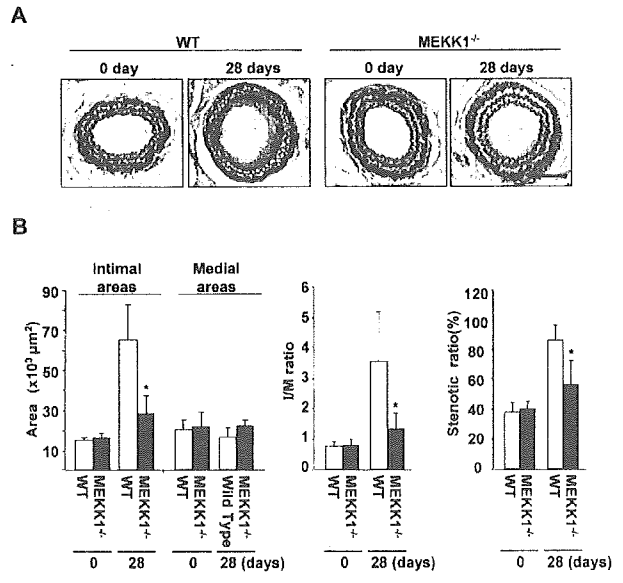

Figure 1. Photomicrographs and morphometry of intimal formation after ligation of carotid arteries. A, Representative cross sections of unligated and ligated carotid arteries on day 28 (elastica van Gieson's stain). Bar indicates 100 μm. B, Quantitative analysis of intimal and medial areas, I-M ratio, and stenotic ratio of ligated arteries. *$P<0.05$ vs WT mice.

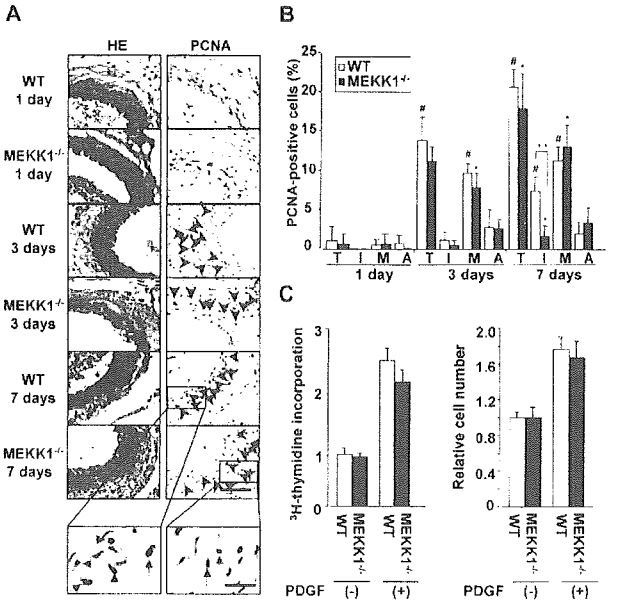

Figure 2. Effects of ablation of MEKK1 on cell proliferation. A, Immunohistochemical analysis of hematoxylin and eosin staining and PCNA staining 1, 3, and 7 days after ligation. Bar indicates 100 μm. Photographs of WT and MEKK1$^{-/-}$ mice 7 days after ligation were enlarged, and bar indicates 30 μm. Red arrowheads indicate PNCA-positive cells. B, Quantification of ratio of PCNA-positive cells to total cell number in arterial wall (T), intima (I), media (M), and adventitia (A) of ligated arteries at 1, 3, and 7 days. Black bars and white bars indicate WT and MEKK1$^{-/-}$ mice, respectively. #$P<0.05$ vs day 1 in WT mice; *$P<0.05$ vs day 1 in MEKK1$^{-/-}$ mice; **$P<0.05$ vs day 7 in WT mice. C, Effects of MEKK1 on PDGF-BB–induced [^{3}H]thymidine incorporation and cell number in AoSMCs. Data are indicated as percentages relative to control group and are mean±SEM of 10 wells in each group of at least 3 replicates.

compared with those 1 day after ligation, but there were no significant differences between WT and MEKK1$^{-/-}$ mice. It is noteworthy that 7 days after ligation, the number of PCNA-positive cells in the intima was significantly less in MEKK1$^{-/-}$ arteries than in WT arteries, although there was no difference within other areas (Figure 2A and 2B). These findings suggest that migration of PCNA-positive cells from the media to the intima was impaired, whereas proliferation was not impaired in MEKK1$^{-/-}$ mice. Consistent with in vivo data, no difference was observed in [^{3}H]thymidine incorporation and cell number between WT and MEKK1$^{-/-}$ AoSMCs after stimulation with PDGF-BB in vitro (Figure 2C). Treatment with EGF or FGF-2 instead of PDGF-BB yielded identical results (data not shown).

Effects of Ablation of MEKK1 on MAPK Activities of AoSMCs

Ablation of MEKK1 does not affect the total protein expression of extracellular signal–regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), or p38. We examined the effects of ablation of MEKK1 on MAPK activities of AoSMCs. FGF-2–induced JNK and ERK but not p38 activation in AoSMCs from MEKK1$^{-/-}$ mice was less than that from WT mice (Figure 3A). We confirmed that addition of MEKK1 restored MEKK1 protein levels in MEKK1$^{-/-}$ AoSMCs (Figure 3B). Addition of MEKK1 restored JNK and ERK activation in response to FGF-2 in MEKK1$^{-/-}$ AoSMCs (Figure 3C and 3D). Treatment with EGF or PDGF-BB instead of FGF-2 yielded identical results (data not shown).

MEKK1 Ablation Inhibited AoSMC Migration and Invasion

In the scrape wound–induced migration assays, the average number of and distance that MEKK1$^{-/-}$ AoSMCs migrated from the wound edge (white dotted line) in the absence of any stimulus was similar to those in WT mice; however, the FGF-2–induced increase in cell number and distance was significantly suppressed in MEKK1$^{-/-}$ AoSMCs compared with WT cells. Addition of MEKK1 restored the number and distance of MEKK1$^{-/-}$ AoSMCs to normal levels (Figure 4A and 4B).

In the aortic explant assays, the number of AoSMCs migrating from MEKK1$^{-/-}$ explants at 10 days was considerably lower than those from WT explants (Figure 4C). Moreover, the number of MEKK1$^{-/-}$ aortic explants that showed migrating cells was significantly smaller than in WT explants (27.2±2.2% versus 60.0±2.5%, $P<0.05$).

In the transwell Matrigel-coated chamber invasion assays, the number of invading MEKK1$^{-/-}$ AoSMCs in response to FGF-2 was suppressed markedly compared with WT cells. Addition of MEKK1 restored the number of MEKK1$^{-/-}$ AoSMCs to normal levels (Figure 4D). Treatment with EGF or PDGF-BB instead of FGF-2 yielded identical results (data not shown).

MEKK1 Ablation Impaired Lamellipodia Formation

There were no morphological differences between WT and MEKK1$^{-/-}$ AoSMCs under basal conditions; however, typi-

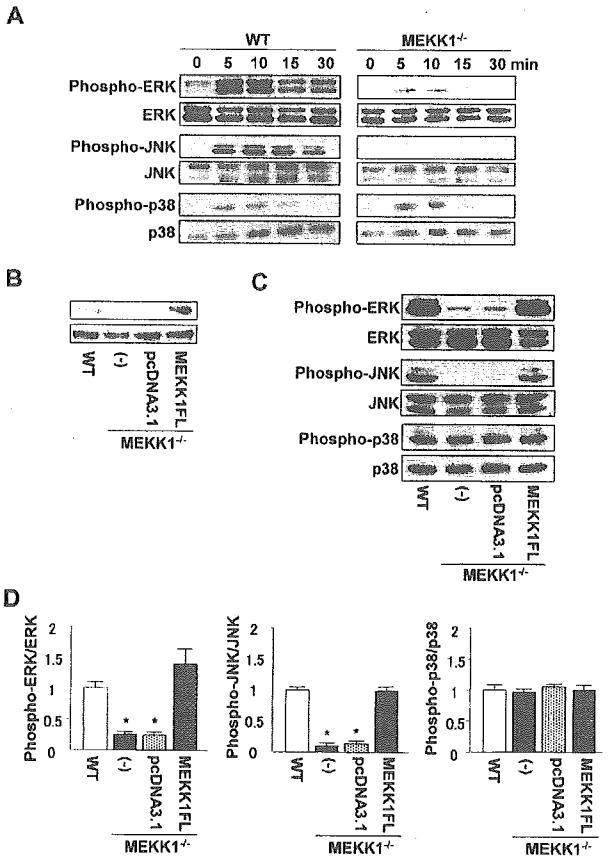

Figure 3. Effects of ablation of MEKK1 on MAPK activities in AoSMCs. A, Time course of JNK, ERK, and p38 activities followed by FGF-2 stimulation. B, Addition of MEKK1 experiments in MEKK1$^{-/-}$ AoSMCs. C, Western blot analysis of MAPKs in MEKK1$^{-/-}$ AoSMCs transfected with empty vector (pcDNA3.1) or full-length forms of MEKK1 vector (MEKK1FL) followed by FGF-2 stimulation. D, Cumulative data for MAPK in Figure 3C. *P<0.05 vs WT AoSMCs.

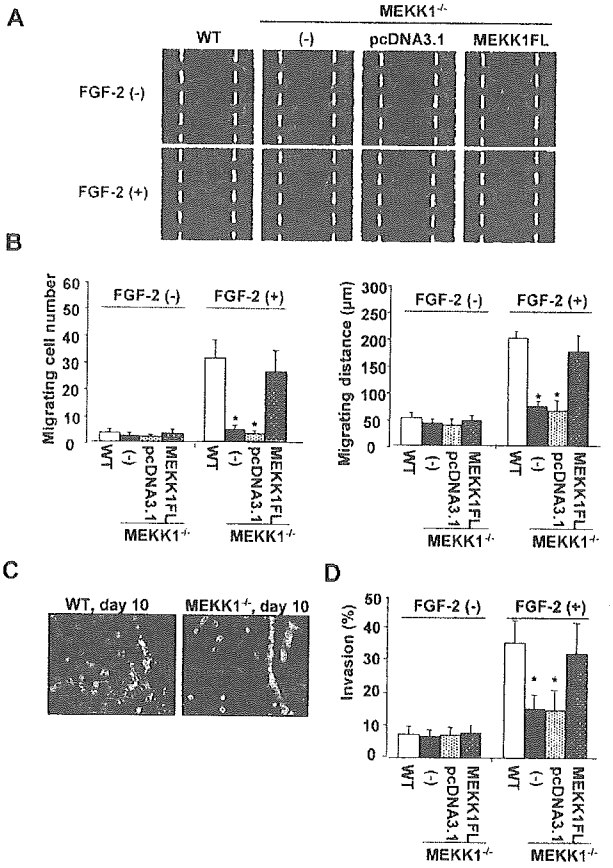

Figure 4. Migration and invasion of AoSMCs. A, Scrape wound–induced migration assay. Immunofluorescence microscopy of migrating cells from wound edge when AoSMCs were cultured for 24 hours with or without FGF-2. F-actin was stained with rhodamine-phalloidin (red), and nuclei were counterstained with DAPI (blue; not visible). B, Quantification of means of number and distance of migrating cells. *P<0.05 vs WT AoSMCs. C, Migration of AoSMCs from aortic explants on day 10. D, Quantification of percent invasion of AoSMCs by transwell Matrigel-coated chamber invasion assay. *P<0.05 vs WT AoSMCs.

cal lamellipodia formation was induced by treatment with EGF in WT AoSMCs but was seldom induced in MEKK1$^{-/-}$ AoSMCs (Figure 5A). The percentage of MEKK1$^{-/-}$ AoSMCs showing lamellipodia was significantly lower than in WT AoSMCs (P<0.05). Addition of MEKK1 restored the lamellipodia-forming capacity of MEKK1$^{-/-}$ AoSMCs (Figure 5B). Treatment with FGF-2 or PDGF-BB instead of EGF yielded identical results (data not shown).

MEKK1 Ablation Decreased uPA Expression

uPA expression began to increase on day 1 and reached a peak on day 3, after which it decreased gradually by 7 days after ligation in WT mice. In contrast, there was only weak positive staining for uPA up to 7 days in MEKK1$^{-/-}$ mice (Figure 6A). In vitro immunofluorescence staining revealed that PDGF-BB–induced uPA expression was decreased in MEKK1$^{-/-}$ AoSMCs compared with those of WT mice (Figure 6B). Western blotting showed a dramatic reduction of FGF-2–induced uPA expression in MEKK1$^{-/-}$ AoSMCs, which was restored by addition of MEKK1 (Figure 6C).

Discussion

Several lines of evidence suggest that MEKK1 is implicated in diverse biological responses.[6–8] Recently, the unique role of MEKK1 in regulating the migration of several cell types has aroused widespread attention[9,12,27] and inspired us to study its involvement in cardiovascular diseases. Up to now, there has been no previous report on the role of MEKK1 in the development of vascular remodeling, during which invasion and proliferation of SMCs play key roles. In the present study, we investigated the mechanism whereby MEKK1 regulates vascular remodeling in a well-established, blood-flow cessation model in MEKK1$^{-/-}$ mice. We found that MEKK1 is essential for intimal hyperplasia after cessation of blood flow.

In this study, we clearly demonstrated that intimal areas, I-M ratios, and stenotic ratios of the ligated arteries were significantly lower in MEKK1$^{-/-}$ mice relative to WT mice, indicating that MEKK1 is implicated in intimal hyperplasia. Although an angioplasty/balloon injury model would have yielded greater applicability to the clinical situation, we used

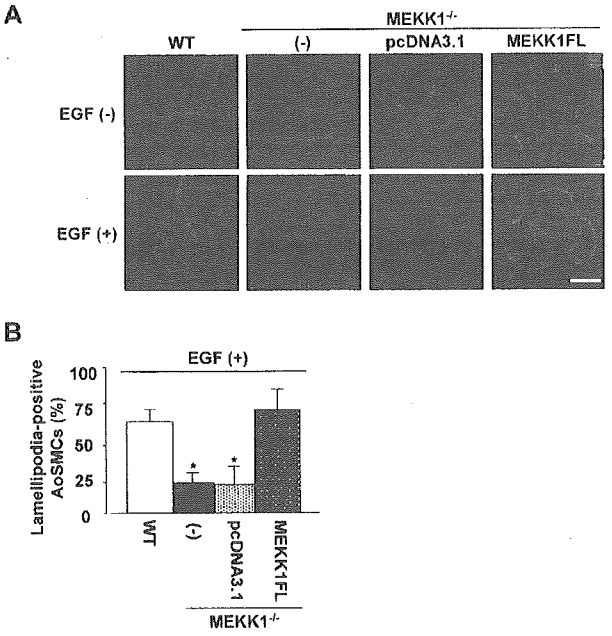

Figure 5. Lamellipodia formation in AoSMCs. A, Representative photomicrographs of lamellipodia formation in AoSMCs with or without EGF stimulation. Bar indicates 25 μm. B, Percentage of lamellipodia-positive cells (n=100) in AoSMCs with EGF. *$P<0.05$ vs WT AoSMCs.

the ligation model because of its excellent reproducibility. To clarify the mechanism(s) of MEKK1 involvement, we first examined cell proliferation within the arterial wall after ligation and found no statistically significant difference in the number of PCNA-positive cells between WT and MEKK1$^{-/-}$ mice. Results from this ligation model are consistent with the fact that MEKK1$^{-/-}$ mice have no overt defects in growth and fertility.[9] There was also no difference in the proliferation of WT and MEKK1$^{-/-}$ AoSMCs evaluated by [^{3}H]thymidine incorporation and cell number. These findings suggest that SMC proliferation might not contribute to the reduced intimal hyperplasia in MEKK1$^{-/-}$ mice. Then we investigated SMC migration and uPA expression, both of which are important factors for intimal hyperplasia.

We demonstrated that there were significantly fewer intimal PCNA-positive cells 7 days after ligation in MEKK1$^{-/-}$ mice than in WT mice. Because PCNA-positive cells are believed to migrate from the media to the intima,[2] this finding suggests that SMC migration is impaired in MEKK1$^{-/-}$ mice. To directly assess the effects of MEKK1 ablation on SMC migration, we used several migration and invasion assays in vitro. We observed comparable migration or invasion of SMCs under control condition between WT and MEKK1$^{-/-}$ AoSMCs; however, significant impairment of migration or invasion was observed after stimulation with FGF-2 in MEKK1$^{-/-}$ AoSMCs, which was restored by addition of full-length MEKK1. These findings indicate that ablation of MEKK1 impairs invasion and migration, both of which may contribute to reduced intimal hyperplasia wherein growth factors may play a vital role.

Lamellipodia formation is essential for cell migration.[13] Exogenous stimuli, such as PDGF or FGF-2, induce lamellipodia formation that can help to complete the first step of the motility cycle.[14,28] Therefore, we examined whether MEKK1 is involved in lamellipodia formation in AoSMCs. Our results showed that the percentage of lamellipodia-positive cells was significantly smaller in MEKK1$^{-/-}$ AoSMCs compared with WT AoSMCs in the presence of EGF. This finding suggests that the impairment of migration in MEKK1$^{-/-}$ AoSMCs may be due to inhibited formation of lamellipodia. Indeed, addition of MEKK1 to MEKK1$^{-/-}$ AoSMCs recovered their capacity to form lamellipodia and migrate. On the other hand, it has been reported that loss of MEKK1 disrupts focal adhesion composition, with decreased vinculin content and focal adhesion kinase (FAK) cleavage.[29] Because disruption of focal adhesion composition will affect cell migration, further investigation will be needed to clarify its role in MEKK1$^{-/-}$ mice.

There is evidence that uPA is induced after arterial injury.[5,28,30,31] uPA enhances vascular remodeling by transforming plasminogen into plasmin, which can activate metalloproteinases and in turn degrade ECM proteins.[5,30,31] We found that uPA expression began to increase at 1 day and reached a peak 3 days after ligation in WT mice, whereas uPA staining appeared to be significantly lower in MEKK1$^{-/-}$ arteries at corresponding times. In vitro, PDGF-BB– and FGF-2–induced uPA expression as detected by immunofluorescence staining and Western blotting was also significantly decreased in MEKK1$^{-/-}$ AoSMCs. Thus, in addition to impaired lamellipodia formation, inhibited uPA expression by MEKK1 ablation may also contribute to impairment of SMC invasion. Addition of full-length MEKK1 restored uPA expression in MEKK1$^{-/-}$ AoSMCs.

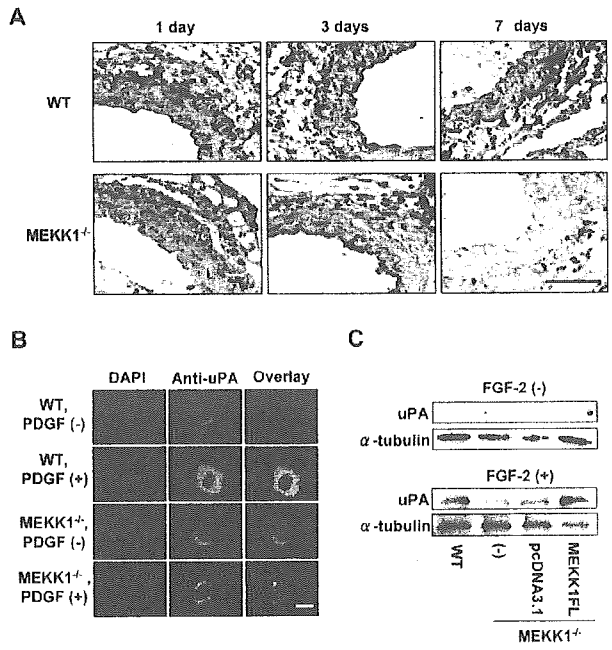

Figure 6. Expression of uPA in AoSMCs. A, Immunohistochemical analysis of time course of uPA expression 1, 3, and 7 days after ligation of carotid arteries. Bar indicates 100 μm. B, Immunofluorescence staining for uPA in AoSMCs stimulated with PDGF-BB. Bar indicates 10 μm. C, Western blots of uPA in AoSMCs stimulated with FGF-2.

Although it has been reported that MEKK1 is required for FGF-2–induced signals to control uPA expression in fibroblasts,[15] further investigations will be needed to elucidate the mechanism by which MEKK1 regulates uPA expression in arteries after ligation.

Recent studies have demonstrated that JNK and ERK transduction pathways may regulate cell migration[32,33] and uPA expression.[34,35] In the present study, we demonstrated that JNK and ERK activation after growth factor stimulation was blunted in MEKK1$^{-/-}$ AoSMCs. Thus, it is possible that ablation of MEKK1 may inhibit cell migration and uPA expression by interfering with the downstream signaling pathways JNK and/or ERK. MEKK1 also has been reported to be associated with cytoskeletal reorganization[11,12] and to be necessary for uPA upregulation,[15] suggesting another possibility that ablation of MEKK1 directly inhibits lamellipodia formation and uPA expression. The stimulus for remodeling after ligation is also influenced by the resultant vascular ischemia. Because MEKK1 is activated by hypoxic stimuli as well as growth factors,[36] we must consider the possibility that the resultant hypoxic stimuli are also important during vascular remodeling in the ligation model.

Izumi et al[22] demonstrated that activation of apoptosis signal–regulating kinase 1 (ASK1), another member of the MAP3K family, also plays a key role during intimal hyperplasia in the carotid artery balloon injury model. Unlike MEKK1, ablation of ASK1 blunted both JNK and p38 but not ERK activation in AoSMCs after serum stimulation. In addition, ablation of ASK1 caused impairment of both SMC migration and proliferation. Thus, although the methods or models used to evaluate functions of MEKK1 and ASK1 were not the same, both MEKK1 and ASK1 may contribute to the development of intimal hyperplasia by different mechanisms.

In conclusion, we have demonstrated that MEKK1 plays a critical role during intimal hyperplasia in a mouse carotid blood-flow cessation model. Intimal hyperplasia is greatly lessened, possibly due to a reduction of SMC invasion by an impairment of their migration and reduced uPA expression. We propose that MEKK1 is a potential target for drug development to prevent vascular remodeling.

Acknowledgments

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Aldosterone Nongenomically Worsens Ischemia Via Protein Kinase C-Dependent Pathways in Hypoperfused Canine Hearts

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Abstract—Rapid nongenomic actions of aldosterone independent of mineralocorticoid receptors (MRs) on vascular tone are divergent. Until now, the rapid nongenomic actions of aldosterone on vascular tone of coronary artery and cardiac function in the in vivo ischemic hearts were not still fully estimated. Furthermore, although aldosterone can modulate protein kinase C (PKC) activity, there is no clear consensus whether PKC is involved in the nongenomic actions of aldosterone on the ischemic hearts. In open chest dogs, the selective infusion of aldosterone into the left anterior descending coronary artery (LAD) reduced coronary blood flow (CBF) in the nonischemic hearts in a dose-dependent manner. Also, in the ischemic state that CBF was decreased to 33% of the baseline, the intracoronary administration of aldosterone (0.1 nmol/L) rapidly decreased CBF (37.4±3.8 to 19.3±5.2 mL/100 g/min; $P<0.05$), along with decreases in fractional shortening (FS) (8.4±0.7 to 5.4±0.4%; $P<0.05$) and lactate extraction rate (LER) (−31.7±2.9 to −41.4±3.7%; $P<0.05$). The decrease in CBF was reproduced by the infusion of bovine serum albumin-conjugated aldosterone. Notably, these aldosterone-induced deteriorations of myocardial contractile and metabolic functions were blunted by the co-administration of GF109203X, an inhibitor of PKC, but not spironolactone. In addition, aldosterone activated vascular PKC. 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 functions in the ischemic hearts. Elevation of plasma or cardiac aldosterone levels may be deleterious to ischemic heart disease through its nongenomic effects. (***Hypertension.*** **2005;46:113-117.)**

Key Words: aldosterone ■ ischemia ■ protein kinases

Aldosterone modulates cardiovascular function in addition to the crucial role in sodium and potassium homeostasis through binding to the intracellular mineralocorticoid receptors (MRs).[1] These receptor-mediated effects with transcriptional modulation of target genes are termed genomic effects.[2] Recently, another pathway possibly mediated by the specific membrane receptors through which aldosterone acts, ie, nongenomic effects of aldosterone, has been investigated.[2–4] Although this nongenomic action of aldosterone was shown in a variety of tissues such as vascular smooth muscle cells or cardiomyocytes, the effects of aldosterone on vascular tone are divergent.[4–9] Aldosterone increased systemic vascular resistance in humans, as shown in the first report on the nongenomic effects of aldosterone, whereas aldosterone inhibits vasoconstriction in renal afferent arterioles.[7–9] Until now, the rapid nongenomic effects of aldosterone on vascular tone of coronary artery and cardiac functions in vivo under ischemia are not still fully estimated. Moreover, several rapid aldosterone-induced changes of the concentration of intracellular second messengers have been described.[6,10,11] Aldosterone is reported to activate protein kinase C (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] However, there is no clear consensus whether PKC is involved in the nongenomic effects of aldosterone on the vascular tone of coronary arteries in the ischemic hearts. Thus, this study was undertaken to investigate the nongenomic effects of aldosterone on coronary vascular tone and cardiac functions in the in vivo ischemic hearts and a role of PKC in this nongenomic effect in canine hearts.

Materials and Methods

Both aldosterone and spironolactone were purchased from Sigma (St. Louis, Mo), and a specific PKC inhibitor, GF109203X, was

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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

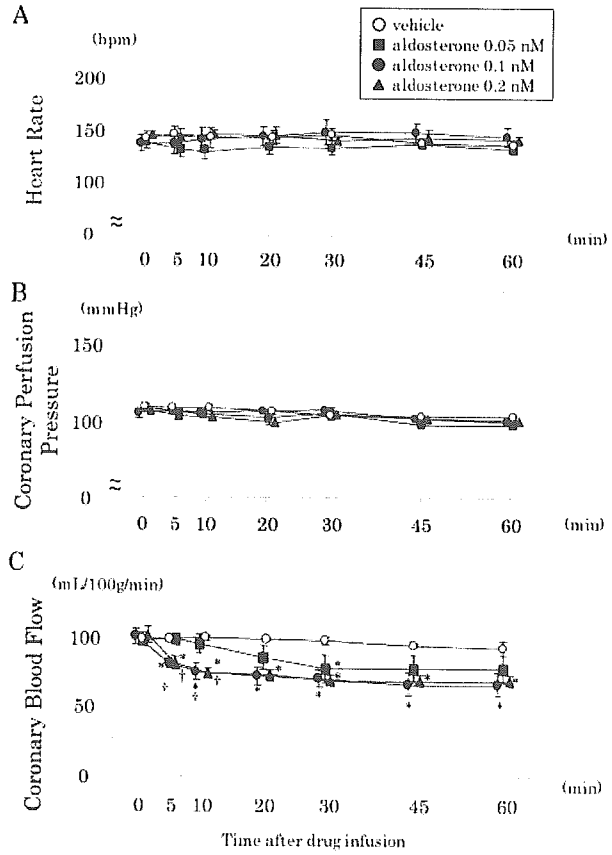

Figure 1. Nongenomic effects of aldosterone on coronary hemodynamics under nonischemic hearts. Plots of changes in heart rate (upper panel, A), coronary perfusion pressure (middle panel, B), and coronary blood flow (lower panel, C) in the nonischemic hearts during intracoronary administration of vehicle 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).

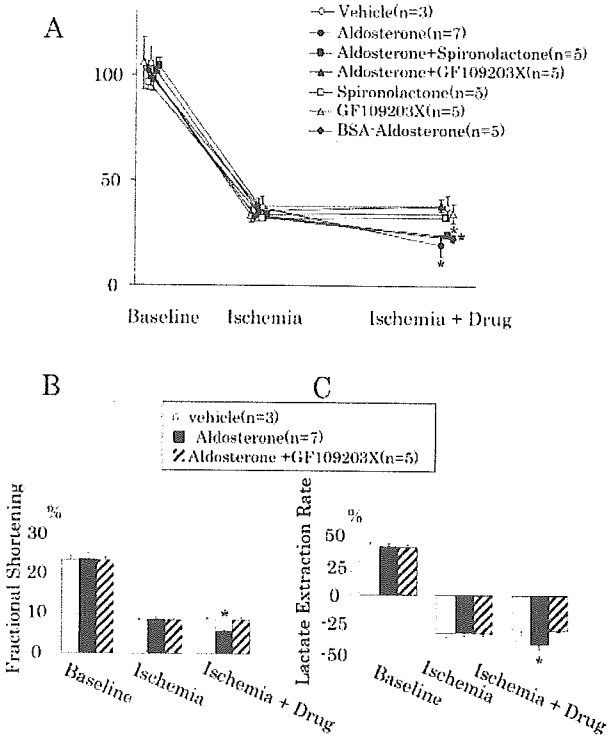

Figure 2. Nongenomic effects of aldosterone on coronary hemodynamics and myocardial contractile and metabolic functions under ischemic hearts. A, Plots of changes in coronary blood flow in the ischemic hearts with intracoronary administration of aldosterone (0.1 nmol/L) with and without either GF109203X 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

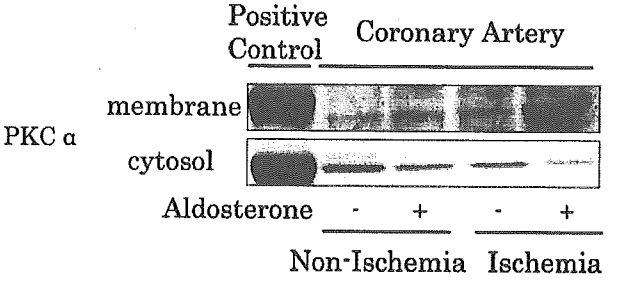

Figure 3. Effects of aldosterone on PKCα activation in coronary arteries. The representative cases in Western blotting against PKCα in the vascular tissues from the LAD.

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). $\mathrm{LVFS}=(\mathrm{LVEDd}-\mathrm{LVESd})/\mathrm{LVEDd}*100$, $\mathrm{LVEF}=[(\mathrm{LVEDd})^3-(\mathrm{LVESd})^3]/(\mathrm{LVEDd})^3*100$.

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.

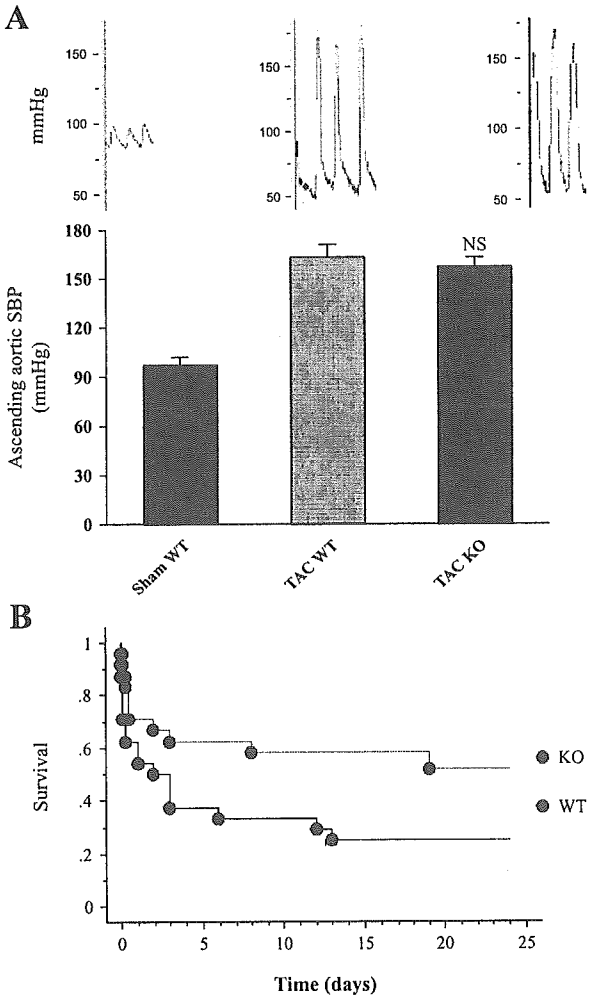

Fig. 1. Left ventricular pressure overload and survival. A) The ascending aortic systolic pressure measured with a 1.4 F catheter was similar in adiponectin KO and WT mice. NS: not significant vs. TAC WT. B) Kaplan–Meier survival analysis showed a significant higher mortality in adiponectin KO mice after 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

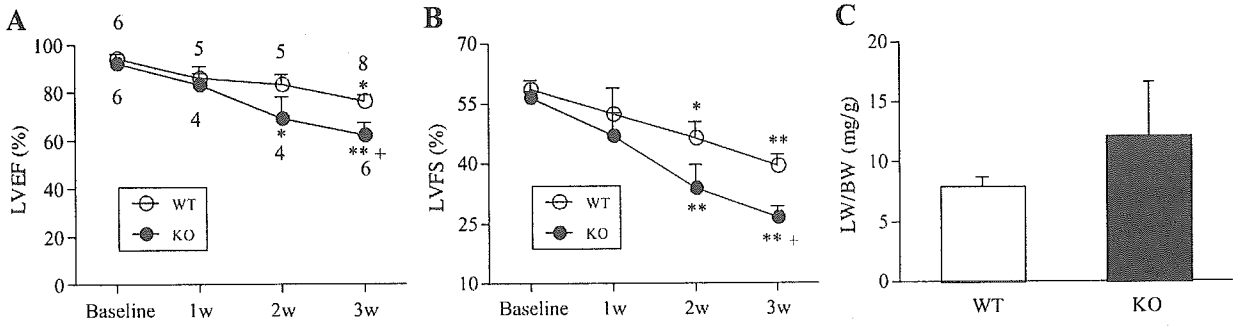

Fig. 2. The transition from hypertrophy to heart failure. A) Left ventricular ejection fraction (LVEF) and B) left ventricular fractional shortening (LVFS) were progressively depressed in adiponectin KO mice after 1 week of TAC, and the transition to heart failure occurred at 2 weeks after TAC in KO 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, $\dagger P<0.05$ 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,

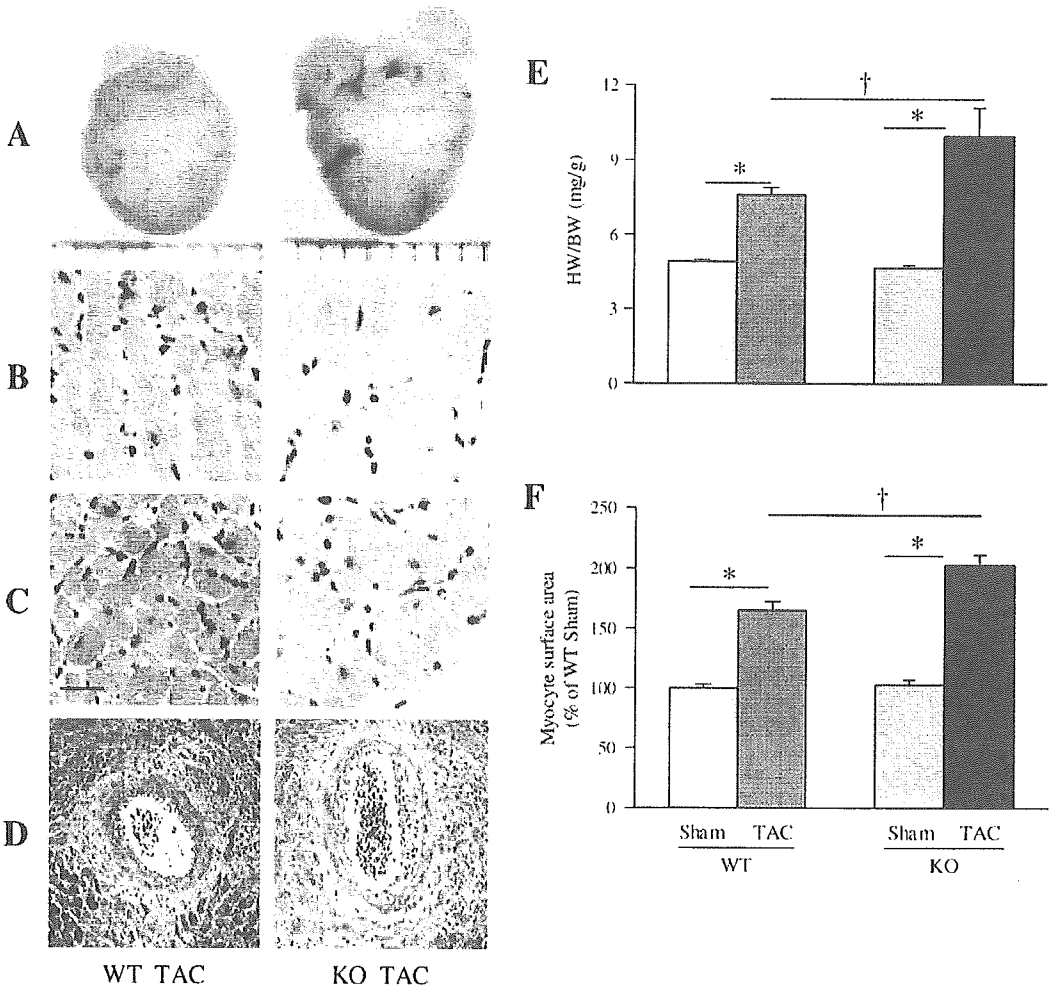

Fig. 3. Cardiac remodeling was more severe in KO mice. A) Representative pictures of cardiac hypertrophy in WT and KO mice at 3 weeks after TAC. B and C) Represent long-axis and cross-sectional views of cardiac myocytes with HE staining. D) Represents cardiac fibrosis with Azan staining (×100 magnification). 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.

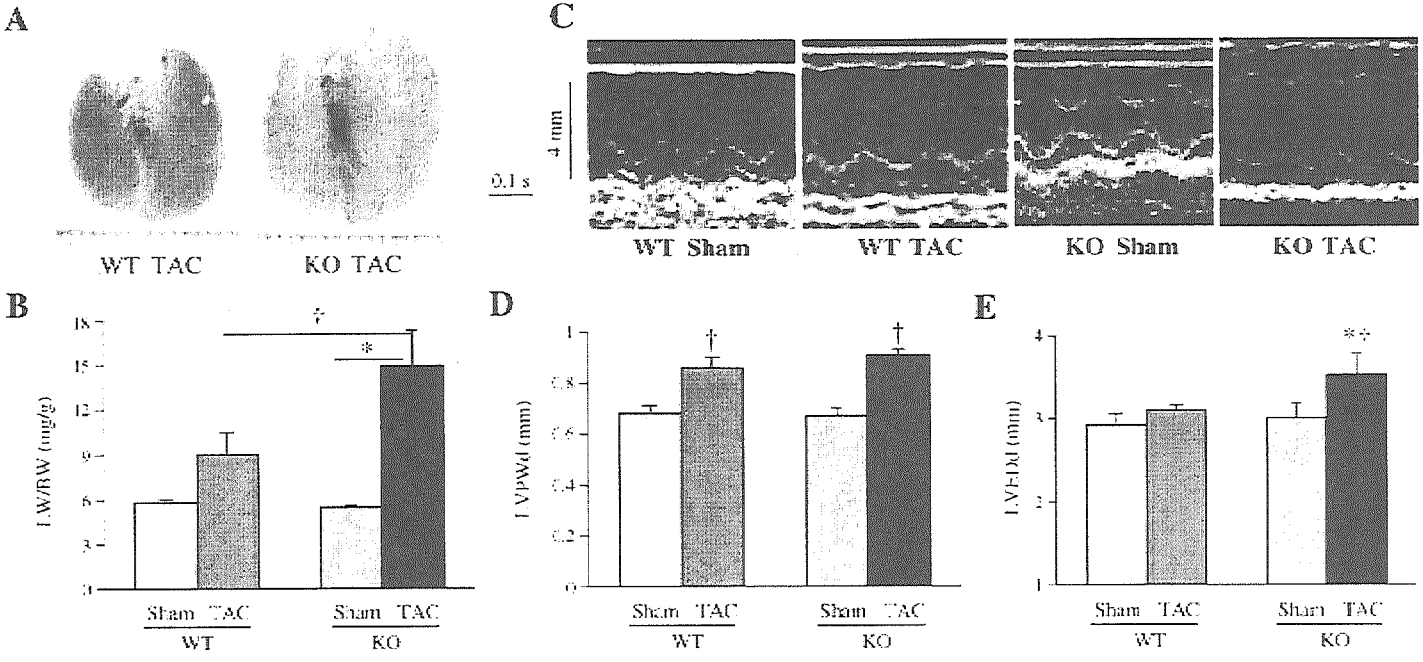

Fig. 4. Pulmonary congestion and echocardiographic findings at 3 weeks after TAC. The lungs of an adiponectin KO mouse were markedly enlarged compared with those of WT mice (A). The lung-to-body weight ratio (LW/BW) was markedly increased in KO mice compared with WT mice (B). $*P<0.01$, $\dagger P<0.05$. 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

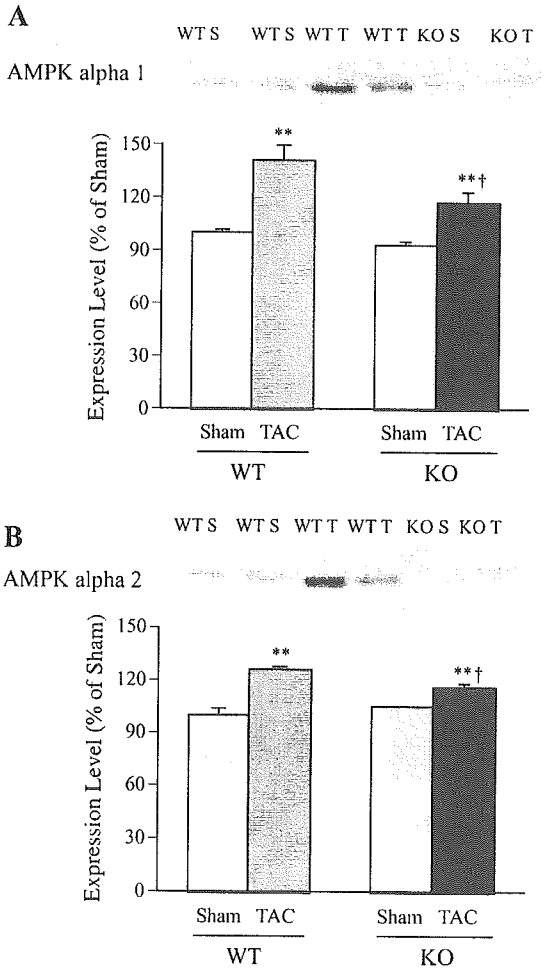

Fig. 5. Myocardial expression of AMPK. AMPKα_1 (A), α_2 (B) were increased in TAC mice, but the change was smaller in KO mice ($n=3$ in each group, $^{**}P<0.01$ vs. responding sham mice; $\dagger P<0.05$ vs. WT TAC). S: sham, T: TAC.

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

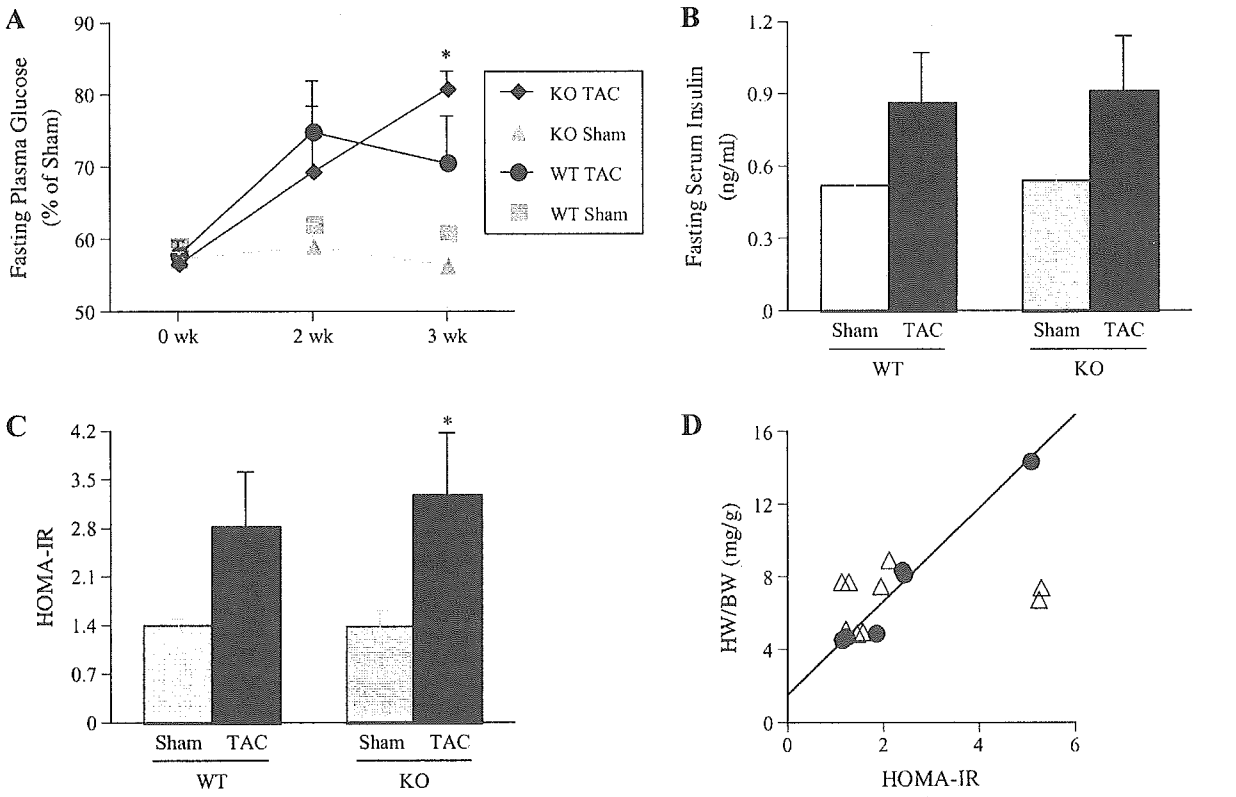

Fig. 6. Changes in glucose metabolism. Fasting glucose levels (A) were increased in adiponectin KO mice at 3 weeks after the onset of TAC, $*P<0.01$ vs. WT TAC ($n=5$ for all the groups at 0 week and for both sham groups at other two time points; $n=4$ for WT and KO TAC mice at 2 weeks, and $n=5$ and 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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