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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.

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

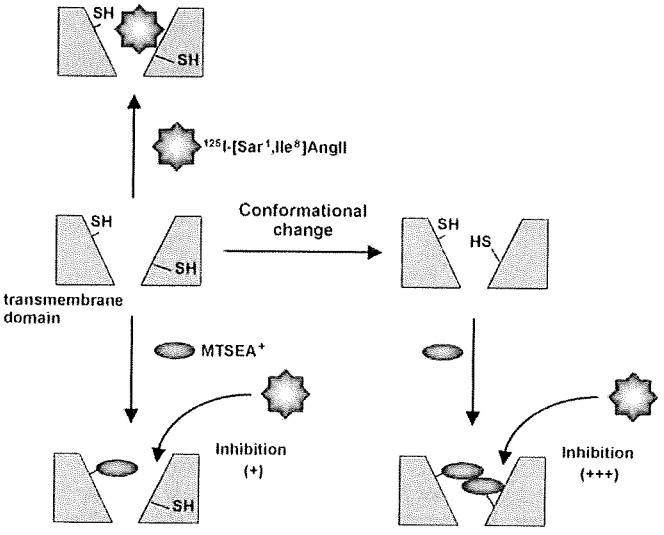

Fig. 1. Substituted cysteine accessibility mappaing (SACM). Sulfhydryl-specific reaction with methanethiosulfonyl ethyl-ammonium ($MTSEA^+$), which reacts a billion times faster with water-exposed and ionized Cys than lipid-exposed and un-ionized Cys. Upon this reaction, a positively charged sulfonylmethylammonium 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-

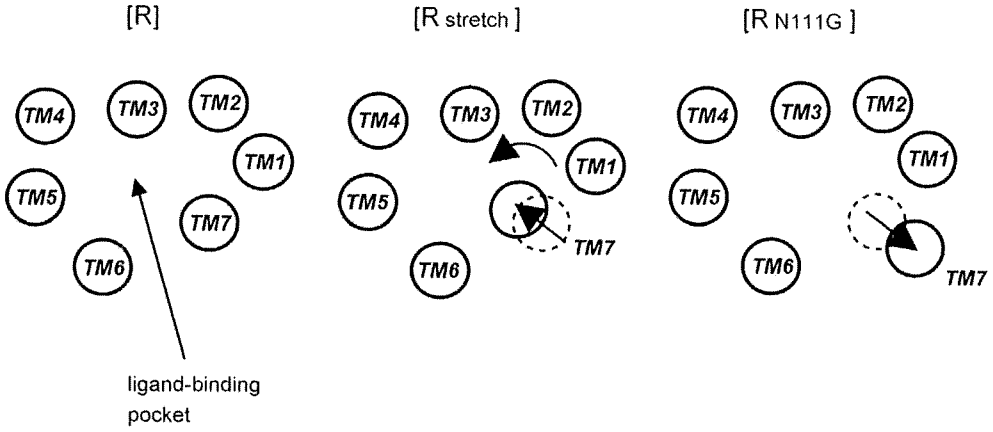

Fig. 2. Helical movements in AT_1 receptor during mechanical stretch-induced activation and in a constitutively active AT_1-N111G receptor. Seven TMs are viewed from the extracellular side. [R] is an unaligned inactive state. [$R_{stretch}$] is an active state stabilized by mechanical stretch. [R_{N111G}] is a state of AT_1-N111G receptor, which mimics a state of AT_1 receptor partially activated by AngII. TM7 rotates counterclockwise and shifts into the ligand-binding pocket in [$R_{stretch}$]. In contrast, TM7 shifts apart from the ligand-binding pocket in [R_{N111G}]. TM, transmembrane domain.

binding pocket in response to mechanical stretch (Yasuda et al., 2008). It is probable that the stabilizing interactions involving TM7 in AT_1 receptor are disrupted by mechanical stress independently of AngII and that counterclockwise rotation of TM7 may cause activation of intracellular signaling pathways. A shift of TM7 to inside the ligand-binding pocket during mechanical stress-induced activation contrasts well with the helical movement observed in a constitutively active AT_1-N111G receptor, because TM7 shifts apart from the ligand-binding pocket in this mutant receptor (Boucard et al., 2003) (Fig. 2). Since AT_1-N111G receptor mimics the state of WT receptor partially activated by AngII (Miura and Karnik, 2002; Le et al., 2003), an active conformation of AT_1 receptor induced by mechanical stress may be substantially different from that by AngII-dependent receptor activation.

Next obvious question is how the AT_1 receptor senses mechanical stress and undergoes a conformational switch. First, membrane tension may directly induce the conformational change of AT_1 receptor. Reconstitution of mechanosensitive channel of large conductance from *Escherichia coli* in synthetic phosphatidylcholines with different chain lengths revealed that thin bilayer favored the open state of channels while thick bilayer stabilized the closed state (Perozo et al., 2002). Likewise, it may be possible that membrane tension causes thinning of the lipid bilayer, which triggers tilting of TM7 of AT_1 receptor to avoid hydrophobic mismatch and to rectify lateral pressure profile (Orr et al., 2006). If so, it follows that AT_1 receptor, a GPCR, functions as a receptor for mechanosensation. It will be intriguing, because GPCRs are involved in mediating senses of vision, olfaction and much of gustation, of Aristotle's five senses (Kung, 2005). Second, mechanical stretch may activate specific mechanosensors, which secondarily activate AT_1 receptor. Potential candidate for mechanosensors, such as muscle LIM protein within the Z-disc (Knoll et al., 2002), integrin-linked kinase (Bendig et al., 2006; White et al., 2006) and melusin (Brancaccio et al., 2003) within the costameres and stretch-sensitive ion channels (Orr et al., 2006; Kung, 2005), might activate the AT_1 receptor, although the underlying mechanism remains to be determined. Recent evidence has shown that mechanical force directly alters conformation or folding of cytoskeletal proteins, which enhances enzymatic activities or susceptibility to enzymatic reactions (Sawada et al., 2006). However, mechanical stretch activated AT_1 receptor even when actin cytoskeleton was disrupted by treatment with cytochalasin D (Yasuda et al., 2008). It will be a great challenge to elucidate the precise mechanism of force sensing by AT_1 receptor.

6. Inverse agonism on agonist-independent activation of AT_1 receptor

Before the early 1990s, GPCR ligands were simply classified as agonists or antagonists (Milligan, 2003; Strange, 2002; Bond and Ijzerman, 2006). Both agonists and antagonists bind to the cognate GPCR with high affinity, but only agonists can activate the receptor. Therefore, agonists possess both high affinity and positive efficacy, whereas antagonists posses high affinity without intrinsic efficacy (Fig. 3). However, some compounds, originally described as antagonists, have been demonstrated to produce effects opposite to those by agonists. First example was ICI174864, a ligand for δ-opioid receptor, which reduces the basal GTPase activity in membranes of NG108-15 cells (Costa and Herz, 1989). Such ligands are classified as "inverse agonists" that have negative efficacy (Fig. 3). An inverse agonist stabilizes inactive conformation of the receptor and reduces constitutive activity of the receptor or the agonist-independent receptor activity.

Several ARBs are already clinically available for the treatment of hypertension. These drugs share a common action, namely blocking AngII-mediated responses, but show a unique pattern of pharmacological properties (Oparil, 2000). The inverse agonist activity of ARBs could be of clinical advantage to inhibition of both AngII-dependent and -independent receptor activation, and thus be a novel and important pharmacological parameter defining the beneficial effects on organ protection. Candesartan reduces the basal activation of *c-fos* gene promoter by AT_1-WT receptor

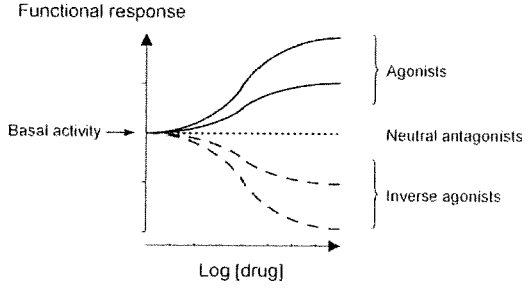

Fig. 3. Classification of GPCR ligands as agonists, neutral antagonists, or inverse agonists. An agonist is a ligand that has a positive efficacy and triggers a functional response. A neutral antagonist is a ligand that has no intrinsic efficacy for a given response, but blocks agonist-induced response. An inverse agonist is a ligand that has a negative efficacy and produces a response opposite to that of the agonist.

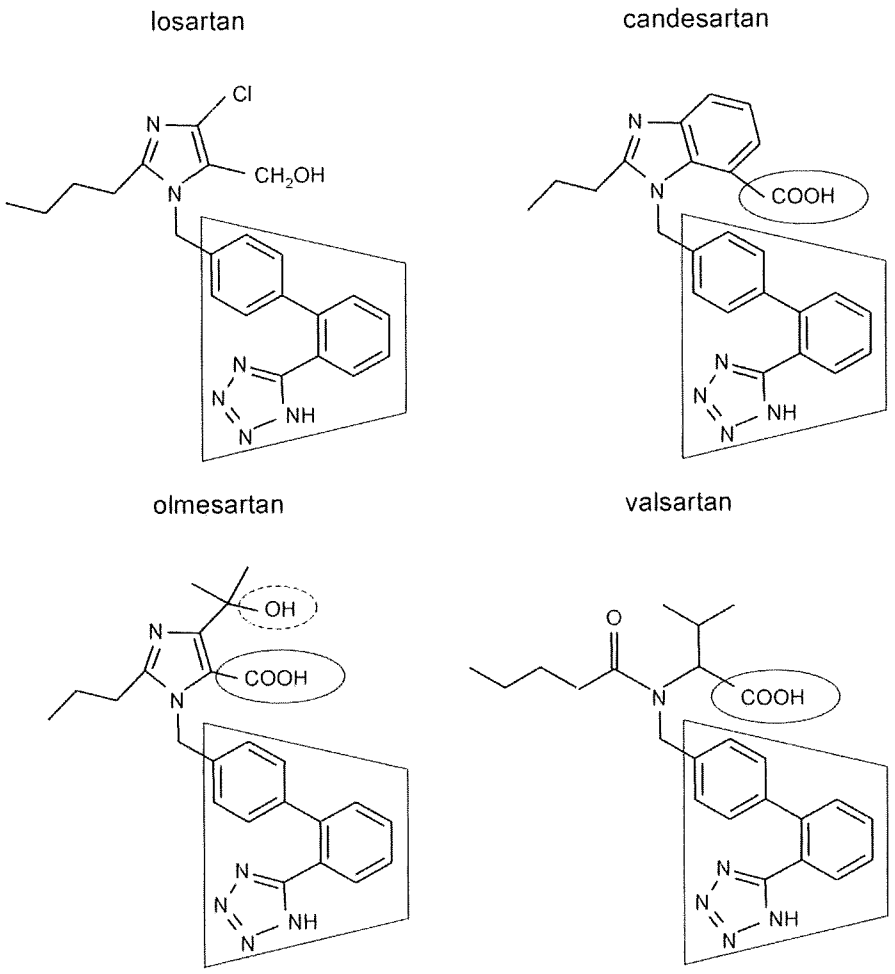

Fig. 4. Chemical structures of losartan, candesartan, olmesartan, and valsartan. A trapezoid indicates biphenyltetrazole ring, a common structure of most ARBs. A circle and a dotted circle indicate carboxyl group and hydroxyl group, respectively. The carboxyl groups and the hydroxyl group in circles are responsible for inverse agonist activity of ARBs.

or a constitutively active AT_1-N111G mutant receptor, suggesting that candesartan is an ARB with potent inverse agonist activity (Yasuda et al., 2008). According to recent papers, olmesartan, valsartan and EXP3174 (active metabolite of losartan) also reduce the constitutive GTPase stimulating activity of AT_1 mutant receptor, while losartan does not reduce it (Miura et al., 2003a, 2006, 2008). Furthermore, candesartan suppressed mechanical stretch-induced helical movement of AT_1 receptor (Yasuda et al., 2008), and thereby inhibited receptor activation (Zou et al., 2004). Inverse agonism of candesartan is especially relevant to its ability to attenuate load-induced cardiac hypertrophy, because pressure overload by constricting the transverse aorta induced cardiac hypertrophy even in *angiotensinogen*-deficient mice as well as in WT mice, which was significantly inhibited by candesartan (Zou et al., 2004).

Although the inverse agonist activity of individual ARBs has not been fully evaluated, we should consider that the distinctive activity of inverse agonism is primarily determined by chemical structure of the drug. Most of ARBs have a biphenyltetrazole ring structure in common (Fig. 4), which interacts with Lys^{199} and His^{256} in the AT_1 receptor (Noda et al., 1995b). It was reported that the carboxyl group at the benzimidazole ring of candesartan (Fig. 4) is an important structure for insurmountable inhibition of AngII-induced receptor activation (Noda et al., 1993; Takezako et al., 2004). Insurmountable ARBs depress the maximal agonist responses, in contrast to surmountable ARBs that produce parallel rightward shifts of agonist concentration–response curves in contraction studies using rabbit aortic strip or cell-based functional studies (Vauquelin et al., 2002). It is interpreted that insurmountable inhibition reflects tight drug-receptor complex formation and slow dissociation (Fierens et al., 1999; Vanderheyden et al., 1999). We recently found that the bindings of the carboxyl group of candesartan to Gln^{257} in TM6 and Thr^{287} in TM7 are responsible for the potent inverse agonism in inhibiting mechanical stretch-induced activation of AT_1 receptor (Yasuda et al., 2008). It is reasonable that the tight binding to AT_1 receptor is prerequisite for an inverse agonist to stabilize the receptor in an inactive conformation, as well as to exert insurmountable inhibition of AngII-induced receptor activation. Besides candesartan, ARBs with potent inverse agonist activity form a complex with AT_1 receptor through tight drug-receptor interactions. For example, olmesartan and valsartan robustly suppresses constitutive production of inositol phosphate by AT_1-N111G receptor (Miura et al., 2006, 2008). Although the interactions of olmesartan with Tyr^{113}, Lys^{199}, His^{256}, and Gln^{257} in the AT_1 receptor are important for the tight drug-receptor binding, its potent inverse agonist activity requires cooperative interactions between the hydroxyl group and Tyr^{113} in TM3 and between the carboxyl group and His^{256} in TM6 (Miura et al., 2006) (Fig. 4). Interestingly, differential interactions of valsartan to Ser^{105} and Ser^{109} in TM3 and Lys^{199} in TM5 are critical for producing inverse agonism (Miura et al., 2008). Among these docking residues, Ser^{105} binds to the carboxyl group of valsartan (Fig. 4). Thus, the chemical structure of an ARB governs the spatial and kinetic pattern of contacts to the

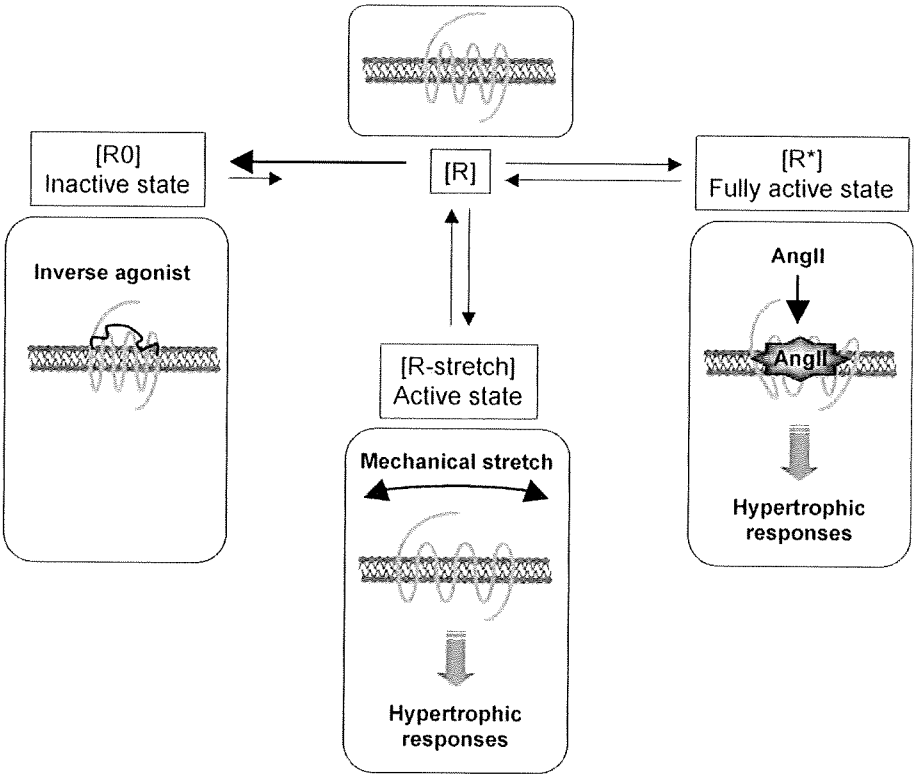

Fig. 5. Distinct conformations of the AT_1 receptor. [R] is an unaligned inactive state, and [R_0] is an inactive state stabilized by an inverse agonist. [R*] is an active state stabilized by the agonist AngII. Mechanical stretch stabilizes AT_1 receptor to another active state [$R_{stretch}$], independently of AngII. An inverse agonist forcibly induces a distinct transition from [R] to [R_0], and prevent a shift of equilibrium to [R*] or [$R_{stretch}$].

AT_1 receptor, which will determine the potency of inverse agonist activity.

According to a sequential binding and conformational model for the molecular mechanism of ligand action on GPCRs (Gether, 2000; Perez and Karnik, 2005), the unaligned receptor in a state [R] can undergo transition to at least two other stabilized states [R_0] and [R*]. [R_0] is an inactive state stabilized by an inverse agonist, and [R*] is an active state stabilized by an agonist. It is consistent with the result of a recent study using a fluorescence resonance energy transfer approach, demonstrating that agonists and inverse agonists for α_{2A}-adrenergic receptor induced distinct conformational changes of the receptor (Vilardaga et al., 2005). With regard to AT_1 receptor, mechanical stretch stabilizes the receptor to another active state [$R_{stretch}$] (Fig. 5). Molecular modeling using the crystal structure of bovine rhodopsin (Palczewski et al., 2000) as a template indicates that, in the inactive state [R_0] in the presence of candesartan, TM6 and 7 move with clockwise rotation, as a consequence of the bindings of the carboxyl group of candesartan to Gln^{257} in TM6 and Thr^{287} in TM7 (Yasuda et al., 2008). The clockwise rotations of TM6 and 7 in this model are consistent with the result of a SCAM experiment demonstrating a decrease in the accessibility of His^{256}, an increase in that of Ile^{290} and a decrease in that of Ala^{291} to the ligand-binding pocket (Yasuda et al., 2008). Therefore, candesartan, as an inverse agonist, forcibly induces a distinct transition from [R] to an inactive conformation [R_0], and prevents a shift of equilibrium to an active conformation [$R_{stretch}$] or [R*] (Fig. 5).

7. Conclusions

The structure–function analyses of the AT_1 receptor have advanced our understanding of the molecular mechanism underlying receptor activation and inverse agonism. Although the structural flexibility of AT_1 receptor, like other GPCRs, may underlie the AngII-independent activation, mechanical stress-induced activation seems to be a phenomenon peculiar to AT_1 receptor. Future investigations with biophysical, biochemical, and pharmacological approaches will elucidate the precise mechanism of force sensing by AT_1 receptor and define the molecular events that link conformational switch of the receptor to the regulation of specific signaling pathways.

Although inverse agonism is now a well-recognized phenomenon in the field of receptor pharmacology, clinical importance of inverse agonist activity of ARBs is still speculative. It is of particular significance to verify whether the drug efficacy assayed in recombinant systems is related to the pharmacological properties *in vivo*. At least, in an experimental animal model, inverse agonist activity of ARBs is relevant to its ability to attenuate load-induced cardiac hypertrophy (Zou et al., 2004). Given that inverse agonist activity is a potential determinant of clinical benefits, molecular dissection of the structure-activity relationship will contribute to the development of a novel and desirable ARB.

We have just taken a first step toward the full understanding of AT_1 receptor activation without the involvement of AngII, and further studies will be required to elucidate the exact molecular mechanisms of receptor activation and to clarify the clinical relevance of inverse agonist activity of ARBs. Recently, crystallizing of native opsin has determined its structure to 2.9 Å resolution, which provides insights into biological process of ligand binding to GPCRs (Park et al., 2008). In addition, crystal structures of squid rhodopsin (Murakami and Kouyama, 2008) and β_1- and β_2-adrenergic receptors (Rasmussen et al., 2007; Cherezov et al., 2007; Rosenbaum et al., 2007) have been obtained, and they reveal several key dif-

ferences with that of bovine rhodopsin. Clearly, crystal structural information of AT_1 receptor will improve our understanding of receptor activation and inactivation at a molecular level.

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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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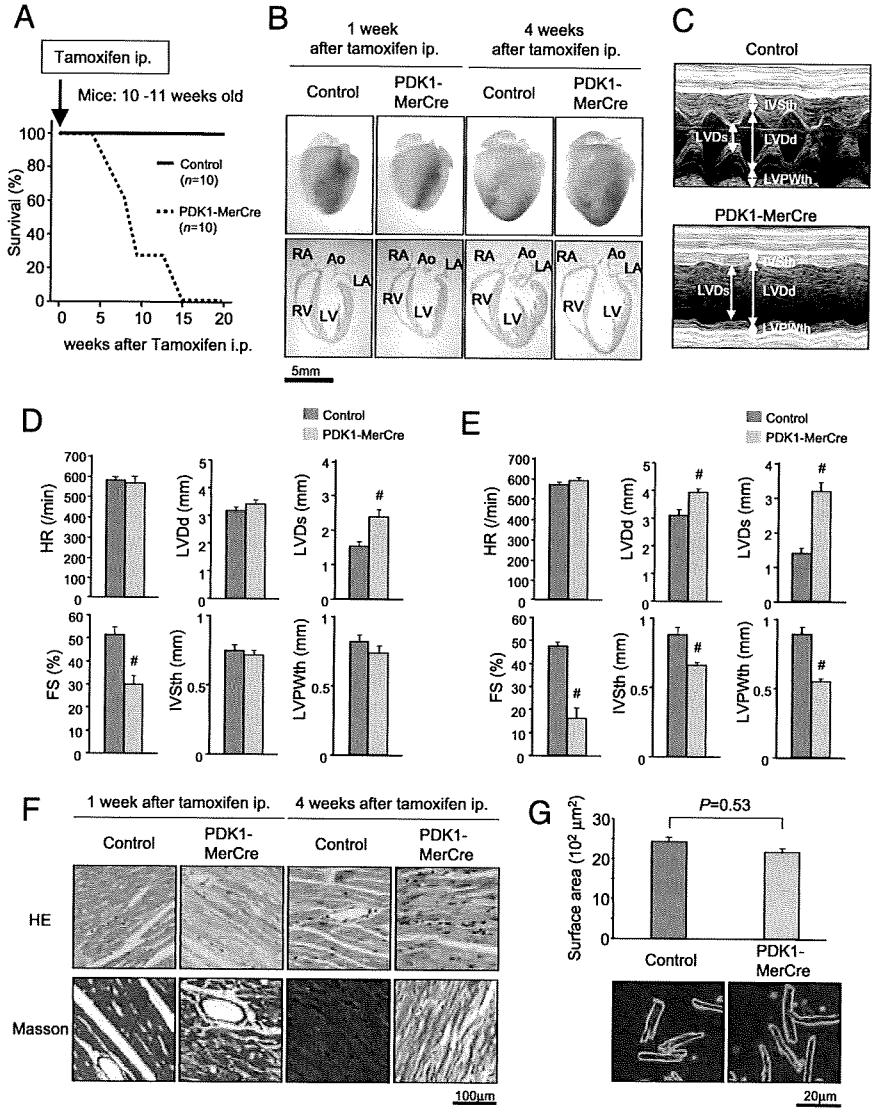

Fig. 1. Severe heart failure observed in PDK1-MerCre mice. (*A*) Kaplan-Meier survival curves of PDK1-MerCre mice (n = 10) and control mice (n = 10). Mice were injected with tamoxifen at the age of 10–11 weeks. (*B*) Macroscopic findings and 4-chamber sections of the hearts from PDK1-MerCre and control 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

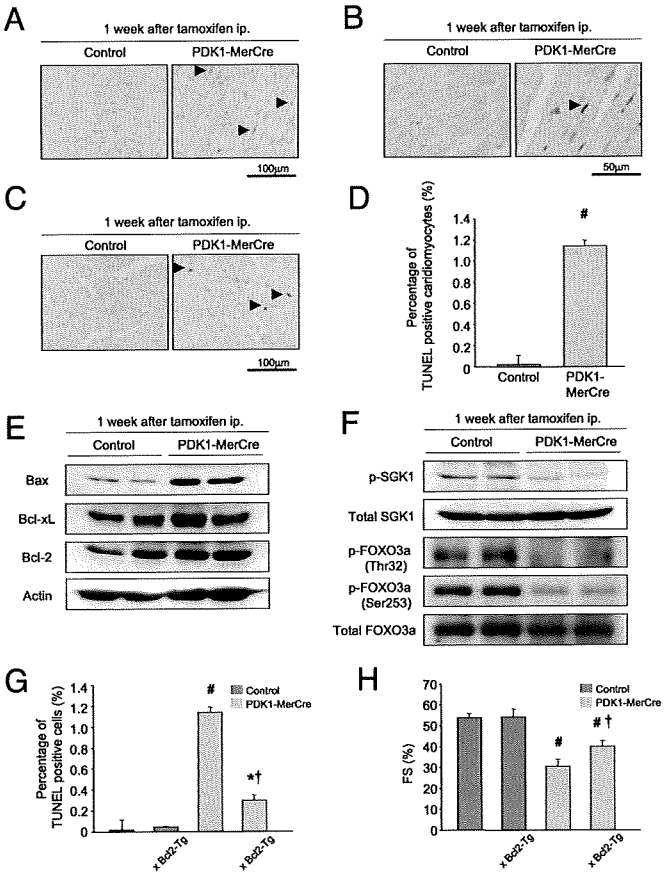

Fig. 2. Cardiomyocyte apoptosis in the pathogenesis of heart failure in PDK1-MerCre mice. (*A*) TUNEL staining. Arrowheads indicate TUNEL-positive cardiomyocytes. (*B*) Double staining for TUNEL staining (brown) and sarcomeric α-actinin (red). Arrowheads indicate TUNEL-positive cardiomyocytes. (*C*) 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

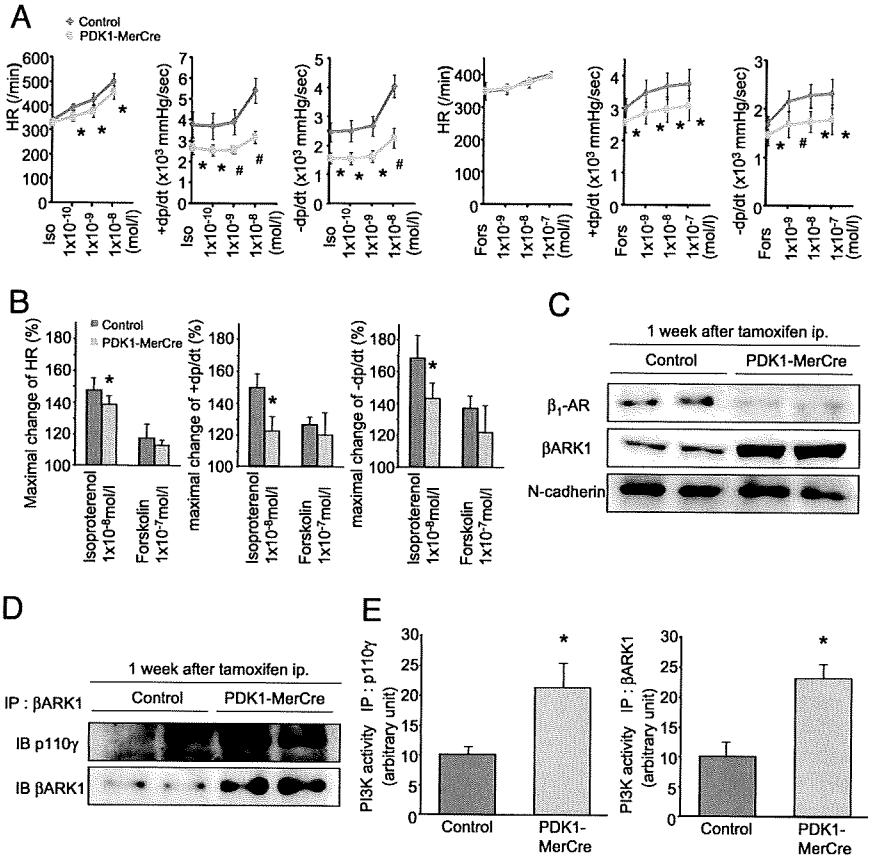

Fig. 3. Impaired β-adrenergic responsiveness in PDK1-MerCre mice. (*A*) Effects of isoproterenol and forskolin on heart rate, contraction, and relaxation in Langendorff-perfused mouse hearts 1 week after tamoxifen treatment. +dp/dt, maximum rate of LV pressure development; −dp/dt, maximum rate of LV 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.

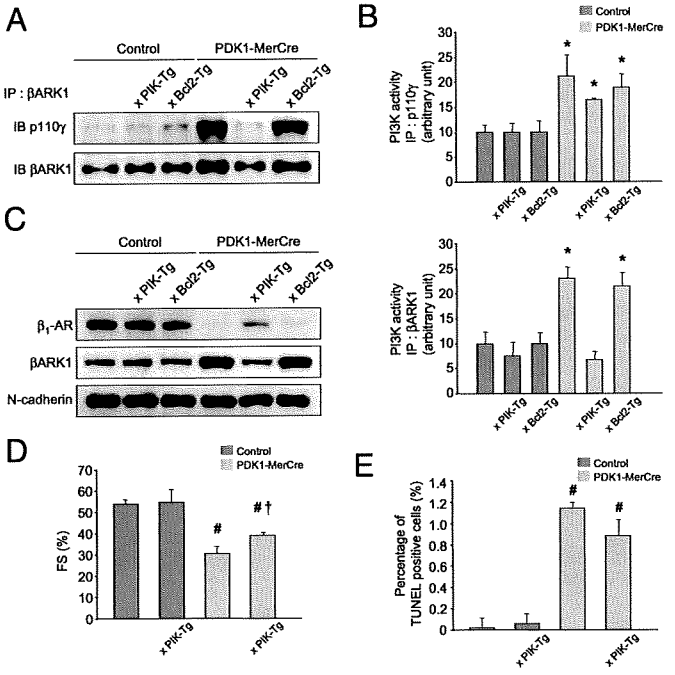

Fig. 4. Alleviated cardiac dysfunction in PDK1-MerCre mice by overexpression of PIK domain or Bcl-2 protein. (*A*) Immunoblot analysis of βARK1-associated p110γ protein in the hearts. (*B*) Kinase assays for PI3-K activity in the hearts. The hearts were subjected to immunoprecipitaion with antibody to 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 ± 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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Long-Term Outcome of Therapeutic Neovascularization Using Peripheral Blood Mononuclear Cells for Limb Ischemia

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Background—Injection of bone marrow mononuclear cells has been reported to promote neovascularization of ischemic tissues effectively. We found that peripheral blood mononuclear cells were as efficient as bone marrow mononuclear cells for the treatment of limb ischemia in animals and showed that this treatment was feasible and safe in no-option patients with limb ischemia. However, the long-term outcome of such therapy has not been investigated.

Methods and Results—We retrospectively analyzed the data for 42 patients who were treated between July 2002 and December 2005 by using the log-rank test, the Kaplan-Meier method, and the Cox proportional hazard model. Improvement of ischemic symptoms was observed in 60% to 70% of the patients. The annual rate of major amputation was decreased markedly by treatment. Improvement of ischemic symptoms was less marked in arteriosclerosis obliterans (ASO) patients on dialysis compared with nonhemodialysis ASO or thromboangiitis obliterans patients. Indeed, the survival rate of these patients was lower than that of nonhemodialysis ASO or thromboangiitis obliterans patients. Major adverse events such as death, major amputation, and cardiovascular events occurred mostly in ASO patients, and most of them were on dialysis. There was no significant difference in the cardiovascular event-free rate between responders and nonresponders. The survival rate of younger responders was better than that of nonresponders.

Conclusions—Although this study was not placebo-controlled and these initial results were from a retrospective analysis, injection of peripheral blood mononuclear cells might be safe and potentially effective for the treatment of limb ischemia, but caution is needed when managing ASO patients on dialysis. **(*Circ Cardiovasc Intervent.* 2009;2:245-254.)**

Key Words: angiogenesis ■ cell therapy ■ hemodialysis ■ peripheral vascular disease

Peripheral vascular disease (PVD) is mainly caused by atherosclerosis, and it results in obstruction of the blood supply to the lower or upper extremities. PVD is known to affect 10% to 15% of the adult population in developed countries and is often associated with coronary artery disease.[1] Arteriosclerosis obliterans (ASO) is the most common cause of PVD affecting the lower limbs. Peripheral ischemia can also arise from various types of vasculitis, including thromboangiitis obliterans (TAO) or Buerger's disease, which affects small- and medium-sized arteries and is related to tobacco use and male sex but not to other coronary risk factors. The 2 cardinal symptoms of limb ischemia are intermittent claudication and rest pain; the latter symptom occurs in patients with critical limb ischemia and often coincides with ischemic ulceration and/or gangrene. Treatment of PVD includes pharmacotherapy, percutaneous transluminal angioplasty, and vascular surgery, with the modality selected depending on the severity of symptoms and the arteries involved.[2] However, 30% to 50% of patients with critical limb ischemia require limb amputation within 1 year because of a poor response to treatment.[1,2]

Clinical Perspective on p 254

Recent progress in understanding the mechanisms underlying vessel formation in adults as well as during embryogenesis has opened up a potential therapeutic avenue for patients without any current options.[3] In particular, the role of bone marrow-derived circulating endothelial progenitors has been studied extensively because these cells were identified in the peripheral blood,[4,5] and it has been demonstrated that

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such cells contribute to both physiological and pathological angiogenesis in adults.[6,7] These findings have led to the development of therapeutic neovascularization techniques using endothelial progenitor cells. Preclinical studies have indicated that injection of bone marrow mononuclear cells (BM-MNC), which contain endothelial progenitors, into ischemic limbs is very effective.[8–10] Consequently, this therapeutic strategy has been tried for no-option patients with critical limb ischemia. The first clinical trial showed that injection of BM-MNC significantly improved tissue oxygenation and blood flow in ischemic limbs, resulting in a decrease of rest pain and the involution of ischemic ulcers.[11] More recent clinical studies have also demonstrated that intracoronary infusion of autologous BM-MNC in conjunction with standard treatment may improve left ventricular function after acute myocardial infarction.[12–16] However, these findings remain controversial because the studies were done on a relatively small scale and partially differed in design. A meta-analysis of 5 randomized controlled clinical trials[12–16] was published recently.[17] When the overall effect on the left ventricular ejection fraction at baseline and follow-up was assessed, there was significant improvement in the BM-MNC-treated group compared with the control group. Thus, transplantation of BM-MNC may be a safe and beneficial adjunctive treatment for acute myocardial infarction. Despite these promising results in the initial trials, the long-term outcome has not yet been reported.

We previously examined the possibility of therapeutic revascularization using peripheral blood mononuclear cells (PB-MNC) and found that injection of PB-MNC was as efficient as injection of BM-MNC for the treatment of limb ischemia in animal studies.[18] We then conducted a pilot trial of therapeutic revascularization using PB-MNC in no-option patients with critical limb ischemia and demonstrated that this treatment modality was both feasible and safe.[18,19] In this study, we assessed the long-term outcome in patients receiving treatment with PB-MNC.

Methods

Patients and Study Design

We treated patients aged 20 to 80 years who had severe intermittent claudication, ischemic rest pain, or nonhealing ischemic ulcers caused by ASO or TAO, and who had not responded to conventional therapy that included nonsurgical and surgical revascularization. Before treatment, we performed a physical examination, laboratory tests, x-rays, electrocardiography, echocardiography, carotid ultrasonography, cardiac scintigraphy, and coronary angiography in all candidates. They also underwent arteriography of the lower extremities, measurement of ankle-brachial blood pressure index (ABPI), a laser Doppler study, and thermography to confirm the existence of PVD that was unsuitable for nonsurgical or surgical revascularization. We excluded patients with proliferative diabetic retinopathy and those with malignancy during the previous 5 years. We also excluded patients with limb ischemia resulting from causes other than ASO or TAO, such as collagen vascular diseases. We obtained written informed consent from all patients, and the ethics committee of Chiba University Graduate School of Medicine reviewed and approved the study protocol.

We retrospectively enrolled 42 patients who were treated between July 2002 and December 2005 and analyzed the data for them in December 2007. The data analyzed include the incidence of major amputation of treated limbs, improvement of rest pain, walking distance, ischemic ulcers (at least 25% reduction in size), and ABPI (an increase of more than 0.1). Rest pain was assessed as follows: +4, severe pain that was not controlled by morphine, pentazocine, or nonsteroidal antiinflammatory drugs; +3, moderate pain that required nonsteroidal antiinflammatory drugs; +2, slight pain that did not need nonsteroidal antiinflammatory drugs; +1, very slight pain; and 0, complete resolution. A responder was defined as a patient who showed at least 1 improvement of the following; rest pain, walking distance, ischemic ulcers, or ABPI at 6 months after treatment. A nonresponder was defined as a patient who showed no improvement of the above at 6 months after treatment. We performed follow-up examinations at 1, 6, 12, 24, and 36 months after treatment. We also monitored relapse of ischemic symptoms, adverse events, and the progression of atherosclerosis by the examinations described earlier. Estimation of ischemic myocardium was performed by single-photon emission computed tomography (SPECT) imaging, as described previously.[20]

Table 1. Patient Background (Overall Population)

Age, y	61.2±13.5 (23–81; 62.0)
Gender	
Male	34 (81.0)
Female	8 (19.0)
Diagnosis	
ASO	28 (66.7)
TAO	14 (33.3)
Ischemic status	
Fontaine 2	6 (14.3)
Fontaine 3	6 (14.3)
Fontaine 4	30 (71.4)
ABPI	0.76±0.32 (0.0–1.39; 0.76)
Rest pain scale	2.4±1.4 (0.0–4.0; 3.0)
Complications	
Diabetes	21 (50.0)
Dyslipidemia	19 (45.2)
Hypertension	25 (59.5)
CRF on HD	19 (45.2)
CAD	19 (45.2)
CVD	14 (33.3)
$CD34^+$ cells, $\times 10^7$	3.78±5.30 (0.09–22.7; 1.46)
Serum VEGF, pg/mL	176±105 (0.0–481; 142)

Data are shown as mean±SD (minimum–maximum; median) or n (%). CRF indicates chronic renal failure; HD, hemodialysis; CAD, coronary artery disease; CVD, cerebrovascular disease; VEGF, vascular endothelial growth factor.

Injection of PB-MNC

PB-MNC were harvested by peripheral blood apheresis with a COBE Spectra Apheresis System (Gambro, Lakewood, Colo). Patient underwent apheresis for 240 minutes while awake and resting quietly. When the patient had severe anemia (hemoglobin <7.4 g/dL) or left ventricular dysfunction (ejection fraction <45%), the apheresis time was reduced to 180 minutes. The total leukocyte count and mononuclear cell count in the apheresis product was 1.5 to 2.0×10^{10} and 1 to 1.5×10^{10}, respectively. The product was concentrated to 20 mL by centrifugation at 2000*g* for 10 minutes and then was injected on the same day. PB-MNC were injected intramuscularly into the ischemic lower extremities at sites 1 to 3 cm apart, with each injection having a volume of 0.1 to 0.3 mL.

Statistical Analysis

All data were analyzed according to the intention-to-treat principle. For baseline variables, the groups were compared by using Fisher's

Table 2. Patient Background (ASO Versus TAO)

	ASO (n=28)	TAO (n=14)	OR (95% CI)*	P
Age, y	65.7±9.2	52.3±16.4	2.32† (1.04, 6.10)	0.011
Gender				
Male	23 (82.1)	11 (78.6)		1.00
Female	5 (17.9)	3 (21.4)	0.90 (0.36, 2.47)	
Ischemic status				
Fontaine 2	3 (10.7)	3 (21.4)		0.13
Fontaine 3	6 (21.4)	0	1.85 (0.64, ∞)	
Fontaine 4	19 (67.9)	11 (78.6)	0.91 (−∞, 2.23)	
ABPI	0.73±0.35	0.84±0.24	0.31 (0.03, 2.63)	0.74
Rest pain scale				
Baseline	2.2±0.26	2.8±0.39		0.73
6 months	1.1±0.28	1.1±0.35		
24 months	1.0±0.27	0.57±0.25		
Walking distance				
Baseline	118±71	23±12		
24 months	233±129	529±209		0.36
Complications				
Diabetes	19 (67.9)	2 (14.3)	3.44 (1.42, 11.4)	0.0025
Dyslipidemia	14 (50.0)	5 (35.7)	1.33 (0.64, 2.93)	0.514
Hypertension	23 (82.1)	2 (14.3)	4.96 (1.97, 17.1)	3.6×10^{-5}
CRF on HD	18 (64.3)	1 (7.1)	4.67 (1.61, 32.3)	9.0×10^{-4}
CAD	18 (64.3)	1 (7.1)	4.67 (1.61, 32.3)	9.0×10^{-4}
CVD	11 (39.3)	3 (21.4)	1.53 (0.68, 3.99)	0.43
$CD34^+$ cells, $\times10^7$	3.89±5.96	3.60±4.10	1.06 (0.66, 1.72)	0.81
Serum VEGF, pg/mL	179±122	169±67.9	0.84 (0.16, 4.57)	0.85

Data are shown as mean±SD or n (%). OR indicates odds ratio; CRF, chronic renal failure; HD, hemodialysis; CAD, coronary artery disease; CVD, cerebrovascular disease; VEGF, vascular endothelial growth factor.

*OR and 95% CI for OR.

†OR for the elderly aged 65 years or older versus younger patients.

Table 3. Patient Background (HD Versus Non-HD)

	HD (n=19)	Non-HD (n=23)	OR (95% CI)*	P
Age, y	62.8±9.3	59.9±16.3	0.99 (0.50, 1.98)	0.30
Gender				
Male	16 (84.2)	18 (78.3)		0.71
Female	3 (15.8)	5 (21.7)	0.83 (0.30, 2.04)	
Diagnosis				
ASO	18 (94.7)	10 (43.5)		6.8×10^{-4}
TAO	1 (5.3)	13 (56.5)	0.21 (0.03, 0.62)	
Ischemic status				
Fontaine 2	1 (5.3)	5 (21.7)		0.38
Fontaine 3	3 (15.8)	3 (13.0)	1.57 (0.26, 9.41)	
Fontaine 4	15 (78.9)	15 (65.3)	1.60 (0.50, 7.40)	
ABPI	0.73±0.39	0.79±0.25	5.16 (0.48, 56.0)	0.15
Rest pain scale	2.5±1.4	2.3±1.5	0.92 (0.24, 3.55)	0.91
Complications				
Diabetes	12 (63.2)	9 (39.1)	1.61 (0.81, 3.36)	0.21
Dyslipidemia	8 (42.1)	11 (47.8)	0.89 (0.44, 1.78)	0.76
Hypertension	14 (73.7)	11 (47.8)	1.72 (0.84, 3.78)	0.17
CAD	13 (68.4)	6 (26.1)	2.42 (1.16, 5.41)	0.012
CVD	4 (21.1)	10 (43.4)	0.60 (0.26, 1.27)	0.19
$CD34^+$ cells, $\times10^7$	4.49±6.92	3.20±3.55	1.04 (0.66, 1.66)	0.87
Serum VEGF, pg/mL	189±123	166±94.7	1.51 (0.30, 7.74)	0.64

Data are shown as mean±SD or n (%). OR indicates odds ratio; CRF, chronic renal failure; HD, hemodialysis; CAD, coronary artery disease; CVD, cerebrovascular disease; VEGF, vascular endothelial growth factor.

*OR and 95% CI for OR.

exact test or logistic regression for categorical outcomes and with *t* tests for continuous variables, as appropriate. For time-to-event outcomes, the lengths of time to a first event were compared by using the log-rank test, the Kaplan-Meier method was used to estimate the absolute risk of each event for each group, and hazard ratio and 95% confidence interval were estimated by the Cox proportional hazards model.

All comparisons were planned and tests were 2-sided, and a *P* value <0.05 was considered to indicate statistical significance. All statistical analyses were performed with the use of SAS software, version 9.1.3 (SAS Institute, Cary, NC).

Results

Patient Profile

After obtaining approval from the ethics committee of Chiba University Graduate School of Medicine, we conducted a pilot clinical trial in patients with critical limb ischemia caused by ASO or TAO for whom there were no conventional treatment options since July 2002. We found that therapeutic neovascularization by injection of PB-MNC was feasible and potentially effective for limb ischemia. Consequently, this therapy was approved as advanced medical care by the Japanese government in 2005, and we have treated about 70 patients with limb ischemia so far. Among them, we retrospectively analyzed the long-term outcome in 42 patients who were treated between July 2002 and December 2005. Most of these patients had been recommended to undergo major amputation before receiving PB-MNC because of chronic ischemic ulcers or gangrene resistant to conventional therapy, whereas some of them had severe intermittent claudication (Table 1). About half of the patients also suffered from chronic renal failure and were on hemodialysis 3 times weekly, whereas most of them had 1 or more complications (Table 1).

Although the ASO patients were significantly older and had more risk factors for coronary artery disease than the TAO patients, the pretreatment ischemic status and rest pain did not differ between the 2 groups (Table 2). More than 60% (18 of 28) of the ASO patients were on dialysis, whereas there were only 7.1% (1 of 14) on hemodialysis in the TAO group (Table 2), and the hemodialysis group included more patients with ASO than the nonhemodialysis group (Table 3). Before treatment, the severity of ischemia did not differ between the hemodialysis and nonhemodialysis groups (Table 3). When the ASO patients were divided into groups with or without hemodialysis, there were no significant differences of the pretreatment ischemic status and rest pain or the number of risk factors (supplemental Table I).

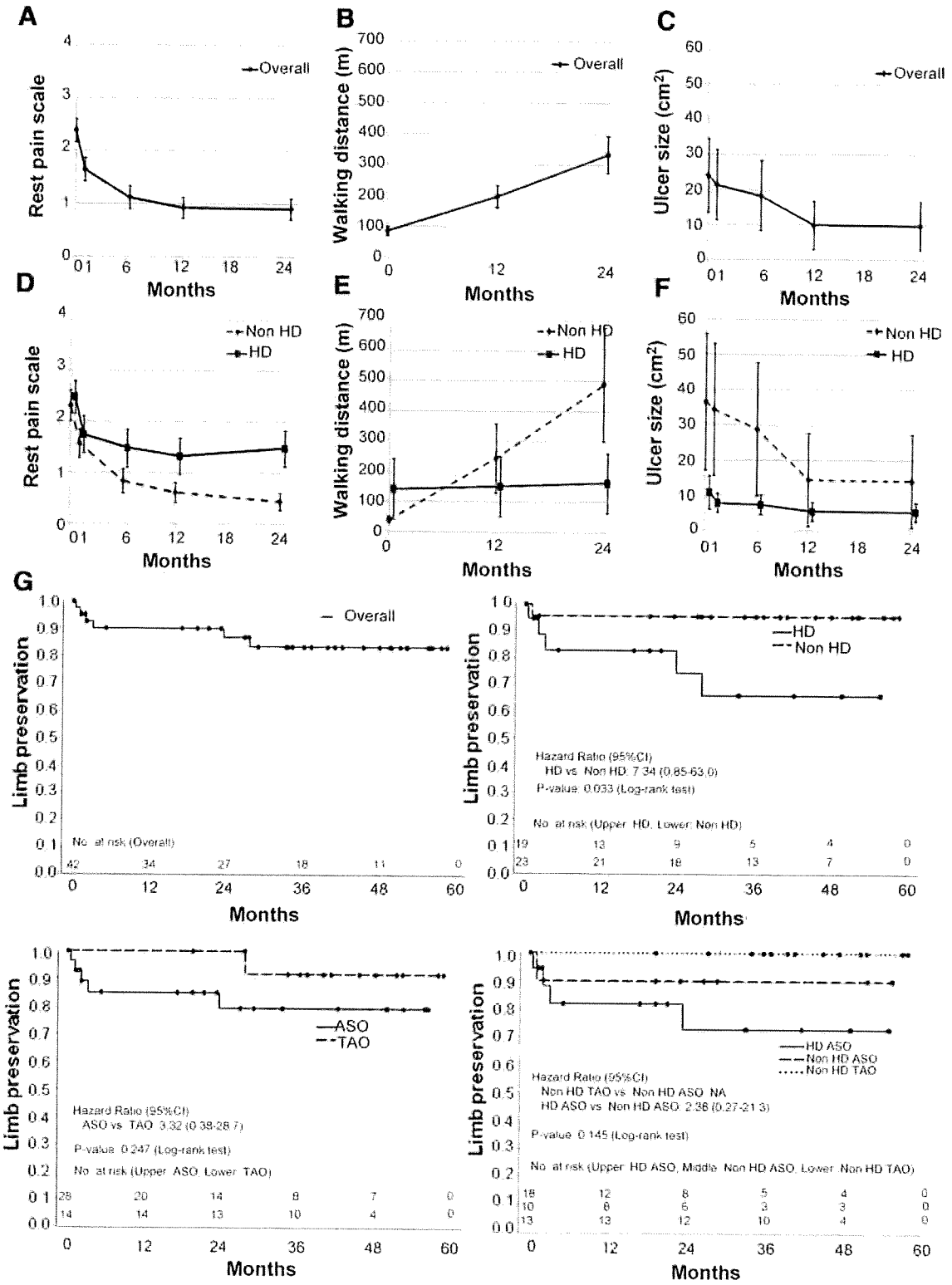

Figure 1. Effect of PB-MNC injection. A through F, The effect of therapeutic revascularization with PB-MNC was estimated by improvement of the rest pain scale (A, D), walking distance (B, E), and ischemic ulceration (C, F) during a 2-year follow-up period (1, 6, 12, and 24 months) in overall (n=42), HD (n=19), and non-HD (n=23) patients. It is noted that the data were omitted when patients died or underwent major amputation. Data are shown as mean±SE. G, Limb preservation rate. HD indicates hemodialysis.

Because the ASO and TAO populations might not have been comparable, we compared background factors among 3 groups, which were ASO patients on hemodialysis, nonhemodialysis ASO patients, and nonhemodialysis TAO patients. There were no differences in the pretreatment ischemic status and rest pain among the 3 groups, but the TAO patients had fewer coronary risk factors than the other groups (supplemental Table II).

Table 4. Cause of Death

Case	Onset, mo	Cause of Death	CRF	Diagnosis
1	2	Cerebral infarction	HD	ASO
2	2	CAD	CRF	ASO
3	2	CRF	HD	ASO
4	3	Sepsis	HD	ASO
5	5	Liver failure	HD	ASO
6	18	Sepsis	HD	ASO
7	20	Gastric cancer	None	ASO
8	20	CAD	HD	ASO
9	21	CAD	HD	ASO
10	24	Cerebral hemorrhage	HD	ASO
11	25	CRF	HD	ASO
12	29	Pneumonia	None	ASO
13	29	Gastric cancer	None	ASO
14	33	CRF	HD	ASO
15	33	CRF	HD	ASO

CRF indicates chronic renal failure; HD, hemodialysis; CAD, coronary artery disease.

Effect of Therapeutic Neovascularization With PB-MNC

Improvement of rest pain was observed in 72.2% (26 of 36) of the patients. The pain score decreased significantly and was nearly normalized by 12 months after treatment ($P<0.05$ versus before treatment in 1, 6, 12, 24 months, Figure 1A). The maximum walking distance in 24 months after treatment also improved, although the increase was not significant ($P=0.07$ versus before treatment in overall patients, Figure 1B). Improvement of ischemic ulcers was observed in 66.7% (20 of 30) of the patients, and ulcer size showed a decrease after treatment in surviving patients without major amputation (Figure 1C and 1F).

Among patients on hemodialysis, the improvement of rest pain and walking distance was less marked (Figure 1D and 1E). Likewise, the improvement of ischemic status was smaller in ASO patients compared with TAO patients (Table 2). Consequently, ASO patients on hemodialysis showed less improvement of ischemia than either nonhemodialysis ASO patients or TAO patients (supplemental Table II). Four patients (9.5%) underwent major amputation within 1 year after treatment (Figure 1G), which was a much lower frequency than the reported annual amputation rate for patients with critical limb ischemia (30% to 50%). One TAO patient underwent major amputation during the observation period, whereas 5 major amputations were performed in ASO patients (4 of 5 were in ASO patients on dialysis; Figure 1G).

Adverse Events After Therapeutic Revascularization With PB-MNC

Fifteen patients (35.7%) died of the causes unrelated to cell therapy, including cerebral infarction, sepsis, and chronic renal failure (Table 4). In these patients, limb ischemia was caused by ASO, and most of them were on hemodialysis (Table 4). The proportion of overall survival was 78.2% at 2 years and 63.9% at 3 years in our study population (Figure 2A). Additionally, the proportions of overall survival for patients on hemodialysis were significantly lower (57.9% at 2 years and 39.5% at 3 years) than those for nonhemodialysis patients, who had a survival rate of 84.4% at 3 years (Figure 2A). None of the TAO patients died during follow-up, whereas the overall survival proportion of ASO patients was 67.3% at 2 years and only 45.7% at 3 years (Figure 2A). The survival rate of ASO patients on hemodialysis was lower than that of nonhemodialysis ASO patients or TAO patients (Figure 2A).

Major adverse cardiovascular events (MACE) occurred in 13 out of 42 patients (31.0%; supplemental Table III), and the proportion of MACE-free rate was 74.6% at 2 years and 71.5% at 3 years in our study population. The proportions of MACE-free rate were significantly lower in patients on hemodialysis (58.7% at 2 years and 51.3% at 3 years) than in nondialysis patients (86.5% at 3 years; Figure 2B). MACE mostly occurred in ASO patients (Figure 2B), and most of them were on dialysis (Figure 2B and supplemental Table III).

Factors Associated With the Response to Treatment

We next investigated the factors that influenced the response to treatment. We divided the patients into responders (n=32) and nonresponders (n=10) according to a response score based on the improvement of rest pain, ischemic ulceration, walking distance, and ABPI. When we compared their background factors, there were no significant differences of the age, gender, Fontaine stage, coronary risk factors, and the number of CD34-positive cells between responders and nonresponders (Table 5). Consistent with our previous findings, the peak plasma level of vascular endothelial growth factor after treatment was significantly higher in responders than in nonresponders (Table 5). Although the association was not significant, nonresponders included more ASO patients and had more complications such as dyslipidemia, hemodialysis, and coronary artery disease (Table 5). We also found that nonresponders tended to have more risk factors and lower vascular endothelial growth factor levels after treatment in the subgroups of ASO, TAO, dialysis, and nondialysis patients (supplemental Tables IV through VII).

Relapse of rest pain occurred in 11.5% (3 of 26) of the patients whose rest pain was improved by treatment, and recurrence of ischemic ulcers was observed in 35.0% (7 of 20) of the patients whose ulcers had improved. Likewise, a decrease of ABPI and walking distance was found in 25.0% (4 of 16) and 12.5% (2 of 16) of the patients, respectively. When all of these parameters were combined, about 40% of the responders suffered from relapse of ischemic symptoms at 3 years after treatment (Figure 3A). There was no significant difference of the relapse rate between hemodialysis and nonhemodialysis patients or between ASO and TAO patients (Figure 3A). Likewise, the relapse rate did not differ among ASO patients on hemodialysis, nonhemodialysis ASO patients, and TAO patients (Figure 3A).

There were no significant differences of the survival rate and the MACE-free rate between responders and nonresponders (Figure 2B and data not shown). When patients

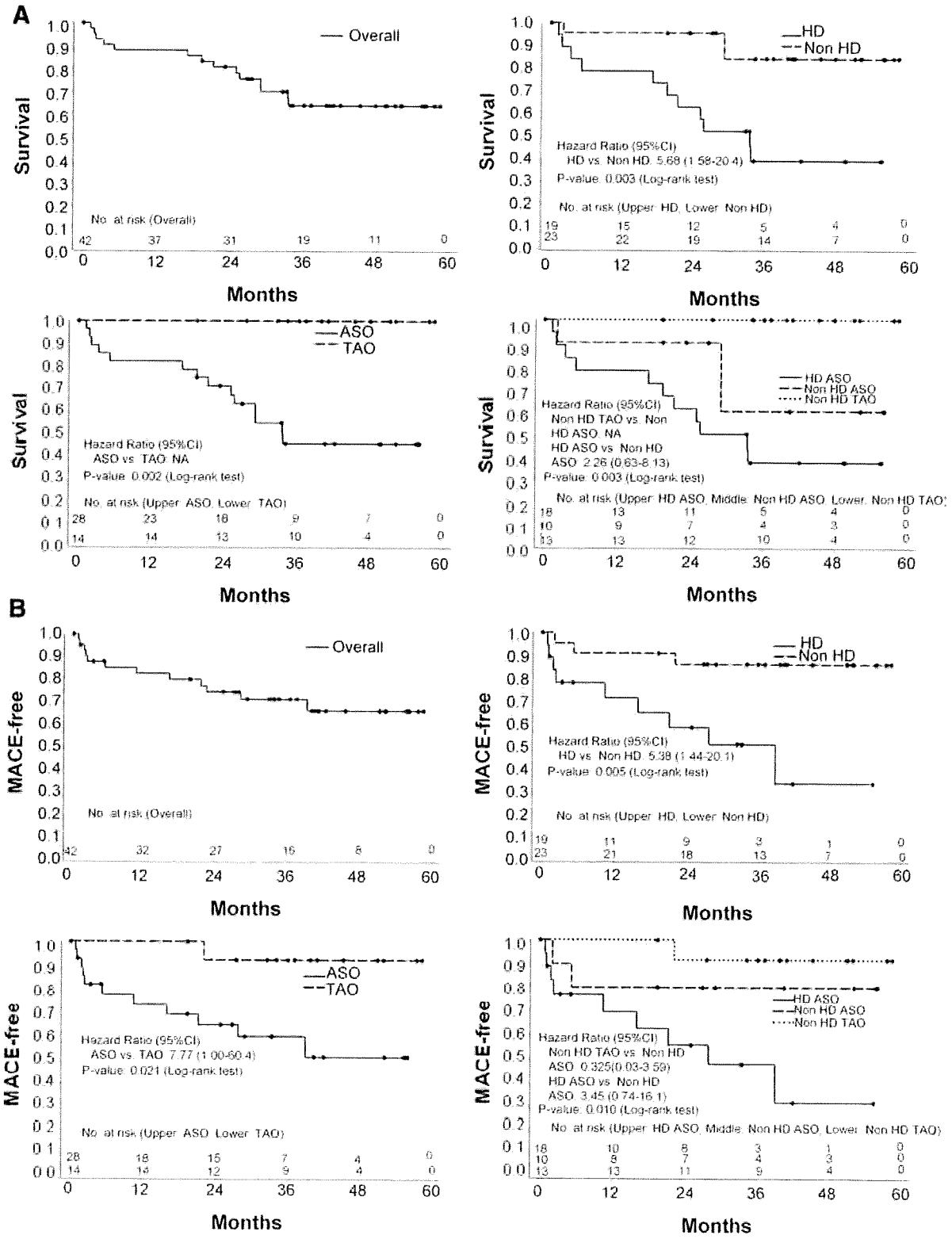

Figure 2. Survival rate and cardiovascular events after treatment. A, Survival rate after treatment. B, MACE-free rate.

younger than 70 years old in the 2 groups were compared, however, the survival rate tended to be higher in responders than in nonresponders until 3 years after treatment (Figure 3B). We performed myocardial perfusion SPECT imaging to estimate myocardial ischemia before and after treatment. The extent of myocardial ischemia showed a significant decrease after treatment that persisted for 6 months (Figure 3C and data not shown). This improvement was significantly associated with the peak plasma level of vascular endothelial growth factor (r=0.76, P<0.05 at 8 weeks) after treatment, suggesting that injection of PB-MNC into ischemic limbs increased the production of vascular endothelial growth