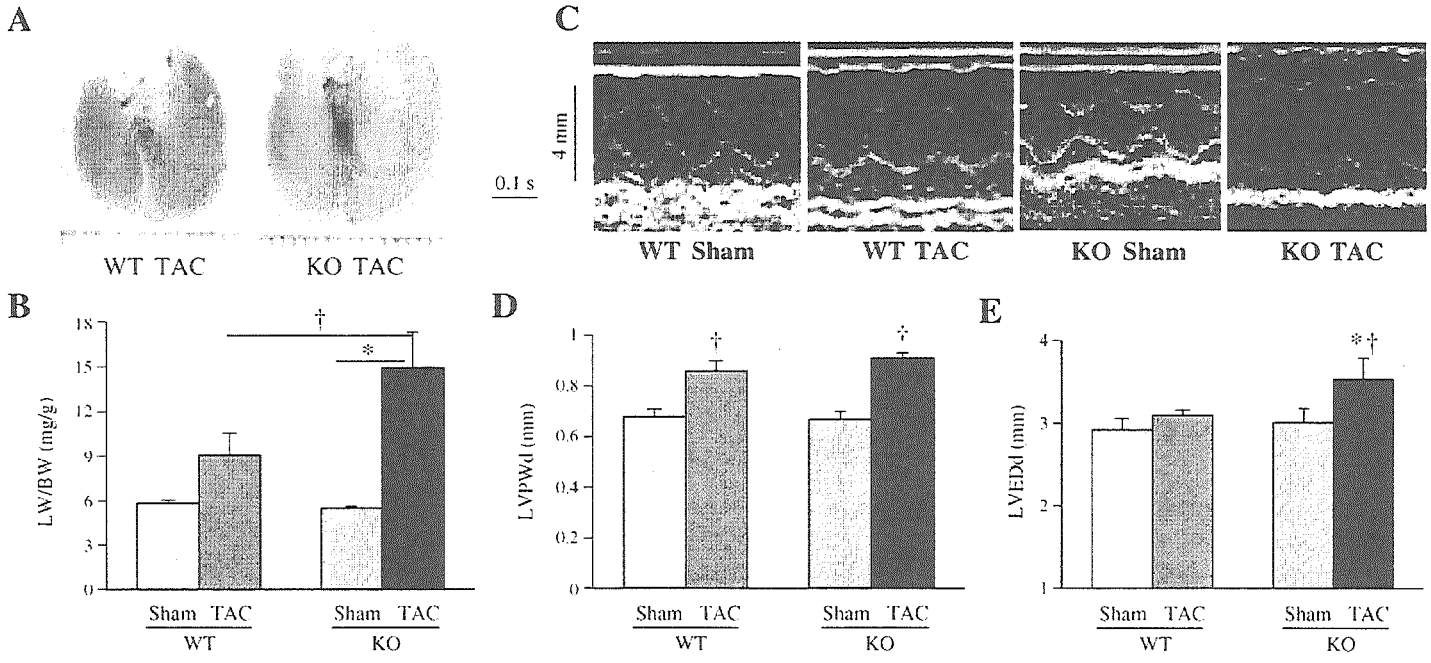

Fig. 4. Pulmonary congestion and echocardiographic findings at 3 weeks after TAC. The lungs of an adiponectin KO mouse were markedly enlarged compared with those of WT mice (A). The lung-to-body weight ratio (LW/BW) was markedly increased in KO mice compared with WT mice (B). $*P<0.01$, $\dagger P<0.05$. Echocardiography (C) shows that the LV posterior wall diastolic thickness (LVPWd) (D) is similar in KO and WT TAC mice. The LV end-diastolic dimension (LVEDd) (E) is significantly increased in KO mice compared with WT mice $*P<0.05$ vs. TAC WT. $\dagger P<0.01$ vs. responding sham mice. The number of animals is the same as Fig. 3 in each group for analysis of LW/BW and echocardiography.

whereas there was only a 55% increase in WT TAC littermates (Fig. 4A, B). There was no significant difference in LW/BW between KO and WT sham mice. We did not evaluate LV hemodynamics using a Millar pressure catheter because most of the KO mice appeared to be too weak to endure this procedure (including anesthesia) at 3 weeks after TAC.

3.5. Echocardiography findings

Because anesthesia has a significant influence on echocardiography data in mice [25] and most of the KO TAC mice were too weak for anesthesia at 3 weeks after TAC, we developed a method of performing echocardiographic examination in conscious mice. Compared with WT TAC mice, there was a significant decrease in both LV fractional shortening (LVFS) and the LV ejection fraction (LVEF) in KO TAC mice (Fig. 2A, B), and marked LV chamber dilation was observed in KO TAC mice (Fig. 4C, D). In contrast, there were no significant differences in these parameters between WT sham and KO sham mice. These findings indicate an increase in cardiac remodeling under pressure overload in adiponectin KO mice.

3.6. Myocardial AMPK expression

AMPK consists of one catalytic subunit (α) and two noncatalytic subunits (β and γ). Because AMPKα was reported to be activated by adiponectin [12], we examined the AMPKα_1 and α_2 protein expression in the hearts of WT and KO mice. As shown in Fig. 5, in the presence of LV pressure overload, AMPKα expression increased significantly, but the increment of AMPKα protein was less in KO than in WT hearts. These findings suggested that adiponectin deficiency means that the expression of AMPK cannot be increased sufficiently enough to provide adequate cardiac protection under stress conditions.

3.7. Increase of fasting glucose and IR

As IR is closely associated with cardiac remodeling [4–7] and adiponectin deficiency can lead to diet-induced IR [19], we determined the influence of adiponectin deficiency on glucose metabolism and IR in mice with LV pressure overload. As shown in Fig. 6A, fasting glucose levels increased by 40% in KO mice at 3 weeks after TAC, but rose by only about 20% in WT littermates, suggesting that the glucose metabolisms were more impaired in the adiponectin KO mice. Meanwhile, a similar increase in serum insulin was noted in both WT and KO TAC mice (Fig. 6B). As an index of IR, HOMA-IR was more increased in adiponectin KO mice than in WT mice at three weeks after TAC (Fig. 6C). Furthermore, we found a significant positive correlation between IR and the heart weight-to-body weight ratio in adiponectin KO mice rather than in WT

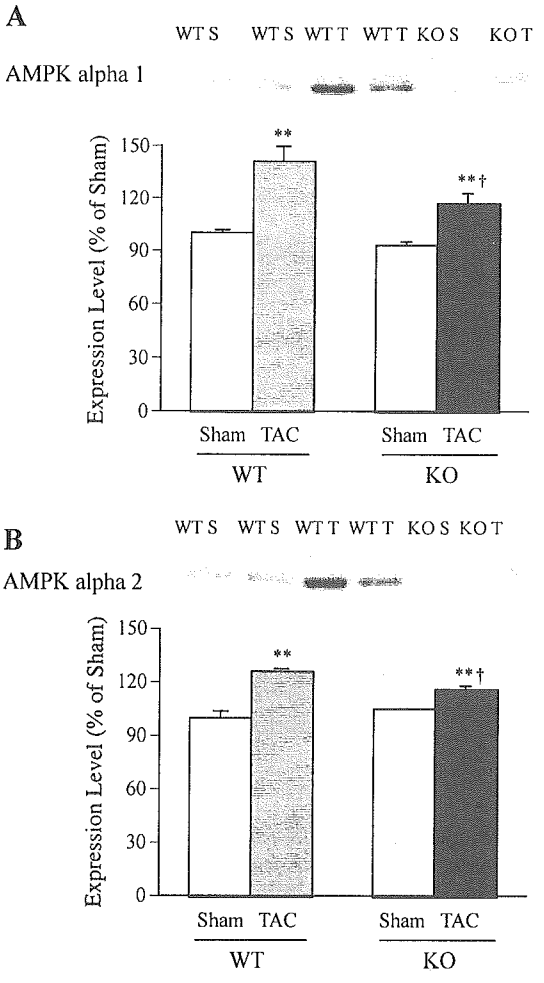

Fig. 5. Myocardial expression of AMPK. AMPKα_1 (A), α_2 (B) were increased in TAC mice, but the change was smaller in KO mice ($n=3$ in each group, $^{**}P<0.01$ vs. responding sham mice; $\dagger P<0.05$ vs. WT TAC). S: sham, T: TAC.

mice (Fig. 6D), indicating that IR might also be involved in cardiac remodeling in adiponectin KO mice.

4. Discussion

In this study, we found that adiponectin deficiency worsens cardiac remodeling induced by LV pressure overload, and this change was associated closely with a decrease in the expression of AMPK, and an increase in IR. These results are consistent with a recent study by Shibata et al. [18] showing that pressure overload for one week in adiponectin KO mice resulted in greater cardiac hypertrophy and higher mortality. Differently, this study further investigated the potential role of adiponectin-deficiency on the development of cardiac hypertrophy and chronic heart failure. We demonstrated that the transition from hypertrophy to heart failure proceeded in adiponectin KO mice. Additionally, we investigated the influence of adiponectin

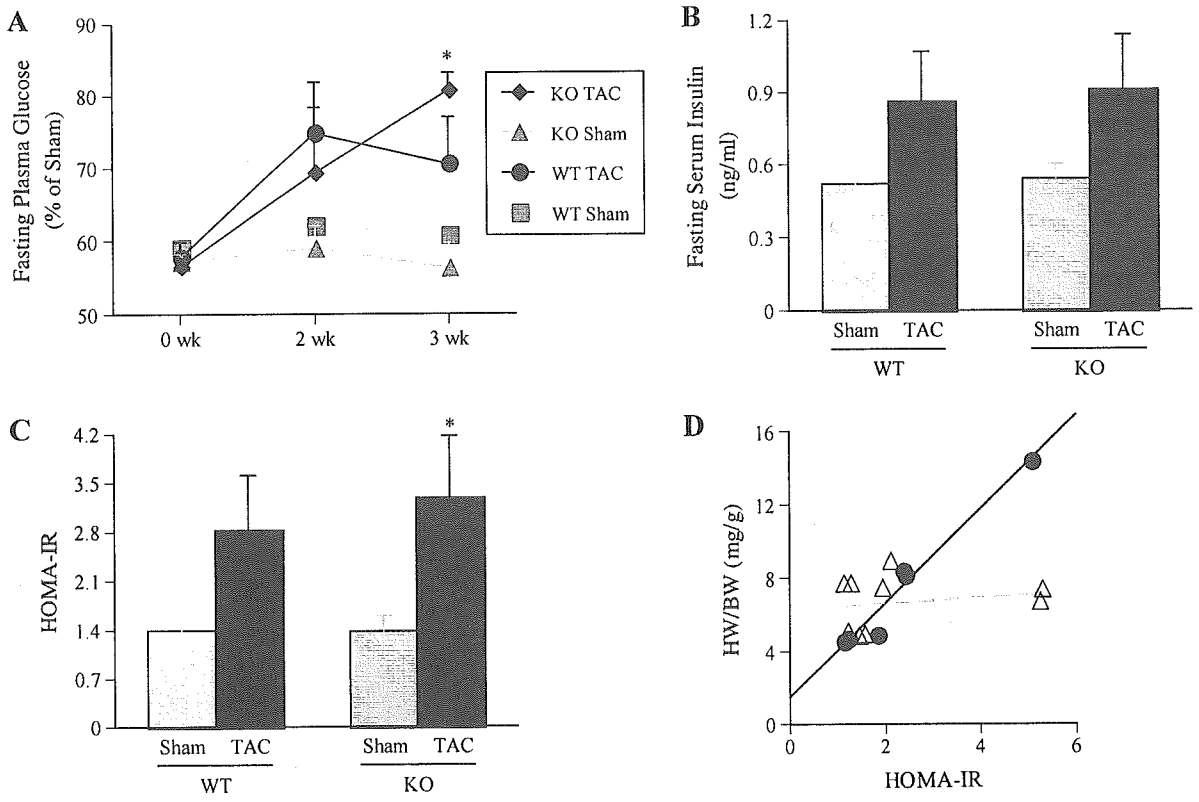

Fig. 6. Changes in glucose metabolism. Fasting glucose levels (A) were increased in adiponectin KO mice at 3 weeks after the onset of TAC, $*P<0.01$ vs. WT TAC ($n=5$ for all the groups at 0 week and for both sham groups at other two time points; $n=4$ for WT and KO TAC mice at 2 weeks, and $n=5$ and 3 for WT and KO TAC mice at 3 weeks, respectively). Serum insulin (B) was increased after TAC, but no significant difference was found between WT and KO mice, while the insulin resistance index HOMA-IR (C) was increased in KO mice. $\dagger P<0.05$ vs. KO sham ($n=3$ in both KO sham and TAC groups, $n=3$ in WT sham and $n=6$ in TAC groups). Linear correlation between HOMA-IR and HW/BW in both WT and KO mice groups (D) irrespective of TAC, $r=0.982$, $P<0.0001$, $n=6$ for KO mice (solid circle), while no significant correlation was found for WT mice ($n=9$, open triangle).

on glucose metabolism and addressed the important relation between metabolism and cardiac remodeling.

An increase in IR, glucose intolerance, and a proinflammatory state are among the six components of the MetS related to CVD, which is viewed as the primary outcome of this syndrome. In the present study, we noted that adiponectin deficiency induced an increase in IR and fasting glucose levels in the presence of pressure overload, suggesting that adiponectin has a strong influence on MetS and subsequently on cardiac remodeling. An increase in IR appears to downregulate adiponectin receptor expression via the phosphoinositide 3-kinase/Foxo1-dependent pathway [26]. In addition, Foxo1 is recognized as a negative regulator of insulin sensitivity [27], so it is theoretically acceptable that adiponectin knockout leads to MetS or that adiponectin KO mice are more susceptible to MetS under pathological stress. Although the exact relationship between MetS and CVD is not clear, both genetic and environmental factors may be involved. There is evidence that neuroendocrine factors [28] or the RAS (review [29]) may play an important role in MetS. We previously showed that plasma concentrations of catecholamines and renin were increased by LV pressure overload in mice [23]. In the present study, in addition to endogenous adiponectin deficiency, activation of the sympathoadrenal system and renin–angiotensin system (RAS) may have contributed to the onset of MetS.

The impact of MetS on CVD mortality has been investigated in several clinical studies [30–32]. It is generally agreed that CVD mortality is higher in subjects with MetS than in those without it. We found a positive correlation between IR and cardiac hypertrophy in adiponectin KO mice rather than in WT mice in this study, with both IR and HW/BW higher in adiponectin KO mice than in WT mice, suggesting that deficiency of adiponectin contributed to enhanced cardiac remodeling. Consistent with our results, a recent case-control study found that abnormal LV geometry and LV dysfunction were related to MetS [33]. Additionally, it is well known that type 2 diabetic patients are susceptible to diabetic cardiomyopathy, and the fasting plasma insulin level was reported to be the strongest independent predictor of LV mass in type 2 diabetes [34]. Taken together, these findings support the concept that MetS has an impact on cardiac remodeling. Although IR is known to be an important contributor to the

progression of heart failure, our data reported here are not enough to delineate the causal relationship between IR and cardiac remodeling. In spite of an increase tendency of IR showing in mice with cardiac hypertrophy, we did not find a significant correlation between IR and heart-to-body weight ratio in a relatively small sample of wild-type mice. In accordance with this study, previous clinical observations have shown IR to be related to the thickness of LV walls rather than LVH [35,36].

Adiponectin was reported to reduce the production of TNFα, and to improve both glucose metabolism and IR via the AMPK signaling pathway [12], suggesting that it may improve MetS. Evidence is emerging to demonstrate a critical role of AMPK in cardiac remodeling. Mutation of the gamma 2 subunit of AMPK has been shown to cause glycogen storage cardiomyopathy, and the influence of AMPKα on cardiac remodeling is another attractive research field. Both AMPKα_1 and AMPKα_2 expression were increased in hypertrophied hearts in the present study, which is only partially consistent with a previous investigation by Tian et al. [8]. They reported that α_1 was increased, α_2 expression was decreased, whereas activity of both AMPKα_1 and α_2 was increased in pressure overload rats. The reasons for this discrepancy are not clear. Generally, the activity of both AMPKα_1 and α_2 was reported to increase under stress conditions such as ischemia and pressure overload [8,10,18]. The protein expression of myocardial AMPK was seldom investigated and the reports are inconsistent. Acute ischemia [37] or short-term pressure overload [18] stimulates activity of myocardial AMPK without changing the AMPK protein expression, whereas both AMPKα_2 activity and expression were decreased at three weeks following volume-overload [38]. AMPK deficiency is reported to result in depressed LV function, increased myocardial necrosis, and apoptosis following ischemia/reperfusion injury [10]. The finding that AMPKα protein expression was increased in WT mice after TAC suggests that the augmentation of AMPKα signaling is a compensatory mechanism that attempts to maintain energy homeostasis in the heart under pressure overload. This mechanism may be partly controlled by adiponectin, because AMPK signaling was impaired in adiponectin KO mice and there was consequent progression of cardiac remodeling. Thus, this study provided a new link between adiponectin and AMPK in the process of cardiac remodeling. Apart from its influence on IR, AMPK, and TNFα, other mechanisms may also be involved in the beneficial effect of adiponectin on cardiac remodeling. Adiponectin has been reported to suppress superoxide generation and enhance eNOS activity [39], to have an antiproliferative effect [40], and to counteract beta adrenergic stimulation [41], all of which are closely related to cardiac remodeling [42]. Interestingly, AMPK and eNOS co-localize in hearts and AMPK was reported to activate eNOS [43,44]. Thus, it is reasonable for adiponectin deficiency to lead to progressive cardiac remodeling in response to pressure overload, as we showed in this study.

Acknowledgments

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Original article

A calcium channel blocker amlodipine increases coronary blood flow via both adenosine- and NO-dependent mechanisms in ischemic hearts

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Abstract

Amlodipine reduces oxidative stress that decreases NO and adenosine release. This study was undertaken to examine whether amlodipine mediates coronary vasodilation and improves myocardial metabolism and contractility in ischemic hearts via either adenosine- or NO-dependent mechanisms. In open-chest dogs, amlodipine (2 μg kg per min) was infused at the minimum dose that caused maximal coronary vasodilation. The perfusion pressure was reduced in the left anterior descending coronary artery so that coronary blood flow (CBF) decreased by 50%. Amlodipine increased the difference of the adenosine level (VAD (Ado): 119 ± 14 to 281 ± 46 nM) and the nitrate + nitrite level (VAD (NOx): 7.8 ± 1.3 to 16.1 ± 1.1 μM) between coronary venous and coronary arterial blood, and also increased CBF (50 ± 3 to 69 ± 6 ml/100 g/min). These changes were partially reversed by either 8-sulfophenyeltheophylline (8SPT) or L$^{\omega}$-nitro arginine methyl ester (L-NAME), and were completely blocked by both 8SPT and L-NAME. The reduction of CBF increased VAD (8-iso-prostaglandin $F_{2\alpha}$), and this increase was reduced by amlodipine (10.8 ± 1.1 to 5.0 ± 0.5 pg/ml). In addition, pretreatment with superoxide dismutase mimicked the coronary effects of amlodipine and blunted the response to amlodipine administration. Amlodipine-induced coronary vasodilation via both adenosine- and NO-dependent mechanisms. Adenosine and NO may interact in ischemic hearts to mediate coronary vasodilation by amlodipine.

Keywords: Calcium channel blocker; Adenosine; Ischemia; Coronary circulation

1. Introduction

Ca channel blockers are often used for the treatment of ischemic heart disease, because coronary vasodilation [1,2] is promoted via inhibition of Ca^{2+} entry into smooth muscle cells [3]. Long-acting dihydropyridine Ca channel blockers were recently reported to protect the endothelium of renal resistance arteries in hypertensive rats [4] and the mesenteric arteries in rats with circulatory shock [5]. Interestingly, amlodipine increases NO production by coronary arterial endothelial cells [6], and we have reported that other long-acting Ca channel blockers (benidipine and nifedipine) have the potential to increase NO production in ischemic heart [7,8]. Since oxidative stress inactivates NO and amlodipine suppresses oxidative stress [9,10], this drug may promote NO release. On the other hand, we have reported that oxidative stress inactivates ecto-5′-nucleotidase [11,12], the enzyme

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responsible for adenosine production, because it is located on the cell membrane and may be targeted by oxygen-derived free radicals. These lines of evidence support the hypothesis that amlodipine increases coronary blood flow (CBF) in ischemic myocardium via both adenosine- or NO-dependent mechanisms.

The aim of this study was to determine whether 1) amlodipine increases either adenosine or NO release from ischemic myocardium in canine hearts, and 2) whether coronary vasodilation mediated by amlodipine is attenuated by either 8-sulfophenyltheophylline (8SPT) or L^{ω}-nitro arginine methyl ester (L-NAME). To approach the goal, we examined the difference in the concentrations of adenosine (VAD (Ado)) and nitrate + nitrite (VAD (NOx)) between coronary venous blood and coronary arterial blood during infusion of amlodipine with 8SPT or L-NAME treatment. Furthermore, we tested whether amlodipine could increase VAD (Ado), VAD (NOx), and CBF in the presence of superoxide dismutase (SOD). We showed that amlodipine could increase both adenosine and NO release in ischemic canine myocardium, thus contributing to coronary vasodilation. We also detected a role of amlodipine-induced attenuation of oxygen-derived free radical generation in these actions.

2. Materials and methods

2.1. Instrumentation [13]

The hybrid dogs (HBD) mated with the Beagle, the American Fox Hound and the Labrador Retriever for the laboratory use (body weight: 15–21 kg) were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg), intubated, and ventilated using room air mixed with oxygen (100% O_2 at a flow rate of 1.0–1.5 l/min). The chest was opened through the left fifth intercostal space, and the heart was suspended a pericardial cradle. After intravenous administration of heparin (500 U/kg), the left anterior descending (LAD) coronary artery was cannulated and perfused with blood from the left carotid artery through an extracorporeal bypass tube. CBF in the perfused region was measured with an electromagnetic flow probe attached to the bypass tube, and coronary perfusion pressure (CPP) was monitored at the tip of the coronary artery cannula. We obtained coronary vascular resistance (CVR) by calculation of CPP/CBP.

A thin (1 mm) and short (70 mm) cannula connected to a thin tube was inserted into a small coronary vein near the center of the perfused region to sample coronary venous blood. The draining venous blood was collected in a reservoir at the level of the left atrium and then was returned to the jugular vein. Hydration was maintained by slow infusion of normal saline. The pH, pO_2, and pCO_2 of systemic arterial blood before the protocol was 7.39 ± 0.02, 106 ± 3, and 38.0 ± 2.0 mmHg, respectively. A pair of ultrasonic crystals was placed at the inner one-third of the myocardium about 10 mm apart in order to measure myocardial segment length with an ultrasonic dimension gauge (5 MHz, 2 mm in diameter; Schuessler, Cardiff-by-the Sea, CA). Hemodynamic parameters were recorded on a multi-channel recorder (Rm-6000; Nihon Kohden, Tokyo, Japan). End-diastolic length (EDL) was measured at the R wave of the electrocardiogram and end-systolic length (ESL) was measured at the minimal value of the first derivative of left ventricular pressure. Then fractional shortening (FS) was calculated as [(EDL − ESL)/EDL] × 100%.

Agents were administered into the LAD via the bypass tube. We purchased sodium pentobarbital, heparin, 8SPT, L-NAME, adenosine, bradykinin, and SOD from Sigma (St. Louis, MO, USA). Amlodipine was kindly provided by Pfizer Pharmaceutical Inc. (Sandwich, UK). The investigation conforms with the *Guide for the Care and Use Laboratory Animals* published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 1996).

2.2. Experimental protocols

2.2.1. Protocol I

After hemodynamic stabilization, coronary arterial and venous blood were sampled for blood gas analysis and for measurement of the lactate, nitrate + nitrite [14], and adenosine [15] concentrations, allowing the calculation of VAD (NOx), VAD (Ado), myocardial oxygen consumption. Myocardial oxygen consumption (ml/100 g/min) is calculated by CBF (ml/100 g/min) × the oxygen difference between coronary arterial and venous blood (ml/dl).

Lactate concentrations were measured by an enzymatic assay, and lactate extraction ratio (LER) was calculated as the coronary arteriovenous difference of the lactate concentration multiplied by 100 and divided by the arterial lactate concentration.

We used 29 dogs for Protocol I. Hemodynamic parameters (systolic and diastolic aortic blood pressure, and heart rate) were monitored. To examine whether either adenosine- or NO-dependent mechanisms were involved in amlodipine-induced coronary vasodilation in ischemic heart, we infused saline (n = 9), 8SPT (25 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 1.5 mg/ml, n = 5), L-NAME (10 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.6 mg/ml, n = 5), or L-NAME + 8SPT (n = 5) into the bypass. We dissolved amlodipine in 0.15% DMSO with saline and either L-NAME or 8SPT in saline. After we confirmed that either systemic or coronary hemodynamics did not change after 5 min of the infusion, we reduced CPP so that CBF decreased to 50% of the baseline value for 5 min. Then amlodipine (2 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.12 mg/ml) was infused and continued for 120 min while CPP was maintained at the set low constant value. As for the control of the amlodipine treatment, we infused the solvent of amlodipine (at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.12 mg/ml, n = 5). The times for the measurements of coro-

nary and systemic hemodynamics, and adenosine and NO concentrations after the onset of coronary hypoperfusion were 10, 30, 60, 90 and 120 min after the onset of coronary hypoperfusion. As a marker of oxidative stress, the 8-iso-prostaglandin $F_{2\alpha}$ (8-Iso-$F_{2\alpha}$) concentration was measured in coronary arterial and venous blood in nine dogs in the amlodipine-treated dogs without either 8SPT or L-NAME, and we calculated the arteriovenous difference of 8-Iso-$F_{2\alpha}$ (VAD(8-Iso-$F_{2\alpha}$)).

In a preliminary study, we confirmed that the doses of 8SPT and L-NAME were the minimum doses that prevented coronary vasodilation induced by intracoronary administration of adenosine (2 μg/kg per min) and bradykinin (20 ng/kg per min), respectively. The dose of amlodipine (2 μg/kg per min) for an intracoronary infusion was the lowest dose that caused maximal coronary vasodilation.

2.2.2. Protocol II

To examine whether elimination of oxidative stress was involved in amlodipine-induced adenosine- or NO-dependent coronary vasodilation in ischemic hearts, we infused recombinant human SOD (5340 IU/mg, > 99% purity) (25 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 1.5 mg/ml, $n = 10$) into the bypass tube at a rate of 1.5 ml/kg per min after hemodynamic stability was achieved. Then we reduced CPP so that CBF decreased to 50% of baseline. At 5 min after the onset of ischemia, infusion of amlodipine was initiated and continued for 120 min while keeping CPP at the 50% value. We previously confirmed that this dose of SOD did not affect systemic and coronary hemodynamic parameters [11]. Adenosine, NO and 8-Iso-$F_{2\alpha}$ concentrations were measured in coronary arterial and venous blood. In the preliminary study, we tested the coronary effects of the solvent of amlodipine (at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.12 mg/ml, $n = 5$) as the control of the amlodipine treatment.

2.2.3. Protocol III

This protocol tested the effects of amlodipine in the constant flow model because the increases in CBF due to amlodipine may secondarily alter the adenosine or NO release in ischemic hearts. We used five dogs in Protocol III. Hemodynamic parameters (systolic and diastolic aortic blood pressure, and heart rate) were monitored. After the hemodynamic stabilization, we reduced CPP so that CBF decreased to 50% of the baseline value for 5 min, and we kept CBF constant at this low level thereafter. Then an infusion of amlodipine (2 μg/kg per min at an infusion rate of 0.0167 ml/kg per min and a concentration of 0.12 mg/ml) was initiated and continued for 120 min while CBF was maintained at the 50% value. The times for the measurements of coronary and systemic hemodynamics, and adenosine and NO concentrations were same as in Protocol I.

2.2.4. Protocol IV

Adenosine and NO were produced via ecto-5′-nucleotidase and NOS, respectively. To test the cellular mechanisms of amlodipine-induced production of either adenosine or NO, we tested whether amlodipine restores the deactivation of ecto-5′-nucleotidase and NOS caused by the oxygen-derived free radicals in rat cardiomyocytes. In rat cardiomyocytes with xanthine (1×10^{-4} M) and xanthine oxidase (1.6×10^{-3} U/ml), that produces oxygen-derived free radicals, the time courses of the changes in both ecto-5′-nucleotidase and NOS activities were observed with and without the concomitant administration of amlodipine of 1×10^{-7} and 1×10^{-8} M for 60 min in rat cardiomyocytes ($n = 6$ each). We obtained the rat cardiomyocytes following the previously reported method [16]. The methods to measure both myocardial ecto-5′-nucleotidase [17] and NOS [18] activities have been reported previously.

Since the calculated amlodipine levels in the hearts in Protocols I–III correspond to 1×10^{-6} M of amlodipine, we employed 1×10^{-7} and 1×10^{-8} M of amlodipine. This is because in in vivo experiments, amlodipine may be conjugated by protein in the blood, and amlodipine concentrations at the site of small coronary smooth muscles may be decreased by the barrier of endothelial cells, which suggest that we need to use a smaller dose of amlodipine to exert the comparable effects of amlodipine in vivo.

2.3. Statistical analysis

Statistical analysis was performed using two-way ANOVA [19,20] to compare data among the groups. When ANOVA reached significance, paired data were compared using Bonferroni's test. Changes of the hemodynamic and metabolic parameters over time were compared by ANOVA for repeated measures. Values were expressed as the mean ± S.E.M., with $P < 0.05$ indicating significance.

3. Results

Either mean systemic blood pressure (101 ± 2 mmHg) and heart rate (134 ± 2 per min) did not differ significantly among all of the groups in Protocols I–III. Before and during coronary hypoperfusion with or without pharmacological intervention, these systemic hemodynamic parameters did not change significantly. Before coronary hypoperfusion, CPP was not different among the groups (104 ± 6 mmHg in the amlodipine group, 99 ± 5 mmHg in the solvent group, 102 ± 4 mmHg in the amlodipine + 8SPT group, 103 ± 4 mmHg in the amlodipine + L-NAME group, 99 ± 6 mmHg in the amlodipine + 8SPT + L-NAME group, 100 ± 6 mmHg in the SOD with or without amlodipine groups and 101 ± 6 mmHg in the constant CBF group), and the additional intracoronary infusion of amlodipine, the solvent of amlodipine, 8SPT, L-NAME or 8SPT + L-NAME did not significantly alter CPP. In the 8SPT, L-NAME, and 8SPT + L-NAME groups, CPP (57 ± 2 mmHg, 58 ± 3 mmHg, and 61 ± 3 mmHg, respectively) was higher (all $P < 0.05$) than in the solvent group (51 ± 3 mmHg) during coronary hypoperfusion, while CPP was lower ($P < 0.05$) in the SOD groups (42 ± 1 mmHg).

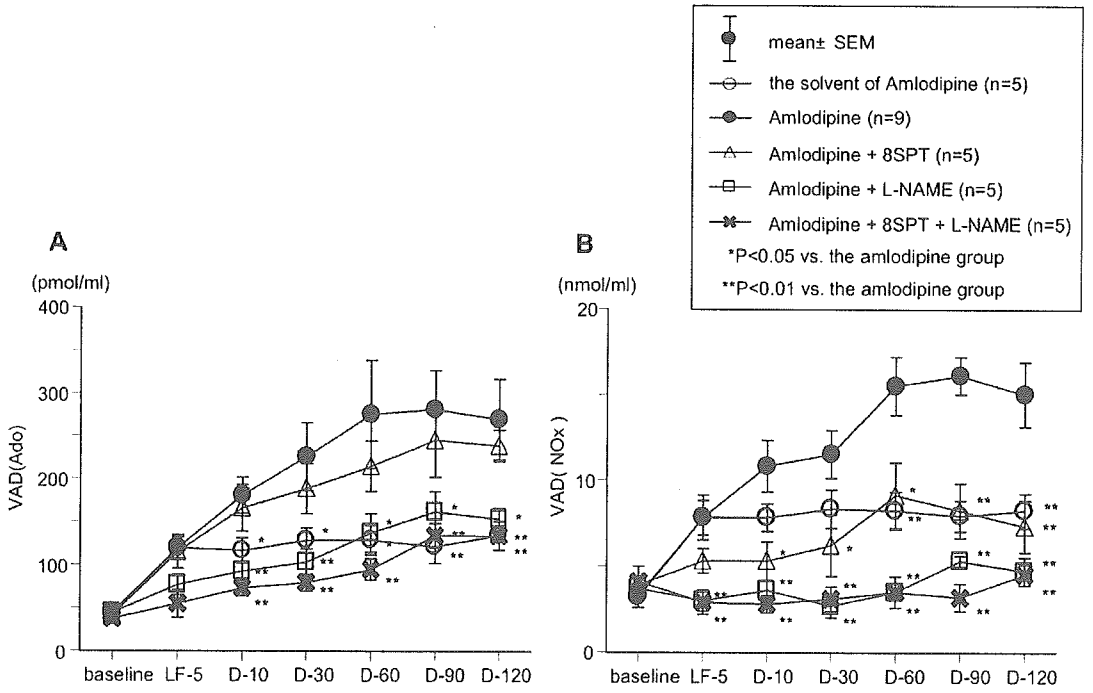

Fig. 1. Effects of either amlodipine or solvent of amlodipine on VAD (Ado) (A) and VAD (NOx) (B) in canine ischemic myocardium. VAD (Ado) and VAD (NOx) = the differences of adenosine and nitrate + nitrite concentrations between coronary venous and arterial blood, respectively, LF-5 = at 5 min of low flow, D-10, 30, 60, 90 and 120 = at 10, 30, 60, 90 and 120 min after the onset of either amlodipine or solvent of amlodipine infusion, respectively. Data are mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. the amlodipine group.

Fig. 1 shows VAD (Ado) (A) and VAD (NOx) (B) in the amlodipine, the solvent, amlodipine + 8SPT, amlodipine + L-NAME, and amlodipine + 8SPT + L-NAME groups. Both VAD (Ado) and VAD (NOx) increased ($P < 0.05$) in response to decreases of both CBF and CPP, and the amlodipine infusion further increased both VAD (Ado) and VAD (NOx) (both $P < 0.01$). The increase of VAD (Ado) was attenuated by L-NAME ($P < 0.05$), and that of VAD (NOx) was attenuated by both L-NAME ($P < 0.01$) and 8SPT ($P < 0.01$). The solvent for amlodipine did not alter either of these parameters. Fig. 2 shows that amlodipine increased CBF ($P < 0.01$) and decreased CVR ($P < 0.05$) in ischemic hearts, while either 8SPT or L-NAME attenuated the increase of CBF (both $P < 0.05$), and 8SPT + L-NAME almost completely blocked the changes of both CBF ($P < 0.01$) and CVR ($P < 0.05$). The solvent for amlodipine did not alter either CBF or CVR. Fig. 3 shows that FS (A), LER (B) and myocardial oxygen consumption (C) decreased after the onset of coronary hypoperfusion, while all increased (both $P < 0.01$) along with the amlodipine infusion. The infusion of the solvent for amlodipine changed none of these parameters during coronary hypoperfusion. These results indicate that amlodipine increased CBF and ameliorated myocardial ischemia via either adenosine- or NO-dependent mechanisms.

We also found that ischemia increases VAD (8-Iso-$F_{2\alpha}$) from 5.0 ± 0.71 to 10.8 ± 1.1 pg/ml ($P < 0.01$), and amlodipine decreases ($P < 0.01$) VAD (8-Iso-$F_{2\alpha}$) (7.4 ± 0.9, 6.8 ± 1.2, 5.8 ± 1.0 5.4 ± 0.9, 5.0 ± 0.5 pg/ml at 10, 30, 60 90 and 120 min after the onset of amlodipine administration, respectively). Figs. 4 and 5 show the effect of SOD on the amlodipine-induced increase of VAD (Ado) (Fig. 4A), VAD (NOx) (Fig. 4B) and VAD (8-Iso-$F_{2\alpha}$) (Fig. 4C), CPP (Fig. 5A), CBF (Fig. 5B), and CVR (Fig. 5C). Treatment with SOD increased both VAD (Ado) ($P < 0.01$) and VAD (NOx) ($P < 0.05$) and reduced VAD (8-Iso-$F_{2\alpha}$) ($P < 0.01$), while amlodipine did not further increase VAD (Ado), VAD (NOx), or CBF, or decrease either CVR or VAD (8-Iso-$F_{2\alpha}$). Fig. 6 shows that amlodipine did not further increase either FS (A) or LER (B) in the presence of SOD during coronary hypoperfusion.

We examined the effects of the solvent for amlodipine in ischemic myocardium. We observed no additional effects of the solvent for amlodipine on VAD (Ado) (125 ± 17 and 123 ± 14 pmol/ml at LF-5 and D-60, ns), VAD (NOx) (7.9 ± 1.7 and 8.1 ± 1.2 nmol/ml at LF-5 and D-60, ns), VAD (8-Iso-$F_{2\alpha}$) (11.4 ± 1.2 and 12.0 ± 1.5 pg/ml at LF-5 and D-60, ns), CPP (51 ± 2 and 51 ± 2 mmHg at LF-5 and D-60, ns), CBF (51 ± 5 and 50 ± 5 ml/100 g/min at LF-5 and D-60, ns), CVR (1.0 ± 0.1 and 1.0 ± 0.1 mmHg/ml/100 g/min at LF-5 and D-60, ns), FS (7.7 ± 1.2 and 7.7 ± 1.1% at LF-5 and D-60, ns), and LER (−29 ± 11 and −25 ± 5% at LF-5 and D-60, ns) during coronary hypoperfusion.

In Protocol III, we kept CBF constant at the 50% of the control levels to keep the severity of myocardial ischemia constant. We observed that amlodipine decreased CPP, and increased the ischemia-induced increase of VAD (Ado) and VAD (NOx) despite unchanged CBF and myocardial oxygen consumption (Table 1).

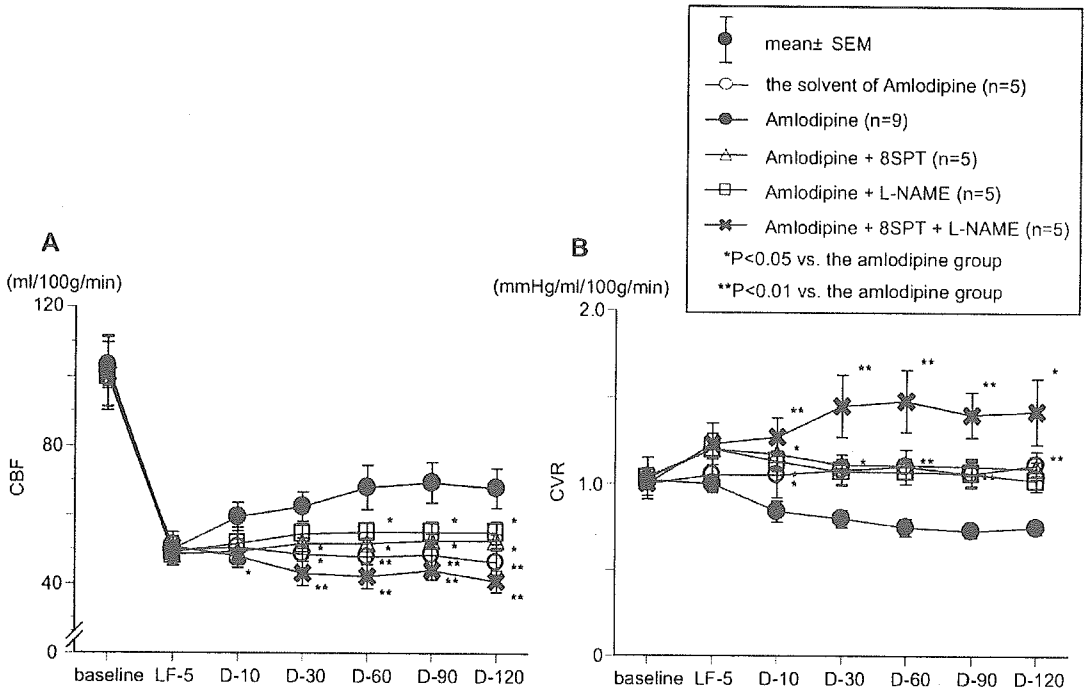

Fig. 2. Effects of either amlodipine or a solvent of amlodipine in CBF and CVR in ischemic myocardium. Either 8SPT or L-NAME attenuated the increases in CBF, and both 8SPT + L-NAME almost completely attenuated the increases in CBF. Abbreviations are same as in Fig. 1. Data are mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. the amlodipine + saline group.

In Protocol IV, we observed the effects of amlodipine on the activities of ecto-5′-nucleotidase and NOS. Xanthine + xanthine oxidase deactivated ecto-5′-nucloetidase (control; 24.2 ± 2.4, 15 min; 16.8 ± 2.7 ($P < 0.01$ vs. control), 60 min; 14.5 ± 1.1 ($P < 0.01$ vs. control) nmol/mg protein per min, $n = 6$ each) and NOS (control; 100%, 15 min; 78 ± 3% ($P < 0.01$ vs. control), 60 min; 73 ± 3% ($P < 0.01$ vs. control), $n = 6$ each). We confirmed that these decreases are stable for 2 h. The exposure to xanthine + xanthine oxidase with 1×10^{-7}amlodipine prevented the deactivation of ecto-5′-nucleotidase (control; 23.6 ± 1.9, 30 min; 25.3 ± 1.1 ($P < 0.01$ vs. the condition without amlodipine), 60 min 25.9 ± 1.8 ($P < 0.01$ vs. the condition without amlodipine) nmol/mg protein/min, $n = 6$ each) and NOS (control; 100%, 30 min; 92 ± 5% ($P < 0.01$ vs. the condition without amlodipine), 60 min; 97 ± 4% ($P < 0.01$ vs. the condition without amlodipine), $n = 6$ each) of rat cardiomyocytes. These effects were also the case when we used 1×10^{-8} M amlodipine (ecto-5′-nucleotidase activity (control; 25.2 ± 2.3, 30 min; 22.5 ± 1.7 ($P < 0.01$ vs. the condition without amlodipine), 60 min 22.5 ± 1.3 ($P < 0.01$ vs. the condition without amlodipine) nmol/mg protein per min, $n = 6$ each) and NOS activity (control; 100%, 30 min; 92 ± 3% ($P < 0.01$ vs. the condition without amlodipine), 60 min; 92 ± 3% ($P < 0.01$ vs. the condition without amlodipine), $n = 6$ each)).

4. Discussion

The present study revealed that amlodipine, a long-acting Ca channel blocker, could increase CBF and ameliorate myocardial ischemia by increasing adenosine or NO release in ischemic hearts. Additionally, we found that elimination of oxygen-derived free radicals by amlodipine plays an important role in its induction of coronary vasodilation and in the increase of both VAD (Ado) and VAD (NOx), since these effects of amlodipine were blunted by treatment with SOD. Furthermore, we showed amlodipine attenuated the deactivation of ecto-5′-nucleotidase and NOS due to oxygen-derived free radicals in rat cardiomyocytes.

4.1. Adenosine, NO, and amlodipine in ischemic hearts

Under ischemic conditions, both alpha-adrenoceptor stimulation and H^+ increase the activity of ecto-5′-nucleotidase [21,22] and NOS [23,24]. Since oxygen-derived free radicals attenuate the ischemia-induced activation of ecto-5′-nucleotidase and enhance the degradation of NO, elimination of oxidative stress may increase adenosine or NO release in the myocardium. Since amlodipine has been reported to reduce oxidative stress, amelioration of oxidative stress by this drug may explain the present findings. Indeed, in the present study, we observed that 8-Iso-$F_{2\alpha}$ concentration, an index of oxidative stress, is attenuated by amlodipine, and amlodipine restores the decreased activities of ecto-5′-nucleotidase and NO synthase due to oxidative stress in rat cardiomyocytes.

Nevertheless, we need to consider other possibilities to explain the present findings. First of all, if amlodipine caused an increase of catecholamine or H^+ production, adenosine and NO release could be enhanced, but this is unlikely because

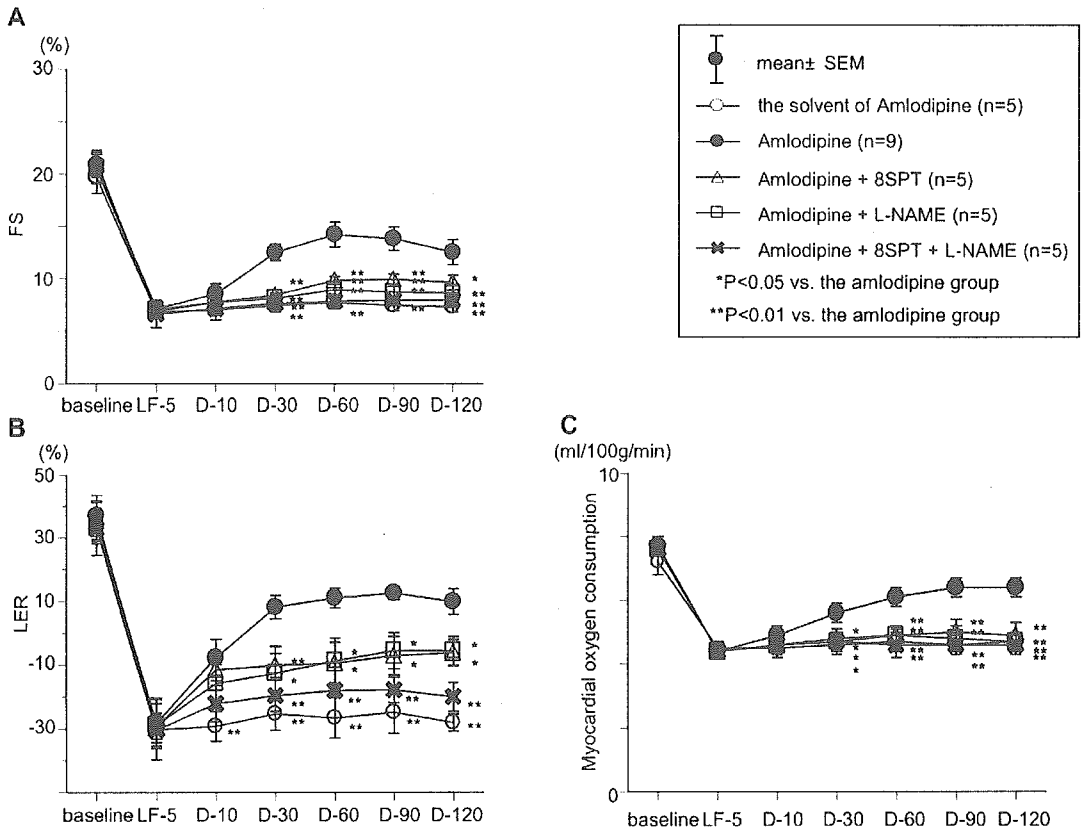

Fig. 3. Effects of either amlodipine or a solvent of amlodipine on FS (A), LER (B) and myocardial oxygen consumption (C) in ischemic myocardium. Abbreviations are same as in Fig. 1. Data are mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. the amlodipine + saline group.

there have been no reports that amlodipine can modulate these parameters. Secondly, amlodipine may affect myocardial oxygen consumption, and secondarily increase CBF. We observed that amlodipine mediates no changes in myocardial oxygen consumption during constant low levels of CBF in Protocol III.

Although amlodipine increased both adenosine and NO production, thus improving CBF in ischemic hearts, the cellular signal transduction pathways that lead to coronary vasodilation by adenosine or NO are completely different. Adenosine and NO are reported to elevate cyclic AMP and GMP concentrations, respectively, which independently cause coronary vasodilation. The former mainly causes detachment of actin from myosin [25], while the latter mainly promotes re-uptake of Ca^{2+} into the SR [26]. Therefore, the increases of both adenosine and NO release independently leads to coronary vasodilation, as revealed by the fact that the attenuation of increased CBF was greater in the 8SPT + L-NAME group than in the 8SPT or L-NAME groups.

It was intriguing to find that L-NAME reduces adenosine release and 8SPT reduces NO release, because this observation implies that NO contributes to adenosine release, and that adenosine contributes to NO release. Interactions between adenosine and NO have been reported previously [27], and we have found that blockade of adenosine receptors activates NO synthase (unpublished data). Indeed, adenosine increases

Table 1
Changes in CPP, VAD (Ado), VAD (NOx), and myocardial oxygen consumption in CBF constant model

Baseline	LF-5	D-10 D-30	D-60	D-90	D-120
CPP (mmHg)					
101 ± 6**	51 ± 3	43 ± 2** ± 2**	38 ± 2**	36 ± 2**	36 ± 3**
VAD (Ado) (pmol/ml)					
41 ± 7**	115 ± 14	161 ± 17** ± 23**	256 ± 26**	262 ± 28**	255 ± 25**
VAD (NOx) (nmol/ml)					
3.4 ± 0.7**	8.0 ± 1.2	9.5 ± 0.8** ± 1.6**	13.2 ± 1.4**	13.9 ± 1.6**	13.4 ± 1.3**
Myocardial oxygen consumption (ml/100 g/min)					
7.1 ± 0.3**	4.4 ± 0.2	4.5 ± 0.3** ± 0.5	5.5 ± 0.6	5.5 ± 0.7	5.5 ± 0.6

Abbreviations: CPP = coronary perfusion pressure, VAD (Ado) and VAD (NOx) = the differences of adenosine and nitrate + nitrite concentrations between coronary venous and arterial blood, respectively, LF-5 = at 5 min of low flow, D-10, 30, 60, 90 and 120 = at 10, 30, 60, 90 and 120 min after the onset of the amlodipine infusion, respectively. Data are mean ± S.E.M. *$P < 0.05$, **$P < 0.01$ vs. the value of LF-5.

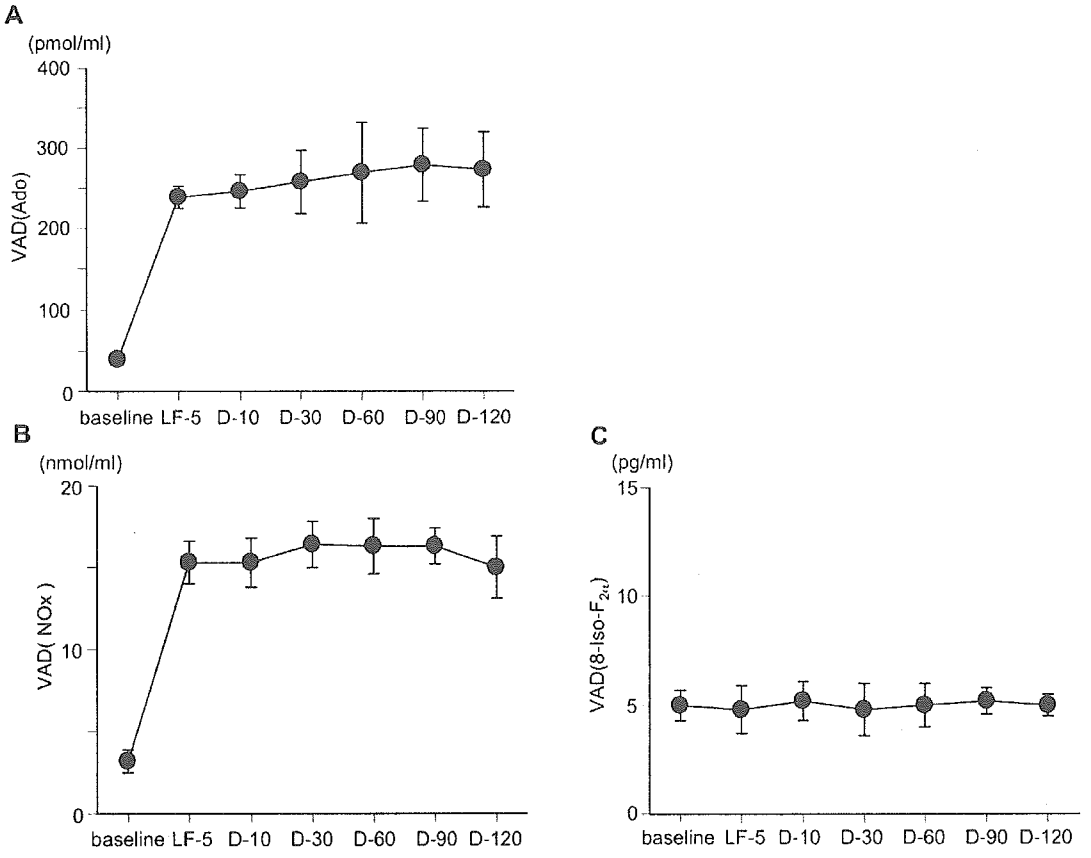

Fig. 4. Effects of amlodipine on VAD (Ado) (A), VAD (NOx) (B) and VAD(8-Iso-$F_{2\alpha}$) (C) in the presence of SOD in ischemic myocardium. Abbreviations are same as in Fig. 1.

the cyclic GMP concentration in vascular smooth muscle cells [28]. We also found that inhibition of NO synthase activates ecto-5′-nucleotidase in endothelial cells [29], suggesting that NO may reduce adenosine production. This may not agree to the present finding, and this difference may be attributable to the variations in experimental conditions (in vitro vs. in vivo, non-ischemic vs. ischemic hearts, and endothelial cells vs. whole hearts). We conclude that at least in ischemic hearts, both endogenous adenosine and NO mutually enhance their production in the in vivo hearts.

Our observation that both concentrations of adenosine and NO increase during an infusion of amlodipine meets the therapeutic requirements of ischemic hearts. Both adenosine and NO are believed to ameliorate myocardial ischemia [27] because both adenosine and NO 1) increase CBF, 2) reduce anaerobic metabolism, 3) inhibit platelet and leukocyte activation, and 4) reduce sympathetic activity in ischemic hearts. Indeed, the present study indicated that amlodipine could decrease the severity of myocardial ischemia (shown by FS and LER), with its effect being blunted by either 8SPT or L-NAME.

The present study suggested that elimination of oxygen derived-free radicals in ischemic hearts by amlodipine contributes to elevation of cardiac adenosine and NO concentrations, coronary vasodilation, and amelioration of myocardial ischemia because SOD blunted the beneficial effects of amlodipine. However, this result of SOD suggests two logical possibilities. One is that amlodipine increases oxygen-derived free radicals and that oxygen-derived free radicals induces coronary vasodilation. Indeed, SOD seems to decrease CBF, and thus, FS and LER in Figs. 5 and 6. However, in this protocol, we treated SOD prior to the time to decrease CPP, and in such a condition, oxygen-derived free radials are already eliminated in non-ischemic or ischemic myocardium. Since amlodipine has no more additional effects on ischemic hearts, the effects of amlodipine may be explained by the scavenging effects of oxygen-derived free radicals. In fact, another possibility that amlodipine attenuates oxygen-derived free radicals and oxygen-derived free radicals induced coronary vasoconstriction is more likely than the former possibility. We found that amlodipine attenuates the production of 8-Iso-F2α, supporting the latter hypothesis.

Since ecto-5′-nucleotidase is susceptible to oxygen derived-free radicals and oxygen derived-free radicals deactivate NO, it is likely that the beneficial effect of amlodipine on ischemic hearts in the present study was attributable to a combination of its anti-oxidant effect, the actions of adenosine and NO, and the Ca channel-blocking effects of amlodipine. However, if amlodipine works as a free radical scavenger, we need to consider the reason that almost 30 min is