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), β₁-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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Journal of Molecular and Cellular Cardiology

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 home-ostasis, 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. Polyubiquitinated 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
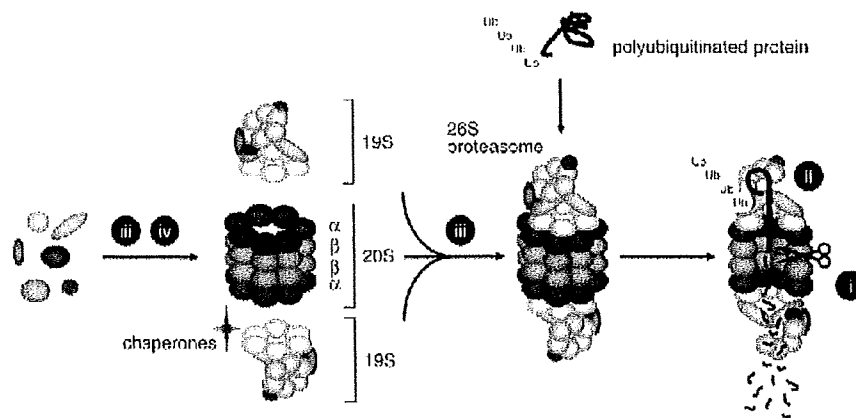
Fig. 1. Potential change in 26S proteasome bought about by PKA. PKA enhances the activity of 26S proteasome potentially i) by increasing proteolytic activity through phosphorylation of 20S subunit, ii) by facilitating translocation of polyubiquitinated substrates through phosphorylation of 19S subunit, 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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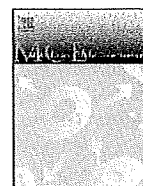
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Review

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

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ARTICLE INFO	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. © 2008 Elsevier Ireland Ltd. All rights reserved.

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
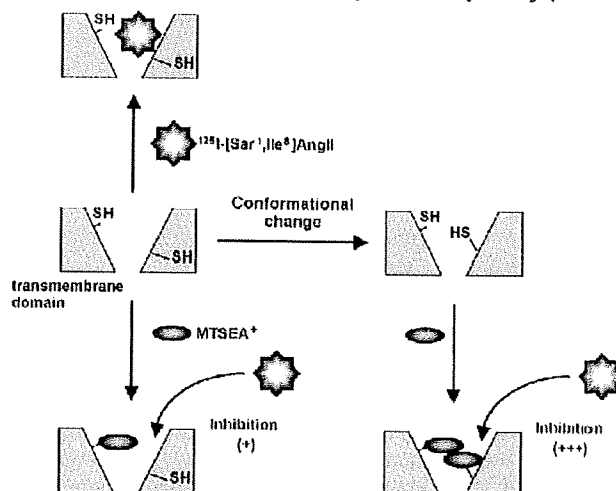

Fig. 1. Substituted cysteine accessibility mapping (SACM). Sulfhydryl-specific reaction with methanethiosulfonyl ethyl-ammonium (MTSEA$^+$), which reacts a billion times faster with water-exposed and ionized Cys than lipid-exposed and un-ionized Cys. Upon this reaction, a positively charged sulfonylmethylammonium 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 upregulated 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
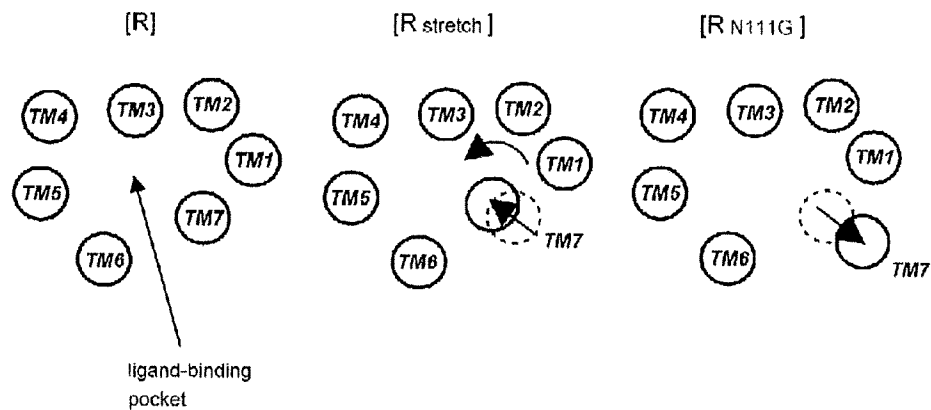
responses induced by stretching (Sadoshima et al., 1993; Yamazaki et al., 1995). These results indicate that mechanical stress induces cardiac hypertrophy through the activation of AT_1 receptor. However, it has been a challenging problem to solve how AT_1 receptor is activated by mechanical stress. 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-

[R]   [R stretch]   [R N111G]

TM4   TM3   TM2   TM4   TM3   TM2   TM4   TM3   TM2

TM1   TM1   TM1

TM5   TM7   TM5   TM7   TM5   TM7

TM6   TM6   TM6

ligand-binding
pocket

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

Functional response

Agonists

Basal activity

Neutral antagonists

Inverse agonists

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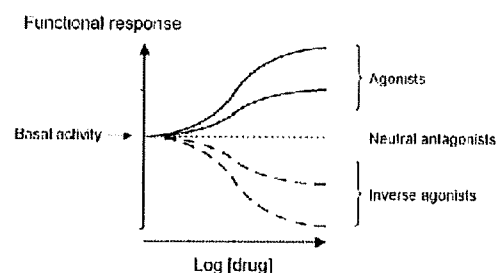
Fig. 3. Classification of GPCR ligands as agonists, neutral antagonists, or inverse agonists. An agonist is a ligand that has a positive efficacy and triggers a functional response. A neutral antagonist is a ligand that has no intrinsic efficacy for a given response, but blocks agonist-induced response.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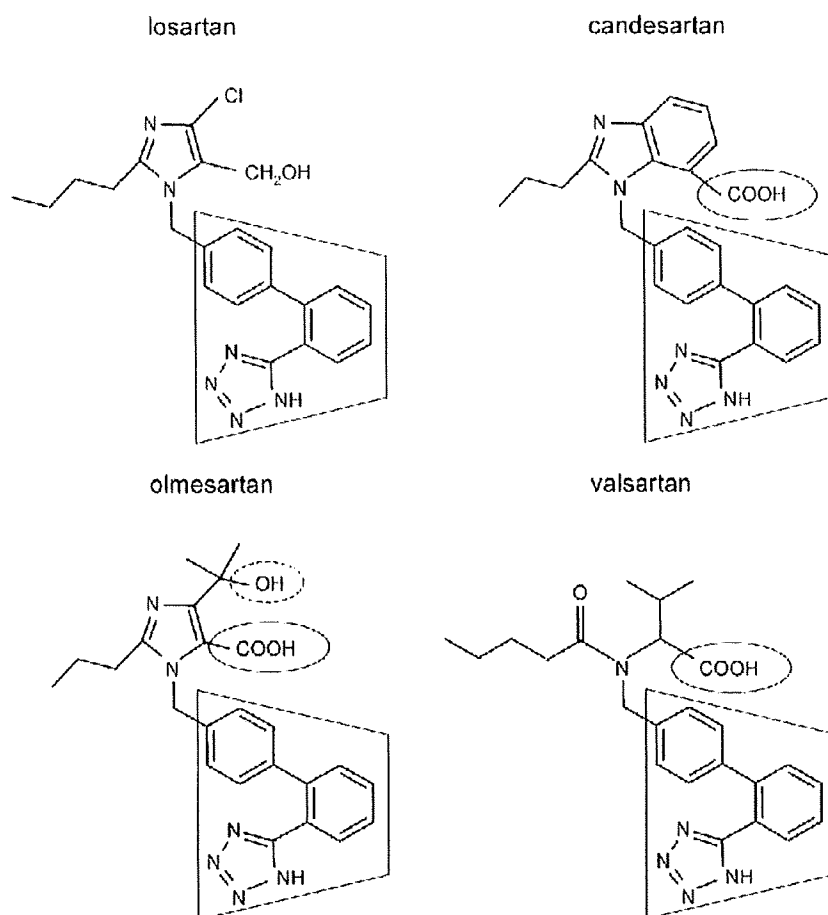
losartan   candesartan

olmesartan   valsartan

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 con-

traction 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
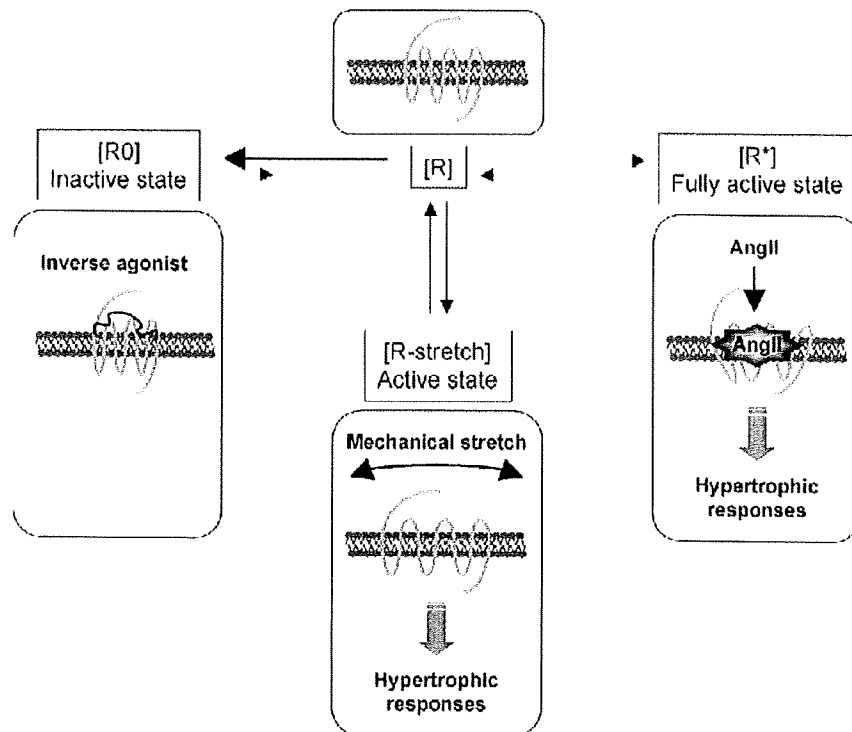
Fig. 5. Distinct conformations of the AT₁ receptor. [R] is an unaligned inactive state, and [R₀] is an inactive state stabilized by an inverse agonist. [R*] is an active state stabilized by the agonist AngII. Mechanical stretch stabilizes AT₁ receptor to another active state [Rstretch], independently of AngII. An inverse agonist forcibly induces a distinct transition from [R] to [R₀], and prevent a shift of equilibrium to [R*] or [Rstretch].

AT₁ 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 [R₀] and [R*]. [R₀] 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 α2A-adrenergic receptor induced distinct conformational changes of the receptor (Vilardaga et al., 2005). With regard to AT₁ receptor, mechanical stretch stabilizes the receptor to another active state [Rstretch] (Fig. 5). Molecular modeling using the crystal structure of bovine rhodopsin (Palczewski et al., 2000) as a template indicates that, in the inactive state [R₀] 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 Gln²⁵⁷ in TM6 and Thr²⁸⁷ 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 His²⁵⁶, an increase in that of Ile²⁹⁰ and a decrease in that of Ala²⁹¹ 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 [R₀], and prevents a shift of equilibrium to an active conformation [Rstretch] or [R*] (Fig. 5).

7. Conclusions

The structure–function analyses of the AT₁ receptor have advanced our understanding of the molecular mechanism under-

lying receptor activation and inverse agonism. Although the structural flexibility of AT₁ receptor, like other GPCRs, may underlie the AngII-independent activation, mechanical stress-induced activation seems to be a phenomenon peculiar to AT₁ receptor. Future investigations with biophysical, biochemical, and pharmacological approaches will elucidate the precise mechanism of force sensing by AT₁ 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 AT₁ 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 β1- and β2-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.

Acknowledgements

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A crucial role for adipose tissue p53 in the regulation of insulin resistance

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Various stimuli, such as telomere dysfunction and oxidative stress, can induce irreversible cell growth arrest, which is termed 'cellular senescence'[1,2]. This response is controlled by tumor suppressor proteins such as p53 and pRb. There is also evidence that senescent cells promote changes related to aging or age-related diseases[3–6]. Here we show that p53 expression in adipose tissue is crucially involved in the development of insulin resistance, which underlies age-related cardiovascular and metabolic disorders. We found that excessive calorie intake led to the accumulation of oxidative stress in the adipose tissue of mice with type 2 diabetes–like disease and promoted senescence-like changes, such as increased activity of senescence-associated β-galactosidase, increased expression of p53 and increased production of proinflammatory cytokines. Inhibition of p53 activity in adipose tissue markedly ameliorated these senescence-like changes, decreased the expression of proinflammatory cytokines and improved insulin resistance in mice with type 2 diabetes–like disease. Conversely, upregulation of p53 in adipose tissue caused an inflammatory response that led to insulin resistance. Adipose tissue from individuals with diabetes also showed senescence-like features. Our results show a previously unappreciated role of adipose tissue p53 expression in the regulation of insulin resistance and suggest that cellular aging signals in adipose tissue could be a new target for the treatment of diabetes.

Cellular senescence was originally defined as the finite replication of human somatic cells in culture. As a consequence of semiconservative DNA replication, the ends of the chromosomes (telomeres) are not duplicated completely, resulting in successive shortening of the telomeres with each cell division[7]. Telomerase is a ribonucleoprotein that adds telomeres to the ends of chromosomes. Telomeres that have shortened beyond a critical threshold, resulting in cell death or senescence, are thought to cause DNA damage that induces cellular senescence. It is now apparent that senescence can be induced by various stresses independently of cell replication, such as chromatin damage related to oxidative stress, and cellular senescence

is believed to be a potent anticancer mechanism. Accumulating evidence also suggests a potential relationship between cellular senescence and aging of organisms[8,9].

Aging is known to increase the prevalence of metabolic disorders such as diabetes. Therefore, we hypothesized that cellular aging might influence insulin resistance and accelerate the development of diabetes. To test our hypothesis, we used Ay mice with ectopic expression of agouti peptide, which leads to perturbation of the central melanocortin pathway and thereby induces excessive calorie intake, resulting in obesity and diabetes. It has been reported that production of reactive oxygen species (ROS) is selectively increased in the adipose tissue of obese mice and that increased oxidative stress in fat is a key mechanism underlying the occurrence of insulin resistance related to obesity[10]. Consistent with previous studies, Ay mice on a normal diet for 20 weeks showed higher adipose tissue amounts of ROS compared with wild-type mice on the same diet (Supplementary Fig. 1a). Because increased stress due to ROS can induce DNA damage and subsequent activation of p53, leading to telomere-independent senescence[3,4], we tested whether adipose tissue of Ay mice shows a senescence-like phenotype. The adipose tissue of these mice showed senescence-like changes, including enhanced activity of senescence-associated β-galactosidase (SA-β-gal; Fig. 1a). Ay mice also showed higher adipose tissue amounts of p53 on the protein level and cyclin-dependent kinase inhibitor-1A (*Cdkn1a*) expression on the mRNA level compared to wild-type mice (Fig. 1b,c), suggesting excessive caloric intake can induce senescence-like changes in adipose tissue.

It has been reported that increased secretion of proinflammatory cytokines by adipose tissue exacerbates insulin resistance in people with metabolic disorders[11–13]. Senescent cells are known to secrete molecules that can alter the local microenvironment, such as proinflammatory cytokines[3,5]. We therefore speculated that senescence-like changes might be associated with increased expression of proinflammatory cytokines by adipose tissue that could induce insulin resistance. Consistent with this concept, expression of proinflammatory cytokines such as tumor necrosis factor-α (*Tnf*) and chemokine (C-C motif) ligand-2 (*Ccl2*), also known as monocyte chemoattractant protein-1, was upregulated in association with an increase

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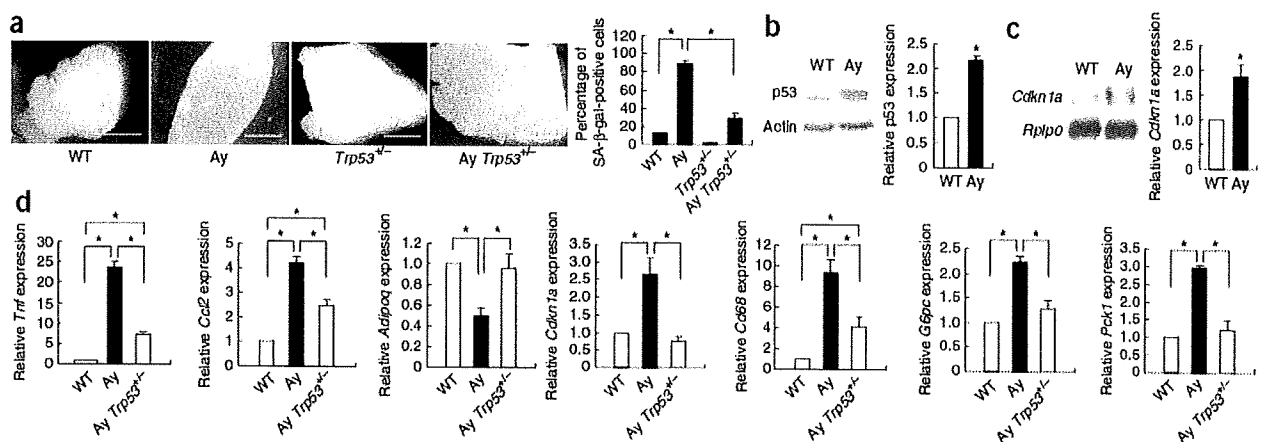
Figure 1 Role of p53 in diabetic mice. (a) Left, photographs showing adipose tissue after SA-β-gal staining. Right, the number of cells positive for SA-β-gal activity in adipose tissue of wild-type mice (WT), Ay mice, Trp53+/− mice and Ay Trp53+/− mice. Scale bar, 5 mm. (b) Expression of p53 in the adipose tissue of WT mice and Ay mice, as determined by western blot analysis. Actin was used as an equal loading control. The graph indicates relative expression of p53 protein. (c) Expression of Cdkn1a in the adipose tissue WT mice and Ay mice, as determined by northern blot analysis. Ribosomal protein, large, P0 (Rplp0) was used as an equal loading control. The graph indicates relative expression of Cdkn1a mRNA. (d) Real-time PCR assessing the expression of cytokines, Cdkn1a and Cd68 in adipose tissue and the expression of G6pc (encoding glucose-6-phosphatase) and Pck1 (encoding phosphoenolpyruvate carboxykinase) in the livers of WT mice, Ay mice and Ay Trp53+/− mice. (e) Plasma insulin concentrations in WT mice, Ay mice, Trp53+/− mice and Ay Trp53+/− mice. *P < 0.05; n = 4–6 for a and d; n = 3 for b; n = 4 for c and e. (f) Insulin tolerance test (ITT) and glucose tolerance test (GTT) in WT mice, Ay mice, Trp53+/− mice and Ay Trp53+/− mice. *P < 0.05 versus WT; #P < 0.05 versus Ay; n = 7. Data are shown as the means ± s.e.m.

of macrophage marker expression, whereas expression of anti-inflammatory cytokines (including adiponectin, (Adipoq)) was downregulated in the adipose tissue of Ay mice (Fig. 1d). We detected upregulation of inflammatory cytokines, as well as of p53 protein and Cdkn1a expression, in both the stromal vascular fraction (macrophage-rich fraction) and the adipose-rich fraction (Supplementary Fig. 1b), suggesting that senescence of both macrophages and adipocytes causes an inflammatory response that leads to insulin resistance.

We next investigated whether inhibition of p53 could reverse insulin resistance and glucose intolerance in Ay mice. The number of SA-β-gal–positive cells and the expression of Cdkn1a were significantly lower in adipose tissue from Ay Trp53+/− mice than in tissue from Ay Trp53+/+ mice (Fig. 1a,d), whereas there was no significant difference in food intake between the two groups (Supplementary Fig. 1c). The fat weight of Ay Trp53+/− mice was slightly lower than that of Ay Trp53+/+ mice (Supplementary Fig. 2a). Reduced activation of p53 led to lower plasma insulin concentrations in Ay mice and also to normalization of cytokine and macrophage marker expression by adipose tissue (Fig. 1d,e). Hepatic expression of gluconeogenic enzymes was also lower in Ay Trp53+/− mice (Fig. 1d). Consistent with these changes, Ay Trp53+/− mice showed significantly better insulin sensitivity and glucose tolerance compared with Ay Trp53+/+ mice as determined by insulin and glucose tolerance tests (Fig. 1f).

Because Ay Trp53+/− mice have p53 haploinsufficiency throughout the whole body, improvement of insulin resistance might be attributable to inhibition of p53 activity in other tissues aside from the white adipose tissue. To investigate the role of adipose tissue p53 in the regulation of insulin resistance, we generated mice with adipocyte-specific p53 deficiency (adipo-p53-deficient mice), using transgenic mice

that express Cre recombinase under control of the mouse fatty acid–binding protein-4 (Fabp4) promoter, and fed these mice a high-fat, high-sucrose (HF-HS) diet for 4 months. Expression of p53 protein and Cdkn1a mRNA in adipose tissue was significantly upregulated in littermate control mice on the HF-HS diet, whereas this increase was significantly attenuated in adipo-p53-deficient mice (Trp53loxP/loxP Fabp4-Cre) receiving the same diet (Fig. 2a,b and Supplementary Fig. 1d). These mice had a slightly smaller increase of fat weight (Supplementary Fig. 2b) and normalized expression of adipokines and hepatic gluconeogenic enzymes (Fig. 2b), whereas hepatic p53 protein expression was unchanged (Fig. 2a). Insulin-induced phosphorylation of Akt was also restored in adipo-p53-deficient mice (Supplementary Fig. 1e). Consequently, insulin resistance induced by the HF-HS diet was lower in mice with adipocyte-specific ablation of p53 compared to control mice (Fig. 2c), indicating that p53 expression in adipose tissue has a crucial role in the development of insulin resistance.

It has been reported that Fabp4 is expressed in hematopoietic cells and has considerable influence on various biological responses[14,15]. To examine the possible involvement of p53 in hematopoietic cells, we transplanted wild-type bone marrow cells into adipo-p53-deficient mice or littermate control mice and induced dietary obesity in these mice. Adipo-p53-deficient mice transplanted with wild-type bone marrow cells showed better glucose tolerance than littermate control mice transplanted with wild-type marrow cells, but their glucose tolerance was impaired compared with that of adipo-p53-deficient mice without bone marrow transplantation (Supplementary Fig. 1f). In adipose-p53-deficient mice, expression of p53 protein and Cdkn1a was considerably lower in both the stromal vascular fraction and the
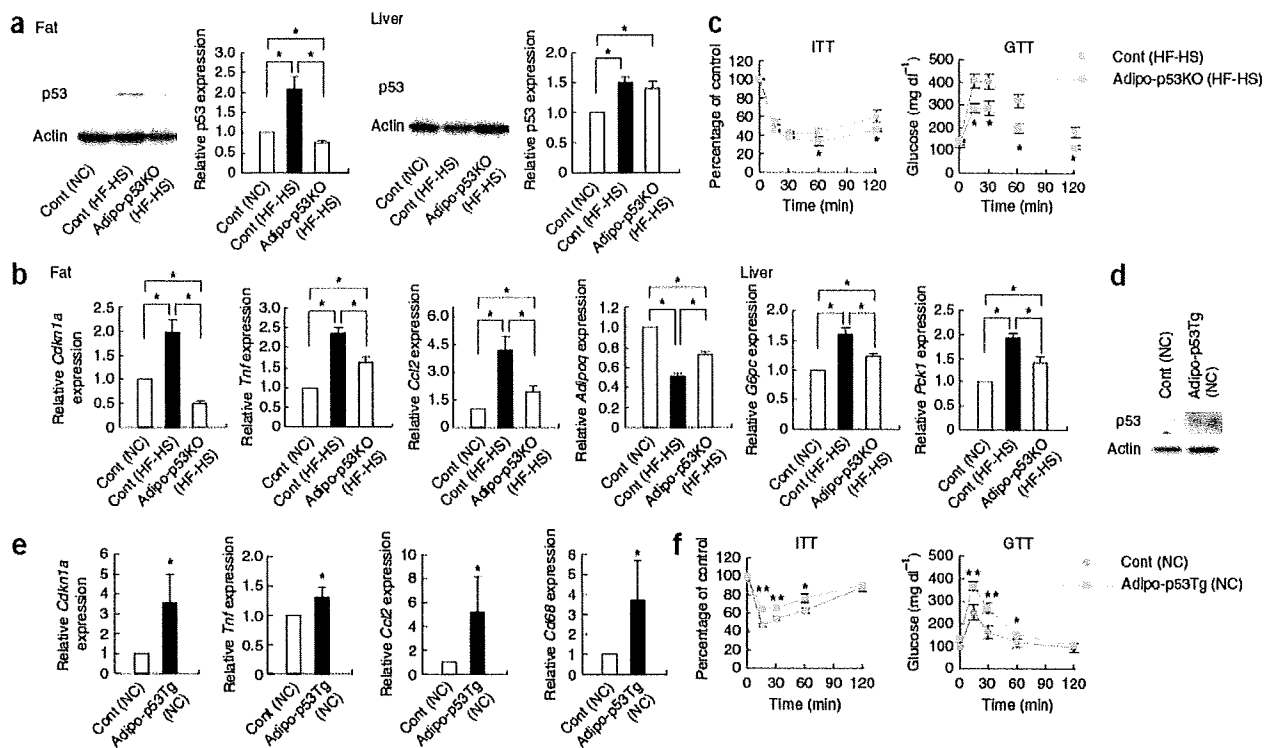
Figure 2 Adipose tissue p53 expression contributes to insulin resistance in mice with dietary obesity. (a) Western blot analysis for p53 in the fat and liver of littermate controls (Cont) on a normal diet (normal chow, NC), littermate controls (Cont) on an HF-HS diet (HF-HS), and adipo-p53–deficient mice (Adipo-p53KO) on an HF-HS diet (HF-HS). The graph indicates relative expression of p53 protein. (b) Real-time PCR assessing the expression of *Cdkn1a*, *Tnf*, *Ccl2* and *Adipoq* in adipose tissue and *G6pc* and *Pck1* in liver of the same mice as in a. *$P < 0.05$; $n = 5$ mice for a and b. (c) ITT and GTT in Adipo-p53KO mice and littermate controls (Cont) after 4 months on a HF-HS diet or a normal diet. *$P < 0.05$ versus control (HF-HS); $n = 8$. (d) Western blot analysis for p53 in adipose tissue of littermate controls (Cont) and adipo-p53–transgenic (Adipo-p53Tg) mice on a normal diet (NC). (e) Real-time PCR assessing the expression of *Cdkn1a*, *Tnf*, *Ccl2* and *Cd68* in adipose tissue of the same mice as in d. *$P < 0.05$; $n = 5$. (f) ITT and GTT in Adipo-p53Tg mice and littermate controls (Cont) on a normal diet (NC). *$P < 0.05$, **$P < 0.01$ versus control; $n = 8$. Data are shown as the means ± s.e.m.

adipose-rich fraction compared with the levels seen in littermate control mice (Supplementary Fig. 1d). These results suggested that both macrophages and adipocytes contribute to the senescence-like changes of adipose tissues in mice with dietary obesity and that upregulation of p53 expression in adipose tissue has a pathological role in producing insulin resistance.

To further investigate the role of adipose tissue p53, we established transgenic mice that showed an increase of p53 protein and *Cdkn1a* mRNA expression in adipose tissue (Fig. 2d,e). Consistent with the results found in adipose-p53–deficient mice, these transgenic mice on a normal diet showed higher expression of proinflammatory cytokines and a macrophage marker (Fig. 2e), which was associated with impairment of insulin sensitivity and glucose tolerance (Fig. 2f), providing further evidence for a major role for adipose tissue p53 in insulin resistance.

Insulin resistance has been reported to correlate with enhanced telomere shortening in young adults[16], whereas aging is known to accelerate telomere dysfunction in various human tissues[1,5]. It is well accepted that dysfunctional (that is, critically short) telomeres resemble damaged DNA and thus trigger a p53-dependent response[17,18]. To investigate a potential relationship between telomere-dependent p53 activation and insulin resistance, we used telomerase reverse transcriptase (*Tert*)-deficient mice. These mice have a normal phenotype

in the first generation (G1), presumably because mice possess very long telomeres[19,20]. However, their telomeres become shorter with successive generations, and the mice eventually become infertile by the fourth to sixth generation (G4–G6), owing to impairment of the reproductive system[20]. We fed an HF-HS diet to G4 mice for 8 weeks (from 4 to 12 weeks of age) and examined the effects of cellular aging on glucose metabolism. Although the insulin sensitivity and glucose tolerance of G4 mice were similar to those of wild-type mice on a normal diet, insulin resistance and glucose intolerance became more prominent in G4 mice than in wild-type mice after feeding with the HF-HS diet (Fig. 3a). There were no significant differences in weight gain, food intake and oxygen consumption between the two groups (Fig. 3b). Expression of proinflammatory cytokines such as *Tnf* and *Ccl2* was increased in the adipose tissue of G4 mice on the HF-HS diet, and this increase was evident in mice with shorter telomeres in adipose cells (Fig. 3c,d and Supplementary Fig. 3a). Shorter telomeres also promoted the infiltration of macrophages into adipose tissue (Fig. 3c and Supplementary Figs. 2c and 3b). Expression of hepatic gluconeogenic enzymes was upregulated in G4 mice (Fig. 3c). Insulin-induced phosphorylation of Akt was markedly lower in the liver of G4 mice compared to wild-type mice, and in skeletal muscle to a lesser extent (Supplementary Fig. 3c). The adipose tissue of G4 mice on the HF-HS diet showed senescence-like changes, including

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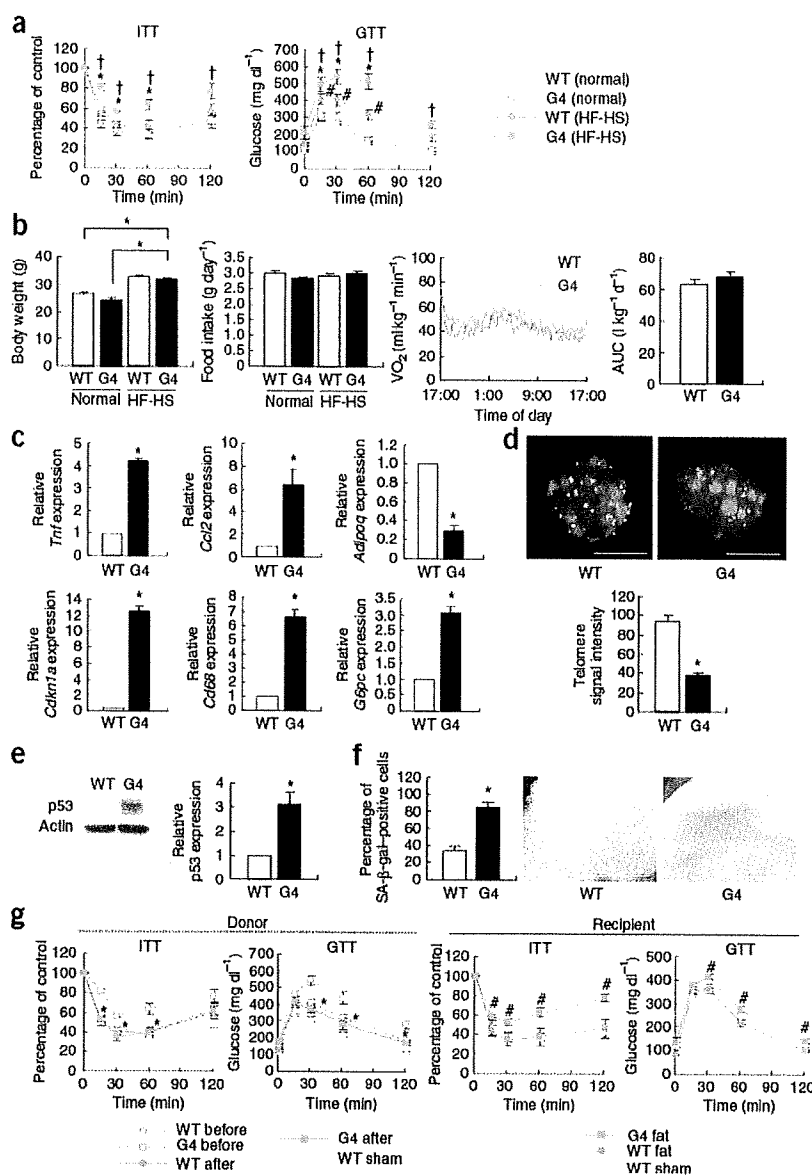
Figure 3 Adipose tissue p53 expression and insulin resistance in G4 mice. (a) ITT and GTT after 8 weeks on a HF-HS diet or a normal diet (normal) in G4 and WT mice. *$P < 0.05$ versus WT (HF-HS); #$P < 0.05$ versus WT (normal); †$P < 0.05$ versus G4 (normal); $n = 7$. (b) Body weight, food intake and oxygen consumption (VO_2) in WT and G4 mice. AUC, area under the curve. (c) Real-time PCR analysis of the expression of cytokines, *Cdkn1a* and *Cd68* in adipose tissue and the expression of *G6pc* in the livers of WT mice and G4 mice. All mice were fed on the HF-HS diet. (d) Top, telomeric fluorescence (yellow) *in situ* hybridization of adipocytes from WT and G4 mice. The signal intensity of the X chromosome (red) was used as an internal control. Bottom, estimation of the length of telomeres in adipose cells by quantification of telomeric fluorescence *in situ* hybridization images. Representative of 30 nuclei (images) for each genotype. Scale bar, 10 μm. (e) Expression of p53 in the adipose tissue of WT mice and G4 mice on the HF-HS diet, as determined by western blot analysis. The graph indicates relative expression of p53 protein. (f) The number of cells positive for SA-β-gal activity in the adipose tissue of WT mice and G4 mice. Photographs show adipose tissue after SA-β-gal staining. Scale bar, 5 mm. *$P < 0.05$; $n = 6$ for b; $n = 5$ for c; $n = 30$ nuclei for d; $n = 3$ for e and f. (g) Left, ITT and GTT in WT and G4 donor mice before and after fat pad removal. Right, ITT and GTT in recipients of fat pads (1 g) from WT mice (WT fat) or G4 mice (G4 fat) and in sham-operated WT mice (WT sham). *$P < 0.05$ versus G4 before, #$P < 0.05$ versus WT fat; $n = 6$. Data are shown as the means ± s.e.m.

increased expression of *Cdkn1a* mRNA and p53 protein, as well as enhanced activity of SA-β-gal (**Fig. 3c,e,f** and **Supplementary Fig. 3d**). These results suggest that telomere-dependent senescence of adipose tissue can also promote an inflammatory response, thereby leading to insulin resistance.

To investigate the influence of adipose tissue senescence on the insulin resistance of G4 mice receiving a HF-HS diet, we transplanted epididymal fat pads subcutaneously into wild-type mice and examined the changes of insulin sensitivity and glucose tolerance in the donor and recipient mice. Two weeks after fat pad removal, insulin resistance and glucose intolerance were both markedly improved in G4 mice on the HF-HS diet (**Fig. 3g** and **Supplementary Fig. 2d**). The insulin level of donor G4 mice was also normalized by 2 weeks after fat pad removal (**Supplementary Fig. 3e**). Conversely, implantation of adipose tissue from G4 mice on this diet significantly impaired the insulin sensitivity and glucose tolerance of wild-type recipient mice, whereas implantation of adipose tissue from other

wild-type mice fed the same diet had no effect (**Fig. 3g**). Implantation of adipose tissue from G4 mice also lowered insulin-induced phosphorylation of Akt in the liver (**Supplementary Fig. 3f**). Histological examination showed that the implanted adipose tissue was viable and vascularized (**Supplementary Fig. 3g**). Moreover, implantation of fat pads collected from G4 *Trp53*^{+/−} mice into wild-type mice had less influence on the insulin resistance and glucose tolerance of the recipients (**Supplementary Fig. 3h**) compared with fat pads from G4 *Trp53*^{+/+} mice. These results indicate that telomere-dependent p53 activation in adipose tissue also leads to insulin resistance.

We noted that expression of histone γ-H2AX, a marker of double-stranded DNA breaks, was increased in the adipose tissue of Ay mice as well as G4 mice (**Fig. 4a**), suggesting a potential role of the ROS-induced DNA damage pathway in the development of type 2 diabetes. To further investigate the relationship between ROS-induced DNA damage and diabetes, we examined the effect of oxidative stress on the expression of inflammatory cytokines in primary cultures of human preadipocytes. Treatment with hydrogen peroxide led to a marked increase of p53 protein expression (**Fig. 4b**). Hydrogen peroxide treatment significantly upregulated expression of *TNF* and *CCL2*, whereas this upregulation was inhibited by p53 knockdown (**Fig. 4c**). This treatment also increased the activity of nuclear factor-κB (NF-κB;
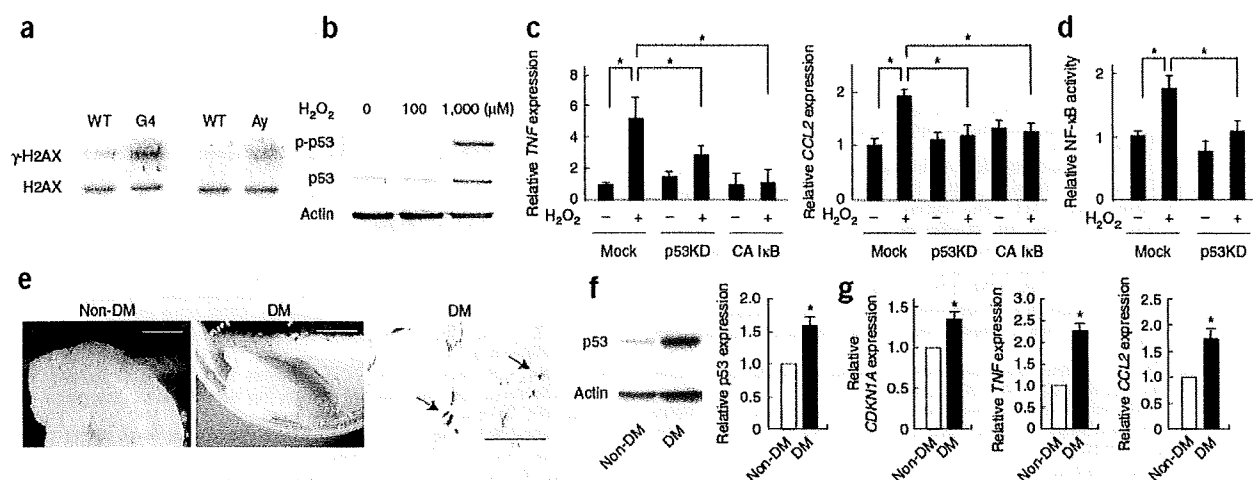
Figure 4 Senescence-like features of adipose tissue from subjects with diabetes. (a) Western blot analysis of γ-H2AX expression in adipose tissue of WT mice and G4 mice on a HF-HS diet and WT mice and Ay mice on a normal diet. (b) Effect of hydrogen peroxide (H_2O_2) on p53 expression in human preadipocytes by western blot analysis. p-p53, phosphorylated p53. (c) Hydrogen peroxide–induced expression of *TNF* and *CCL2* in human preadipocytes transfected with siRNA targeting p53 (p53KD) or the vector encoding constitutively active inhibitor of κB (CA IκB). (d) Effect of p53 knockdown (p53KD) on hydrogen peroxide–induced activation of NF-κB. (e) Adipose tissue from subjects without diabetes (non-DM) or subjects with diabetes (DM) after SA-β-gal staining. Scale bar, 10 mm. The photograph on the right shows adipose tissue obtained from a subject with diabetes (DM). Arrows indicate SA-β-gal–positive cells. Scale bar, 50 μm. (f,g) Expression of p53, *CDKN1A* and cytokines in adipose tissue obtained from subjects without diabetes or subjects with diabetes, as determined by western blot analysis (f) or real-time PCR (g). The graphs indicate relative expression of p53 protein (f) and relative mRNA levels of *CDKN1A*, *TNF* and *CCL2* (g). *$P < 0.05$; $n = 5$ for c, d, f and g. Data are shown as the means ± s.e.m.

Fig. 4d), a key transcription factor that regulates the induction of cytokines, including *TNF* and *CCL2*, whereas inhibition of NF-κB activation suppressed oxidative stress–induced upregulation of these cytokines (Fig. 4c). In agreement with previous reports that induction of p53 causes activation of NF-κB[21,22], we found that p53 deficiency led to a decrease in oxidative stress–induced NF-κB activation (Fig. 4d), indicating that ROS-induced p53 activation causes NF-κB–dependent induction of inflammatory cytokines and thus accelerates the development of diabetes.

To determine whether or not senescence-like changes occur in human adipose tissue, we examined visceral fat obtained from subjects undergoing abdominal surgery for primary gastric cancer or colon cancer. Adipose tissue from subjects with diabetes showed increased SA-β-gal activity and higher levels of p53 protein and *CDKN1A* mRNA expression compared with tissue from nondiabetic subjects (Fig. 4e–g). Moreover, expression of inflammatory cytokines was significantly increased in diabetic adipose tissue (Fig. 4g), suggesting that aging of fat cells has a major role in human diabetes.

Recent studies have shown that longevity signals generated in adipose tissue are crucial in regulating the lifespan of various species, ranging from worms to mice, and suggested that aging is non–cell-autonomously regulated by adipose tissue[23–26]. Consistent with these reports, subcutaneous implantation of senescent adipose tissue from G4 mice accelerates the senescence of epididymal fat in wild-type recipients (T.M., unpublished data). Senescence of adipose tissue may increase the local production of proinflammatory molecules, and it also promotes systemic inflammation and insulin resistance via non–cell-autonomous mechanisms. In contrast, low circulating insulin concentrations are generally associated with longevity, and the activation of longevity signals in adipose tissue has been reported to lower the circulating insulin level and extend the lifespan[27,28]. We found that inhibition of p53 activity in adipose tissue improved insulin resistance and decreased the plasma insulin level. Thus, p53 activation in adipose tissue may be a proaging

signal with a negative influence on longevity, whereas inhibition of cellular aging may become a new strategy for the treatment of diabetes as well as aging and its associated diseases.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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ONLINE METHODS

Animal models. The study protocol was approved by the Chiba University Institutional Animal Care and Use Committee. The creation of mice deficient in *Tert* has been described previously[29]. We backcrossed heterozygous mice with wild-type C57BL/6 mice (SLC) for six generations and intercrossed them to produce G1 *Tert*^{-/-} mice. Mating of G1 *Tert*^{-/-} mice with each other generated G2 mice, after which we produced *Tert*^{-/-} mice up to the fourth generation (G4). We purchased p53-deficient mice (with a C57BL/6 background) from Jackson Laboratories and mated them with *Tert*^{+/-} mice to generate double heterozygotes (*Tert*^{+/-} *Trp53*^{+/-}). We intercrossed these mice to generate G1 *Tert*^{-/-} *Trp53*^{+/-} mice. We mated G1 *Tert*^{-/-} *Trp53*^{+/-} mice with other G1 mice to produce G2 *Tert*^{-/-} *Trp53*^{+/-} mice, after which we repeated this mating strategy to generate G4 *Tert*^{-/-} *Trp53*^{+/-} mice. We fed the mice a HF-HS diet (Oriental Yeast)[30] or normal chow from 4 to 12 weeks of age before we performed metabolic analyses. We purchased Ay mice (with a C57BL/6 background) from Jackson Laboratories and mated them with *Trp53*^{+/-} mice to generate *Trp53*^{+/+}, Ay *Trp53*^{+/+}, *Trp53*^{+/-} and Ay *Trp53*^{+/-} mice. We fed these mice normal chow and analyzed them at 20 weeks of age. We purchased mice that express Cre recombinase in adipocytes (Fabp4-Cre) from Jackson Laboratories. We then crossed Fabp4-Cre mice (with a C57BL/6 background) with mice that carry floxed *Trp53* alleles (with a C57BL/6 background)[31] to generate adipocyte-specific p53-knockout mice. We fed these mice a HF-HS diet or normal chow for 4 months before we performed metabolic analyses. Littermate controls had the genotype Cre⁻*Trp53*[loxP/-] or Cre⁻*Trp53*[loxP/loxP]. We also generated transgenic mice (with a C57BL/6 background) that carry the *loxP-LacZ-loxP* cassette flanked by the *TP53* complementary DNA fragment under the control of the cytomegalovirus enhancer–chicken actin promoter. Expression of transgene-derived *TP53* was prevented by the *loxP-LacZ-loxP* cassette. When we bred these transgenic mice with Fabp4-Cre mice, the floxed *LacZ* cassette was excised in the resulting offspring (Cre⁺*LacZ-TP53*⁺), and we observed upregulation of p53 expression in adipose tissue (adipo-p53–transgenic mice). We fed these mice normal chow and analyzed them at 10–12 weeks of age. Littermate controls had the genotype Cre⁻*LacZ-TP53*⁺.

Cell culture. We purchased human preadipocytes from Sanko, and we cultured them according to the manufacturer's instructions.

Western blot analysis. We resolved whole-cell lysates (30–50 µg) by SDS PAGE. We transferred the proteins onto a polyvinylidene difluoride (PVDF) membrane (Millipore) incubated them with the primary antibody (Supplementary Methods), followed by incubation with rabbit IgG–specific horseradish peroxidase–conjugated antibody (111-035-003) or mouse IgG–specific horseradish peroxidase–conjugated antibody (115-035-003; Jackson). We detected specific proteins by enhanced chemiluminescence (Amersham).

Human subjects. The ethical committee of Chiba University Graduate School of Medicine reviewed and approved the study protocol. We enrolled 10 subjects (56–68 years old; six males and four females) who were admitted to Chiba University Hospital and underwent surgery for primary gastric or colon cancer. We obtained informed consent from all subjects before inclusion in the study.

Statistical analyses. Data are shown as the means ± s.e.m. We examined differences between groups by Student's *t* test or analysis of variance followed by Bonferroni's correction for comparison of means. For all analyses, we considered $P < 0.05$ as statistically significant.

Additional methods. Detailed methodology is described in the Supplementary Methods.

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ORIGINAL ARTICLE

Multivalent ligand–receptor interactions elicit inverse agonist activity of AT$_1$ receptor blockers against stretch-induced AT$_1$ receptor activation

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Type 1 angiotensin II (AT$_1$) receptor has a critical role in the development of load-induced cardiac hypertrophy. Recently, we showed that mechanical stretching of cells activates the AT$_1$ receptor without the involvement of angiotensin II (AngII) and that this AngII-independent activation is inhibited by the inverse agonistic activity of the AT$_1$ receptor blocker (ARB), candesartan. Although the inverse agonist activity of ARBs has been studied in terms of their action on constitutively active AT$_1$ receptors, the structure–function relationship of the inverse agonism they exert against stretch-induced AT$_1$ receptor activation has not been fully elucidated. Assays evaluating *c-fos* gene expression and phosphorylated extracellular signal-regulated protein kinases (ERKs) have shown that olmesartan has strong inverse agonist activities against the constitutively active AT$_1$ receptor and the stretch-induced activation of AT$_1$ receptor, respectively. Ternary drug–receptor interactions, which occur between the hydroxyl group of olmesartan and Tyr^{113} and between the carboxyl group of olmesartan and Lys^{199} and His^{256}, were essential for the potent inverse agonist action olmesartan exerts against stretch-induced ERK activation and the constitutive activity of the AT$_1$-N111G mutant receptor. Furthermore, the inverse agonist activity olmesartan exerts against stretch-induced ERK activation requires an additional drug–receptor interaction involving the tetrazole group of olmesartan and Gln^{257} of the AT$_1$ receptor. These results suggest that multivalent interactions between an inverse agonist and the AT$_1$ receptor are required to stabilize the receptor in an inactive conformation in response to the distinct processes that lead to an AngII-independent activation of the AT$_1$ receptor.
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INTRODUCTION

The type 1 angiotensin II (AT$_1$) receptor is a member of the G protein-coupled receptor (GPCR) family and mediates most of the actions that angiotensin II (AngII) exerts on the cardiovascular system.[1] AT$_1$ receptor blockers (ARBs) are non-peptide compounds that selectively bind to the AT$_1$ receptor and inhibit AngII-induced receptor activation. At present, several ARBs are clinically available as a highly effective and well-tolerated class of drugs for the management of hypertension. In addition, clinical trials have indicated that ARBs provide cardiovascular protection that extends beyond blood pressure lowering.[2] Treatment with ARBs effectively prevents cardiac hypertrophy and improves cardiovascular outcomes in patients with hypertension.[2,3] Structurally, most ARBs have a common biphenyl-tetrazole ring and unique side chains, which contribute to drug-specific differences in their pharmacokinetic and pharmacodynamic proper-

ties.[2,4] These structural and pharmacological differences among ARBs may have an impact on long-term cardiovascular outcomes, although the clinical significance of these differences remains to be determined in large-scale trials.

Recent studies have shown that most GPCRs, including the AT$_1$ receptor, show spontaneous activity even in the absence of an agonist.[5] The AT$_1$ receptor is also activated by the mechanical stress of cellular stretching without the involvement of AngII.[6,7] A ligand capable of suppressing the agonist-independent activities of a receptor is defined as an inverse agonist.[5,8] We have previously reported that pressure overload induces cardiac hypertrophy in angiotensinogen-deficient mice as well as in wild-type (WT) mice and that hypertrophy is significantly attenuated by the inverse agonist, candesartan.[6] Therefore, the inverse agonist activities of ARBs have potential therapeutic benefits, at least in the prevention of load-induced cardiac hypertro-

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