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Review Article

Vascular cell senescence and vascular aging☆

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Abstract

Vascular cells have a finite lifespan when cultured in vitro and eventually enter an irreversible growth arrest called "cellular senescence". A number of genetic animal models carrying targeted disruption of the genes that confer the protection against senescence in vitro have been reported to exhibit the phenotypes of premature aging. Similar mutations have been found in the patients with premature aging syndromes. Many of the changes in senescent vascular cell behavior are consistent with the changes seen in age-related vascular diseases. We have demonstrated the presence of senescent vascular cells in human atherosclerotic lesions but not in non-atherosclerotic lesions. Moreover, these cells express increased levels of pro-inflammatory molecules and decreased levels of endothelial nitric oxide synthase, suggesting that cellular senescence in vivo contributes to the pathogenesis of human atherosclerosis. One widely discussed hypothesis of senescence is the telomere hypothesis. An increasing body of evidence has established the critical role of the telomere in vascular cell senescence. Another line of evidence suggests that telomere-independent mechanisms are also involved in vascular cell senescence. Activation of Ras, an important signaling molecule involved in atherogenic stimuli, induces vascular cell senescence and thereby promotes vascular inflammation in vitro and in vivo. It is possible that mitogenic-signaling pathways induce telomere-dependent and telomere-independent senescence, which results in vascular dysfunction. Further understanding of the mechanism underlying cellular senescence will provide insights into the potential of antisenescence therapy for vascular aging.
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1. Introduction

Cellular senescence is the limited ability of primary human cells to divide when cultured in vitro and is accompanied by a specific set of phenotypic changes in morphology, gene expression and function. These changes in phenotype have been implicated in human aging [1]. This hypothesis, the hypothesis of cellular aging, was first described by Hayflick [2] and supported by evidence that cellular senescence and the division potential of human primary cultures are dependent on donor age [3] and that the growth potential of cultures correlates well with mean maximum lifespan of the species from which the cultures are derived [4]. Human primary cultures derived from the patients with premature aging syndromes, such as Werner syndrome and Bloom syn-

drome, are known to have shorter lifespan than the cultures from age-matched healthy populations [5], thus supporting the hypothesis of aging. However, until recently, little attention has been paid on the potential impact of vascular cell senescence in vivo on age-related vascular disorders.

In the past decades, significant progress has been made in our understanding of the mechanisms underlying cellular senescence. One widely discussed hypothesis is the telomere hypothesis of senescence [6]. A growing body of evidence has demonstrated a critical role of telomere and telomerase in regulating not only cellular lifespan but also organismal aging. However, recent findings suggest that cellular senescence can also be induced by DNA damage, cellular stress or oncogenic activation, which is independent of replicative age [7]. For example, the constitutive activation of mitogenic stimuli by expression of oncogenic Ras induces senescent phenotypes [8–10]. Thus, it is possible that atherogenic stimuli increase cell turnover at the sites of atherosclerosis, thereby promoting telomere shortening, whereas it also re-

sults in activation of proliferative signals that potentially induce senescence independent of telomere shortening.

In this review, we will describe recently accumulating evidence that supports the hypothesis of cellular aging in the vasculature and discuss the potential of antisenescence therapy for age-related human vascular disorders.

2. Occurrence of vascular cell senescence in vivo

Vascular cells have a finite lifespan in vitro and eventually enter an irreversible growth arrest called cellular senescence. Flattened and enlarged cell morphology is known to be one of the characteristics of cellular senescence [11]. Expression of negative regulators for the cell cycle machinery, such as p53 and p16, is increased with cell division and thereby promotes growth arrest [12]. Primary cultured cells undergoing cellular senescence in vitro express the increased activity of β-galactosidase (β-gal) when assayed at pH 6, which is distinguishable from endogenous lysosomal β-gal activity that can be detected at pH 4. This activity, senescence-associated β-gal (SA β-gal) activity, has been shown to correlate with cellular aging and thus is regarded as a biomarker for cellular senescence [13]. The in vitro growth properties of vascular cells derived from human atherosclerotic plaque are impaired, and they develop senescence earlier than those from normal lesions [14,15]. The histology of the lesions of human atherosclerosis has been extensively studied, and these studies have demonstrated that there are endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) that exhibit the morphological features of cellular senescence [16,17]. These suggest the occurrence of cellular senescence in vivo. Recently, this notion has been confirmed by cytochemical analysis in vivo using SA β-gal activity. Fenton et al. [18] have successfully detected SA β-gal-positive vascular cells in rabbit carotid arteries subjected to vascular injury. With repeated denudation, the accumulation of SA β-gal-positive cells was markedly enhanced. The authors have recently demonstrated SA β-gal-positive vascular cells in human atherosclerotic plaque of the coronary arteries obtained from the patients who had ischemic heart disease [19]. SA β-gal-positive cells were predominately localized on the luminal surface of atherosclerotic plaque and identified as ECs, but no positive cells were observed in the internal mammary arteries from the same patients where atherosclerotic changes were minimally observed. In advanced plaque, however, SA β-gal-positive VSMCs were detected in the intima but not in the media [20], which may represent extensive replication in the lesions, as observed in the arteries subjected to double-denudation (Fig. 1). SA β-gal-positive cells exhibit increased expression of p53 and p16, alternative markers for cellular senescence, in human atheroma, suggesting the further evidence of in vivo senescence. These cells also show impaired function, such as the decreased expression of endothelial nitric oxide synthase (eNOS) and the increased expression of pro-inflammatory molecules
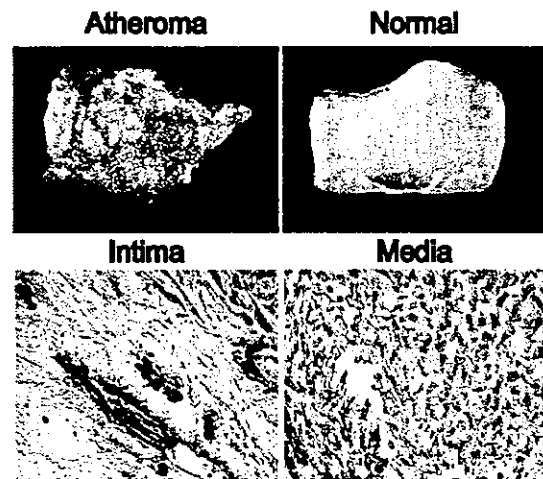

Fig. 1. Senescent vascular cells in human atheroma. Photographs of the luminal surface of human atheroma (atheroma, left) and non-atherosclerotic normal artery (normal, right) stained with SA β-gal staining. SA β-gal activity was observed in human atheroma but not in normal arteries (upper panel). A double staining for SA β-gal and α-smooth muscle actin of the sections of atheroma identified SA β-gal-positive cells as VSMCs in the intima but not in the media (lower panel).

[20]. Thus, cellular senescence in vivo may contribute to the pathogenesis of vascular aging.

3. Role of cellular senescence in vascular pathophysiology

Age-associated changes in the blood vessels include a decrease in compliance and an increase in inflammatory response that promote atherogenesis [21]. It has also been reported that angiogenesis is impaired with advanced age [22,23], whereas aging decreases the antithrombogenic property of the endothelium [24]. A number of studies have reported that many of the changes in senescent vascular cell behavior are consistent with known changes seen in age-related vascular diseases, suggesting a critical role of cellular senescence in vascular pathophysiology. The production of nitric oxide (NO) and the eNOS activity are reduced in senescent human ECs [25]. Induction of NO production by shear stress is also decreased in senescent ECs [26]. A decline in the eNOS activity of senescent ECs is attributable to a decrease in expression of eNOS protein as well as in eNOS phosphorylation mediated by Akt [27]. The levels of prostacyclin production are significantly decreased with in vitro aging of ECs [28], whereas senescent ECs upregulate plasminogen activator inhibitor-1 [29]. All these alterations are likely involved in the impairment of endothelium-dependent vasodilation but also increased sensitivity of thrombogenesis in human atherosclerosis. The interaction between monocytes and ECs is enhanced by EC senescence [30], thereby promoting atherogenesis. This appears to be mediated by upregulation of adhesion molecules and pro-inflammatory

cytokines as well as a decrease in the production of NO in senescent ECs [19,26]. It is reported that the ability to form capillary structures in vitro is reduced in senescent ECs [31]. Bone marrow-derived circulating endothelial progenitor cells (EPCs) are known to participate in postnatal neovascularization and vascular repair [32,33]. The in vitro growth property and function of bone marrow-derived EPCs are impaired in the patients with coronary artery disease and negatively correlate with risk factors including age [34,35]. Thus, aging may promote senescence of EPCs as well as ECs, resulting in decreases in angiogenesis and vascular healing.

4. Telomere-dependent vascular cell senescence

Telomeres are non-nucleosomal DNA–protein complexes at the end of chromosomes that serve as protective caps and are substrates for specialized replication mechanisms. As a consequence of semi-conservative DNA replication, the extreme termini of chromosomes are not duplicated completely, resulting in successive shortening of telomeres with each cell division. Critically short telomere was thought to trigger the onset of cellular senescence, but recent studies suggest that the single-strand telomeric overhang and associated proteins are key components for signals of senescence [36,37]. Telomerase is a ribonucleoprotein that adds telomeres onto chromosome ends with its RNA moiety as a template. Forced expression of the catalytic component of telomerase TERT in telomerase-negative human fibroblasts results in the stabilization of telomere length and extension of cell lifespan [38]. This observation has established the importance of telomere shortening in human cellular senescence. It has been also reported that there is another mechanism of telomere maintenance without telomerase activity, called telomerase-independent alternative lengthening of telomeres (ALT) [39]. In the field of vascular biology, it has been shown that telomere shortening with cell divisions occurs in human-cultured ECs and VSMCs, and that introduction of TERT extends cell lifespan of human ECs and VSMCs, suggesting a critical role of telomere shortening in vascular cell senescence as well [31,40,41]. It is likely that shortened telomeres are in some way sensed in a cell, and that a pathway is activated that results in exit from cell cycle (Fig. 2). The p53, p21 and p16 proteins and their downstream effectors are important for cellular senescence, and thus are likely to be a part of the telomere-response pathway. However, less is known about what links telomeres and these factors. Until present, several telomeric-binding proteins have been identified that contribute to the integrity of telomere functions and are potentially involved in the telomere-response pathway [42]. These include protection of telomeres-1 (Pot-1) [43], Ku [44], telomeric repeat-binding factor 1 and 2 (TRF1 and TRF2, respectively) [45]. Pot-1 is identified as a telomeric protein that binds to a tip of telomere and is thought to constitute the telomere shortening signal [46]. The protein
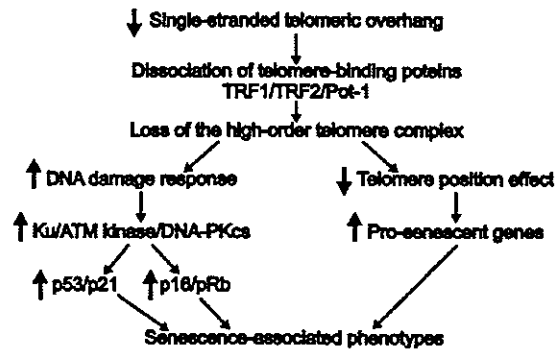

Fig. 2. Putative telomere-responsible pathways. As cells age, single-stranded telomeric overhangs are eroded, resulting in the dissociation of telomeric proteins. Loss of the high-order telomere complex is recognized as DNA breaks, thereby activating the molecules involving DNA repair that also play a critical role for telomere maintenance. These signals of DNA damage response are conducted to the cell cycle machinery. Telomere dysfunction also results in a release of telomere position effects that have the impact on expression of the genes at subtelomeric regions.

Ku, originally defined by its role in the repair of chromosomal DNA breaks, is found at telomeres and is necessary for normal telomere maintenance and functions. Genetic ablation studies demonstrated an essential role of Ku in mediating the telomere-response pathway [47]. Likewise, the catalytic subunit of the DNA-dependent protein kinase complex [48] and ATM kinase [49], both of which function in the double-strand break repair, have been implicated in the signal pathway of telomere shortening. Recently, it has been proposed that telomeres form large duplex loops, and telomeric proteins, TRF1 and TRF2, are essential for their formation [50,51]. Particularly, inhibition of TRF2 function is reported to cause cellular senescence in a p53/p16-dependent manner [19,52]. Thus, it is supposed that, as a result of telomere shortening, the efficacy of forming a high-order telomeric complex is impaired, leading to a release of various telomeric proteins from telomeres that elicit signals for cellular senescence (Fig. 2). Cytologically, telomeric regions are heterochromatic, implying that local DNA folding is increased. Positioning a gene in a telomeric heterochromatic region can impose telomere position effect on that gene. Telomere position effect, which has been characterized in the yeast and recently in human cells, is known to induce the reversible silencing of the gene [53]. Consequently, the modification of gene expression by telomere position effect may also contribute to the signaling pathway for cellular senescence.

5. Telomere shortening and vascular dysfunction

There is evidence indicating that telomere shortening occurs in human vasculature, which may be related to age-associated vascular diseases [54]. In most of the previous reports mentioned, changes in cell phenotypes associated with senescence were studied in vascular cell populations

undergoing replicative senescence, thus suggesting telomere-dependent vascular dysfunction. However, it remains unclear whether phenotypic changes in senescent vascular cells result from telomere dysfunction. Inhibition of TRF2 has been shown to induce either cellular senescence or apoptosis in various cells by destroying telomere loop structure [51,55]. The authors have demonstrated that the introduction of a dominant-negative form of TRF2 into human ECs induces a growth arrest with phenotypic characteristics of cellular senescence [19]. Telomere dysfunction significantly increases expression of pro-inflammatory molecules and reduces the activity of eNOS, suggesting a causal link between telomere and vascular dysfunction associated with senescence.

Telomerase-deficient mice have been developed and found to reveal a normal phenotype at the first generation presumably because of mice having much longer telomeres [56,57]. Telomeres are shortened with successive generations, and they become infertile at the sixth generation due to the impairment of reproductive system. Some aspects of the late generation mice mimic age-associated phenotypes. They exhibit shortened lifespan and a reduced capacity to respond to stress, such as wound healing and hematopoietic ablation [58]. The ability of neovascularization is reduced in the late generation of telomerase-deficient mice [59]. Decreased vessel formation may be attributable to the impaired function and replicative capacity of ECs induced by telomere shortening. Recently, the heart in late generation of telomerase-deficient mice has been shown to mimic the end-stage-dilated cardiac myopathy in humans [60].

6. Telomerase

Since early studies reported that telomerase activity was detected in cancer cells but not normal somatic cells, the idea emerged that telomerase activity might be essential for tumor growth [61]. Multiple tumor-suppressor pathways are likely to repress telomerase expression in normal somatic cells [62]. Increasing evidence has suggested that telomerase activity regulates cell proliferation in normal somatic cells by telomere lengthening or upregulating growth-controlling genes in a telomere length-independent manner [57,63]. Human ECs and VSMCs express telomerase activity, which is drastically activated by mitogenic stimuli via a protein kinase C-dependent pathway [64] but the activity declined with in vitro aging due to a decrease in expression of TERT, leading to telomere shortening and cellular senescence [41,65]. Introduction of TERT prevents endothelial dysfunction associated with senescence, such as a decrease in eNOS activity and an increase in monocyte binding to ECs [19,26]. Immortalized human ECs (TERT-ECs) have been established by introduction of TERT [31]. TERT-ECs appear to retain EC characteristics including various cell surface markers. When examined in Matrigel, they form capillary-like structures in response to extracellular matrix signals as efficiently as early

passage of ECs, whereas senescent or transformed ECs do not. In addition, TERT-ECs are more resistant to apoptotic induction than pre-senescent ECs. They maintain a normal growth control and exhibit no transformed phenotype. These telomerized human ECs are functional in vivo as demonstrated by the Matrigel implantation mouse model [66]. In this model, whereas primary human EC-derived vessel density decreased with time after implantation, telomerized ECs maintained durable vessels, indicating that telomerase activity is important for the maintenance of a microvascular phenotype.

7. Telomere-independent vascular cell senescence

Signals other than extended proliferation have been shown to result in cells developing a phenotype indistinguishable from that of senescent cells at the end of their replicative lifespan. For example, the constitutive activation of mitogenic stimuli by expression of oncogenic Ras or E2F induce a senescent phenotype [8,67]. Cellular senescence triggered by mitogenic stimuli is independent of replicative age, and these signals act before the replicative limits of cells. Hence, it is apparently telomere independent and thus termed as premature senescence. The constitutive activation of Ras provokes premature senescence in vascular cells, which is associated with accumulation of the proteins p53 as well as p16 [20]. Activation of extracellular signal-regulated kinase (ERK) appears to be critical for Ras-induced senescence since inhibition of ERK restores cell growth arrest elicited by Ras activation [20], whereas introduction of an active form of ERK results in premature senescence involving p53 and p16 [9]. p38 mitogen-activated protein kinase (MAPK) is also implicated in Ras-induced senescence. p38 MAPK is activated in a ERK-dependent fashion, thus indicating that Ras promotes premature senescence by sequentially activating the ERK and p38 MAPK pathway [68].

8. Cell cycle regulators

It is clear that cellular senescence entails the activation of several tumor-suppressor proteins and inactivation of several oncoproteins, each of which ultimately engages either the p53 or pRb pathway and interacts with each other at multiple levels (Fig. 3). p53 activity and in some cases protein levels are increased in senescent cells. The mechanisms responsible for p53 activation in senescent cells remain elusive, however some molecular details are emerging. One cause of p53 activation may be an increase in expression of p14, a tumor-suppressor protein encoded by the INK4a locus. p14 activates p53 through a mechanism involving sequestration of MDM2, a protein that promotes degradation of p53 [69]. p14 is induced by oncogenic Ras and E2F [70], whereas TBX2, a member of T-box family of transcription factors, suppresses p14 expression [71]. Another cause of p53 activation is the
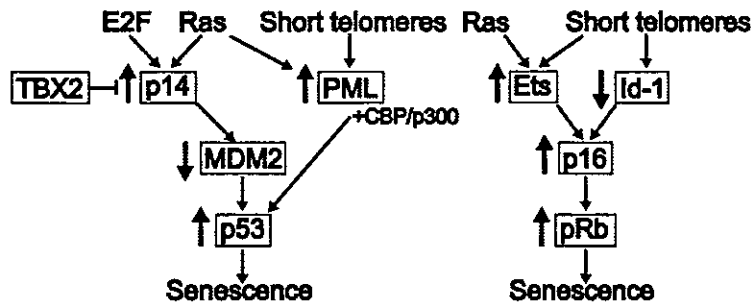
Fig. 3. Control of cellular senescence by the p53/p21 and p16/pRb pathways. Shown are the consequences of senescence-inducing signals on cell cycle regulators in the p53/p21 and p16/pRb pathways. Senescence-inducing signals, such as oncogenic Ras and E2F, increase expression of p14, whereas TBX2 represses p14 promoter activity, thus counteracting with each other. p14 sequesters MDM2, leading to an increase in p53 activity. Signals, such as oncogenic Ras, telomere shortening and possibly other signals increase expression of PML, which interacts with CBP/p300 and stimulates p53 activity. Oncogenic Ras stimulates the activity of Ets that induces p16 transcription by promoting its phosphorylation. Short telomeres lead to an increase in Ets and a decrease in Id, a protein that inhibits Ets activity, resulting in the accumulation of p16. p16 inhibits the cyclin-dependent kinases that phosphorylate pRb, leading to an increase in its active form.

promyelocytic leukemia (PML) tumor suppressor. PML is induced by replicative senescence and Ras activation by unknown mechanisms [72,73]. PML interacts with CBP/ p300 acetyltransferase, which acetylates p53, thus stimulating p53 activity. Recently, p53 is found at telomeres and ablation of p53 function restores adverse effects of telomere loss [74], suggesting active roles of p53 in telomere maintenance as well as the telomere-response pathway. pRb exists in hypophosphorylated form that binds to E2F and inhibits cell cycle progression in senescent cells because of high levels of the cyclin-dependent kinase inhibitors, p21 and p16. p21 is transcriptionally induced at least partly by p53, although p53-independent, post-transcriptional mechanisms also contribute to an increase in p21 expression in senescent cells [75]. p16, another tumor-suppressor protein encoded by INK4a locus, increases in part because Ets, a transcription factor that stimulates p16 expression, is induced by senescent signals including telomere shortening and Ras activation, whereas Id1, a protein that inhibits Ets activity, is decreased in senescent cells [76]. It is demonstrated that ectopic expression of the cyclin-dependent kinase inhibitors, such as p21, p16 and p14, causes premature senescence [77], suggesting pivotal roles in the signaling pathways of senescence.

p53 immunoreactivity is present in vascular cells in areas with chronic inflammation of human atheroma, while a few cells positive for p53 immunoreactivity are found in control arteries [78]. p21 immunoreactivity is also detected in human atheroma but not in normal lesions and is colocalized with p53. Forced expression of cyclin-dependent kinase inhibitors induces premature senescence that is associated with cell dysfunction in cultured vascular cells (Minamino et al. unpublished data). These observations suggest a pathological role of p53 and p21 in atherogenesis. However, their precise roles remain unclear. It is demonstrated that atherosclerosis is aggravated in p53/apolipoprotein E (ApoE) double-knockout mice through an increase in p53-controlled proliferation [79]. In contrast, the study using perivascular collar model in ApoE-knockout mice shows that p53 overexpres-

sion results in a marked decrease in the cellular and extracellular contents in the cap lesions, leading to spontaneous plaque rupture [80]. Thus, in the clinical settings, elevated expression of p53 and cyclin-dependent kinase inhibitors may be deleterious in human atherosclerosis.

9. Ras-induced senescence and vascular inflammation

It has been demonstrated that various molecules including growth factors, vasoactive peptides and oxidative stresses, such as ROS and oxidized low-density lipoproteins, are induced during the lesion formation and regulate numerous critical cell functions, thereby contributing to atherogenesis [81]. These stimuli function as mitogens for vascular cells through the signaling cascades that activate Ras [81]. Inhibition of Ras has been reported to prevent intimal formation after vascular injury, suggesting a critical role of Ras activation in VSMC proliferation [82]. In addition to its role in cell proliferation, we have found that constitutive activation of Ras induces vascular cell senescence that is associated with vascular inflammation [20]. Activation of Ras drastically increased expression of pro-inflammatory cytokines partially through ERK activation in cultured vascular cells. Introduction of Ras into balloon-injured arteries enhanced vascular inflammation as well as senescence compared with control-injured arteries (Fig. 4). Moreover, senescent cells express inflammatory molecules in human atherosclerotic plaque, and ERK is activated in these cells, suggesting that telomere-independent mechanisms may also contribute to vascular cell senescence in human atherosclerosis. Consistent with our findings, functional inhibition of Ras has been demonstrated to suppress pro-inflammatory molecules, thereby reducing lesion formation in ApoE-deficient mice [83]. Moreover, angiotensin II, an important atherogenic molecule that activates the Ras-signaling pathway, has been demonstrated to promote vascular cell senescence as well as vascular inflammation [84]. Thus, it is assumed that atherogenic stimuli may
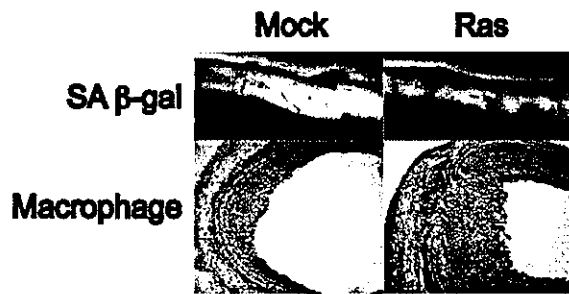
Mock   Ras

SA β-gal

Macrophage

Fig. 4. Activation of Ras induces senescence and inflammation in vivo. The adenoviral vector encoding H-rasV12 (Ras) or the empty vector (Mock) was transduced into rat carotid arteries injured by a balloon catheter. It is known that accumulation of macrophages is minimally involved in the lesion formation in this model. Whereas only a little SA β-gal activity was found in mock-infected injured arteries, transduction of adeno-Ras into injured arteries increased SA β-gal activity (upper panel). The area of accumulated macrophages (brown) in the intima was markedly increased in Ras-infected injured arteries compared with mock-infected injured arteries (lower panel), indicating a causal relationship between Ras activation and vascular inflammation. Adapted from Ref. [20] with permission.

initially promote cell proliferation, and when overstimulated to proliferate, mitogenic-signaling pathways may induce telomere-dependent and telomere-independent senescence, which results in vascular dysfunction. Vascular inflammation is known to induce degradation of extracellular matrix by various proteinases, such as collagenases and gelatinases, and by inhibition of matrix production [85]. Therefore, decreased cellularity and enhanced inflammation associated with vascular cell senescence may contribute to plaque vulnerability.

10. Genetic models for aging

Many molecular mechanisms have been suggested to contribute to human aging and age-associated disease. Recent genetic analyses have demonstrated that reduction-of-function mutations of the signaling pathway of insulin/insulin-like growth factor-1 (IGF-1)/phosphatidylinositol-3 kinase (PI3K)/Akt (also known as protein kinase B) extends the longevity of the nematode *Caenorhabditis elegans* [86–92]. The forkhead transcription factor DAF-16, which is phosphorylated and thereby inactivated by Akt, plays an essential role in this longevity pathway [93,94]. More recently, it has been reported that the genes regulating longevity are conserved in organisms ranging from yeast to mice. The mutation of *Sch9*, which is homologous to *Akt*, extends the lifespan of yeast [95] and mutations that decrease the activity of insulin/IGF-1-like pathway increase the longevity of fruit flies [96] and mice [97,98]. These mutations that extend the lifespan are associated with increased resistance to oxidative stress, which is mediated in part by an increase in expression of antioxidant genes [99–101]. In mammalian cells, activation of Akt has been reported to induce cell proliferation and survival toward tumorigenesis [102–104].

The insulin pathway has also been shown to be essential for the maintenance of normal metabolic homeostasis [105]. Restriction of caloric intake extends the longevity of yeast, worms, fruit flies, mice and probably humans and postpones or prevents age-dependent deterioration and chronic diseases [91]. Since calorie restriction associates with the persistent decrease in the insulin signals, one might think that the insulin pathway could be involved in human aging and age-associated diseases, especially in the patients with diabetes.

Mice models that exhibit an early onset of phenotypes associated with aging have been reported. These include mouse mutants carrying targeted disruption of the genes involving DNA damage repair, such as *ku86* [106], *XPD* [107] and *BRCA1* [108]. Activation of p53 [109] as well as telomerase deficiency [58] also cause premature aging, which is characterized by reduced longevity, osteoporosis, organ atrophy and a diminished stress tolerance. All these molecules have been implicated in cellular senescence. More importantly, cellular senescence in vivo has been detected in premature aging mice [108]. Thus, these results provide in vivo evidence that links cellular senescence to organismal aging.

11. Conclusion

Accumulating evidence indicates a critical role of cellular senescence in organismal aging and age-related disease including atherosclerosis. Young adult bone marrow-derived EPCs have been shown to restore aging-impaired angiogenic function [110]. Chronic treatment with EPCs from young non-atherosclerotic ApoE-deficient mice prevents atherosclerosis progression in ApoE-deficient recipients despite persistent hypercholesterolemia [111]. Moreover, introduction of telomerase into EPCs has been reported to extend cell lifespan and to increase the efficacy of vasculogenesis in vivo [112]. These reports indicate that progressive progenitor cell deficits contribute to age-associated vascular dysfunction and suggest the potential utility of cell-based antisenescence therapy as a novel therapeutic strategy for vascular aging. Further understanding of mechanisms underlying cellular senescence will provide new insights into the pathogenesis of age-associated vascular disorders.

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Perspecticve

Akt-Induced Cellular Senescence
Implication for Human Disease

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ABSTRACT

Reduction-of-function mutations in components of the insulin/insulin-like growth factor-1/Akt pathway have been shown to extend the lifespan in organisms ranging from yeast to mice. It has also been reported that activation of Akt induces proliferation and survival of mammalian cells, thereby promoting tumorigenesis. We have recently shown that Akt activity increases with cellular senescence and that inhibition of Akt extends the lifespan of primary cultured human endothelial cells. Constitutive activation of Akt promotes senescence-like arrest of cell growth via a p53/p21-dependent pathway, leading to endothelial dysfunction. This novel role of Akt in regulating the cellular lifespan may contribute to various human diseases including atherosclerosis and diabetes mellitus.

Normal human somatic cells have a finite lifespan in vitro and eventually show irreversible growth arrest called cellular senescence. As cells age in vitro, significant phenotypic changes occur. The cells become flattened and enlarged, as well as expressing different genes such as the tumor suppressor gene p53 and cyclin-dependent kinase inhibitors including p21$^{Wafl/Cip1}$ and p16^{Ink4a}.[1] Signals other than extended proliferation have also been shown to produce a phenotype which is indistinguishable from that of senescent cells at the end of their replicative lifespan.[2] For example, constitutive activation of mitogenic stimuli, DNA damage, or oxidative stress can all prematurely induce cellular senescence.[3-5] In senescent vascular cells, expression of pro-inflammatory molecules is increased and production of vasodilators is decreased,[6-9] both of which are well-known changes in the vascular tissues of elderly persons that contribute to the pathogenesis of vascular aging.[10] There is also in vivo evidence cellular senescence.[11,12] Vascular cells with a similar phenotype to that of senescent vascular cells in vitro have been detected in human atheroma tissues, but not in normal arteries.[6,7] Such reports suggest that cellular senescence may play an important role in human aging and age-associated diseases. Alternatively, the mechanisms underlying cellular senescence may also regulate the lifespan of the entire organism and vice versa. This concept is supported by recent studies on mice that exhibit early onset of phenotypes associated with aging. These include mouse mutants with targeted disruption of the genes that are involved in the repair of DNA damage, such as ku86,[13] XPD[14] and BRCA1.[15] Activation of p53 as well as telomerase-deficiency also cause premature aging,[16,17] which is characterized by reduced longevity, osteoporosis, organ atrophy and a diminished stress tolerance. All of these molecules have been implicated in cellular senescence. More importantly, cellular senescence has been detected in the tissues of mice with premature aging.[15] These results provide further in vivo evidence that links cellular senescence to aging of the organism.

Restriction of calorie intake is known to extend longevity in organisms ranging from yeast to mice and to prevent age-dependent deterioration such as cancer, impaired immune function, and increased inflammation.[18,19] Calorie restriction decreases the plasma levels of glucose, insulin, and insulin-like growth factor-1 (IGF-1). Recent genetic analyses have demonstrated that reduction-of-function mutations in the glucose or IGF-1-like signaling pathways also extend the lifespan of many organisms, suggesting that these pathways may underlie the mechanism of longevity related to calorie restriction.[18,20] Among the molecules that exist in these pathways, the Ser/Thr kinase Akt is remarkably well conserved across a broad range of species and is involved in a diverse array of cellular processes.[21] Although mutations that reduce Akt activity prolong the longevity of yeast and nematodes,[22,23] activation of Akt has been reported to induce proliferation and survival of mammalian cells, thereby promoting tumorigenesis.[21,24,25] In a study that was recently published in *EMBO J*,[26] we showed that Akt activity increases with cellular senescence and that inhibition of Akt extends the lifespan of primary cultured human endothelial cells.
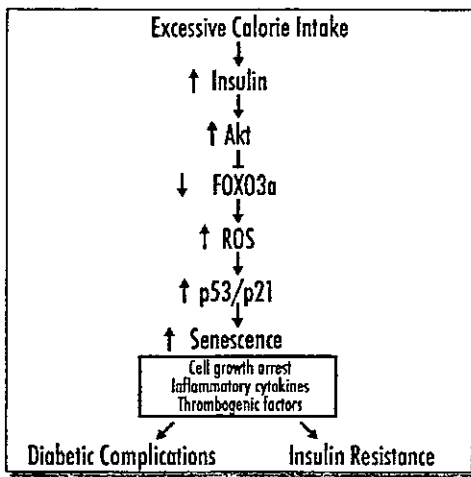
Figure 1. Signaling pathway of insulin/Akt-induced senescence. Excessive calorie intake increases the levels of insulin and results in activation of Akt in the target tissues. Constitutive activation of Akt induces cellular senescence via a p53/p21-dependent pathway, which is mediated by the forkhead transcription factor (FOXO3a), which regulates cellular levels of reactive oxygen species. Accumulation of senescent cells in the target organs causes diabetic complications and promotes insulin resistance because of their senescent phenotypes such as increased production of inflammatory cytokines and thrombogenic factors.

Constitutive activation of Akt promotes senescence-like arrest of cell growth via a p53/p21-dependent pathway. This action is at least partly mediated by the forkhead transcription factor, which regulates cellular levels of reactive oxygen species. Our findings reveal a novel role of Akt in regulating the cellular lifespan and suggest that the mechanism of longevity is conserved in primary cultured human cells.

What is the implication of these findings for human disease? Since various growth factors that contribute to atherosclerosis have been shown to increase Akt activity,[27] atherogenic stimuli may activate Akt in the vasculature and thus promote atherogenesis. Consistent with this notion, activation of Akt is observed in human atheroma tissues, but not in normal arteries.[26] Constitutive activation of Akt in human endothelial cells not only leads to cellular senescence but also to vascular dysfunction such as impaired angiogenesis and increased inflammation.[26] In a similar way, Akt-induced senescence may contribute to other age-associated diseases. Cell division is essential for the survival of multicellular organisms that contain various renewable tissues, but it also puts the organism at risk of developing cancer. Thus, complex organisms have evolved at least two cellular mechanisms to prevent oncogenesis, which are apoptosis and cellular senescence.[2] In this respect, Akt-induced senescence can be seen as an anti-tumorigenesis mechanism since gain-of-function mutations in the Akt signaling pathway are common in human cancers. Finally, Akt is known to be involved in signaling pathways that mediate the metabolic effects of insulin in several physiologically important target tissues.[28] Insulin regulates energy metabolism after food intake by promoting the uptake and storage of glucose, amino acid and fat, while simultaneously antagonizing the catabolism of fuel reserve. The basic role of insulin as a signal that informs the organism of nutritional abundance appears to be well conserved among species.[28] We have shown that insulin also increases p53 activity and expression of p21 and that it promotes cellular senescence in an Akt-dependent manner.[26] Since hyperinsulinemia is a basic feature of type 2 diabetes

mellitus, insulin-induced senescence may have the direct impact on diabetic complications such as vasculopathy and nephropathy. An inevitable feature of type 2 diabetes mellitus is insulin resistance.[28] Some previous studies have suggested that a decline of Akt activity in response to insulin might lead to insulin resistance.[29] In contrast, some recent studies have shown that basal Akt activity in the tissues of diabetic patients tends to be higher than in normal subjects and that there is no significant difference in the response of Akt to insulin stimulation.[30] Increased plasma and tissue levels of pro-inflammatory cytokines and pro-thrombogenic factors have been demonstrated to exaggerate insulin resistance and to contribute to diabetic complications,[31,32] and both of these changes are well-known features of senescent cells in vitro.[12] Accordingly, we propose that type 2 diabetes mellitus can be regarded as a premature aging syndrome in which the dysregulation of insulin/Akt signaling promotes cellular senescence, leading to various complications. This suggests that anti-senescence therapy might be effective for the treatment of diabetic complications and insulin resistance.

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ARTICLES

Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II

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The angiotensin II type 1 (AT1) receptor has a crucial role in load-induced cardiac hypertrophy. Here we show that the AT1 receptor can be activated by mechanical stress through an angiotensin-II-independent mechanism. Without the involvement of angiotensin II, mechanical stress not only activates extracellular-signal-regulated kinases and increases phosphoinositide production *in vitro*, but also induces cardiac hypertrophy *in vivo*. Mechanical stretch induces association of the AT1 receptor with Janus kinase 2, and translocation of G proteins into the cytosol. All of these events are inhibited by the AT1 receptor blocker candesartan. Thus, mechanical stress activates AT1 receptor independently of angiotensin II, and this activation can be inhibited by an inverse agonist of the AT1 receptor.

Cardiac hypertrophy is not only an adaptational state before cardiac failure, but also an independent risk factor of major cardiac events[1]. It is thus very important to understand the molecular mechanism that underlies the development of cardiac hypertrophy. Although various humoral factors such as vasoactive peptides, catecholamines, cytokines and growth factors can contribute to the development of cardiac hypertrophy during the increase in haemodynamic load, the initial stimulus mechanical stress is the most important contributory factor[2].

To dissect the mechanism of how mechanical stress induces cardiac hypertrophy, we and others have developed an *in vitro* device by which stretch stimuli can be imposed on cultured cardiomyocytes[3,4]. Mechanical stretch induces the activation of many protein kinases including extracellular-signal-regulated kinases (ERKs), reprogramming of gene expression, and cardiomyocyte hypertrophy in cultured cardiomyocytes[5]. Pretreatment of cardiomyocytes with angiotensin II (AII) type (AT1) receptor blockers significantly attenuates all of these mechanical-stretch-induced events[6,7]. Furthermore, many animal and clinical studies have shown that AT1 receptor blockers induce regression of cardiac hypertrophy and prevent progression of heart failure, resulting in a reduction in cardiac morbidity and mortality[8–12].

These basic and clinical studies have suggested that the local renin–angiotensin system (RAS) is activated by haemodynamic overload and that the AT1 receptor has a crucial role in the development of load-induced cardiac hypertrophy; however, it

remains unclear how the AT1 receptor is activated by mechanical stress. It has been reported that AII is stored in cardiomyocytes and that mechanical stretch induces the secretion of stored AII into the culture medium, resulting in the induction of cardiomyocyte hypertrophy by the autocrine mechanism[6]. Here we have examined the possibility that mechanical stress can directly activate the AT1 receptor without the involvement of AII.

RESULTS
Marginal roles of endogenous AII in cardiomyoctes
Mechanically stretching cardiomyocytes by 20% for 8 min activated ERKs, and this activation was significantly inhibited by an AT1 receptor blocker, candesartan, as reported previously[7] (Fig. 1a, lanes 2 and 4). The magnitude of the stretch-induced activation of ERKs was the same as that induced by exposing the cardiomyocytes to 10^{-8} to 10^{-7} M AII (Fig. 1a, lanes 7, 8). These results indicate that the AT1 receptor is crucially involved in the stretch-induced activation of ERKs in cardiomyocytes and suggest that AII is secreted from cardiomyocytes into the culture medium by stretch.

We first used radioimmunoassay to measure AII in culture medium conditioned by stretching cardiomyocytes for 8 min, but did not detect a significant increase in AII concentration after stretch (AII without stretch, $0.7 \pm 1.6 \times 10^{-12}$ M; AII with stretch, $2.0 \pm 3.5 \times 10^{-12}$ M; not significant; Fig. 1b). We then carried out a bioassay using human embryonic kidney 293 cells expressing the

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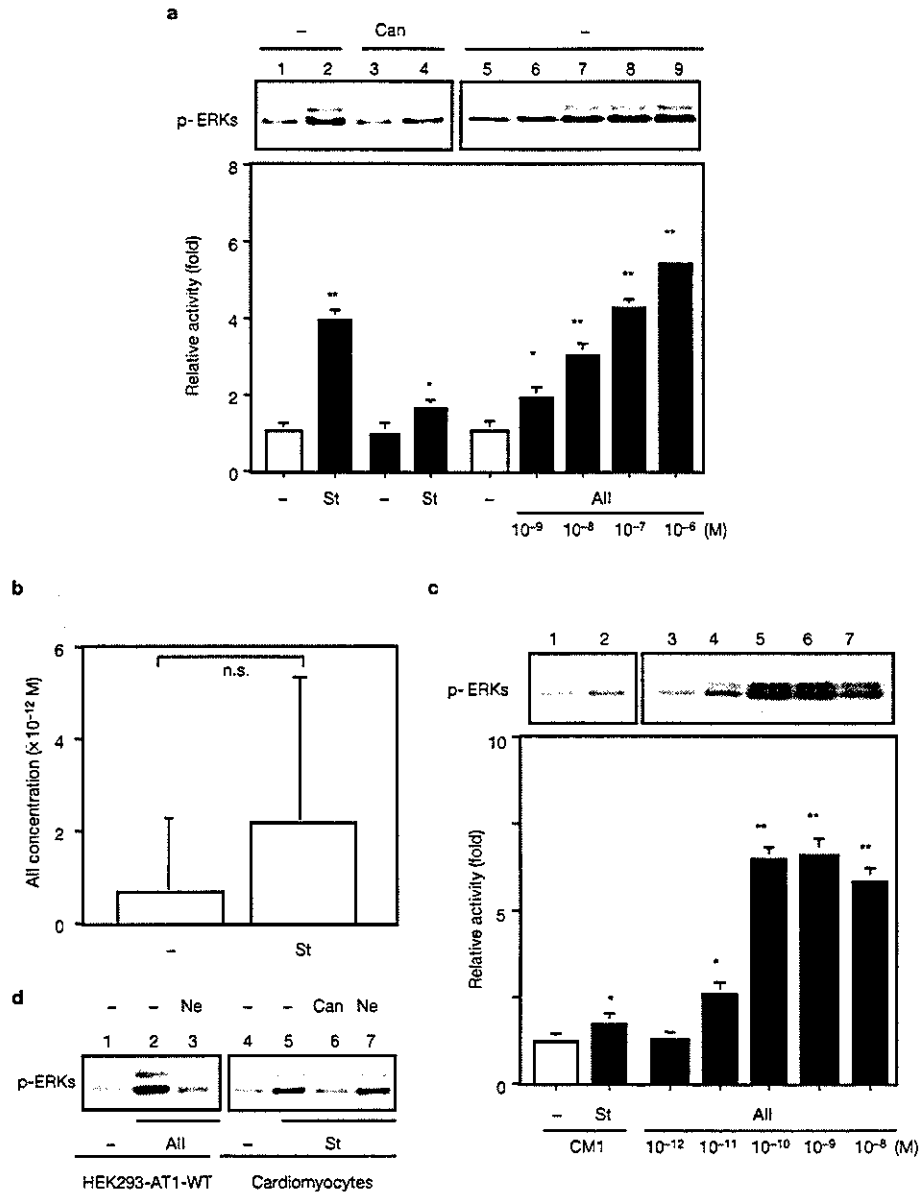
Figure 1 Activation of ERKs by mechanical stretch, AII and conditioned medium. (a) Cultured cardiomyocytes of neonatal rats were pretreated with 10^{-7} M candesartan (Can) or vehicle (–), and either stretched by 20% (St) or incubated with the indicated concentrations of AII for 8 min. Activation of ERKs was determined by using antibodies against phosphorylated ERKs (p-ERKs). The relative kinase activities of an ERK of relative molecular mass 42,000 (M_r 42K) were determined by scanning each band with a densitometer. Results are shown as the fold increase in activity over unstimulated cells and are the mean ± s.e.m. of three independent experiments. $*P < 0.05$, $**P < 0.01$ versus vehicle.

(b) The culture medium from cardiomyocytes conditioned by stretch (St) or no stretch (–) was measured for AII by radioimmunoassay. Results are the mean ± s.e.m. of six samples. n.s., not significant. (c) HEK293-AT1-WT cells were exposed to conditioned medium collected before (–) or after stretching (St) cardiomyocytes for 8 min (CM1) or to the indicated concentrations of AII. Relative kinase activities of the 42K ERK were determined as in a. $*P < 0.05$, $**P < 0.01$, as compared with vehicle. (d) HEK293-AT1-WT cells or cardiomyocytes were subjected to AII or stretch for 8 min without (–) or with (Ne) the presence of a neutralizing antibody to AII (10 μg ml^{-1}; Cortex Biochem).
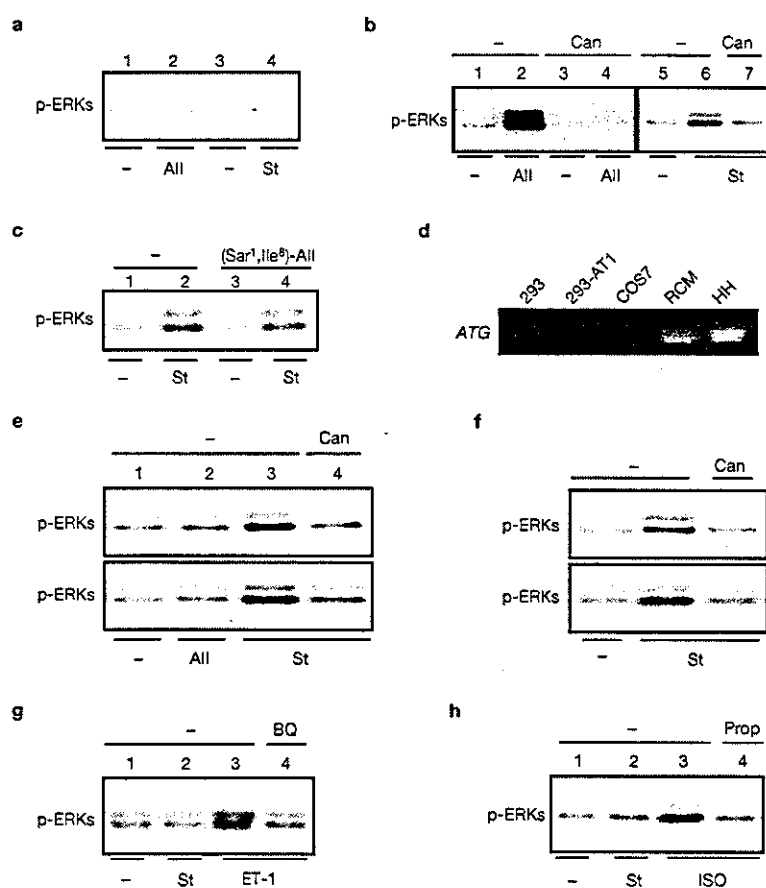
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Figure 2 All-independent activation of ERKs by mechanical stretch in cells overexpressing AT1 receptors. (a) HEK293 cells were stretched by 20% (St) or exposed to 10^{-7} M All for 8 min. (b) HEK293-AT1-WT cells were stimulated by mechanical stretch or All in the absence (-) or presence of 10^{-7} M candesartan (Can). (c) HEK293-AT1-WT cells were stretched in the absence (-) or presence of 10^{-7} M (Sar^1,Ile^8)-All. (d) Expression of the *ATG* gene analysed by RT-PCR. 293, HEK293 cells; 293-AT1, HEK293-AT1-WT cells; RCM, cardiomyocytes of neonatal rats; HH, human heart. (e) HEK293 (top) and COS7 cells

(bottom) were transiently transfected with AT1-mut1, which does not bind to All. Cells were stimulated with mechanical stretch or All without (-) or with candesartan (Can). (f) Cardiomyocytes prepared from neonatal (top) and adult (bottom) *ATG*$^{-/-}$ mice were pretreated with candesartan, and then stimulated with mechanical stretch. (g, h) COS7 cells transiently transfected with ET1A (g) or β2-AR (h) receptors were pretreated with an ET1A antagonist BQ123 (BQ), a β-AR blocker propranolol (Prop) or vehicle (-), and then stimulated with mechanical stretch, ET-1 or ISO.

wild-type mouse AT1a receptor (hereafter denoted HEK293-AT1-WT cells). The medium conditioned by stretching cardiomyocytes for 8 min only slightly activated ERKs in HEK293-AT1-WT cells (Fig. 1c, lane 2). Its activity was equivalent to that induced by incubating HEK293-AT1-WT cells with 10^{-12} to 10^{-11} M All (Fig. 1c, lanes 3 and 4), a value that was similar to the concentration of All measured in the medium by radioimmunoassay. These results suggest that All, even if secreted, would not be enough to induce the full activation of the AT1 receptor observed after stretch.

To confirm whether secreted All has a marginal role in the stretch-induced activation of ERKs, we blocked All activity with a neutralizing antibody to All. Although the antibody abolished the activation of ERKs induced by 10^{-7} M All (Fig. 1d, lane 3), it did not significantly suppress the stretch-induced activation of ERKs (Fig. 1d, lane 7).

Stretch activates ERKs through the AT1 receptor without All

Neither mechanical stretch nor All (10^{-7} M) activated ERKs in HEK293 cells (Fig. 2a). In HEK293-AT1-WT cells, however, both mechanical stretch and All activated ERKs (Fig. 2b, lanes 2 and 6). Pretreatment with the AT1 receptor blocker candesartan inhibited the activation of ERKs induced not only by All (Fig. 2b, lane 4) but also by mechanical stretch in HEK293-AT1-WT cells (Fig. 2b, lane 7). Basal ERK activity was also decreased by candesartan (Fig. 2b, lane 3) in HEK293-AT1-WT cells, and similar results were obtained in COS7 cells transiently expressing AT1-WT.

Mechanical stretch activated ERKs in COS7 cells expressing AT1-WT (see Supplementary Information Fig. S1) but not in COS7 cells (data not shown). Candesartan also reduced the basal and the stretch-enhanced ERK activities in these cells (see Supplementary Information Fig. S1). However, a competitive inhibitor for All, (Sar^1,Ile^8)-All, did
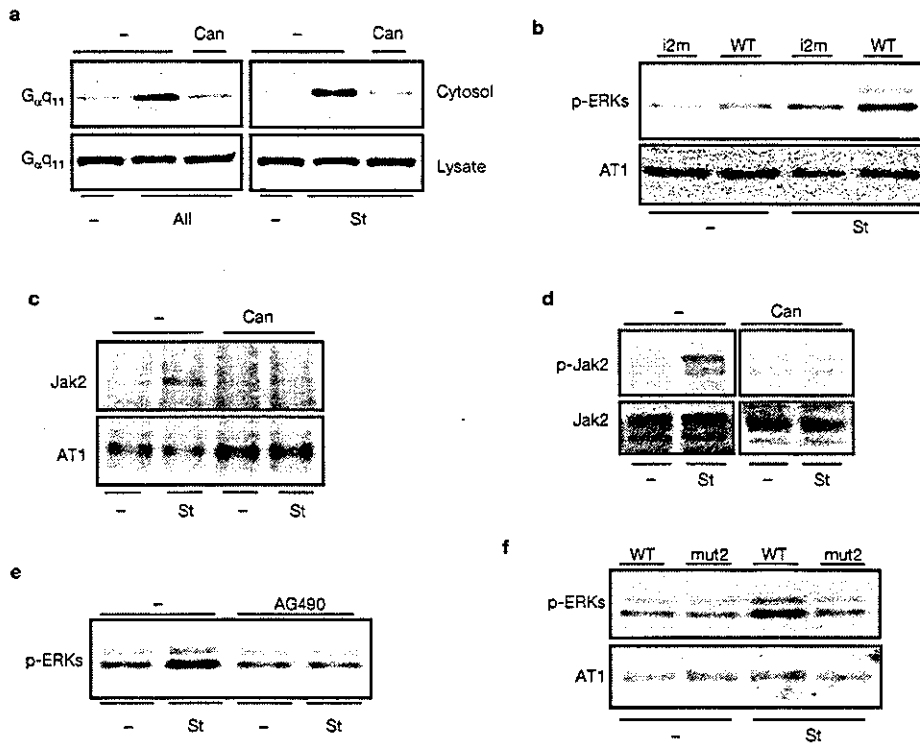
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Figure 3 Activation of G proteins and Jak2 by mechanical stretch, and their effects on ERK activation. (a) HEK293T-AT1-WT cells, pretreated with candesartan (Can) or vehicle (–), were stimulated with AII or mechanical stretch (St) for 5 min. Aliquots of the cytosol (top) and whole lysates (bottom) were resolved by SDS–PAGE. Blotted membranes were incubated with an antibodies against $G_{\alpha}q_{11}$. (b) COS7 cells were transfected with AT1-i2m (i2m), which lacks a binding domain for G proteins, or the wild-type AT1 receptor (WT), and stretched for 8 min (top). Receptor transfection was verified by an antibody against the AT1 receptor (bottom). (c) HEK293T-AT1-WT cells, pretreated with candesartan (Can) or vehicle (–), were subjected to mechanical stretch for 5 min. Total cell lysates were immunoprecipitated by an antibody against the AT1 receptor and the immunocomplexes were subjected to immunoblotting with antibodies against Jak2 (top) or the AT1 receptor (bottom). (d) HEK293T-AT1-WT cells, pretreated with candesartan (Can) or vehicle (–), were subjected to mechanical stretch. Blotted membranes were incubated with antibodies against phosphorylated Jak2 (top) or total Jak2 (bottom). (e) HEK293T-AT1-WT cells, pretreated with tyrphostin AG490 or vehicle (–), were stretched by 20% for 8 min. (f) COS7 cells were transfected with AT1-mut2 (mut2), which lacks a binding domain for Jak2, or the wild-type AT1 receptor (WT), and stimulated by mechanical stretch for 8 min (top). Receptor transfection was verified by an antibody against the AT1 receptor (bottom).

not attenuate stretch-induced ERK activation in HEK293-AT1-WT cells (Fig. 2c, lane 4), although this inhibitor abolished AII-induced ERK activation in the same cells (data not shown).

These results indicate that expression of the AT1 receptor provides HEK293 and COS7 cells with the ability to respond to stretch, for which there might be two possible mechanisms. First, AII is stored in these cells and secreted by stretch, but the cells do not respond to secreted AII because they lack the AT1 receptor. Second, the AT1 receptor is activated by stretch without the involvement of AII.

The medium conditioned by stretching HEK293-AT1-WT cells did not activate ERKs in HEK293-AT1-WT cells (see Supplementary Information Fig. S2). Analysis by polymerase chain reaction with reverse transcription (RT–PCR) did not detect the transcript of the angiotensinogen gene (*ATG*) in HEK293, COS7 or HEK293-AT1-WT cells even after 50 cycles, although it was detected in cardiomyocytes of neonatal rats and human hearts (Fig. 2d). Taken together, these results suggest that mechanical stretch activates the AT1 receptor in both HEK293-AT1-WT cells and COS7 cells expressing the AT1 receptor without the secretion of AII.

To confirm whether or not AT1 receptor is activated by mechanical stretch without involving its ligand, we stretched HEK293 and COS7 cells expressing an AT1 receptor whose binding site for AII was mutated by the replacement of Lys 199 with glutamine (AT1-mut1)[13]. AII did not activate ERKs in cells expressing AT1-mut1 (Fig. 2e, lane 2), indicating a lack of coupling between the mutated receptor and AII. In the same AT1-mut1 cells, however, ERKs were strongly activated by mechanical stretch, and this activation was inhibited by candesartan (Fig. 2e, lanes 3 and 4).

To confirm further activation of the AT1 receptor by mechanical stretch without the involvement of AII, we stretched cardiomyocytes prepared from *ATG*-deficient (*ATG*^{−/−}) mice, in which AII is not detected[14]. Mechanical stress activated ERKs in the cardiomyocytes prepared from both neonatal (Fig. 2f, top) and adult *ATG*^{−/−} mice (Fig. 2f, bottom). Pretreatment of these cells with candesartan markedly suppressed the activation of ERKs (Fig. 2f). These results indicate that mechanical stress may activate ERKs in cardiomyocytes through the AT1 receptor even in the absence of AII.
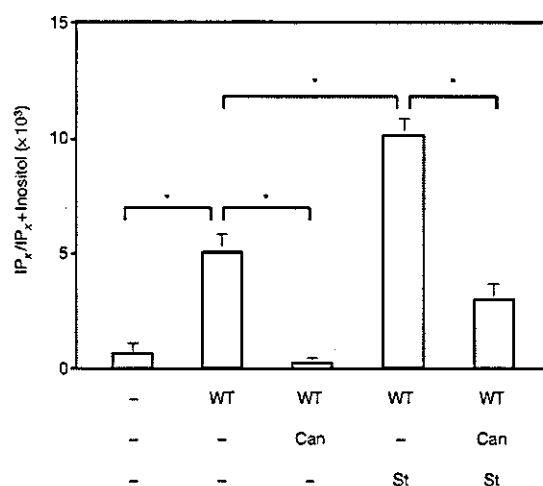
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Figure 4 Mechanical-stretch-stimulated production of inositol phosphates through the AT1 receptor. COS7 cells (−) or COS7 cells transfected with AT1-WT (WT) were labelled with myo-[^{3}H]inositol 24 h after transfection. After 24 h of labelling, cells were incubated with vehicle (−) or candesartan (Can) for 5 h at 37 °C. The accumulation of inositol phosphates was measured as described in Methods. Some COS7 cells transiently transfected with AT1-WT (WT) were subjected to stretching (St) for 45 min or no stimulus (−) in the presence of 5 mM LiCl. $^*P < 0.05$.

The AT1 receptor is a guanine-nucleotide-binding protein (G-protein)-coupled receptor (GPCR), a member of a large family of cell-surface receptors that contain common structural features characterized by seven transmembrane helices essential for signal transduction[15–17]. Activation of other GPCRs, such as the receptors of endothelin 1 (ET-1) and catecholamines, also induces cardiomyocyte hypertrophy[18,19]. We therefore tested whether mechanical stretch can activate these receptors in a ligand-independent manner. We stretched COS7 cells overexpressing either the wild-type ET-1 type A (ET1A) receptor (Fig. 2g) or the wild-type β2-adrenoceptor (β2-AR; Fig. 2h). Whereas ET-1 and isoproterenol (ISO) activated ERKs, mechanical stretch did not evoke significant activation of ERKs in these transfected cells. These results suggest that the activation of GPCRs by mechanical stretch without the involvement of their ligands is not a general phenomenon but specific to some GPCRs including the AT1 receptor.

G proteins and Jak2 are activated by stretch

As a member of the GPCR family, the AT1 receptor evokes intracellular signals through G proteins[20,21]. We therefore examined whether mechanical stress could activate G proteins through the AT1 receptor. Stimulation with either AII or mechanical stretch induced the redistribution of $G_\alpha q_{11}$ subunits into the cytosol of HEK293-AT1-WT cells and this redistribution was inhibited by pretreatment with candesartan (Fig. 3a), suggesting that $G_\alpha q_{11}$ is activated by mechanical stretch as well as by AII.

To determine whether an interaction between the AT1 receptor and G proteins has a role in the activation of ERKs, we transfected an AT1 receptor mutant that does not couple to G proteins (AT1-i2m)[22] into COS7 cells. Activation of ERKs by mechanical stretch was weaker in the AT1-i2m-transfected cells than in those overexpressing AT1-WT (Fig. 3b), suggesting that coupling of G proteins to the AT1 receptor is partly involved in the stretch-induced activation of ERKs.

Non-receptor-type tyrosine kinases such as the Janus kinase (Jak) family and the Src family may be important in AT1 receptor

signalling[22,23]. The AT1 receptor activates the Src–Ras–ERK pathway independently of G-protein coupling through the association and activation of Jak2 (refs 22,24,25). Mechanical stretch induced association of Jak2 with the AT1 receptor (Fig. 3c) and phosphorylation of Jak2 (Fig. 3d) in HEK293-AT1-WT cells. Pretreatment of the cells with candesartan significantly suppressed association with the AT1 receptor and phosphorylation of Jak2 (Fig. 3c, d). Mechanical stretch did not activate ERKs in HEK293-AT1-WT cells that had been pretreated with AG490, a specific inhibitor of Jak2 (Fig. 3e), or in COS7 cells expressing an AT1-mutant (AT1-mut2)[25] that lacks a binding domain for Jak2 (Fig. 3f). These results suggest that activation of Jak2 is crucially involved in the stretch-induced activation of ERKs.

Mechanical stretch upregulates inositol phosphates

To identify other stretch-induced events, we examined the accumulation of inositol phosphates in COS-7 cells expressing AT1-WT. Overexpression of AT1-WT resulted in a roughly fivefold increase in basal inositol phosphates, as compared with untransfected cells (Fig. 4). Mechanical stretch of these AT1-WT-expressing cells further upregulated inositol phosphate production by about twofold (Fig. 4). Stretching the parental COS7 cells did not increase inositol phosphate production (data not shown). Candesartan inhibited the accumulation of inositol phosphates in COS7 cells expressing AT1-WT, as well as the stretch-induced increase in inositol phosphate production (Fig. 4).

Load-induced cardiac hypertrophy through AT1 receptor

We examined whether mechanical stress could induce cardiac hypertrophy *in vivo* through the AT1 receptor in the absence of AII. We imposed a pressure overload on the heart by constricting the transverse aorta (TAC) of adult male $ATG^{-/-}$ mice. Pressure overload for 2 weeks induced significant hypertrophy in the heart of the $ATG^{-/-}$ mice (Fig. 5a, b). Heart weight was increased from 110 ± 12 mg to 189 ± 14 mg after 2 weeks of pressure overload (Fig. 5b).

Although treatment with candesartan did not reduce blood pressure in the right carotid artery (sham operated, 78 ± 10 mmHg; TAC plus saline, 166 ± 15 mmHg; TAC plus candesartan, 160 ± 17 mmHg), the development of cardiac hypertrophy was significantly attenuated by candesartan (heart weight, 145 ± 21 mg; Fig. 5). These results suggest that mechanical stress can induce cardiac hypertrophy *in vivo* by activating the AT1 receptor without the involvement of AII.

DISCUSSION

Many basic and clinical studies have shown that RAS is crucially involved in the development of various cardiovascular diseases[9–12]. Much evidence has indicated that RAS exists in various organs, as well as in the circulation, and that local RAS has an important role in organ damage including cardiac hypertrophy[26,27]. All components of RAS, such as angiotensinogen, renin, angiotensin-converting enzyme and receptors, are present in the heart[26,27], and AII induces hypertrophy of cultured cardiomyocytes[27]. It has been reported that AII is stored in cardiomyocytes and that mechanical stretch induces the secretion of stored AII into the culture medium, resulting in the induction of cardiomyocyte hypertrophy by the autocrine mechanism[6]. Taking these observations together, haemodynamic overload has been thought to promote cardiac hypertrophy by inducing the secretion of AII in the heart. In this study, however, we have shown that mechanical stress can induce cardiomyocyte hypertrophy both *in vitro* and *in vivo* through the AT1 receptor without the involvement of AII.

Although mechanical stretch has been reported to induce the release of endogenous AII from cardiomyocytes[6], by radioimmunoassay we did not detect a significant increase in AII in the cul-
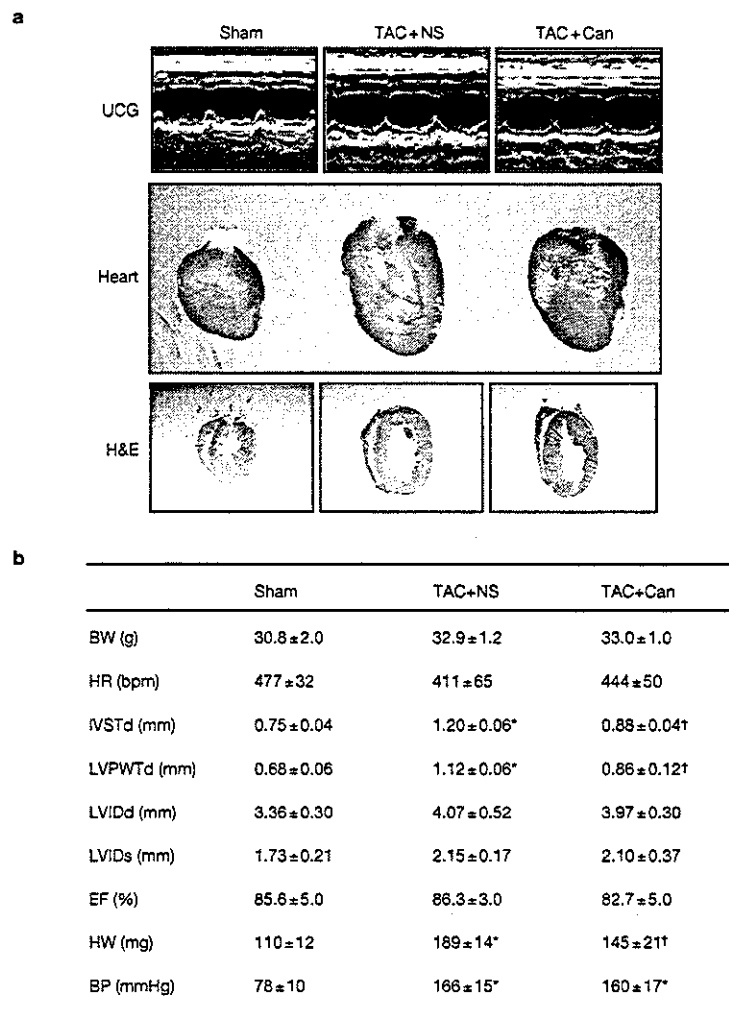
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a

b

	Sham	TAC+NS	TAC+Can
BW (g)	30.8±2.0	32.9±1.2	33.0±1.0
HR (bpm)	477±32	411±65	444±50
IVSTd (mm)	0.75±0.04	1.20±0.06*	0.88±0.04†
LVPWTd (mm)	0.68±0.06	1.12±0.06*	0.86±0.12†
LVIDd (mm)	3.36±0.30	4.07±0.52	3.97±0.30
LVIDs (mm)	1.73±0.21	2.15±0.17	2.10±0.37
EF (%)	85.6±5.0	86.3±3.0	82.7±5.0
HW (mg)	110±12	189±14*	145±21†
BP (mmHg)	78±10	166±15*	160±17*

Figure 5 Cardiac hypertrophy in $ATG^{-/-}$ mice induced by pressure overload. Ten-week-old male $ATG^{-/-}$ mice, treated with saline (NS) or candesartan (Can), were subjected to a sham or TAC operation. Echocardiography and catheterization were done 2 weeks later. (a) Top, M-mode echocardiograms; middle, gross appearance of the heart; bottom, sections stained by H&E. (b) Echocardiographic results and haemodynamic parameters, shown as the mean ± s.e.m. ($n = 3$). *$P <$ 0.05 versus sham operated; †$P <$ 0.05 versus saline. BW, body weight; HR, heart rate; IVSTd, thickness of interventricular septum at diastole; LVPWTd, posterior wall thickness of LV at diastole; LVIDd and LVIDs, LV internal dimension at end diastole and systole, respectively; EF, ejection fraction; HW, heart weight; BP, systolic blood pressure.

ture medium after stretch. AII concentration varied considerably in the conditioned media, whereas the degree of ERK activation induced by stretch was constant, which also suggests that secreted AII is not involved in the stretch-induced activation of ERKs. A very sensitive bioassay, as well as radioimmunoassay, showed that the concentration of AII in medium conditioned by stretch was less than 10^{-11} M, which is far too low to evoke a hypertrophic response in cardiomyocytes. The reason for the difference in the role of secreted AII by stretch between the previous report[6] and this study is not clear at present. Many reports have shown that cardiomyocytes contain about 10^{-13} mol of AII per gram of cells[28–30]. Thus, even if all of the AII stored in cardiomyocytes were secreted by stretch, at most about 10^{-11} M AII would be detected in the culture medium. We detected roughly 2×10^{-12} M AII in culture medium conditioned by stretch for 8 min, consistent with the theoretical concentrations.

Although it is evident that mechanical stress is the primary trigger of cardiac hypertrophy, it is not clear how mechanical stress is received and converted into the active intracellular signalling responsible for the development of cardiac hypertrophy. Muscle LIM protein, integrins and their associated signalling machinery have been reported to be sensors for mechanical stress[31,32]. We propose that the AT1 receptor is also a receptor for mechanical stress. Mechanical stretch did not activate ERKs in HEK293 cells or COS7 cells, but expression of the AT1 receptor gave these cells the ability to respond to stretch. Bioassays using conditioned medium and RT–PCR analysis showed that there is little or no AII in HEK293 cells or COS7 cells. These results suggest that the AT1 receptor is a 'mechanical sensor' and converts mechanical stress into biochemical signals inside the cells.

This hypothesis was confirmed by results from cells expressing the AT1-mut1, which cannot bind AII, and from cardiomyocytes pre-

pared from $ATG^{-/-}$ mice. Although AII did not activate ERKs in cells expressing AT1-mut1, mechanical stretch activated ERKs in these cells and this activation was inhibited by an AT1 receptor blocker, candesartan. Mechanical stretch activated ERKs in cardiomyocytes prepared from $ATG^{-/-}$ mice, which do not express AII, and candesartan inhibited this activation.

Because mechanical stretch did not activate ERKs in cells expressing the ET1A or β2-AR receptors, not all GPCRs are necessarily a mechanical sensor. Although we do not know at present why the AT1 receptor, but not the ET1A or β2-AR receptor, is significantly activated by mechanical stretch, there are a few possibilities. First, specificity may be due to molecules that associate with the AT1 receptor. We found that in the response to mechanical stretch, some unknown molecules bind to the AT1 receptor (our own unpublished results). Second, diversity in the structures and expression of the receptors may also determine their responsibility to mechanical stress[16,27,33].

After binding to AII, the AT1 receptor changes its conformation into an active form and stimulates G proteins through its intracellular domains[15,20,33,34]. The intracellular loops and the region between residues 312 and 318 in the carboxy-terminal tail of the AT1 receptor have been reported to be essential for coupling and activating G proteins[35]. After activation, G proteins dissociate into α- and βγ-subunits, and the α-subunit is translocated into the cytosol[34]. In addition, ligand binding to the AT1 receptor induces association of the C terminus of the AT1 receptor with Jak2, thereby resulting in activation of the Jak2–STAT3 pathway[22–25]. Although there is no direct evidence, our results suggest that, similar to AII binding to the AT1 receptor, mechanical stress induces a conformational change in the AT1 receptor by a mechanism independent of AII binding, resulting in the association and activation of G proteins and Jak2.

There are a few mechanisms by which mechanical stress might activate the AT1 receptor without the involvement of AII. First, stretching the cell membrane may directly change the conformation of the AT1 receptor. Many receptors can change their conformation between the active and inactive state under basal conditions without ligands[36,37]. Candesartan reduced the basal activity of ERKs, suggesting that part of the wild-type AT1 receptor is in an active state, and mechanical stress may increase the number of AT1 receptors in the active state. Second, mechanical stretch might activate specific mechanical sensors, which then activate the AT1 receptor from inside the cells. Potential stretch sensors, such as muscle LIM protein, integrins and stretch-sensitive ion channels, might activate the AT1 receptor, though the underlying mechanism remains to be determined.

Strong pressure overload induced cardiac hypertrophy in $ATG^{-/-}$ mice, indicating that mechanical stress can induce cardiac hypertrophy without AII. Treatment with candesartan significantly attenuated the development of cardiac hypertrophy without reducing blood pressure, suggesting that mechanical stress activates the AT1 receptor and induces cardiac hypertrophy without the involvement of AII *in vivo*. We previously reported that pressure overload induces cardiac hypertrophy in AT1a receptor knockout mice[38]. The activity of tyrosine kinases is upregulated before stretch and more strongly enhanced by mechanical stretch in AT1a-receptor-deficient cardiomyocytes as compared with wild-type cells through epidermal growth factor (EGF) receptor tyrosine kinases[39], suggesting that the AT1 receptor is not indispensable for stretch-induced cardiac hypertrophy and that some compensatory mechanisms operate and induce cardiac hypertrophy even in the absence of the AT1 receptor[38,39].

Candesartan reduced the basal activity of ERKs and inositol phosphates in cells overexpressing AT1-WT and inhibited the

stretch-induced activation of ERKs and increase in inositol phosphates independently of AII, suggesting that candesartan works as an inverse agonist of the AT1 receptor. An inverse agonist of the AT1 receptor is defined as an agent that stabilizes the AT1 receptor in an inactive conformation, thereby inhibiting signals evoked by the wild-type or active AT1 receptor. As an inverse agonist, candesartan may inhibit changes in conformation of AT1 receptor and thus may efficiently suppress its activation induced by both mechanical stress and AII. Much evidence suggests that local RAS has a crucial role in injury to various organs[26,27,40]. It remains to be determined whether activation of the AT1 receptor without AII occurs in other organs, and whether inverse agonists prevent organ damage more effectively than do competitive antagonists. □

METHODS

cDNA constructs. The AT1a receptor mutants lacking binding activity with AII (AT1-mut1) or the Jak2 coupling domain (AT1-mut2) were generated by PCR from the wild-type mouse AT1 receptor (GenBank accession number S37484)[41] by replacement of Lys 199 with glutamine[13] or truncation of the C terminus (residues 312–359)[22], respectively. AT1-i2m[22], β2-AR[42] and ET1A[43] were gifts from J. Sadoshima, R J. Lefkowitz and S. Kimura, respectively. The complementary DNAs used in this study are summarized in Supplementary Information, Table 1.

Cell culture and transfection. We prepared primary cultures of cardiomyocytes from the ventricles of 1-day-old Wistar rats or $ATG^{-/-}$ mice as described[19]. Adult and neonatal cardiomyocytes of $ATG^{-/-}$ mice were prepared as described[44]. HEK293 and COS7 cells were cultured in Dulbecco's modified Eagle's medium with 10% serum. cDNAs were transfected by the calcium phosphate method as described[19]. Stable transformants were selected by the addition of hygromycin B to the cells 3 d after transfection and for all subsequent passages of the cells[41]. All cultures were transferred to serum-free conditions 48 h before stimulation.

Western blotting. Total proteins (50 μg) were size-fractionated by SDS–PAGE and transferred to Immobilon-P membranes (Millipore). The blotted membranes were incubated with antibodies against phosphorylated ERKs, phosphorylated Jak2, Jak2, AT1 or $G_\alpha q_{11}$ (Santa Cruz).

ATG gene expression. Expression of the ATG gene was examined by RT–PCR using specific primers (sense, 5'-TTCAGGCCAAGACCTCCC-3'; antisense, 5'-CCAGC-CGGGAGGTGCAGT-3')[45]. We separated the PCR products on 1.2% agarose gels and visualized them by using ethidium bromide.

Detection of inositol phosphates. Accumulation of inositol phosphates was assayed in COS7 cells as described[46,47]. In brief, 24 h after transfection by the DEAE–adenovirus method[48], cells were replated in 24-well plates at 1.5×10^5 cells per well and labelled for 24 h with myo-[^{3}H]inositol (2 μCi ml^{-1}; Amersham). The cells were washed in medium containing 5 mM LiCl for 10 min, incubated with vehicle or candesartan for 5 h, and then subjected to mechanical stretch for 45 min in the presence of 5 mM LiCl. Inositol phosphates and total inositol fractions were resolved on a Dowex AG 1-X8 formate column (Bio-Rad), and inositol phosphate accumulation was estimated by determining the ratio of inositol phosphate radioactivity to the sum of inositol phosphate plus inositol radioactivity.

AII in the medium of cultured cardiomyocytes. Culture medium (2 ml per dish) was collected from dishes before and after stretching the cells by 20% for 8 min. We measured AII concentration by radioimmunoassay using two antibodies specific for AII (SRL Co.).

TAC operation. TAC operation was done on 10-week-old male $ATG^{-/-}$ mice and wild-type C52/BL6 mice[14]. A mini-osmotic pump (Alzet) filled with saline or candesartan was implanted subcutaneously in mice 3 d before TAC. All mouse protocols were approved by the guidelines of Chiba University.

Haemodynamic parameters. Transthoracic echocardiography (UCG) was done as reported[38] using a Agilent sonos 4500 (Agilent Technologies Co.) equipped

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with a 11-MHz imaging transducer. Haemodynamic measurements were taken by inserting a micronanometer catheter (Millar 1.4F, SPR 671, Millar Instruments) from the right common carotid artery into the aorta and then the left ventricle (LV). The transducer was connected to the MacLab system (AD Instruments) to record the pressure. For heart morphometry, hearts were perfused with 10% buffered formalin and subsequently embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E).

Statistics. Data are shown as mean ± s.e.m. Multiple group comparison was done by a one-way analysis of variance (ANOVA), followed by the Bonferroni procedure for comparison of means. A two-tailed Student's t-test was used to compare drug-treated and vehicle-treated specimens under identical conditions. Values of $P < 0.05$ were considered statistically significant.

Note: Supplementary Information is available on the Nature Cell Biology website.

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