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Peroxisome Proliferator-Activated Receptor γ and Cardiovascular Diseases

Hiroyuki Takano, MD; Issei Komuro, MD

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and form heterodimers with retinoid X receptor. Three PPAR isoforms have been isolated and termed α, β (or δ) and γ. Although PPARγ is expressed predominantly in adipose tissue and associated with adipocyte differentiation and glucose homeostasis, PPARγ is also present in a variety of cell types. Synthetic antidiabetic thiazolidinediones (TZDs) are well known as ligands and activators for PPARγ. After it was reported that activation of PPARγ suppressed production of pro-inflammatory cytokines in activated macrophages, medical interest in PPARγ has grown and there has been a huge research effort. PPARγ is currently known to be implicated in various human chronic diseases such as diabetes mellitus, atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, and Alzheimer's disease. Many studies suggest that TZDs not only ameliorate insulin sensitivity, but also have pleiotropic effects on many tissues and cell types. Although activation of PPARγ seems to have beneficial effects on cardiovascular diseases, the mechanisms by which PPARγ ligands prevent their development are not fully understood. Recent data about the actions and its mechanisms of PPARγ-dependent pathway in cardiovascular diseases are discussed here. (*Circ J* 2009; **73:** 214–220)

Key Words: Atherosclerosis; Cardiac hypertrophy; Heart failure; PPARγ; Thiazolidinedione

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily that heterodimerize with the retinoid X receptor (RXR) and bind to specific response elements termed PPAR responsive elements (PPREs) in target gene promoters. The PPREs are direct repeats of the hexameric consensus sequence AGGTCA, separated by 1 nucleotide. These nuclear receptors are ligand-dependent transcription factors, and activation of target gene transcription depends on the binding of the ligand to the receptor. PPARs have 3 isoforms, α, β (or δ) and γ. PPAR α regulates genes involved in fatty acid oxidation, whereas PPARγ promotes adipocyte differentiation and glucose homeostasis. The main function of PPARβ/δ has yet to be ascertained, but involvement in the regulation of fatty acid oxidation seems likely. PPARα is present mainly in the liver, kidney, and muscle, whereas PPARγ is expressed predominantly in adipose tissue. PPARβ/δ is almost ubiquitously expressed. It was recently demonstrated that PPARγ is also expressed in a variety of cell types. After it was reported that activation of PPARγ suppresses production of inflammatory cytokines in activated macrophages, medical interest in PPARγ has grown, along with a huge research effort.

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PPARγ

Peroxisome is a subcellular organelle that plays a crucial role in cellular metabolism. Peroxisome enzymes are implicated in a broad range of catabolic and anabolic enzymatic pathways, such as fatty acid oxidation, biosynthesis of both glycerolipids and cholesterol, and metabolism of reactive oxygen species. Peroxisome proliferation induced in rodents is associated with cellular responses to a range of chemical compounds. In 1990, Issemann and Green reported that peroxisome proliferators activate a member of the steroid hormone receptor superfamily in mouse liver! This nuclear receptor was named PPAR. Soon after, 3 major types of PPAR (α, β/δ, and γ) were recognized. PPARγ is associated with adipocyte differentiation and glucose homeostasis. PPARγ is expressed in a variety of cell types, including adipocytes, macrophages, vascular smooth muscle cells (VSMCs), endothelial cells (ECs), and cardiomyocytes[2–7] Several lines of evidence have demonstrated the functional significance of PPARγ in atherosclerotic lesions[8,9]

Activity of PPARγ is depressed by phosphorylation of a serine residue (Ser^{112}) in the N-terminal domain, mediated by a member of the mitogen-activated protein (MAP) kinase family, extracellular signal-regulated protein kinase (ERK). In addition, another member of MAP kinase family, c-Jun N-terminal kinase (JNK) also phosphorylates PPARγ at Ser^{82} and reduces the transcriptional activity of PPARγ. The association of PPARγ polymorphism with metabolic syndrome has also been examined![10,11] In the presence of ligand, PPARγ binds to coactivator complexes, resulting in the activation of target genes. In the absence of ligand, PPARγ binds to the promoters of several target genes and associates with a corepressor complex, leading to active repression of target genes. This process is referred to as active repression (**Fig 1**). The corepressor complex constitutes corepressor proteins, such as nuclear receptor corepressor

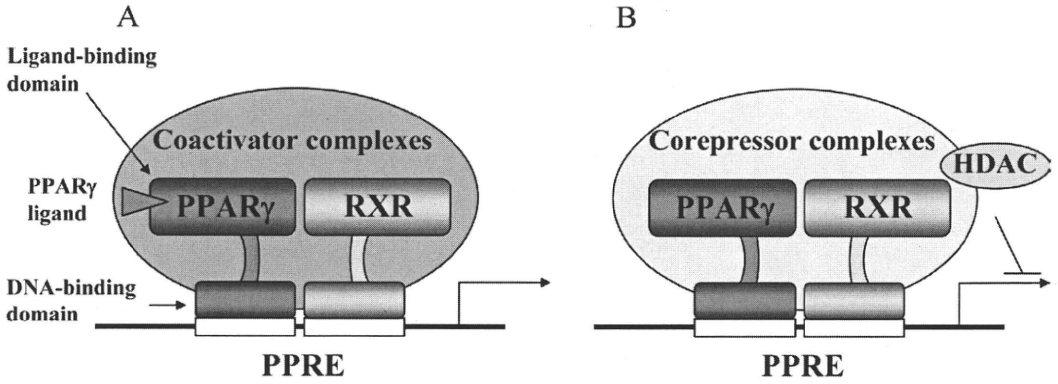

Fig 1. Transactivation and active repression. PPARγ functions as a heterodimer with RXR. (**A**) In the presence of ligand, PPARγ binds to coactivator complexes, resulting in the activation of target genes. (**B**) In the absence of ligand, PPARγ binds to the promoters of several target genes and associates with corepressor complexes, leading to active repression of target genes. HDAC, histone deacetylase; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR responsive element; RXR, retinoid X receptor.

(NCoR) and silencing mediator of retinoid and thyroid hormone receptors, histone deacetylases (HDACs) and transducin β-like protein 1 (TBL1). HDACs are essential in maintaining repressed chromatin structure and TBL1 exchanges a corepressor complex for a coactivator complex in the presence of ligand![12]

Many nuclear receptors are proposed to sequester inflammatory transcription factors, such as nuclear factor-κB (NF-κB) and AP-1, by inhibiting their DNA-binding activities, resulting in inhibition of inflammatory target genes. In the presence of ligand, PPARγ also interacts with inflammatory transcription factors and inhibits their DNA-binding activities. PPARγ blocks clearance of the corepressor complex in a ligand-dependent manner, and PPARγ stabilizes the corepressor complex bound to the promoter of inflammatory genes![13] It was demonstrated that PPARγ associates with the protein inhibitor of activated STAT1 (PIAS1), which is a small ubiquitin-like modifier (SUMO)-E3 ligase, in a ligand-dependent manner. PIAS1-induced SUMOylation of the ligand-binding domain of PPARγ enables the receptor to maintain NCoR on the promoter of inflammatory genes![14] These are the suggested mechanisms of PPARγ transrepression.

PPARγ Ligands

Natural and synthetic ligands bind to PPARγ, resulting in conformational change and activation of PPARγ. The PGD_2 metabolite, 15d-PGJ_2, was the first endogenous ligand for PPARγ to be discovered. Although 15d-PGJ_2 is the most potent natural ligand of PPARγ, the extent to which its effects are mediated through PPARγ in vivo remains to be determined. Two components of oxidized low density lipoprotein (ox-LDL), the 9-hydroxy and 13-hydroxy octadecadienoic acids (HODE), are also potent endogenous activators of PPARγ![15,16] Activation of 12/15-lipoxygenase induced by interleukin (IL)-4 also produced endogenous ligands for PPARγ;[17] however, whether these natural ligands act as physiological PPARγ ligands in vivo remains unknown. The antidiabetic thiazolidinediones (TZDs), such as troglitazone, pioglitazone, ciglitazone and rosiglitazone, which are used to control glucose concentration in patients with diabetes mellitus (DM), are pharmacological ligands of PPARγ. They bind PPARγ with various affinities and it is conceivable that their insulin-sensitizing and hypoglycemic effects are exerted by activating PPARγ. However, the molecular mechanisms by which TZDs affect insulin resistance and glucose homeostasis are not fully understood. They seem to mediate their effects primarily through adipose tissue, because TZDs alter the expression level of genes that are involved in lipid uptake, lipid metabolism and insulin action in adipocytes. TZDs enhance adipocyte insulin signaling and reduce the release of free fatty acids. TZDs also decrease the inflammation of adipose tissue that is induced by obesity and contributes to increased insulin resistance. There is a possibility that TZDs improve insulin sensitivity in skeletal muscle and liver, the main insulin-sensitive organs, through these multiple adipocentric actions. PPARγ has been demonstrated to have an antiinflammatory effect, leading to initiation of treatment trials for patients with inflammatory diseases. RXR, which interacts with the PPARs, is activated by 9-cis retinoic acid. When combined as a PPAR:RXR heterodimer, the PPAR ligands and 9-cis retinoic acid act synergistically on PPAR responses.

PPARγ and Atherosclerosis

Atherosclerosis is a complex process to which many different factors contribute. Injury of the endothelium, proliferation of VSMCs, migration of monocytes/macrophages, and the regulatory network of growth factors and cytokines are important in the development of atherosclerosis. In addition, chronic inflammation of the vascular wall is also involved. As mentioned earlier, PPARγ has antiinflammatory effect. PPARγ ligands have been shown to reduce production of inflammatory cytokines, such as IL-1β, IL-6, inducible nitric oxide synthase and tumor necrosis factor-α (TNF-α), by inhibiting the activity of transcription factors such as activator protein-1 (AP-1), signal transducers and activators of transcription (STAT), and NF-κB in monocytes/macrophages![2,3] Those findings suggest that PPARγ activation may have beneficial effects in modulating inflammatory responses in atherosclerosis. Interestingly, expression of PPARγ has been demonstrated in atherosclerotic plaques![8] Macrophages affect the vulnerability of plaque to rupture and they are implicated in the secretion of matrix metalloproteinases (MMPs), enzymes that are important in the degradation of extracellular matrix. In macrophages and VSMCs, PPARγ

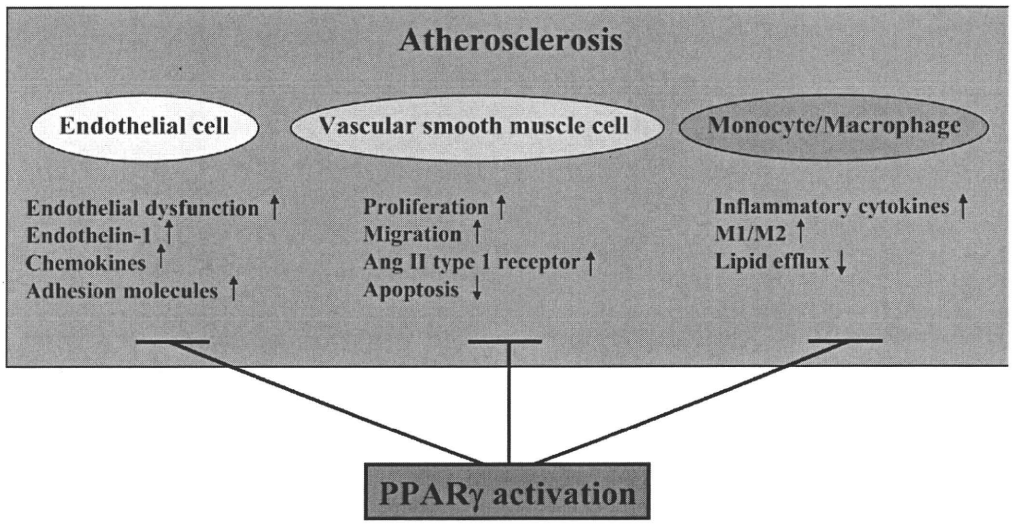

Fig 2. In atherosclerosis, PPARγ inhibits progression of the atherosclerotic lesion. PPAR, peroxisome proliferator-activated receptor.

ligands have been shown to reduce the expression of MMP-9, resulting in the inhibition of migration of VSMCs, and plaque destabilization[3,4] Although activation of T lymphocytes represents a critical step in atherosclerosis, PPARγ ligands also reduce the activation T lymphocytes.[18] Recently, it was reported that PPARγ is a key regulator of M1/M2 polarization.[19] Classically activated macrophages (M1) express a high level of pro-inflammatory cytokines and reactive oxygen species, whereas alternatively activated macrophages (M2) play an antiinflammatory role in atherosclerosis. PPARγ agonists prime monocytes into M2 and PPARγ expression is enhanced by M2 differentiation.[20]

VSMC proliferation and migration are also critical events in atherosclerosis and vascular-intervention-induced restenosis. TZDs inhibit both these changes in the VSMCs and neointimal thickening after vascular injury.[21–24] Furthermore, TZDs induce apoptosis of VSMCs via p53 and Gadd45.[25,26] Angiotensin II (AngII) plays an important role in vascular remodeling via the AngII type 1 receptor (AT1R) and accelerates atherosclerosis. Although AngII induces transcriptional suppression of PPARγ, activation of PPARγ inhibits AT1R gene expression at a transcriptional level in VSMCs.[27–29] Expression of adhesion molecule by ECs, leading to adhesion of leukocytes, is a critical early step in atherosclerosis. PPARγ ligands inhibit the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 and decreased production of chemokines, such as IL-8 and monocyte chemotactic protein-1 (MCP-1) via suppressions of AP-1 and NF-κB activities in ECs.[30–32] PPARγ ligands also inhibit MCP-1-induced monocytes migration.[33] Endothelin-1 (ET-1) is involved in the regulation of vascular tone and endothelial functions, and induces proliferation of VSMCs. In bovine aortic ECs, PPARγ ligands suppressed transcription of the ET-1 promoter by interfering with AP-1.[34]

PPARγ activation by major oxidized lipid components of ox-LDL, 9-HODE and 13-HODE has an important role in the development of lipid-accumulating macrophages through transcriptional induction of CD36, a scavenger receptor.[35] These findings suggest that atherogenic ox-LDL particles could induce their own uptake through activation of PPARγ and expression of CD36, leading to atherosclerosis. However, several studies have demonstrated that activation of PPARγ does not promote lipid accumulation in either mouse or human macrophages.[36–38] Liver X receptor α (LXRα) is an oxysterol receptor that promotes cholesterol excretion and efflux by modulating expression of ATP-binding cassette transporter 1 (ABCA1).[37,38] LXRα was recently identified as a direct target of PPARγ in mouse and human macrophages.[39,40] Although the PPARγ-induced increase in CD36 expression might accelerate lipid uptake in macrophages, subsequent activation of LXRα and upregulation of ABCA1 appear to induce lipid efflux.

Diep et al have demonstrated that rosiglitazone and pioglitazone attenuate the development of hypertension and structural abnormalities, and improve endothelial dysfunction in AngII-infused rats.[41] These TZDs also prevented upregulation of AT1R, cell cycle proteins, and inflammatory mediators. Rosiglitazone, but not the PPARα ligand fenofibrate, prevented hypertension and endothelial dysfunction in DOCA-salt hypertensive rats.[42] It has been reported that serum levels of the soluble CD40 ligand are elevated in acute coronary syndrome and associated with increased cardiovascular risk. Treatment with rosiglitazone decreased the serum levels of soluble CD40 and MMP-9 in type 2 diabetic patients with coronary artery disease.[43] Taking all the evidence together, PPARγ ligands may prevent the progression of atherosclerotic lesions, particularly in patients with DM (**Fig 2**).

PPARγ and Ischemic Heart Disease

As the effects of PPARγ on the heart are not fully understood, we and others have examined whether PPARγ is involved in various heart diseases. Although the expression of PPARγ in cardiac myocytes is low compared with adipocytes, PPARγ ligands seem to act on cardiac myocytes.[7,44] We demonstrated that PPARγ ligands inhibited the cardiac expression of TNF-α at the transcriptional level, in part by antagonizing NF-κB activity.[7] Because TNF-α expression is elevated in the failing heart and has a negative inotropic effect on cardiac myocytes, treatment with PPARγ ligands may prevent the development of congestive heart failure. Diabetic cardiomyopathy, which is characterized by

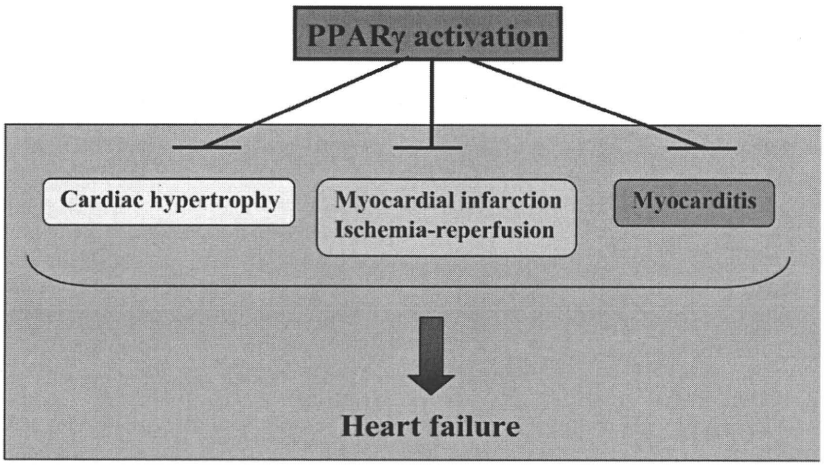

Fig 3. Actions of PPARγ in heart diseases. PPARγ inhibits the progression of heart failure following cardiac hypertrophy, myocardial infarction, ischemia–reperfusion injury, and myocarditis. PPAR, peroxisome proliferator-activated receptor.

systolic and diastolic dysfunction, is a major complication of DM, and therefore TZDs seem to be beneficial for the impaired cardiac function in patients with DM. Following our study, the role of PPARγ in myocardial ischemia–reperfusion (IR) injury has been elucidated.[45–48] In animal models, PPARγ ligands reduced the size of the myocardial infarct and improved contractile dysfunction after IR through inhibition of the inflammatory response. IR injury activates JNK, and subsequently JNK induces increases in both AP-1 DNA-binding activity and apoptotic cells. It has been shown in rats that rosiglitazone inhibits the activation of JNK and AP-1 after myocardial IR.[46] Furthermore, pioglitazone has been reported to attenuate left ventricular remodeling and heart failure after myocardial infarction (MI) in mice.[49] Both of these effects of TZDs ligands were associated with decreases in inflammatory cytokines and chemokines.[49,50]

PPARγ and Cardiac Hypertrophy

The PPARγ ligands, troglitazone, pioglitazone and rosiglitazone, inhibited AngII-induced hypertrophy of neonatal rat cardiac myocytes.[51–53] Because generalized PPARγ gene deletion causes embryonic lethality, we examined the role of PPARγ in the development of cardiac hypertrophy in vivo using heterozygous PPARγ-deficient ($PPAR\gamma^{+/-}$) mice.[53] Pressure overload-induced cardiac hypertrophy was more prominent in heterozygous $PPAR\gamma^{+/-}$ mice than in wild-type (WT) mice. Treatment with pioglitazone strongly inhibited the pressure overload-induced cardiac hypertrophy in WT mice and moderately in $PPAR\gamma^{+/-}$ mice.[53] Thereafter, 2 other groups examined the role of PPARγ in the heart by using cardiomyocyte-specific PPARγ knockout mice.[54,55] Duan et al reported that these mice develop cardiac hypertrophy through elevated NF-κB activity;[54] and unexpectedly, rosiglitazone induced cardiac hypertrophy in both the WT mice and cardiomyocyte-specific PPARγ knockout mice through activation of p38 MAP kinase independent of PPARγ. Ding et al reported that cardiomyocyte-specific PPARγ knockout mice displayed cardiac hypertrophy from approximately 3 months of age and then progress to dilated cardiomyopathy;[55] most mice died from heart failure within 1 year after birth. Mitochondrial oxidative damage and reduced expression of manganese superoxide dismutase were recognized in the cardiomyocyte-specific PPARγ knockout mice.[55] These mice models demonstrate that PPARγ is essential for protecting cardiomyocytes from stress and oxidative damage, although the expression level of PPARγ in cardiomyocytes is low. On the other hand, Son et al demonstrated that cardiomyocyte-specific PPARγ transgenic mice develop dilated cardiomyopathy associated with increased uptake of both fatty acid and glucose.[56] Rosiglitazone increased this glucolipotoxicity in cardiomyocyte-specific PPARγ transgenic mice. If PPARγ in the heart is expressed at a high level, rosiglitazone may cause cardiotoxic effects; however, as noted earlier the expression level of PPARγ in the heart is quite low.

PPARγ and Myocarditis

Experimental autoimmune myocarditis (EAM) is a T-cell-mediated disease characterized by infiltration of T cells and macrophages, leading to massive myocarditis necrosis, which develops into heart failure in the chronic phase.[57] The onset of EAM in rats occurs approximately 2 weeks after the first immunization with porcine cardiac myosin. At this time, small numbers of $CD4^+$ T cells and macrophages start to infiltrate into the myocardium and various cytokines are expressed. Macrophage inflammatory protein-1α (MIP-1α) is a C-C chemokine that induces leukocyte accumulation in tissue sites of inflammation. We previously demonstrated that MIP-1α mRNA and protein are highly expressed in the hearts of rats with EAM from day 11 after first immunization.[57] Th1 cells produce interferon-γ (IFN-γ), which is mainly involved in cell-mediated immune responses, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which participate in humoral responses. Immune dysfunction associated with autoimmune disease is known to involve an imbalance between Th1 and Th2 cells.

It has been reported that pioglitazone treatment markedly reduces the severity of myocarditis in a rat model of EAM.[58,59] Pioglitazone suppressed expression of inflammatory cytokines and activation of myocardiogenic T cells in the myocardium of EAM rats.[58] The mRNA levels of MIP-1γ were upregulated in the hearts of EAM rats, but not in the hearts of those in the pioglitazone group. Furthermore, treatment with pioglitazone decreased the expression levels of pro-inflamatory cytokine (TNF-α and IL-1β) genes and Th1 cytokine (IFN-γ) genes, and increased the expression levels of Th2 cytokine (IL-4) gene.[59] These results suggest that PPARγ ligands may have beneficial effects on myocarditis by inhibiting MIP-1α expression and modulating the Th1/Th2 balance (**Fig 3**).

Efficacy and Safety of TZD Treatment in the Clinical Setting

Despite the beneficial effects of TZDs in the basic experiments, their propensity to cause fluid retention is a serious side-effect. Clinical studies report TZD-induced peripheral fluid retention, and an increase in plasma volume in 2–5% of patients on monotherapy.[60] Fluid retention was more likely to occur with concomitant insulin use, and in patients with underlying cardiac dysfunction or renal insufficiency. The exact mechanisms for TZD-induced fluid retention are not well understood, and it remains unclear whether TZDs directly cause the development of de novo congestive heart failure. It is known that the level of vascular endothelial growth factor is increased in the patients who develop fluid retention with TZD therapy[61] and this may lead to peripheral edema through increased vascular permeability. The insulin-sensitizing action of TZDs also induces water and salt retention. PPARγ is highly expressed in the kidney and collecting-duct-specific PPARγ knockout mice demonstrated no effects of TZD on fluid retention or the expression level of sodium channel ENaC-γ.[62,63] These findings suggest that activation of the sodium channel in the collecting duct cells expressing PPARγ may be a mechanism of fluid retention. In patients without evidence of heart failure, careful examination did not reveal any worsening of left ventricular function by TZDs.[64] There are very few studies investigating the safety of TZDs in patients with preexisting heart failure. Although a recent study demonstrated that there is not a direct association between the risk of fluid retention and the baseline degree of severity of heart failure in diabetic patients treated with TZDs, the prescription of TZDs for patients with established heart failure should be avoided at present.[60,65]

The PROactive (Prospective Pioglitazone Clinical Trial in Macrovascular Events) study has shown that pioglitazone significantly decreases the occurrence of all-cause mortality, nonfatal MI, and nonfatal stroke in patients with type 2 DM and macrovascular diseases.[66] Pioglitazone significantly reduced the occurrence of fatal and nonfatal MI by 28% in the PROactive study.[66] Although there was a 1.6% absolute increase in heart failure hospitalizations in the pioglitazone group compared with the placebo group, the number of heart-failure-related deaths was almost identical. In contrast to the PROactive study, it has been recently reported that rosiglitazone treatment is associated with increased incidence of MI by meta-analysis.[67,68] Although meta-analysis has a number of limitations and the increased risk in MI is still controversial, those results attracted the attention of many clinicians. There are some differences in the actions of pioglitazone and rosiglitazone. Pioglitazone has more beneficial effects on the lipid profile than rosiglitazone.[69] As mentioned earlier, rosiglitazone, but not pioglitazone, induced cardiac hypertrophy by a non-PPARγ-mediated pathway.[54] Pioglitazone represses NF-κB activation and VCAM-1 expression in a PPARα-dependent manner.[70] Pioglitazone was recently reported to increase the number and function of endothelial progenitor cells (EPCs) in patients with stable coronary artery disease and normal glucose tolerance.[71] Pioglitazone may induce angiogenesis by modulating EPC mobilization and function. In the future, more mechanistic studies are required to investigate the differences in action between pioglitazone and rosiglitazone.

Conclusions

The American Heart Association (AHA) and American Diabetes Association (ADA) have released a consensus statement that advises caution regarding the use of TZDs in patients with known or suspected heart failure.[72] Because there is a possibility that TZDs may unmask asymptomatic cardiac dysfunction by increasing plasma volume, they should be avoided in patients with congestive heart failure of New York Heart Association (NYHA) class III or IV. The data from in vitro studies suggest that TZDs exert direct actions on vascular cells and cardiomyocytes, independent of their glucose-mediated mechanisms. Further studies using tissue-specific gene targeting mice are necessary to address in vivo the pleiotropic effects of PPARγ on the cardiovascular system. If the beneficial roles of PPARγ can be solved, modulation of PPARγ may become a promising therapeutic strategy for cardiovascular diseases. Because cardiac hypertrophy can be seen even in normotensive diabetic patients, and diabetic cardiomyopathy is a major complication of DM, antidiabetic agents such as the TZDs would be expected to have beneficial effects on cardiac hypertrophy and dysfunction in patients with DM. It has been already clarified that 3-hydroxy-3-methyglutaryl-coenzyme A reductase inhibitors, statins, have pleiotropic effects in cardiovascular diseases. The effects of PPARγ ligands are similar to those of statins in many respects. A recent study demonstrated that statins activate PPARγ through ERK and p38 MAP-kinase-dependent cyclooxygenase-2 expression in macrophages.[73] Further studies are needed to elucidate the molecular mechanisms of the pleiotropic effects of PPARγ ligands in cardiovascular disease.

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PDK1 coordinates survival pathways and β-adrenergic response in the heart

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The 3-phosphoinositide-dependent kinase-1 (PDK1) plays an important role in the regulation of cellular responses in multiple organs by mediating the phosphoinositide 3-kinase (PI3-K) signaling pathway through activating AGC kinases. Here we defined the role of PDK1 in controlling cardiac homeostasis. Cardiac expression of PDK1 was significantly decreased in murine models of heart failure. Tamoxifen-inducible and heart-specific disruption of *Pdk1* in adult mice caused severe and lethal heart failure, which was associated with apoptotic death of cardiomyocytes and β_1-adrenergic receptor (AR) down-regulation. Overexpression of Bcl-2 protein prevented cardiomyocyte apoptosis and improved cardiac function. In addition, PDK1-deficient hearts showed enhanced activity of PI3-Kγ, leading to robust β_1-AR internalization by forming complex with β-AR kinase 1 (βARK1). Interference of βARK1/PI3-Kγ complex formation by transgenic overexpression of phosphoinositide kinase domain normalized β_1-AR trafficking and improved cardiac function. Taken together, these results suggest that PDK1 plays a critical role in cardiac homeostasis in vivo by serving as a dual effector for cell survival and β-adrenergic response.

AGC kinase | apoptosis | heart failure | receptor internalization

Heart failure, a major cause of morbidity and mortality worldwide, is a clinical syndrome in which the heart is incapable of pumping blood at a rate commensurate with systemic demands (1). Injurious stresses from extrinsic or intrinsic origins trigger the complex intracellular signaling pathways in cardiomyocytes and thereby activate the compensatory mechanisms involving alterations in survival and growth signals, calcium handling, and energy production (2). Simultaneously, the sympathetic nervous, renin-angiotensin-aldosterone, and cytokine systems are activated to cope with a decline in cardiac performance. Although these compensatory systems initially maintain cardiac function within a physiological range, prolonged activation of these systems paradoxically leads to cardiac damage and worsens clinical prognosis (2). Therefore, for the elucidation of the pathophysiology of heart failure, it is very important to dissect the inherent complexity of intracellular signaling pathways that coordinate the cellular homeostasis and neurohumoral responses in cardiomyocytes.

The 3-phosphoinositide-dependent protein kinase-1 (PDK1) is a member of the AGC serine/threonine kinase family that functions downstream of phosphoinositide 3-kinase (PI3-K) and activates several AGC kinases, including Akt, p70 ribosomal S6 kinase (p70S6K), and serum- and glucocorticoid-induced protein kinase 1 (SGK1), by phosphorylating these enzymes at their activation loops (3). The physiological functions of PDK1 have been investigated by targeted disruption of *Pdk1* gene. Mouse embryos systemically deficient for *Pdk1* were lethal during early embryogenesis, displaying multiple abnormalities that included lack of somites, forebrain, and neural crest-derived tissues (4). Alessi et al. (5) recently generated striated muscle-specific PDK1 conditional knockout mice (PDK1-MCKCre) by crossing mice harboring a "floxed" *Pdk1* allele with transgenic mice expressing Cre recombinase under the control of the *muscle creatine kinase* (*MCK*) promotor. PDK1-MCKCre mice died of heart failure by 11 weeks of age. Interestingly, PDK1-MCKCre mice showed attenuation of cardimyocyte cell growth and impairment of left ventricular (LV) contraction. It was reported that cardiomyocytes deficient for *Pdk1* were sensitive to hypoxia (5), and that ischemic preconditioning failed to protect *Pdk1*-hypomorphic mutant mice against myocardial infarction (MI) (6). However, the mechanisms of how PDK1 deficiency induces these cardiac abnormalities remain to be resolved.

In this study, we found that the expression levels of PDK1 protein were significantly decreased in the failing hearts of murine models. We generated tamoxifen-inducible and heart-specific PDK1 conditional knockout mice (PDK1-MerCre) to elucidate the relevance of PDK1 to the pathogenesis of heart failure. We disrupted the *Pdk1* gene in the adulthood and demonstrated that PDK1 plays a role in the regulation of normal cardiac function by preventing cardiomyocyte apoptosis and by preserving responsiveness to β-adrenergic stimulation.

Results

Generation of Tamoxifen-Inducible and Heart-Specific PDK1 Knockout Mice. We examined alterations in the expression levels of PDK1 in failing hearts. Heart failure was induced in mice by producing myocardial infarction or administering doxorubicin i.p. Two weeks after operation of myocardial infarction or doxorubicin injection, expression levels of PDK1 were significantly decreased in the failing hearts, compared with control hearts (Fig. S1).

To assess the pathophysiological significance of PDK1 down-regulation, we created a model of temporally regulated inactivation of *Pdk1* specifically in the adult hearts. We crossed *Pdk1*$^{flox/flox}$ mice (7, 8) with transgenic mice expressing tamoxifen-inducible Cre recombinase protein fused to two mutant estrogen-receptor ligand-binding domains (MerCreMer) under the control of the *α-myosin heavy chain* promoter (9). In the resulting *Pdk1*$^{flox/flox}$/*MerCreMer*$^{+}$ mice (PDK1-MerCre) at the age of 10 weeks, we administered tamoxifen successively for 5 days and confirmed by immunoblot analysis that functional PDK1 expression was almost undetectable specifically in the hearts on day 7 after the initiation of tamoxifen treatment (Fig. S2*A*).

Next, we examined whether the activation of kinases downstream of PDK1 were suppressed in the hearts of PDK1-MerCre. In

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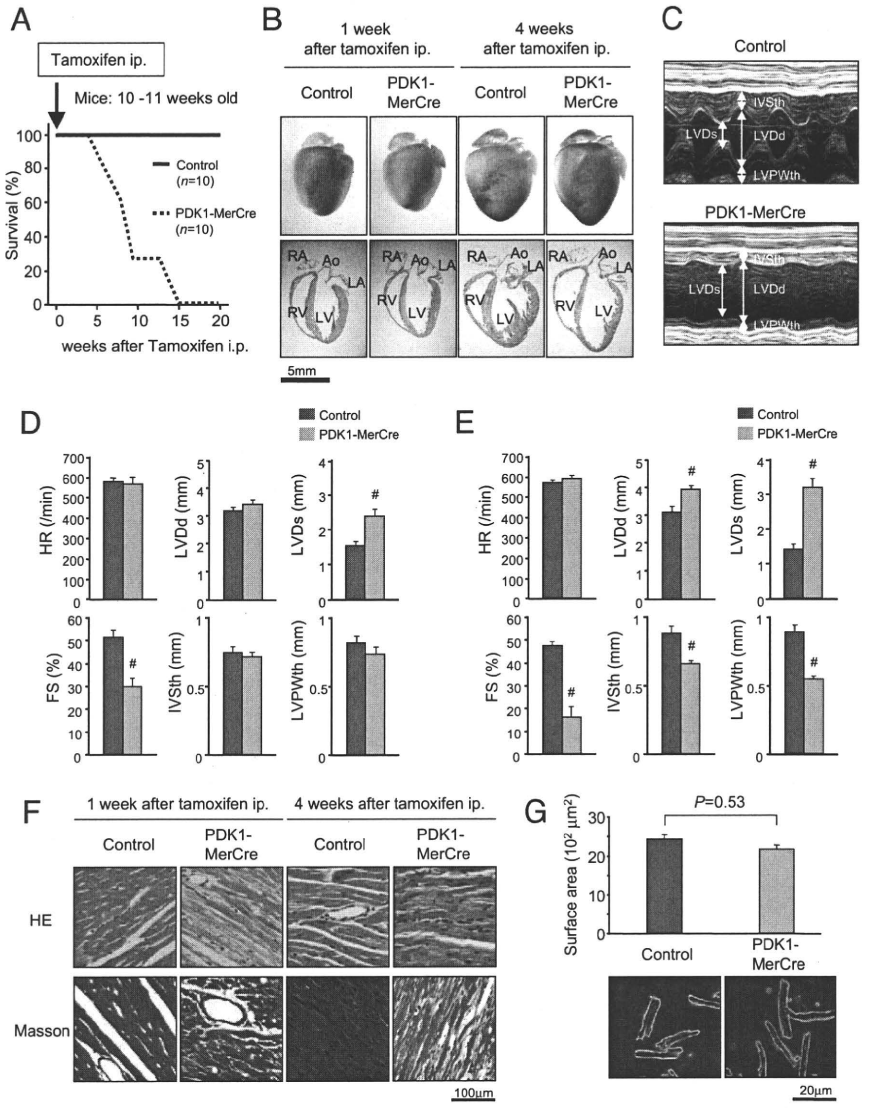

Fig. 1. Severe heart failure observed in PDK1-MerCre mice. (*A*) Kaplan-Meier survival curves of PDK1-MerCre mice (n = 10) and control mice (n = 10). Mice were injected with tamoxifen at the age of 10–11 weeks. (*B*) Macroscopic findings and 4-chamber sections of the hearts from PDK1-MerCre and control mice 1 and 4 weeks after the initiation of tamoxifen treatment. Ao, aorta; LA, left atrium; LV, left ventricule; RA, right atrium; RV, right ventricule. (*C*) Representative M-mode echocardiograms of mice 1 week after tamoxifen treatment. (*D*) Echocardiographic measurements of PDK1-MerCre and control mice 1 week after tamoxifen treatment. HR, heart rate; LVDd, LV dimension in diastole; LVDs, LV dimension in systole; FS, fractional shortening; IVSth, interventricular septum thickness; LVPWth, LV posterior wall thickness. Values represent the mean ± SEM of data from 10 mice in each group. #, $P < 0.01$ versus control group. (*E*) Echocardiographic measurements of PDK1-MerCre and control mice 4 weeks after tamoxifen treatment. Values represent the mean ± SEM of data from 6 mice in each group. #, $P < 0.01$ versus control group. (*F*) Histological sections with hematoxylin and eosin (HE) staining and Masson's trichrome (Masson) staining of PDK1-MerCre and control mice 1 and 4 weeks after tamoxifen treatment. (*G*) Surface areas of isolated cardiomyocytes (57 individual cardiomyocytes in each group) and sample pictures of isolated cardiomyocytes from PDK1-MerCre and control mice 1 week after tamoxifen treatment. Values represent the mean ± SEM.

mammalian cells, Akt is fully activated through PDK1-dependent phosphorylation of Thr-308 and PDK1-independent phosphorylation of Ser-473 (10). Insulin-induced phosphorylation of Akt at Thr-308 in PDK1-MerCre hearts was significantly attenuated, compared with control hearts, while phosphorylation level at Ser-473 was unchanged (Fig. S2*B*). As a consequence, Akt kinase activity was markedly reduced in PDK1-MerCre hearts (Fig. S2*C*). Consistently, insulin-induced phosphorylation levels of glycogen synthase kinase (GSK) 3β at Ser-9, mammalian target of rapamycin (mTOR) at Ser-2448, and p70S6K at Thr-389 (11) were attenuated in the PDK1-MerCre hearts (Fig. S2*B*). Collectively, these results indicate that Akt signaling is inhibited in PDK1-MerCre hearts.

Lethal Heart Failure in PDK1-MerCre Mice. Without tamoxifen treatment, PDK1-MerCre mice survived normally and were indistinguishable in appearance from control littermates. Strikingly, all PDK1-MerCre mice died from 5 to 15 weeks after the initiation of tamoxifen treatment (Fig. 1*A*).

One week after tamoxifen treatment, cardiac sizes were not significantly different between PDK1-MerCre mice and control mice (Fig. 1*B*). Echocardiographic examination revealed a significant decrease in the percent of fractional shortening (%FS), a parameter for contractile function, as early as 1 week after tamoxifen treatment in PDK1-MerCre mice (Fig. 1 *C* and *D*). During this period, there was no increase in LV dimension or thinning of LV wall, which was consistent with the macroscopic findings (Fig. 1 *B* and *D*). However, 4 weeks after tamoxifen treatment, progression of contractile dysfunction together with global chamber dilatation and wall thinning was observed in PDK1-MerCre mice (Fig. 1 *B* and *E*). Histologically, interstitial fibrosis was increased at 1 week in

PDK1-MerCre hearts and further enhanced at 4 weeks after tamoxifen treatment (Fig. 1*F*). These results suggest that PDK1-MerCre mice exhibited cardiac dysfunction as early as 1 week after tamoxifen treatment and LV remodeling at 4 weeks.

It was reported that PDK1-MCKCre showed marked reduction both in the heart size and in cardiac contractility (5). Since the *MCK* promoter directs expression of Cre recombinase before birth (5, 12), retardation of heart growth that was not proportional to somatic growth after birth might lead to cardiac dysfunction. However, the surface areas of caridomyocytes were not significantly different between PDK1-MerCre mice and control mice 1 week after tamoxifen treatment (Fig. 1*G*). Given that LV dysfunction was already observed as early as 1 week after tamoxifen treatment (Fig. 1 *C* and *D*), we suppose that reduction of cardiomyocyte size is not critically involved in the impairment of LV contraction observed in PDK1-MerCre hearts.

Increased Cardiomyocyte Apoptosis in PDK1-MerCre Mice. We next examined whether cardiomyocyte apoptosis was involved in the pathogenesis of heart failure in PDK1-MerCre mice. TUNEL staining revealed that the number of apoptotic cells was dramatically increased in PDK1-MerCre hearts 1 week after tamoxifen treatment (Fig. 2*A*). TUNEL-positive cells were cardiomyocytes, because these cells were positively stained with anti-sarcomeric α-actinin antibody (Fig. 2*B*). In addition, immunostaining revealed an increase in cardiomyocytes positively stained for cleaved caspase-3 in PDK1-MerCre hearts (Fig. 2*C*). The prevalence of TUNEL-positive cardiomyocytes was 1.14 ± 0.05% of total cardiomyocytes (Fig. 2*D*). Therefore, cardiomyocyte loss through apoptotic cell death may play an important role in the pathogenesis of heart failure in PDK1-MerCre mice.

In the hearts of PDK1-MerCre, the expression level of proapoptotic Bax was increased, whereas those of anti-apoptotic molecules such as Bcl-2 and Bcl-xL were unchanged (Fig. 2*E*). SGK1 has been reported to be functionally anti-apoptotic in the hearts (13). The basal level of phosphorylated SGK1 was reduced in PDK1-MerCre hearts (Fig. 2*F*). It has been reported that SGK1, in concert with Akt, mediates cell survival by phosphorylating and inactivating the Forkhead transcription factor FOXO3a (13, 14). FOXO3a is phosphorylated at Thr-32 and Ser-315 by SGK1, and Akt favors the phosphorylation of Thr-32 and Ser-253 (14). In PDK1-MerCre hearts, phosphorylation levels of FOXO3a at Thr-32 and Ser-253 were significantly decreased (Fig. 2*F*). Collectively, these results suggest that up-regulation of Bax protein and reduction of Akt and SGK1 activity were potentially involved in enhancing susceptibility of cardiomyocytes to apoptosis in PDK1-MerCre mice.

Overexpression of Bcl-2 Protein Prevented Cardiomyocyte Apoptosis and Partially Rescued Cardiac Dysfunction in PDK1-MerCre Mice. To examine whether cardiomyocyte apoptosis plays a causative role in the pathogenesis of heart failure in PDK1-MerCre mice, we crossed PDK1-MerCre with transgenic mice with cardiac-specific overexpression of Bcl-2 (Bcl2-Tg mice) (15). In PDK1-MerCre × Bcl2-Tg hearts, the number of TUNEL-positive cardiomyocytes was significantly decreased in comparison with PDK1-MerCre hearts (Fig. 2*G*), and the %FS showed partial but significant improvement (Fig. 2*H*). These results suggest that cardiac dysfunction is caused in part by cardiomyocyte loss through apoptosis in PDK1-MerCre mice.

Impairment of β-adrenergic Responsiveness in PDK1-MerCre Hearts. Incomplete restoration of cardiac function by prevention of cardiomyocyte apoptosis implies that some functional abnormalities persist in viable cardiomyocytes in PDK1-MerCre mice. To determine whether β-adrenergic responsiveness was changed in PDK1-MerCre hearts, we carried out Langendorff perfusion analysis in the hearts 1 week after tamoxifen treatment, and evaluated responsiveness to isoproterenol, a β-AR agonist, and forskolin, an activator of adenylate cyclase that increases cAMP independently

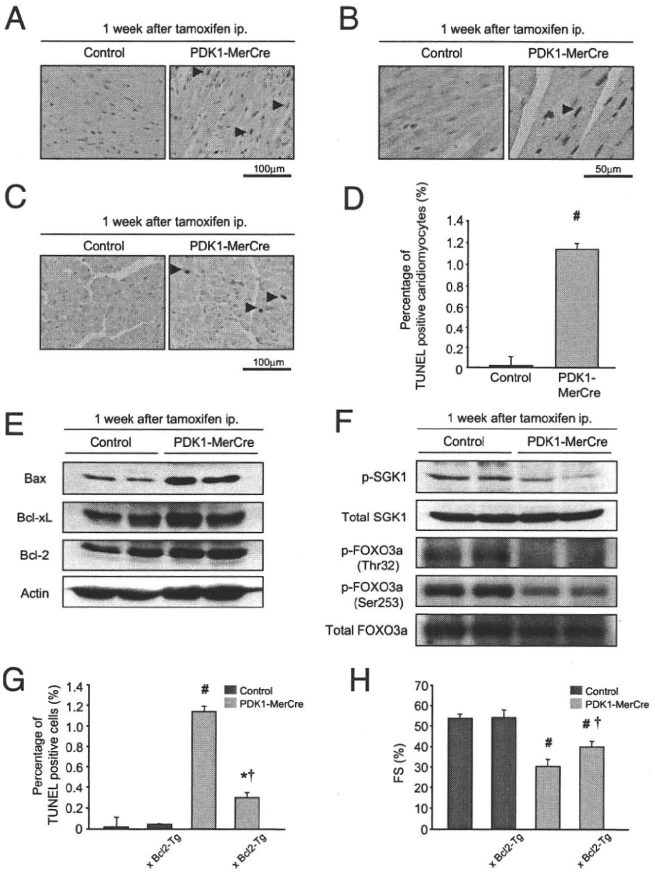

Fig. 2. Cardiomyocyte apoptosis in the pathogenesis of heart failure in PDK1-MerCre mice. (*A*) TUNEL staining. Arrowheads indicate TUNEL-positive cardiomyocytes. (*B*) Double staining for TUNEL staining (brown) and sarcomeric α-actinin (red). Arrowheads indicate TUNEL-positive cardiomyocytes. (*C*) Immunostaining for cleaved caspase-3. Arrowheads indicate cardiomyocytes positively stained for cleaved caspase-3. (*D*) Percentage of TUNEL-positive caridomyocytes. Values represent the mean ± SEM (3,000 cardiomyocytes in each group). #, $P < 0.01$ versus control group. (*E*) Immunoblot analysis of Bcl-2 family proteins in the hearts. (*F*) Immunoblot analysis of phosphorylated-SGK1 at Ser-78, total SGK1, phosphorylated-FOXO3a at Thr-32 or at Ser-253, and total FOXO3a in the hearts. (*G*) Percentage of TUNEL-positive caridomyocytes in control, Bcl2-Tg, PDK1-MerCre, and PDK1-MerCre × Bcl2-Tg mice. Values represent the mean ± SEM (3,000 cardiomyocytes in each group). #, $P < 0.01$ versus control group; *, $P < 0.05$, versus control group; †, $P < 0.01$ versus PDK1-MerCre group. (*H*) Measurement of fractional shortening of control, Bcl2-Tg, PDK1-MerCre, and PDK1-MerCre × Bcl2-Tg mice by echocardiography. Values represent the mean ± SEM of data from control mice (n = 10), control × Bcl2-Tg mice (n = 6), PDK1-MerCre mice (n = 10), and PDK1-MerCre × Bcl2-Tg mice (n = 6). #, $P < 0.01$ versus control mice. †, $P < 0.01$ versus PDK1-MerCre mice. FS, % of fractional shortening.

of β-AR. As shown in Fig. 3*A*, the baseline parameters of +dp/dt and −dp/dt were significantly lower in PDK1-MerCre mice than in control mice. Both isoproterenol and forskolin induced positive chronotropic and inotropic responses in control mice (Fig. 3*A*). However, PDK1-MerCre mice showed a significant reduction in the maximal changes in HR, +dP/dt, and −dP/dt after the stimulation of isoproterenol (1×10^{-8} M), compared with control mice (Fig. 3*B*). In contrast, the maximal changes in these parameters after the stimulation of forskolin (1×10^{-7} M) did not differ significantly between PDK1-MerCre and control mice (Fig. 3*B*). These results suggest that the responsiveness of β-AR is impaired in PDK1-MerCre mice.

Next, we measured the amount of β_1-AR in the membrane fraction by immunoblot analysis. In PDK1-MerCre hearts, the expression levels of β_1-AR in membrane fraction were markedly

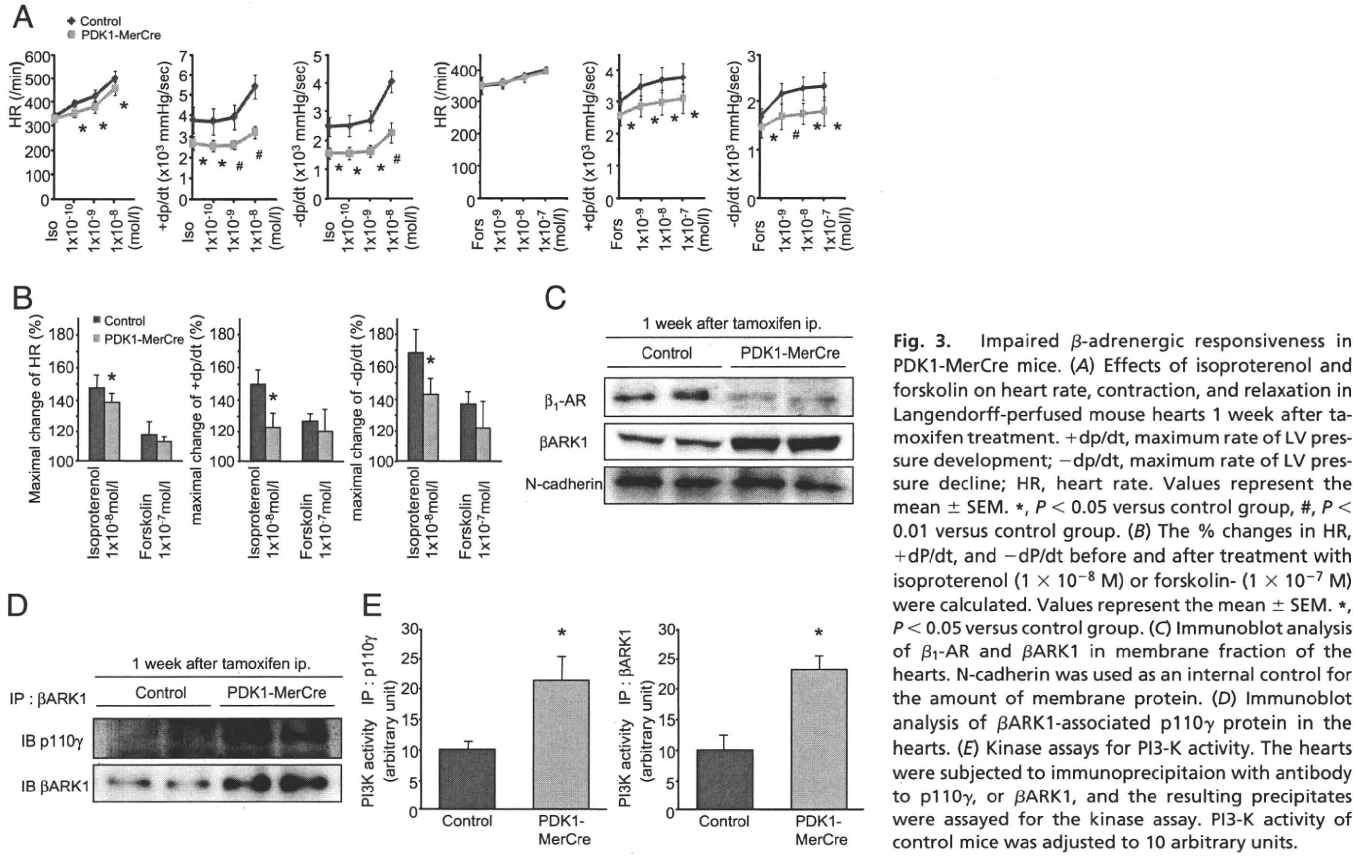

Fig. 3. Impaired β-adrenergic responsiveness in PDK1-MerCre mice. (*A*) Effects of isoproterenol and forskolin on heart rate, contraction, and relaxation in Langendorff-perfused mouse hearts 1 week after tamoxifen treatment. +dp/dt, maximum rate of LV pressure development; −dp/dt, maximum rate of LV pressure decline; HR, heart rate. Values represent the mean ± SEM. *, $P < 0.05$ versus control group, #, $P < 0.01$ versus control group. (*B*) The % changes in HR, +dP/dt, and −dP/dt before and after treatment with isoproterenol (1×10^{-8} M) or forskolin- (1×10^{-7} M) were calculated. Values represent the mean ± SEM. *, $P < 0.05$ versus control group. (*C*) Immunoblot analysis of β_1-AR and βARK1 in membrane fraction of the hearts. N-cadherin was used as an internal control for the amount of membrane protein. (*D*) Immunoblot analysis of βARK1-associated p110γ protein in the hearts. (*E*) Kinase assays for PI3-K activity. The hearts were subjected to immunoprecipitaion with antibody to p110γ, or βARK1, and the resulting precipitates were assayed for the kinase assay. PI3-K activity of control mice was adjusted to 10 arbitrary units.

down-regulated (Fig. 3*C*). Inversely, the amount of β_1-AR in cytosolic fraction was increased in PDK1-MerCre hearts, compared with control hearts, while the total amount of β_1-AR was unchanged (Fig. S3 *A* and *B*), suggesting that receptor internalization underlies β_1-AR down-regulation in membrane fraction of PDK1-MerCre hearts. In response to β-AR simulation, increased cAMP activates protein kinase A (PKA), which directly phosphorylates phospholamban (PLN) at Ser-16. PDK1-MerCre hearts showed a significant decrease in cAMP concentrations (Fig. S3*C*) and phosphorylation level of PLN at Ser-16 (Fig. S3*D*), compared with control hearts. Phosphorylated PLN dissociates from sarcoplasmic reticulum Ca^{2+}-ATPase2 (SERCA2) and thereby enhances Ca^{2+} uptake by SERCA2, which leads to enhancement of cardiac contractility (2). These results suggest that, in PDK1-MerCre hearts, robust β_1-AR internalization leads to contractile dysfunction.

It has been reported that phosphorylation of β-AR by β-AR kinase 1 (βARK1, commonly known as G protein-coupled receptor kinase 2) regulates receptor internalization (16). In the hearts of PDK1-MerCre mice 1 week after tamoxifen treatment, the expression levels of βARK1 (Fig. 3*C*) and βARK1-associated p110γ, a catalytic subunit of PI3-Kγ, were increased (Fig. 3*D*). Notably, PI3-K activity immunoprecipitated with antibodies to either p110γ or βARK1 was enhanced (Fig. 3*E*) in PDK1-MerCre hearts. βARK1 forms complex with PI3-Kγ through the phosphoinositide kinase (PIK) domain, and protein kinase activity of PI3-Kγ in this complex is required for receptor internalization (17). Therefore, these results suggest that enhanced PI3-Kγ activity in PDK1-MerCre hearts increases βARK1/PI3-Kγ complex formation, and that βARK1 phosphorylates β-AR to cause robust receptor internalization.

Disruption of βARK1/PI3-Kγ Complex Restored β-AR Internalization and Partially Rescued Cardiac Dysfunction in PDK1-MerCre Mice. To corroborate that enhanced PI3-Kγ activity promotes β-AR internalization by forming complex with βARK1 and that robust β-AR internalization causes cardiac dysfunction, we examined whether disruption of the βARK1/PI3-Kγ complex normalizes β-AR trafficking and improves cardiac function in PDK1-MerCre mice. For that purpose, we crossed PDK1-MerCre mice with transgenic mice harboring cardiac-specific overexpression of PIK domain (PIK-Tg mice) (16), which competitively inhibits the association between βARK1 and PI3-Kγ. The amount of βARK1-associated p110γ protein was significantly decreased in PDK1-MerCre × PIK-Tg mice, compared with PDK1-MerCre mice (Fig. 4*A*). Importantly, βARK1-associated PI3-K activity was markedly decreased in PDK1-MerCre × PIK-Tg mice, compared with PDK1-MerCre mice (Fig. 4*B*, *Lower*), although total PI3-Kγ activity remained elevated (Fig. 4*B*, *Upper*). As a consequence, in PDK1-MerCre × PIK-Tg mice 1week after tamoxifen treatment, the expression levels of β_1-AR in membrane fraction were restored (Fig. 4*C*). The %FS in echocardiographic examination showed partial but significant improvement (Fig. 4*D*). Overexpression of PIK domain did not influence cardiomyocyte apoptosis, because the prevalence of TUNEL-positive cardiomyocytes (Fig. 4*E*), as well as the amount of cleaved poly(ADP-ribose) polymerase, Bax, and phosphrylated FOXO3a (Fig. S4), was unchanged in PDK1-MerCre hearts. In addition, overexpression of Bcl-2 protein did not influence β-adrenergic response, because the amount of βARK1-associated p110γ protein (Fig. 4*A*), βARK1-associated PI3-K activity (Fig. 4*B*), the expression levels of membranous β_1-AR (Fig. 4*C*), as well as cAMP concentration and phosphorylation levels of PLN at Ser-16 (Fig. S5), were unchanged in PDK1-MerCre hearts. These results suggest that enhancement of βARK1-associated PI3-Kγ activity induces robust β-AR internalization, and thereby contributes to cardiac dysfunction, independently of cardiomyocyte apoptosis, in PDK1-MerCre mice.

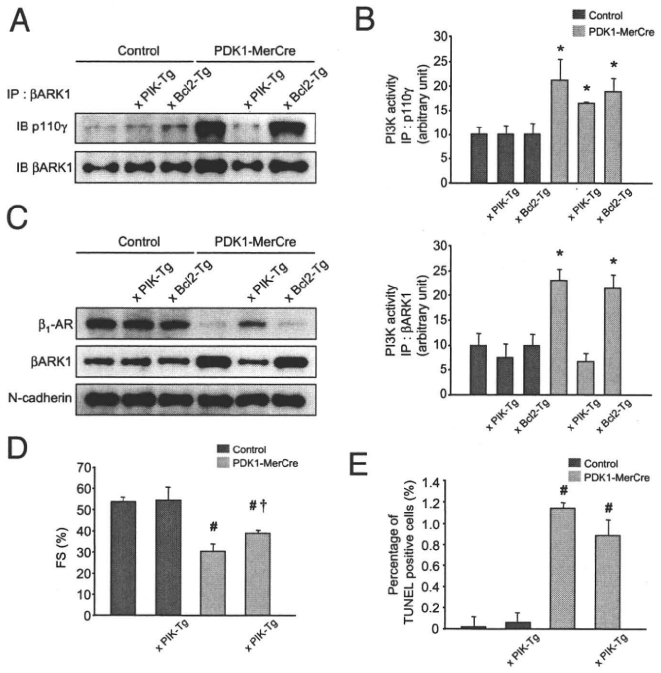

Fig. 4. Alleviated cardiac dysfunction in PDK1-MerCre mice by overexpression of PIK domain or Bcl-2 protein. (*A*) Immunoblot analysis of βARK1-associated p110γ protein in the hearts. (*B*) Kinase assays for PI3-K activity in the hearts. The hearts were subjected to immunoprecipitaion with antibody to p110γ *(Upper)* or βARK1 (*Lower*), and the resulting precipitates were assayed for the kinase assay. PI3-K activity of control mice was adjusted to 10 arbitrary units. (*C*) Immunoblot analysis of β_1-AR and βARK1 in membrane fraction in the hearts. N-cadherin was used as an internal control for the amount of membrane protein. (*D*) Fractional shortening measured by echocardiography. Values represent the mean ± SEM of data from control mice (n = 10), control × PIK-Tg mice (n = 6), PDK1-MerCre mice (n = 10), and PDK1-MerCre × PIK-Tg mice (n = 6). #, $P < 0.01$ versus control mice. †, $P < 0.01$ versus PDK1-MerCre mice. FS, % of fractional shortening. (*E*) Percentage of TUNEL-positive caridomyocytes. Values represent the mean ± SEM (3,000 cardiomyocytes in each group). #, $P < 0.01$ versus control group. †, $P < 0.01$ versus PDK1-MerCre group.

Discussion

Our present study revealed that PDK1 plays an integrative role in normal cardiac function by coordinating survival signals and β-adrenergic response (Fig. S6). Besides the fundamental role in promoting cell growth and survival observed in many tissues in common (18–21), PDK1 uniquely accommodates β-adrenergic response to prevent cardiac decompensation. In addition, decreased expression of PDK1 protein in experimental models of heart failure raises a possibility that functional alterations of PDK1 may be implicated in the pathogenesis of heart failure, although it remains unclear how PDK1 expression is regulated in stressed hearts.

β-AR signaling plays a pivotal role in the chronotropic and inotropic functions in the hearts (22). In PDK1-MerCre hearts, the activity of βARK1-associated PI3-Kγ was enhanced, which enforced robust β_1-AR down-regulation. PDK1 is a direct downstream effector of PI3-K and may participate in the negative feedback regulation of PI3-K signaling pathway (20). Importantly, overexpression of PIK-domain prevented β_1-AR down-regulation by interfering βARK1/PI3-Kγ complex formation, and alleviated cardiac dysfunction in PDK1-MerCre mice. A recent report demonstrated that PI3-Kγ negatively modulates cardiac contractility by promoting phosphodiesterase 3B-mediated destruction of cAMP in a kinase-independent manner (23), but we did not observe significant change in the activity of phosphodiesterase 3B in PDK1-MerCre hearts despite enhanced PI3-Kγ activity (Fig. S7). Therefore, we suppose that impairment of β-adrenergic responsiveness results from intense β-AR down-regulation in PDK1-MerCre hearts.

It remains controversial whether down-regulation and desensitization of β-AR function is beneficial or detrimental in failing hearts. Indeed, clinical trials have indicated that the use of β-AR antagonists improves morbidity and mortality in patients of heart failure (1). Sustained β-AR overstimulation promotes energy consumption and apoptosis in cardiomyocytes (1, 24). But, accumulating evidence has suggested that normalization of β-adrenergic signaling by interfering βARK1 function rescued numerous genetic and experimental models of heart failure in mice (16, 25–28). A possible explanation for this discrepancy is that the therapeutic window for optimal level of β-AR signaling may be narrow in failing hearts (22, 28). It has been reported that the proapoptotic effect of β_1-AR stimulation is dependent on Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (24). The phosphorylation level of CaMKII was decreased in PDK1-MerCre hearts, and restored to a subnormal level by overexpression of PIK domain (Fig. S8). Importantly, normalization of β_1-AR did not induce excessive activation of CaMKII and cardiomyocyte apoptosis (Fig. 4*E* and Fig. S4). Thus, the β_1-AR normalization may improve contractile function without evoking a 'fight or flight' reaction, unlike the simple β_1-AR activation. Alternatively, robust β-AR internalization may activate adverse intracellular signaling pathways through β-arrestins (29) and abrogate the cardioprotective effects mediated by transactivation of epidermal growth factor receptor (30). Further investigations will be required to clarify the entire mechanisms of how normalization of β-AR signaling confers therapeutic benefits on failing hearts.

A growing body of evidence has suggested that cardiomyocyte apoptosis plays an important role in the pathogenesis of heart failure (31). In PDK1-MerCre hearts, the phosphorylation levels of Akt, SGK1 and FOXO3a were reduced, which may give rise to marked increase in cardiomyocyte apoptosis. In addition, PDK1-MerCre hearts showed an increase in expression level of Bax protein, a key molecule that translocates to the mitochondrial membrane and triggers the release of cytochrome *c* into the cytoplasm (31). Overexpression of Bcl-2 attenuated apoptotic loss of cardiomyocytes and alleviated cardiac dysfunction in PDK1-MerCre mice, suggesting that cardiomyocyte apoptosis contributes to the development of heart failure.

The previous paper demonstrated that PDK1-MCKCre mice showed growth retardation and contractile dysfunction of cardimyocytes (5). In our study, PDK1-MerCre mice showed severe heart failure without alterations in cardiomyocyte size. Besides regulation of cell growth, PDK1 controls cardiac homeostasis by promoting cell survival and preserving β-AR response. The phenotypic difference between PDK1-MerCre mice and PDK1-MCKCre mice resulted from the timing of gene disruption. The *Pdk1* gene was deleted within a week in tamoxifen-treated PDK1-MerCre hearts of adult mice, but in contrast, *Pdk1* disruption commenced before birth in PDK1-MCKCre mice. The number of apoptotic cardiomyocytes was pronouncedly increased in PDK1-MerCre hearts, but was unchanged in PDK1-MCKCre hearts (5). Some compensation mechanisms may prevent proapoptotic effects of *Pdk1* disruption in PDK1-MCKCre mice.

In conclusion, PDK1 is a pivotal effector with dual functions to promote survival of cardiomyocytes and to preserve β-AR response in vivo (Fig. S6). In this regard, up-regulation of PDK1 in the hearts may emerge as a potential therapeutic strategy for heart failure.

Methods

Generation of PDK1-MerCre Mice. Mice harboring a *Pdk1*^{flox} allele were previously described (7, 8). Mice expressing MerCreMer under the control of *α-myosin heavy chain* promoter were previously described (9). Details are in *SI Methods*. Bcl2-Tg mice and PIK-Tg mice were kindly gifted by Dr. Michael D. Schneider (Imperial

College, London, U.K.) (15) and Dr. Howard A. Rockman (Duke University Medical Center, Durham, NC) (16). All of the experimental protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

Echocardiography and Isolated Heart Preparation. Transthoracic echocardiography was performed on conscious mice with Vevo 660 Imaging System using a 25-MHz linear probe (Visual Sonics Inc.). For analyses of hemodynamic parameters, hearts were excised rapidly and mounted on a Langendorff perfusion system, and a balloon was inserted into the cavity of the left ventricle (32). Isolated hearts were stabilized for 30 min by perfusion of Krebs-Henseleit buffer followed by perfusion of isoproterenol (NIKKEN Chemical Laboratory) or forskolin (Sigma). For measurement of surface areas of cardiomyocytes, hearts were enzymatically dissociated as described previously (33).

Histological Analysis and Immunohistochemistry. Hearts were excised and immediately fixed in 10% neutralized formalin, embedded in paraffin. Serial sections at 5 μm were stained with hematoxylin and eosin for morphological analysis, and with Masson's trichrome for detection of fibrosis. For immunohistochemistry, Vectastain ABC kit (Vector Laboratories) was used to detect the primary antibodies. TUNEL assay was performed on paraffin sections, using an in situ apoptosis detection kit (Takara Bio Inc.).

Western Blot Analysis and Subcellular Fractionation. Protein samples were fractionated by SDS/PAGE, and immunoblot analysis was performed as described previously (34). The membrane and cytosol fractions were isolated from lysate of the hearts as previously described (35).

Assay for PI3-K Activities. PI3-K acitivity was measured as previously described (36). We determined Akt activity using a Akt Kinase Assay Kit according to the manufacturer's protocol (Cell Signaling Technology).

Antibodies. The following antibodies were used: p110γ, phosphorylated-SGK, and cleaved caspase-3 (Cell Signaling Technology), βARK1, Bax, Bcl-xL, Bcl-2 (Santa Cruz Biotechnology), β_1-AR (Affinity BioReagents), N-cadherin (Zymed Laboratories Inc.), SGK1, FOXO3a, phosphorylated-FOXO3a (Thr-32), phosphorylated-FOXO3a (Ser-253) (Upstate) and actin (Sigma).

Statistical Analysis. All data are presented as means $\pm$ SEM. All data were analyzed by one-way ANOVA followed by the Fisher procedure for comparison of means. A probability value of $P < 0.05$ was considered to be statistically significant.

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Editorial

"Change can happen" by PKA: Proteasomes in in vivo hearts

Rudolf Schoenheimer pioneered the technique to tag amino acids with isotope for tracing their metabolism within living animals [1]. The results of his experiments led to a revolutionary view that the proteins within a body are in a dynamic state of synthesis and degradation. Now, after more than 6 decades, the concept of protein turnover is widely accepted. Especially, to maintain cellular homeostasis, the cells carry out protein quality control through ubiquitin–proteasome system (UPS) and autophagy, and eliminate needless or defective intracellular proteins that are of no use and even hazardous. The UPS is a highly selective degradation process occurring in the cytoplasm, but in contrast, autophagy is a non-selective process that degrades bulk proteins and organelles in the lysosomes to recycle [2]. Insomuch as the UPS participates in proteolysis of thousands of specific proteins, this regulatory system plays an important role in a variety of cellular responses including cell cycle and division, hypoxic response, DNA repair, apoptosis and immune response [3]. Importantly, recent studies have indicated that dysregulation of the UPS is profoundly implicated in human diseases such as inflammatory diseases, neurodegenerative diseases, muscle-wasting disorders, cancer, and cardiovascular diseases [3,4], and the UPS has emerged as a potential therapeutic target for the treatment of these diseases [5].

Postnatal cardiomyocytes scarcely proliferate, and thus are in extraordinary need of removing damaged or misfolded proteins to avoid accumulation of these kinds of garbage within the cells. In addition, since the beating heart is under continuous stress, especially in diseased conditions, myocardial proteins are liable to damaging and misfolding [4]. Furthermore, recent studies have demonstrated that the UPS is dysfunctional in the hearts of rodent models of ischemia/reperfusion (I/R) [6,7] or desmin-related cardiomyopathy [8]. Therefore, elucidation of the regulatory mechanism of the UPS in the heart will be important to understand the pathogenesis of heart diseases. The UPS-mediated proteolysis consists of two sequential steps: covalent attachment of ubiquitin to the protein substrate (ubiquitination) and degradation of the ubiquitinated proteins by 26S proteasome complex [9,10]. Ubiquitination proceed through a series of enzymatic reactions involving ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). Selectivity and specificity of the protein substrate is determined by E3 ligases that have either the RING-finger domain or the HECT domain. Although much knowledge has been accumulated on selective and specific aspects of ubiquitination, the regulatory mechanism of 26S proteasome remains elusive, especially in the heart.

In this issue of Journal of Molecular and Cellular Cardiology, Asai and colleagues have provided unequivocal evidence that protein kinase A (PKA) enhances the assembly and activity of cardiac 26S proteasome both in vitro and in in vivo [11]. The 26S proteasome is a 2.4 MDa multisubunit complex, consisting of a core 670 kDa 20S catalytic subcomplex and two 700 kDa 19S (or PA700) regulatory subcomplexes [9,10,12]. Both ends of the barrel-shaped 20S subunit are capped by 19S regulatory subunits (Fig. 1). The 20S subunit is composed of four axially stacked rings (two identical outer α rings and two identical inner β rings), and each α and β ring contains seven distinct subunits (α1–α7, β1–β7). Three distinct peptidase (chymotrypsin-like, trypsin-like, and caspase-like) activities have been identified, and assigned to three distinct catalytic subunits (β5, β2, and β1, respectively) lining a central lumen. Polyuniquitinated proteins are recognized and unraveled by 19S subunit, which then facilitates the entry and degradation of unfolded polypeptides in the cavity of the 20S subunit.

A couple of studies have shown that PKA can induce serine- or threonine-phosphorylation in 26S proteasome and increase proteolytic activity in vitro [13,14]. The 19S subunit contains six AAA ATPases (Rpt1~6), which contact with outer α rings of the 20S subunit and unfold the polyubiquitinated substrates [12]. According to a recent study, PKA stimulates the proteasome activity by phosphorylating Rpt6 [14]. Ping and colleagues recently delineated a phosphorylation profile of the endogenous 20S subunit of murine hearts and identified phosphorylation in multiple subunits, by using 2-D gel electrophoresis, immunoblotting, and tandem mass spectrometry [13]. In the same study, PKA was identified within the native cardiac 20S complex, and recombinant PKA significantly increased proteasome activity in vitro. The study by Asai et al firstly shows that PKA stimulation enhances the activity of 26S proteasome in in vivo hearts [11]. The proteolytic activities of 26S proteasome in the hearts of anesthetized dogs were significantly increased after PKA stimulation through intracoronary administration of isoproterenol (a β-adrenergic receptor agonist) or forskolin (an activator of adenylate cyclase that increases cyclic AMP and activates PKA) for 30 min. In addition, the 26S proteasome activity was also increased at 30 min after ischemic preconditioning (IP), consisting of 4 cycles of 5 min of ischemia and 5 min of reperfusion. Among a number of signaling pathways involved in IP [15], PKA mediates the enhancement of proteasome activity after IP, because it was attenuated by intracoronary administration of a PKA inhibitor, H-89. As mentioned above, PKA phosphorylates Rpt6 in the 19S subunit and may facilitate diffusion of the polypeptide substrates into the proteolytic cavity of the 20S subunit (Fig. 1), although the precise mechanism remains unclear. The phosphorylations of the 20S subunit may directly or indirectly induce conformational change of the catalytic sites to increase proteolytic activity (Fig. 1).

Alternatively, PKA phosphorylation may be involved in the assembly of proteasome subunits (Fig. 1). Proteasomes with normal function require correct assembly of all subunits, which is orchestrated by multiple proteasome-specific chaperones [16]. Although

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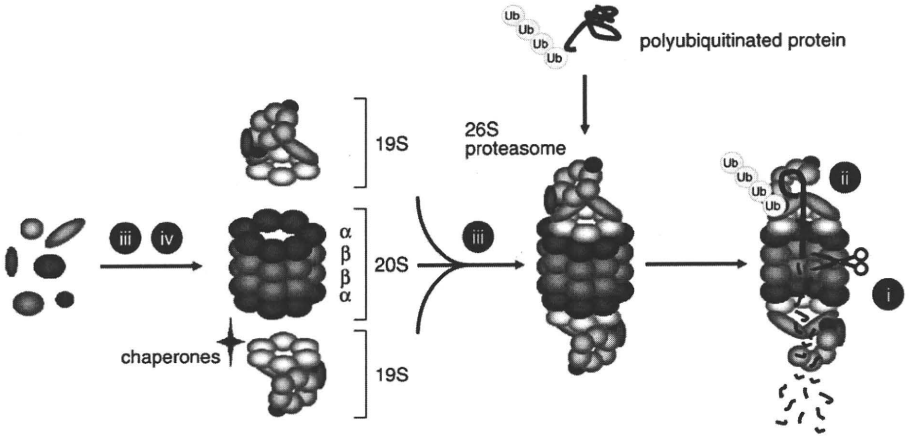

Fig. 1. Potential change in 26S proteasome bought about by PKA. PKA enhances the activity of 26S proteasome potentially i) by increasing proteolytic activity through phosphorylation of 20S subunit, ii) by facilitating translocation of polyubiquitinated substrates through phosphorylation of 19S subunit, iii) by promoting assembly of proteasome subunits through phosphorylation of the subunits or chaperones, or iv) by altering molecular composition of proteasome through an unknown mechanism.

the regulatory kinases are not specified, the phosphorylation of proteasome subunits or chaperones can affect the status of proteasome assembly. For example, the phosphorylation of Rpt6 in the 19S subunit is required for the incorporation of the 19S subunit into 26S proteasome, possibly through the formation of interaction between Rpt6 and α2 subunit [17]. In addition, the phosphorylation of α7 subunit stabilizes the association of the 19S subunit with the 20S subunit to form 26S proteasome [18]. The study by Asai et al shows that PKA stimulation increases the incorporation of Rpt5, α7, and β5 subunits into cardiac 26S proteasome both in vitro and in vivo by immunoblot analysis under non-reducing conditions [11]. Clearly, these results leave many open questions: which subunit phosphorylated by PKA is important in this process? How does the phosphorylation induce an allosteric effect that changes the stability of 26S proteasome? Is the assembly of subunits by PKA critically linked to proteolytic activity of 26S proteasome? Furthermore, recent studies have indicated that certain pathological stress can alter proteasome composition, and that the molecular composition of proteasome is closely related to proteolytic activity [19,20]. It may be possible that PKA alters the proteasome composition, especially in vivo (Fig. 1). Additional studies are necessary to determine the mechanism and consequence of PKA-mediated assembly of 26S proteasome.

Asai et al further investigated the pathophysiological role of proteasome activation by IP in canine hearts subjected to I/R [11]. A significant decrease in the proteasome activity was observed after 90 min of ischemia, which lasted for the following period of reperfusion. It has been reported that free radical-initiated oxidation, such as lipid peroxidation, participates in oxidative modification and inactivation of the 20S proteasome during I/R [6,7]. As a consequence of a decline in the proteasome activity, I/R increased accumulation of ubiquitinated proteins in the hearts. Interestingly, IP counteracted the decline of proteasome activity during I/R, which was associated with a significant reduction in the accumulation of ubiquitinated proteins. Abnormal accumulation of ubiquitinated proteins causes aberrant protein aggregates, and thus is thought to be deleterious to cardiomyocytes [4]. The favorable effect of IP on accumulation of ubiquitinated protein in I/R hearts was abolished by intracoronary administration of a proteasome inhibitor epoxomicin, but surprisingly, the infarct size in I/R hearts was unchanged with or without IP even by epoxomicin at the concentration that reduced proteasome activity by 43%. These results imply that proteasome activation by IP is irrelevant to the alleviative effect of IP on myocardial cell death during I/R. Then, the next question will be whether the beneficial effect of IP on contractile function of viable myocardium is prevented or not by epoxomicin in I/R hearts. Indeed, proteasome inhibitors may lead to deleterious and beneficial outcomes during myocardial ischemia according to the experimental designs [21]. The intracoronary administration of epoxomicin in anesthetized dogs may mitigate the inflammatory response within the hearts, because the NF-κB signaling is regulated by the UPS. Given that the UPS tightly controls turnover of regulatory proteins involved in physiological responses such as intracellular signaling and transcriptional regulation [3], the subtle difference in the concentrations or the pharmacokinetics of the proteasome inhibitors may influence the outcomes in in vivo experiments. In addition, it has been reported that autophagy acts as a compensatory degradation system when the UPS is impaired in a Drosophila model of neurodegenerative disease [22]. Administration of proteasome inhibitors may induce autophagy in I/R hearts, and thereby prevent myocardial cell death by maintaining organelle turnover and energy homeostasis [23]. Further studies are needed to clarify the functional coupling between the UPS and autophagy, especially in I/R hearts.

The proteasome inhibitor bortezomib shows selective cytotoxicity to cancer cells, and is approved for clinical treatment of refractory multiple myeloma [5]. Insomuch as the proteasome activity is hampered in ischemic hearts, pharmacological restoration of the proteasome function has a potential to become a rational strategy for treatment. The study of Asai et al provides an important clue toward this strategy [11]. Manipulation of proteasome function may be applied to treatment of a wide spectrum of heart diseases such as cardiac hypertrophy. Tsukamoto et al revealed that proteasome was dysfunctional in murine hearts of pressure-overloaded hypertrophy [24]. However, Depre et al argued that proteasome function was upregulated during pressure overload in canine hearts and that administration of proteasome inhibitors attenuated cardiac hypertrophy without altering cardiac function [25]. Of course, in-depth assessment of the pathogenic role of the UPS in heart diseases will be a prerequisite for launching a bench-to-bed approach.

Will pharmacological activation of PKA induce "a change we can believe in" in proteasomes of stressed myocardium and produce a clinical benefit in the treatment of heart diseases? Further studies are required to explore the detailed mechanism of proteasome modification and to develop an optimal way in normalization of proteasome function in diseased hearts.

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Review

Mechanisms and functions of agonist-independent activation in the angiotensin II type 1 receptor

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ABSTRACT

The angiotensin II (AngII) type 1 (AT_1) receptor is a seven-transmembrane G protein-coupled receptor, and is involved in regulating the physiological and pathological process of the cardiovascular system. Systemically and locally generated AngII has agonistic action on AT_1 receptor, but recent studies have demonstrated that AT_1 receptor inherently shows spontaneous activity even in the absence of AngII. Furthermore, mechanical stress can activate AT_1 receptor by inducing conformational switch without the involvement of AngII, and induce cardiac hypertrophy *in vivo*. These agonist-independent activities of AT_1 receptor can be inhibited by inverse agonists, but not by neutral antagonists. Considerable attention has been directed to molecular mechanisms and clinical implications of agonist-independent AT_1 receptor activation, and inverse agonist activity emerges as an important pharmacological parameter for AT_1 receptor blockers that will improve efficacy and expand therapeutic potentials in cardiovascular medicine.
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Contents

1. Introduction

Pharmacological inhibitions of the renin–angiotensin system (RAS) are crowned with one of the greatest success in the current field of cardiovascular medicine. During the past quarter of century, a growing body of evidence has accumulated indicating that RAS blockade can prevent progression of cardiac hypertrophy and reduce the morbidity and mortality in patients with heart failure (Zaman et al., 2002; Jessup and Brozena, 2003). In addition to the systemic effects including elevation of blood pressure, sodium and water retention, and activation of sympathetic nervous system, the RAS has unfavorable direct effects on the hearts, especially through a system of local activation in tissues (Re, 2004; Paul et al., 2006). Angiotensin II (AngII) has been considered to be the pivotal bioactive molecule of RAS, and most of the pathophysiological actions of AngII in the cardiovascular system are mainly mediated through AngII type 1 (AT_1) receptor (Timmermans et al., 1993). According to the results from *in vitro* experiments, activation of AT_1 receptor stimulates diverse intracellular signaling cascade cascades and enhances production of reactive oxygen species, which consequently evokes hypertrophic responses in cardiomyocytes and enhances cellular proliferation and production of extracellular matrix proteins in cardiac fibroblasts (Kim and Iwao, 2000; Hunyady and Catt, 2006).

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The AT_1 receptor is a typical member of the G protein-coupled receptor (GPCR) family, the structure of which is characterized by seven-transmembrane spanning α-helices with an extracellular N-terminus and a cytoplasmic C-terminus (Gether and Kobilka, 1998; Gether, 2000; Miura et al., 2003a). As a matter of course, AT_1 receptor is activated upon binding to AngII, the specific and endogenous agonist. AT_1 receptor can also be activated by autoantibodies against the receptor. These agonistic antibodies bind to epitopes on the second extracellular loop of the receptor, and are involved in the pathogenesis of preeclampsia and renal-allograft rejection (Thway et al., 2004; Dragun et al., 2005). It is now believed that agonist binding facilitates isomerization of a GPCR to an active conformation by disrupting the intramolecular interactions that constrain the receptor in an inactive conformation (Gether and Kobilka, 1998; Gether, 2000; Farrens et al., 1996; Hunyady et al., 2003). However, the classical concept that the receptors switch by a simple 'on-off' mechanism has been challenged since the discovery of spontaneous activity of δ-opioid receptor in the absence of agonist (Costa and Herz, 1989). Inherently, GPCRs are structurally flexible and instable, and have significant and varying levels of spontaneous activity in an agonist-independent manner (Leurs et al., 1998; Milligan, 2003). The constitutive activity has been demonstrated when AT_1 receptor is heterologously expressed in recombinant systems, and becomes manifest as a consequence of specific mutations (Hunyady et al., 2003; Noda et al., 1996; Groblewski et al., 1997; Balmforth et al., 1997; Feng et al., 1998). Furthermore, we have recently obtained compelling evidence that AT_1 receptor is activated by mechanical stress independently of AngII (Zou et al., 2004; Yasuda et al., 2008). These observations have in turn led to identification of the ligands that are able to inhibit agonist-independent receptor activity and/or activation, i.e. inverse agonists (Milligan, 2003; Strange, 2002; Bond and Ijzerman, 2006), and now prompt us to re-evaluate pharmacological actions of AT_1 receptor blockers (ARBs). In this article, we will review the current understanding of the structure–function relationship and the pathophysiological or therapeutical relevance of agonist-independent AT_1 receptor activation.

2. Constitutive activity of AT_1 receptor

Constitutive activity of wild-type AT_1 receptor under basal conditions is relatively low, but can be detected when AT_1 receptor is overexpressed in cells even in the absence of endogenous expression of angiotensiogen (Zou et al., 2004; Miura et al., 2006). This phenomenon can be rendered more distinct by introducing specific amino acid substitutions of the receptor (Hunyady et al., 2003; Noda et al., 1996; Groblewski et al., 1997; Balmforth et al., 1997; Feng et al., 1998). The first evidence of constitutively active mutant (CAM) GPCR was obtained in α_{1B}-adrenoreceptor (Cotecchia et al., 1990). Amino acid substitutions of Ala^{293} at the end of the third intracellular loop of the α_{1B}-adrenergic receptor conferred constitutive activity (Kjelsberg et al., 1992). The following studies revealed that the mutational changes in the equivalent residues in this region resulted in constitutive activation of β_2-adrenoreceptor (Samama et al., 1993) and α_2-adrenoreceptor (Ren et al., 1993). These results provided a model that spontaneous signaling is repressed to a low level via the conserved intramolecular constraints, and that agonist binding alters the receptor conformation by relieving these intrinsic constraints (Parnot et al., 2002; Costa and Cotecchia, 2005). CAMs are thought to mimic an active conformation of the wild-type receptor, in which structural constraints are disrupted. In this regard, CAMs have provided plentiful insights into the molecular process of agonist-induced receptor activation (Parnot et al., 2002; Costa and Cotecchia, 2005).

Structure–function analyses have demonstrated that the bindings of AngII to Asn^{111} in transmembrane (TM) 3 and to His^{256} in TM6 of the AT_1 receptor are crucial for receptor activation (Noda et al., 1995a, 1996; Feng et al., 1998; Miura et al., 1999), although two salt bridges between AngII and Lys^{199} in TM 5 or Asp^{281} in the third extracellular loop are important for docking AngII to the receptor (Yamano et al., 1992; Noda et al., 1995b; Feng et al., 1995). Interestingly, substitutions of Asn^{111} to residues of smaller size such as Gly or Ala caused higher constitutive activity in inositol phosphate production, while those to larger residues such as Phe or Tyr resulted in a reduction of the basal activity (Noda et al., 1996; Feng et al., 1998). The mechanism by which the size of the residue at the position of Asn^{111} determines the level of constitutive activity is not clear, but it is likely that the receptor with the activating mutations may emulate the conformational transition that AngII-binding normally induced through altering the van der Waals contact between Asn^{111} and other residues in the AT_1 receptor (Noda et al., 1996; Feng et al., 1998).

The structural transition underlying constitutive activation in AT_1 receptor harboring the Asn^{111} mutations has been explored by studies using the substituted cysteine accessibility mapping (SCAM). The SCAM study is used to probe relative conformational changes of GPCRs by validating the presence of Cys residues within the ligand-binding pocket (Miura and Karnik, 2002; Chen et al., 2002; Boucard et al., 2003; Miura et al., 2003b; Lemaire et al., 2004; Jongejan et al., 2005; Martin et al., 2007) (Fig. 1). The SCAM studies have revealed that the mutations in Asn^{111} confer constitutive activity of the receptor (Groblewski et al., 1997; Feng et al., 1998) by releasing helical constraints involving TM2 (Miura and Karnik, 2002; Miura et al., 2003b), TM6 (Martin et al., 2007) and TM7 (Boucard et al., 2003; Miura et al., 2003b).

3. Pathophysiological relevance of constitutive activity of AT_1 receptor

Activating mutations for several GPCRs are causative of diseases, such as thyrotropin-stimulating hormone receptor in hyperfunctioning thyroid adenoma (Parma et al., 1993) and luteinizing hormone receptor in familial male precocious puberty (Shenker

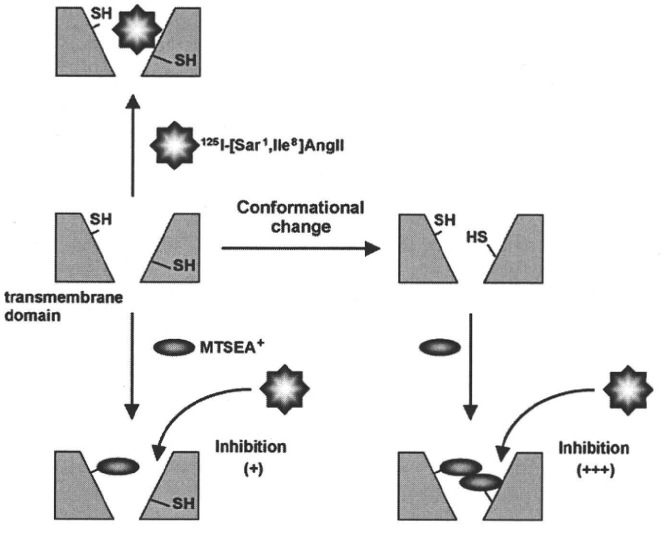

Fig. 1. Substituted cysteine accessibility mappaing (SACM). Sulfhydryl-specific reaction with methanethiosulfonyl ethyl-ammonium ($MTSEA^+$), which reacts a billion times faster with water-exposed and ionized Cys than lipid-exposed and un-ionized Cys. Upon this reaction, a positively charged sulfonylmethylammonium group is added onto water-exposed Cys via a mixed disulfide bond. The chemical modification of Cys in the water-accessible ligand pocket results in interference with the binding of radioisotope-labeled ligand either through steric hindrance or electrostatic repulsion. Thus, changes in the binding of radioisotope-labeled ligand indicate an entry or exit of Cys residues within the ligand pocket, which results from a conformational transition of the GPCR.

et al., 1993). With regard to AT_1 receptor, no germline or somatic mutation has been identified that causes hypertension or primary hyperaldosteronism (Davies et al., 1997; Sachse et al., 1997) through induction of robust constitutive activity.

The question remains to be difficult to solve whether the subtle constitutive activity of native GPCRs fulfills a pathophysiological role. Indeed, constitutive activity of native histamine H_3 receptors is present in rodent brain, and seems to control activities of cerebral histaminergic neurons *in vivo* (Morisset et al., 2000). However, it is still unclear whether this spontaneous activity is crucial to proper function of neurons. Theoretically, higher expression levels of GPCRs are anticipated to increase agonist-independent basal activity in native tissues. The expression of AT_1 receptor is up-regulated in vascular cells by low-density lipoprotein cholesterol (Nickenig et al., 1997), insulin (Nickenig et al., 1998), glucose (Sodhi et al., 2003), progesterone (Nickenig et al., 2000), and inflammatory cytokines such as interleukin-1α or interleukin-6 (Sasamura et al., 1997; Wassmann et al., 2004), providing a potential mechanistic link of enhanced AT_1 receptor expression to atherosclerosis associated with hyperinsulinemia, hypercholesterolemia and estrogen deficiency (Wassmann and Nickenig, 2006; Griendling et al., 1996). However, it is quite difficult to measure the accurate amount of functional AT_1 receptor expression in tissues, and experimental proof is needed that such distinctions of enhanced intrinsic receptor activity contribute to progression of atherosclerosis.

According to recent papers, transgenic overexpression of AT_1 receptor in the hearts induced cardiac hypertrophy and remodeling without alterations in systemic blood pressure (Hein et al., 1997; Paradis et al., 2000). In addition, knockin mice with a constitutively activating mutation (substitution of Asn^{111} to Gly with a C-terminal deletion) showed low-renin hypertension and progressive fibrosis in kidney and heart (Billet et al., 2007). These results may raise a possibility that enhancement of constitutive activity, either through up-regulation of receptor expression or activating mutations, is disease-causing. To corroborate this possibility, further studies will be needed to examine whether enhanced intrinsic activity of AT_1 receptor leads to some phenotypic abnormalities even under circumstances where the production of AngII is pharmacologically or genetically inhibited.

4. Mechanical stress-induced activation of AT_1 receptor

We recently found a novel mechanism whereby mechanical stress activates AT_1 receptor independently of AngII (Zou et al., 2004; Yasuda et al., 2008). Mechanical stress, along with neurohumoral factors, is the primary stimulus for cardiac hypertrophy. In isolated hearts perfused as Langendorff preparations, the increase in protein synthesis was most closely related to stretching of ventricular wall as a consequence of increased afterload (Kira et al., 1984). In addition, an increase in protein synthesis was also observed, when cardiomyocytes cultured on deformable silicone rubber dishes underwent passive stretch even in serum-free media (Mann et al., 1989). Furthermore, mechanical stretching of cultured cardiomyocytes induced hypertrophic responses such as activation of many protein kinases including extracellular signal-regulated protein kinases (ERKs) and reprogramming of gene expression (Komuro and Yazaki, 1993; Sadoshima and Izumo, 1997). These results suggest that mechanical stress *per se* induces hypertrophic responses primarily by stretching cardiomyocytes.

Activation of AT_1 receptor is profoundly involved in the development of load-induced cardiac hypertrophy. Many clinical studies have shown that ARBs have superior effects on left ventricular mass reduction in hypertensive patients (Kjeldsen et al., 2002; Klingbeil et al., 2003; Okin et al., 2004). Furthermore, pretreatment of cardiomyocytes with ARBs significantly attenuated hypertrophic responses induced by stretching (Sadoshima et al., 1993; Yamazaki et al., 1995). These results indicate that mechanical stress induces cardiac hypertrophy through the activation of AT_1 receptor. However, it has been a challenging problem to solve how AT_1 receptor is activated by mechanical stress. There is a possibility that AngII is stored in cardiomyocytes, and that mechanical stretch induces the secretion of stored AngII into the culture medium, resulting in the induction of cardiomyocyte hypertrophy by the autocrine mechanism (Sadoshima et al., 1993). However, direct measurement of AngII concentration in the medium conditioned by stretching cardiomyocytes did not reveal a significant increase in AngII concentration (Zou et al., 2004). Furthermore, a neutralizing antibody to AngII did not suppress the stretch-induced ERKs activation in cardiomyocytes, although the antibody abolished AngII-induced ERKs activation (Zou et al., 2004). These results suggest that AngII, even if secreted from cardiomyocytes, plays a marginal role in stretch-induced ERKs activation, and raise quite a different possibility that mechanical stress can directly activate the AT_1 receptor without the involvement of AngII.

In human embryonic kidney (HEK) 293 cells or COS7 cells which have no detectable expression of AT_1 receptor and angiotensinogen, neither AngII nor mechanical stretch activated ERKs, but forced expression of AT_1 receptor conferred the ability to activate ERKs in response to both AngII and mechanical stretch. Interestingly, candesartan, as an inverse agonist for ARB, inhibited the ERKs activation induced not only by AngII but also by mechanical stretch in HEK293 cells expressing AT_1 receptor. Stretch stimuli also activated ERKs in HEK293 cells expressing AT_1 mutant which did not bind AngII (Yamano et al., 1992) and in cardiomyocytes prepared from *angiotensinogen*-deficient mice (Tanimoto et al., 1994), and these activations were inhibited by candesartan (Zou et al., 2004). Furthermore, mechanical stress can induce cardiac hypertrophy *in vivo* through the AT_1 receptor in the absence of AngII, because pressure overload induced cardiac hypertrophy in *angiotensinogen*-deficient mice as well as in wild-type mice, which was significantly inhibited by candesartan. These experimental data provided compelling evidence that AT_1 receptor is activated in the absence of AngII both *in vitro* and *in vivo*, and that this AngII-independent activation of AT_1 receptor is inhibited by candesartan.

Besides AT_1 receptor, several GPCRs, such as the receptors of endothelin 1 (ET-1) and catecholamines, also contribute to induction of cardiomyocyte hypertrophy (Yamazaki et al., 1996; Zou et al., 1999). However, mechanical stretch did not induce significant activation of ERKs in COS7 cells expressing either ET-1 type A receptor or β_2-adrenoceptor in a ligand-independent manner. A recent study using a fluorescence resonance energy transfer approach demonstrated that fluid shear stress induced a conformational change of bradykinin B_2 receptor in endothelial cells (Chachisvilis et al., 2006). These results suggest that the activation of GPCRs by mechanical stretch without the involvement of their agonists is not a general phenomenon but specific to some GPCRs including the AT_1 receptor.

5. Conformational switch of AT_1 receptor during mechanical stress-induced activation

Insomuch as AT_1 receptor is activated by mechanical stress, AT_1 receptor should undergo a conformational switch that couples mechanical stress-induced activation. We recently demonstrated by a SCAM study that mechanical stretch increased the accessibility of Cys^{289} in TM7 to the ligand-binding pocket in a time-dependent manner (Yasuda et al., 2008). According to the results of a series of SCAM experiments using mutant receptors with substitution of the TM7 residue ranging from Thr^{287} to Asn^{295} to Cys one at a time, TM7 undergoes a counterclockwise rotation and a shift into the ligand-