SIRT1 Promotes Proliferation and Prevents Senescence Through Targeting LKB1 in Primary Porcine Aortic Endothelial Cells

Yi Zu,* Ling Liu,* Mary Y.K. Lee, Cheng Xu, Yan Liang, Ricky Y. Man, Paul M. Vanhoutte, Yu Wang

Rationale: Endothelial senescence causes endothelial dysfunction, promotes atherogenesis and contributes to age-related vascular disorders. SIRT1 is a conserved NAD⁺-dependent deacetylase possessing beneficial effects against aging-related diseases, despite that the detailed functional mechanisms are largely uncharacterized.

Objective: The present study is designed to evaluate the protective effects of SIRT1 on endothelial senescence and to elucidate the underlying mechanisms.

Methods and Results: An in vitro senescence model was established by prolonged culture of primary endothelial cells isolated from porcine aorta. The freshly isolated “young” cells gradually underwent senescence during 1 month of repetitive passages. Both mRNA and protein expressions of SIRT1 were progressively decreased. In contrast, the protein levels of LKB1, a serine/threonine kinase and tumor suppressor, and the phosphorylation of its downstream target AMPK(Thr172) were dramatically increased in senescent cells. Overexpression of LKB1 promoted cellular senescence and retarded endothelial proliferation, which could be blocked by increasing SIRT1 levels. Knocking down of SIRT1 induced senescence and elevated the protein levels of LKB1 and phosphorylated AMPK(Thr172). Regardless of the nutritional status, hyperactivation of AMPK was able to induce endothelial senescence. SIRT1 antagonized LKB1-dependent AMPK activation through promoting the deacetylation, ubiquitination and proteasome-mediated degradation of LKB1. The survival signaling of Akt was also found to be modulated by SIRT1 and LKB1, and could cross-regulate AMPK activity.

Conclusions: SIRT1 and LKB1/AMPK are the 2 key sensor systems for regulating endothelial cell survival, proliferation and senescence. The protective activities of SIRT1 may be achieved at least in part by fine tuning the acetylation/deacetylation status and stabilities of LKB1 protein. (Circ Res. 2010;106:1384-1393.)

Key Words: endothelial senescence ■ SIRT1 ■ LKB1 ■ AMPK ■ acetylation

Endothelium possesses vital functions on regulating vascular homeostasis.1 Under physiological conditions, macrovascular endothelial cells are quiescent and rarely divide, with a turnover rate of approximately once every 3 years.2 Under conditions that cause endothelium injury, such as hypertension, high cholesterol levels and turbulent blood flow, replication of endothelial cells is increased for regenerating the damaged endothelium.3 However, the regenerated endothelial cells usually reach replicative senescence after a finite number of cell replication. Senescent endothelial cells show diminished vasomotor-regulatory activities and elevated expression of proinflammatory molecules, which contribute to the development of age-associated cardiovascular diseases, such as atherosclerosis.4

SIRT1 is a class III deacetylase implicated in a wide range of cellular functions.5 This protein has attracted enormous interest since the discovery of the potential antiaging activities of its yeast homolog, Sir2. In mammalian system, the beneficial activities of SIRT1 against aging diseases, such as those related to neuro- and cardioprotection, have been suggested by many studies.5,6 Cardiovascular aging represents the largest portion of age-related morbidity and mortality. Therefore, it is particularly important to elucidate whether SIRT1 exerts any antiaging effects in the cardiovascular system.
Using affinity chromatography and tandem mass spectrometry analyses, we have identified LKB1, a serine/threonine protein kinase, as a binding partner and potential intracellular target of SIRT1 (Online Figure I, available at http://circres.ahajournals.org). Similar findings have been reported by Lan et al. LKB1 can phosphorylate and activate various signaling molecules involved in cell cycle progression, cell polarity, and chromatin remodeling. One of its downstream targets, AMPK, is a master regulator for energy metabolism. In the present study, using primary endothelial cultures, the interplay between SIRT1 and LKB1 are investigated. The results suggest that LKB1 plays an inhibitory role in endothelial cell proliferation and can promote endothelial senescence. SIRT1 exhibits its protective and antiaging activities at least partly through antagonizing LKB1-mediated AMPK signaling pathways.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Isolation and Culture of Primary Porcine Aortic Endothelial Cells**

The study was approved by the Institutional Committee on the Use of Live Animals in Teaching & Research. The hearts were collected from female pigs (3 to 4 months old; 25 to 30 kg) and aortic endothelial cells collected as described previously. A fixed schedule was adopted for subculturing the cells at a ratio of 1:3 once per week up to 4 weeks (designated as P1, P2, P3, and P4). After transfection, adenoviral infection or drug treatment, the cells were incubated for 2 days before subsequent evaluation. Senescence-associated β-galactosidase (SA-β-gal) staining was performed using Senescence Cells histochemical staining Kit (Sigma). Telomerase activity of primary porcine aortic endothelial cells (PAECs) was determined using TRAPEZE XL Telomerase Detection Kit (Chemicon; Temecula, Calif).

**RNA Interference**

Based on the highly conserved LKB1 sequence regions of human, monkey, bovine, mouse, rat, and dog species, 4 potential small interfering RNA target sites were determined using the Invitrogen design program. The most effective target sequence (GGGACAA-CATCTACAAGCTGTTTGA) (LKB1 RNA interference [RNAi]) was screened out and synthesized. RNAi (100 pmol) was transfected into PAECs using Lipofectamine 2000 (Invitrogen). The oligonucleotide (GGGACCAUCGGGCCUCAGCCCGU) was used as the RNAi control. Downregulation of LKB1 was confirmed by Western blotting.

**Data Analysis**

All the studies were repeated for at least 3 times. Results were presented as means±SEM. Statistical analysis of differences between 2 groups was performed using Student’s t test. Statistical significance was determined at a value of P<0.05. All the results were normalized against and shown as the value of fold change compared to those of the same batch of P1 data set.

**Results**

**SIRT1 Expression Is Downregulated and LKB1 Expression Upregulated in Senescent PAECs**

The characteristics of the primary culture model of endothelial senescence were reported previously. The proliferation rate of PAECs was progressively declined during the passage from P1 to P4 (Online Figure II). After 4 weeks of passage, PAECs almost stopped proliferation and entered a state of dormancy with extremely low levels of telomerase activities and showed severe senescence as revealed by SA-β-gal staining and flow cytometric analysis (Online Figures III and IV). Note that the senescent PAECs were presented as a portion of cells that could not be detected by TUNEL. The percentage of these cells progressively increased during prolonged culture of PAECs. The senescence marker P21 and P16 could be easily detected in P4 but not P1 cells. Real-time quantitative PCR was performed to detect the mRNA levels of SIRT1 and LKB1. The gene expression of LKB1 remained stable from P1 to P4, whereas the mRNA level of SIRT1 gradually decreased during the prolonged culture of PAECs (Figure 1). At P4, the SIRT1 mRNA expression was reduced by more than 70% compared to P1 cells. Western blotting results revealed that the protein level of SIRT1 was also significantly reduced in these cells. After 1 month of culture, the SIRT1 protein amount was less than half of that in P1 cells. On the contrary, LKB1 protein amount was relatively low in “young” P1 cells but sharply upregulated from passage 2. The high level of LKB1 protein was maintained during the subsequent cultures. Phosphorylation of LKB1 at Ser428 was significantly elevated, probably because of the elevation of total LKB1. The results demonstrated an inverse correlation between increased LKB1 and decreased SIRT1 during the progression of endothelial senescence.

**SIRT1 and LKB1 Elicit Opposite Effects on Endothelial Cell Proliferation and Senescence**

To uncover whether there were causal relationships behind the above phenomena, LKB1 was overexpressed in PAECs in the presence or absence of SIRT1 or the deacetylase mutant SIRT1(H363Y) (Figure 2A). Cell growth was monitored by crystal violet staining. Compared to the pcDNA vector (Invitrogen) control group, SIRT1 promoted, whereas LKB1 inhibited the proliferation of PAECs (Figure 2B). On the other hand, SIRT1(H363Y) acted as a dominant-negative inhibitor of endogenous SIRT1 and decreased the proliferation rate of PAECs. SIRT1, but not its mutant, antagonized the inhibitory
effects of LKB1 and restored the proliferative capacity of PAECs close to that of pcDNA control group. When SIRT1(H363Y) was coexpressed with LKB1, the proliferation of these PAECs was further attenuated. Results from SA-/H9252-gal staining (Figure 2C and 2D), as well as flow cytometric analysis (Online Figure V), suggested that SIRT1 alleviated cellular senescence, whereas LKB1 accelerated this process. SIRT1 was able to rescue PAECs from LKB1-induced senescence. Overexpression of SIRT1(H363Y) mutant failed to exert antisenescence function, indicating that the effect of SIRT1 on endothelial senescence was dependent on its deacetylase activity.

**LKB1 Is Downregulated by SIRT1 Through Deacetylation and Proteasome-Mediated Degradation**

As the results demonstrated in Figure 1, during the senescence process, the protein levels of SIRT1 and LKB1 were inversely correlated. In fact, both SIRT1 overexpression and treatment with resveratrol dramatically decreased total LKB1 protein levels (Figures 2A and 2D). On the other hand, inhibition of endogenous SIRT1 by its dominant negative mutant SIRT1(H363Y) and SIRT1 inhibitor nicotinamide resulted in a slightly increased level of LKB1 protein. Because SIRT1 could interact with LKB1 directly, the acetylation status of LKB1 was evaluated by immunoprecipitation experiment. Both Western blotting and radio-labeling with [3H] sodium acetate (data not shown) confirmed that the acetylation of LKB1 protein was reduced by SIRT1 overexpression or resveratrol treatment (Figure 3B). Comparing to PAECs at P1, the amount of SIRT1 interacting with LKB1 was significantly decreased, resulting in an elevated level of acetylated LKB1 in P3 cells. The association between LKB1 and HSP90 was not changed in P3 cells compared to P1 cells (Figure 3C). Treatment with MG132, a specific cell-permeable proteasome inhibitor, largely increased the protein levels of LKB1 in P1 cells (Figure 3D). In P3 cells, the effect of MG132 to increase LKB1 was more prominent when SIRT1 was overexpressed. In the presence of proteasome inhibitor, LKB1 was found to be heavily ubiquitinated in P1 cells, whereas the ubiquitination of this protein decreased dramatically in P3 cells (Figure 3E). Compared to pcDNA transfected cells, SIRT1 overexpression promoted, whereas SIRT1(H363Y) overexpression attenuated ubiquitination of LKB1 in PAECs (Figure 3F). Note that downregulation of SIRT1 by siRNA treatment dramatically increased LKB1 protein levels and AMPK(T172) phosphorylation, which was accompanied by an induction of cellular senescence (Figure 3G and 3H). These findings collectively suggested that SIRT1 could downregulate LKB1 through promoting deacetylation, ubiquitination and proteasome-mediated degradation.

**SIRT1 Counteracts LKB1-Activated AMPK Pathway in PAECs**

The protein level and activation status of AMPK, a major downstream target of LKB1, were checked in PAECs by Western blotting (Figure 4). When PAECs were cultured successively in vitro, phosphorylated AMPK at Thr172 was increased progressively, although the total level was not changed significantly. The ratio of phosphorylated AMPK at Ser485, a site that was believed to have inhibitory effect on AMPK activation, was reduced when calculated against total AMPK (Figure 4). Furthermore, the phosphorylation of acetyl-coenzyme A carboxylase (ACC), the downstream target of AMPK that could serve as an indicator of its activity, was gradually increased during the senescent process. These findings pointed out that AMPK was activated during the senescence process of PAECs.

The effect of SIRT1 on LKB1-induced activation of AMPK was investigated in transiently transfected PAECs (Figure 4). LKB1 overexpression activated AMPK, as indicated by elevated phosphorylations of AMPK(Thr172) and ACC(Ser79). On the contrary, the phosphorylation of AMPK at Thr172 was decreased and the phosphorylation at Ser485 was significantly increased by SIRT1 overexpression. Similarly, treatment with resveratrol, the SIRT1 activator, largely diminished the phosphorylation of AMPK(Thr172) but dramatically augmented the phosphorylation of AMPK at Ser485. Moreover, both SIRT1 overex-

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Figure 1. Expression levels of SIRT1 and LKB1 were inversely correlated during the senescence process of PAECs. A, The mRNA levels of SIRT1 and LKB1 were quantified and compared by real time PCR analysis. B, The protein amounts of SIRT1, total LKB1 (t-LKB1), and phosphorylated LKB1 (p-LKB1, Ser428) were quantified by Western blotting. Actin was used as the internal control for calculating the fold changes. In addition, the comparison of actin levels across different passages of PAECs was also included. *P<0.05 vs P1; n=6.
pression and resveratrol treatment decreased the ACC(Ser79) phosphorylation (Figure 4). Again, the dominant negative effect of SIRT1(H363Y) was observed. The mutant induced phosphorylation of AMPK(Thr172) to a level much higher than those of basal or LKB1-treated samples. Note that these effects could be observed in both serum containing and starvation (data not shown) conditions.

LKB1 Provokes Senescence in PAECs Through Activating AMPK
To verify whether or not SIRT1 exerted its antisenescence activities through targeting LKB1-related pathway, stealth siRNA was used to specifically knockdown the expression levels of LKB1. The results in Figure 5A demonstrated that downregulating LKB1 reversed the senescence phenotype of PAECs and reduced the phosphorylation of AMPK at Thr172, but enhanced the phosphorylation at Ser485 site. The similarity of these effects to those caused by replacement with SIRT1 further suggested that SIRT1 might exert its antisenescence functions through modulating LKB1/AMPK signaling pathway. Moreover, SIRT1 could not further decrease the senescence when LKB1 had already been downregulated (data not shown).

AMPK is the most likely candidate that could mediate the prosenescence effect of LKB1. Therefore, pharmacological activator (AICAR) or inhibitor (compound C), and adenovirus-mediated overexpression system were used to determine whether or not AMPK could induce senescence in PAECs (Figure 5B). Comparing to the respective control samples, treatment with AICAR or the adenoviruses encoding constitutively active AMPK (CA-AMPK) resulted in a remarkably elevated SA-β-gal staining in PAECs. The effects could be observed under both normal culture and serum starvation conditions. Moreover, compound C and dominant negative AMPK could alleviate the senescence of PAECs induced by LKB1 (data not shown). These results confirmed that hyperactivation of AMPK signaling could induce senescence in PAECs.

The phosphorylation of AMPK(Ser485) was reported to be Akt-dependent. Akt is an essential signaling molecule for cell survival and proliferation. The phosphorylation of Akt(Ser473) was dramatically decreased in the senescent P4 cells (Figure 6A). SIRT1 and resveratrol treatment increased Akt(Ser473) phosphorylation in normal cultures (Figure 6A), but not in serum-starved cultures (data not shown). LKB1 overexpression dramatically decreased Akt activity only when endogenous SIRT1 was inhibited by its deacetylase mutant SIRT1(H363Y). Inhibition of Akt by either Akt inhibitor or kinase-dead Akt could induce senescence only when the experiment was performed under serum containing conditions (Figure 6B). These
results largely mirrored the effects of Akt inhibition on AMPK activation (Figure 6C). Akt inhibitor could increase the phosphorylation of AMPK(Thr172) but decrease the phosphorylation of AMPK(Ser485) only in normal culture condition. These results further indicated that hyperactivation of AMPK signaling was the major player in LKB1-induced senescence of PAECs. In fact, dominant-negative AMPK but not constitutively active Akt could antagonize LKB1’s effects on PAECs senescence (data not shown).

**Overexpression of SIRT1 in Endothelial Cells Elicits Protective Effects Against Drug-Induced Vascular Senescence in Mice**

To further confirm the regulation of SIRT1 on LKB1/AMPK signaling in endothelial cells, transgenic mice with endothelial-specific overexpression of SIRT1 or SIRT1(H363Y) were treated with paraquat, a herbicide that could induce vascular senescence.12–14 The SA-/H9252–stained senescent cells were significantly less in SIRT1 transgenic mice (Figure 7A). The vascular senescence in SIRT1(H363Y) mice was much more severe. Importantly, the amount of LKB1 and phospho-AMPK(T172) were significantly lower in SIRT1 transgenic mice (Figure 7B). In addition, decreased SIRT1 expression and elevated LKB1/AMPK levels were also observed in the aorta tissues of old mice by comparing to those in young mice (Figure 7C).

**Discussion**

A number of recent studies have demonstrated the vascular protective activities of SIRT1, especially on endothelial-dependent control of the vascular tone and oxidative stress-induced premature senescence.12–14 SIRT1 is highly expressed in the vasculature during blood vessel growth and disruption of SIRT1 gene expression results in defective blood vessel formation.15 It mediates the effects of calorie restriction on endothelium-dependent control of vasomotor tone by deacetylating endothelial NO synthase and increasing nitric oxide bioavailability.13 Treatment with high glucose reduces the number of endothelial progenitor cells and induces their senescence through a mechanism involving reduced SIRT1 expression and activity.16 Although SIRT1 has been shown to prevent premature senescence of endothelial cells, little information is available on the detailed mechanisms. In the present study, it was found that the mRNA and protein levels of SIRT1 were progressively decreased during the senescence process of PAECs. On the other hand, LKB1, a tumor suppressor, was significantly upregulated in senescent PAECs (Figure 1 and Online Figure II). Overexpression of LKB1 could induce senescence through AMPK activation, whereas SIRT1 antagonized LKB1/AMPK signaling by deacetylating LKB1 and promoting its ubiquitination and degradation (Figures 2 through 6).
vascular defect, possibly attributable to the upregulated expression of vascular endothelial growth factor and the defects of TGFβ-mediated vascular smooth muscle cell recruitment. These limited information suggest that loss of LKB1 increases angiogenic potential at least in certain cell types. Overexpression of LKB1 in HUVEC reduces tube formation and suppresses endothelial angiogenesis. Song et al suggest that reactive nitrogen species induced by hyperglycemia suppresses Akt signaling and triggers apoptosis of endothelial cell by upregulating phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) in an LKB1-dependent manner. On the other hand, there are numerous reports suggesting that LKB1 is a key signaling molecule mediating various cellular stresses (hypoxia-reoxygenation, ischemia, hyperglycemia and shear stress etc)- and pharmacological agents (such as antidiabetic drug metformin and rosiglitazone, and lipid-lowering drug statin)-stimulated AMPK activation in endothelial cells.

Transient activation of LKB1/AMPK signaling protects cells against stress by maintaining energy homeostasis, ensuring a slow consumption of energy storage. However, prolonged overactivation of AMPK can cause irreversible cellular senescence in mammalian systems. Similar to the results in present study (Figure 5 and 6), Bardeesy et al have found that LKB1 deficiency prevents culture-induced senescence in murine embryonic fibroblasts. In senescent fibroblasts, AMP:ATP ratios are 2- to 3-fold higher than those of young fibroblasts, and senescence is accompanied by a marked elevation in AMPK activity.

In mice, caloric restriction downregulates AMPK activity in the liver. Activation of LKB1/AMPK and inhibition of mTOR (mammalian target of rapamycin) contribute to the premature aging phenotype of Zmpste24−/− mice. AMPK hyperactivation has also been reported in the skeletal muscle and liver of old rodents. In aorta of old mice, LKB1 and phosphorylated AMPK (Thr172) levels are much higher and the expression of SIRT1 is significantly lower than those of young mice (Figure 7). Moreover, a decreased aortic LKB1 level has been found in the aorta of transgenic mice with endothelial specific overexpression of SIRT1, but not in those with endothelial specific overexpression of SIRT1(H363Y).

AMPK was activated in senescent PAECs and in those overexpressing LKB1, but inactivated by SIRT1. Western blotting was performed for PAECs collected at different passages (P1, P2, P3, and P4), PAECs transiently transfected with various expression vectors, or PAECs treated with the activator (resveratrol) or inhibitor (nicotinamide) of SIRT1. The molecular masses (kDa) of the detected protein bands were labeled. Note that AMPK activity progressively increased from P1 to P4. SIRT1 and resveratrol inhibited basal AMPK activity. LKB1-induced AMPK activation was abolished by SIRT1. Both SIRT1 and resveratrol enhanced the phosphorylation of AMPK(Ser485). *P<0.05 when compared to P1, pcDNA or vehicle control, respectively; #P<0.05 vs LKB1 group; n=3.
dothelial cells, respectively, and how these may contribute or protect the vascular aging process.

The detailed signaling mechanisms for inducing senescence downstream of LKB1/AMPK are not clear. AMPK activation can cause premature fibroblast senescence through mechanisms that likely involve the reduction of HuR, a RNA-binding protein. Persistent activation of AMPK leads to accelerated P53-dependent cellular senescence. In the present study, increased P53 protein levels have been observed in cells cotransfected with LKB1 and SIRT1(H363Y) (data not shown), in which the phosphorylation of AMPK(T172) is the highest among the 6 transfected groups (Figure 4). However, there is no increase of P53 levels in cells transfected with LKB1 alone (data not shown). AMPK coordinates energy availability through activation of tuberous sclerosis complex 2 (TSC2), which is a negative regulator of the mTOR signaling pathway. A study by Ota et al reveals that sirolimus and everolimus, 2 mTOR inhibitors, cause senescence in human umbilical vein endothelial cells (HUVEC), which could be reversed by SIRT1 activation. mTOR inactivation can increase cellular cyclin-dependent kinase inhibitor P27 and decrease the phosphorylation of retinoblastoma protein, which blocks the cell cycle from G1 to S phase. In addition, mTOR pathway has been implicated in the regulation of autophagy, a new effector mechanism of senescence. On the other hand, mTOR can be activated by Akt via direct phosphorylation and inhibition of tuberous sclerosis complex 2. Note that although AMPK has no obvious actions on Akt activity, Akt can counteract AMPK activity through stimulating the phosphorylation at Ser485, which prevents LKB1 from phosphorylating AMPK at its primary activation site Thr172. The crosstalk between LKB1 and Akt, as well as SIRT1 pathways upstream of mTOR, might represent a fine-tuning mechanism adopted by the cells for determination of their fate under different conditions.

SIRT1 is known as a stress and energy sensor that can be activated by increased NAD/NADH ratio. AMPK senses AMP/ATP levels through its upstream kinase LKB1. Previous works have suggested that there are various interactions between SIRT1 and AMPK pathway. However, the precise connections between these 2 nutrient sensing enzymes are largely uncharacterized. For example, in neuronal system, resveratrol-stimulated AMPK activity depends on LKB1 but does not require SIRT1. In liver, resveratrol protects against metabolic disease through activating SIRT1, which functions as an upstream regulator for LKB1/AMPK signaling. Studies have also suggested a potential regulatory role of AMPK on SIRT1 expression and activity. The results in present study suggest that SIRT1 antagonizes LKB1/AMPK signaling in endothelial cells through downregulating LKB1 protein (Figure 2, 3 and 7). On the other hand, activation or inhibition of AMPK does not affect the expression levels of SIRT1 and the NAD+ biosynthetic enzyme, NAMPT.
(nicotinamide phosphoribosyltransferase) (Online Figure VI). Moreover, NAMPT expression is not altered in senescent PAECs. These results, in contrast to recent reports using mouse skeletal muscle cells,37,42 suggest that there might be other mechanisms involved in the regulation of SIRT1 protein and gene expression levels. Nevertheless, findings of the present study suggest that LKB1/AMPK may be regulated by both NAD/NADH and AMP/ATP ratios. When SIRT1 activity is reduced, such as the PAECs at P4 that have lower NAD/NADH levels (data not shown) or cells transfected with LKB1 and SIRT1(H363Y) (Figures 4 and 6), LKB1/AMPK signaling may dominate the survival signaling, leading to irreversible senescence or apoptosis. In conditions that allow proper NAD/NADH and AMP/ATP sensing, activation of LKB1/AMPK may facilitate cells to survive through the transient stresses. The survival signal of SIRT1 could counterbalance the adverse consequence of prolonged activation of LKB1/AMPK.

Figure 6. Inactivation of Akt caused senescence through AMPK activation in PAECs. A, Under normal culture conditions, the phosphorylation of Akt was decreased in senescent PAECs, increased by SIRT1 or resveratrol treatment, but diminished by LKB1 when endogenous SIRT1 was inhibited. B, Inhibition of Akt caused senescence in normal cultures of PAECs but not in serum starved PAECs (left). The number of senescent cells was counted and presented as a percentage of SA-β-gal-positive cells (right). *P<0.05 vs luciferase control; n=3. C, Akt inhibition was associated with AMPK activation only in PAECs that were cultured in serum-containing media.

Figure 7. Endothelial-specific overexpression of SIRT1, but not the deacetylase mutant SIRT1(H363Y), protected mice from pharmacological drug-induced vascular senescence. A, Endothelial-specific SIRT1 or SIRT1(H363Y) transgenic mice were established as described in the Online Data Supplement. Senescence was induced by injecting paraquat at a dosage of 25 mg/kg. The aorta collected from wide-type or the 2 types of transgenic mice (5 to 6 weeks) were subjected to SA-β-gal staining (A) and Western blotting analysis (B). Note that mice of this age showed no significant differences on vascular senescence at basal status or after vehicle (PBS) treatment (data not shown). C, The protein levels of SIRT1, total and phosphorylated AMPK and LKB1 in aorta tissues were compared between young (4 weeks) and old mice (24 months) by Western blotting.
This beneficial circle can help to integrate nutrient/energy availability and the survival/proliferative capability of the endothelial cells.

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Disclosures
None

References

Novelty and Significance

**What Is Known?**
- The biological age of endothelial cells in patients with cardiovascular disease is often older than their chronological age.
- Endothelial senescence precedes and contributes to the development of abnormal vascular responses and atherosclerosis.
- SIRT1 is a class III deacetylase with beneficial activity against aging-associated diseases.
- SIRT1 promotes endothelium-dependent vascular relaxation and prevents premature senescence in cultured endothelial cells.

**What New Information Does This Article Contribute?**
- Downregulation of SIRT1 represents a key mechanism contributing to endothelial senescence.
- SIRT1 elicits its antiaging activity in endothelial cells largely by antagonizing LKB1-mediated AMPK activation.

Vascular aging contributes to age-related morbidity and mortality. It is of particular importance to elucidate whether or not, and how, SIRT1 exerts antiaging activity in the vascular system. The present study demonstrates that during the progression of endothelial senescence, mRNA and protein expressions of SIRT1 are progressively and significantly decreased, whereas the protein levels of LKB1, a serine/threonine kinase and tumor suppressor, are upregulated. SIRT1 elicits its antiaging activities, in part, by deacetylating and downregulating LKB1 proteins, causing the inactivation of downstream AMPK signaling. This finding is of particular interest in understanding the crosstalk between SIRT1 and AMPK, both extremely important energy sensors and regulators in the vascular system. Under normal conditions, LKB1 is constitutively active. Therefore, a mechanism must be in place for preventing overactivation of AMPK, which promotes endothelial senescence. The regulatory role of SIRT1 on LKB1 protein stability described in this study represents a timely switch-off mechanism for blocking the detrimental influence of prolonged LKB1/AMPK activation. The results suggest that the fate of endothelial cells is tightly regulated by SIRT1 and LKB1/AMPK and that both systems should be taken into account when designing therapeutic interventions targeting SIRT1 and AMPK.
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Online Methods

Materials

Antibodies against phospho-LKB1(Ser428), LKB1, phosphor-AMPK(Thr172), phosphor-AMPK(Ser485), AMPK, phospho-acetyl-coenzyme A carboxylase (ACC) (Ser79), ACC, phospho-Akt(Ser473), and Akt were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against SIRT1 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for phospho-eNOS(Ser1177) and eNOS were from BD Transduction Laboratories (San Jose, CA). Resveratrol, nicotinamide, AICAR and compound C were from Sigma (St. Louis, MO). Akt-1/2 inhibitor was provided by Dr Peter R. Shepherd (The University of Auckland, New Zealand). 1 Mammalian expression vector encoding human SIRT1 tagged with FLAG peptide (SIRT1) was kindly provided by Dr. D. Koya. 2 The plasmid for over-expressing the deacetylase mutant SIRT1(H363Y) was generated by site-directed mutagenesis to replace histone at residue 363 to tyrosine. The expression vector for FLAG-tagged murine LKB1 and recombinant adenoviruses for expression of a constitutive active (CA-AMPK) or dominant-negative version of AMPK (DN-AMPK) were kindly provided by Dr. D. Carling. 3 The recombinant adenoviruses that encode luciferase, constitutive active (CA-Akt) and kinase-dead form of Akt (KD-Akt) were kindly provided by Dr C. Rhodes. 4 Stably-transfected HEK293 cells overexpressing LKB1 were established following the protocol described previously. 5

Isolation and culture of primary porcine aortic endothelial cells (PAECs)

After clearing all the adhering fat, the aorta was cut longitudinally for exposing the luminal area. Isolation of endothelial cells was done by gently scraping the surface with a scalpel blade and collecting the fragments of endothelial lining in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS. The cells were resuspended and washed by repeated centrifugation at 1,000 rpm for three time (five min each), and subsequently plated in 100 mm collagen-coated Petri dish. The cells were cultured at 37°C in humidified 5% CO2 95% air. The attached PAECs were cultured with the medium changed every two days. Primary porcine endothelial cells have a limited life span in culture and gradually entered the status of senescence. In this study, a fixed schedule was adopted for sub-culturing the cells at a ratio of 1:3 once per week up to four weeks (designated as P1, P2, P3 and P4). Cell proliferation during each week was monitored by crystal violet staining and cell numbers counted when seeding and harvesting from each passage for calculating population doubling. The purity of the PAECs is more than 99% as demonstrated by staining of endothelial specific markers CD31 and Von Willbrand factor.

Transfection, adenoviral infection and drug treatment

Transient transfection was performed in PAECs using Effectene® Transfection Reagent (Qiagen; Hilden, Germany) according to manufacturer’s instruction. Cells were cultured either in DMEM with
or without fetal bovine serum. Two days after the transfection, cells were washed with cold PBS and collected with RIPA lysis buffer for subsequent analysis.

For adenovirus infection, initially, the appropriate titer for each recombinant virus was determined by the addition of various dilutions into PAECs in six-well plates, giving a multiplicity of infection (m.o.i.) ranging from 50 to 2000 based on 0.5-2.0 × 10^6 plaque-forming units/ml as measured by A_260. For all infections, the viral stock was replaced with complete medium after 2 h and the cells were incubated for two days before experiment.

For drug treatment, PAECs cultured in the presence or absence of serum were incubated with AICAR (2 mM), compound C (5 μM), Akt inhibitor (10 μM), resveratrol (100 μM) or nicotinamide (10 mM) for 48 hours before harvesting for subsequent experiment.

**Quantitative real-time PCR**

Total RNA was isolated using Trizol Reagent (Invitrogen Life Technologies; Carlsbad, CA) according to the manufacturer's instructions. The purity and concentration of total RNA were measured with a spectrophotometer at 260 nm and 280 nm. Ratios of absorption (260/280 nm) of all samples were between 1.8 and 2.0. cDNA was transcribed with the Superscript III reverse transcription system (Invitrogen). Quantitation of target genes was performed using SYBR Green PCR Master Mix (Qiagen) and an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primers were 5'-AGGAGGTGAATATGCCAAC-3' (forward) and 5'-CTGAAGAAGCTGGTGGTGAA-3' (reverse) for SIRT1; 5'-GCAGATCCGACGAGCGCT-3' (forward) and 5'-GAAAGTCCTGAGTGATGA-3' (reverse) for LKB1; 5'-AATGACCCCTTCCATTGAC-3' (forward) 5'-GCTTCCCATTCTCAGCT-3' (reverse) for GAPDH.

**Co-immunoprecipitation and Western blotting analysis.**

Cells transiently transfected with various expression vectors were solubilized in RIPA lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4 and the complete protease inhibitor cocktail. The resulting lysates were sonicated twice for 10 s in an Ultrasonic Dismembrator with 10% output (Model 500; Fisher Scientific) and then centrifuged at 14,000 x g for 20 min at 4°C. Protein concentration was determined by BCA method (BCA Protein Assay Reagent; Thermo Fisher Scientific, Rockford). The supernatants were subjected to immunoprecipitation with the indicated antibodies conjugated to N-hydroxysuccinimide-activated Sepharose beads. Immunoprecipitates or whole-cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies, as described previously. The proteins were visualized using chemiluminescence detection. The intensities (density x area) of individual bands were measured by densitometry (Model GS-700, Imaging Densitometer, Bio-Rad). The background was subtracted from the calculated area.

**Cell proliferation assay**

PAECs were seeded at a density of 1 x 10^3 in 96-well plates. The culture medium was changed every
two days. Briefly, at each time point, cells were washed with PBS, fixed with 100 µL of methanol, and stained with 0.2% crystal violet for 15 min. Excess dye was removed by gentle washing with double distilled water. After air drying, stained cells were solubilised with 1% SDS and quantified using μQuant MQX200 microplate reader (Biotek Instruments, Inc; Winooski, Vermont). Cell proliferation was also evaluated by ³H-thymidine incorporation assay and similar results have been obtained (data not shown).

Telomerase activity measurement

Telomerase activity of PAECs was determined using TRAPEZE® XL Telomerase Detection Kit (Chemicon; Temecula, CA) according to the manufacturer’s instructions. About 10⁶ PAECs are lysed in CHAPS Buffer. Two microliter of cell lysates equivalent to 1 µg protein (approximately 2 X 10⁴ cells) was used for the assay. The results were presented as the form of Total Product Generated (TPG).

Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal staining was performed using Senescence Cells Histochemical Staining Kit (Sigma) according to the manufacturer’s instruction. Briefly, PAECs were washed with PBS and fixed for six minutes at room temperature. Cells were then kept in Staining Mixture at 37°C for 14 hours. After washing with PBS, 70% glycerol solution was added and cells counted under a microscope.

Cell cycle progression and apoptosis analysis

For cell cycle analysis, cells fixed with 70% ethanol were resuspended in the staining buffer [100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% NP-40 and 2 µg/ml propidium iodide (PI)] and incubated in the dark for 20 minutes on ice. The analysis was carried out with EPICS® Elite ESP Flow Cytometer and EXPO software (Beckman Coulter, Miami, FL). Data was acquired from 1 x 10⁴ cells per sample. PI-stained cells were counted through FL3 channel. Gating of sub G0/G1-, G0/G1-, S-, and G2/M-populations was performed using WinList software (Purdue University Cytometry Laboratories, West Lafayette, IN). For apoptosis analysis, apoptotic cells were stained by TUNEL in situ Cell Death Detection Kit (Roche, Indianapolis, IN) and monitored using Flow Cytometer. Briefly, cells were fixed in 4% paraformaldehyde and the assay conducted according to the manufacturer’s instructions. TUNEL positive cells were counted through FL1 channel. Double staining was performed by first staining with TUNEL and then PI.

Radio-labeling and LKB1 acetylation analysis

HEK293 cells stably-transfected with vector overexpressing LKB1 were used for analyzing the acetylation status of LKB1. Briefly, cells were transiently transfected with pcDNA control vector or vector encoding SIRT1, or treated with vehicle or resveratrol. 24 hours after transfection, 20 µCi/ml of [³H] sodium acetate was added to the culture medium and incubated for another 6 hours. Cells were then lysed in RIPA lysis buffer with freshly added protease inhibitor cocktails. The lysate was centrifuged at 14,000 rpm for 15 min. Supernatants were incubated with anti-FLAG agarose beads overnight at 4°C. Immunoprecipitated proteins were resolved by 8% SDS-PAGE gel. The dried gel was subjected to phosphorimager or autoradiography analysis.
Genera tion of endothelial-specific SIRT1 or SIRT1(H363Y) transgenic mice

Human SIRT1 cDNA (NCBI ref: NM_012238.4) was cloned into pDrive™-ICAM-2 (InvivoGen, San Diego, CA). The Cla1/Bsu361 DNA fragment (~2.5 kb) containing the ICAM-2 promoter, human SIRT1 cDNA, a FLAG-tag sequence and the Poly(A) was purified for oocyte injection and embryo implantation. The endothelial-specific SIRT1(H363Y) transgenic mice were generated at the same time using a DNA fragment containing the mutated human SIRT1(H363Y) cDNA sequence. The F0 offsprings were screened for mice carrying the transgene. The founder lines were continued cross-bred with mice of C57BL/6J background. All animals were kept under 12-hour light-dark cycles at 22 to 24°C. All mice did not show any developmental defects with similar body weights and food intake. Mice used in this study were from the F10 generation. The wide type mice from the same litters were used as controls for all experiment.

Drug-induced endothelial senescence and SA-β gal staining

Mice (6-8 weeks) were randomly assigned to vehicle (PBS) or drug treatment groups. PBS or paraquat (1,1-dimethyl-4,4-bipyridinium, Sigma #36541, 25 mg/kg) was intraperitoneally injected into mice at day one. Three days later, mice were anaesthetized and sacrificed. The aorta was removed for SA-β-gal staining. Briefly, the aorta was opened longitudinally for exposing the luminal area. After fixation at room temperature in 4% formaldehyde for 15 min, the aorta was washed with PBS and incubated for 48 hours at 37°C in SA-β-gal staining solution [containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, Sigma), 5 mmol/l potassium ferrocyanide, 5 mmol/l potassium ferricyanide, 150 mmol/l NaCl, 2 mmol/l MgCl₂, and 40 mM citric acid/sodium phosphate, pH 6.0]. The images were taken under a dissection microscope. Six to 10 random fields of the stained tissues as well as their sections were analyzed under a light microscope. ImageJ Processing and Analysis software was used to quantify areas of abnormal tissue. All animal studies were approved by the Institutional Committee on the Use of Live Animals in Teaching & Research.
Online Figure I. Identification of LKB1 as an interacting protein of SIRT1. HEK293 cells were transfected with pcDNA or expression vector encoding human SIRT1 tagged with FLAG peptide (SIRT1). 48 hours after transfection, cells were collected and subjected to immunoprecipitation using Sepharose beads conjugated with anti-Flag antibody. The bound immune complexes were eluted by glycine-HCl, pH 3.0 and separated by SDS-PAGE for silver staining. The individual bands were cut for in-gel digestion and protein identification by mass spectrometry analysis. The identified SIRT1 and LKB1 were annotated.
Online Figure II. Prolonged culture induced senescence in PAECs. A, Proliferation was monitored by crystal violet assay for different passages of PAECs. *, P < 0.05 vs P2, P3, P4, n = 3. B, The telomerase activities were progressively decreased during prolonged culture of PAECs. PC, Positive control. #, P < 0.05 vs P1, n = 3. C, Representative images of SA-β-gal staining showed severe senescence in P4 cells. Magnification, × 100. D, The senescence markers P21 and P16 were upregulated in P4 cells.
Online Figure III. Identification of senescent PAECs by flow cytometric analysis. 

A, P1 cells were stained with both TUNEL and PI as described in Methods. Note that almost no apoptotic cells could be detected in FL1 channel (for TUNEL staining), whereas two distinct portions of cells could be detected in FL3 channel (for PI staining). The green color dots referred to those with normal cycle distributions (data not shown) and the red color dots were senescent cells. 

B, P1 cells pre-treated with DNase I were stained by TUNEL and PI for flow cytometric analysis. A portion of cells (blue) with fragmented DNA could be stained by TUNEL and detected in both FL1 channel (signal over 10^6 in histogram) and FL3 channel (note that they were different from the red portion of cells). 

C, the color dot plots were shown for cells derived from the same batch of P1 and P3 cultures demonstrating that the senescent cells dramatically increased in later passage of PAECs. There were hardly any cells stained with TUNEL in both P1 and P3 PAECs. Similar results were also obtained (not shown) at excitation 488 nm/emission 512 nm under a fluorescence microscope (Leica Microsystems, Bensheim, Germany).
Online Figure IV. PAECs progressed to severe senescence during the prolonged culture process. PAECs at different passages were serum starved for 24 hours before performing the cycle analysis as in Supplementary Figure 3. Representative result demonstrated that a distinct portion of senescent cells (denoted blue) could be detected by flow cytometry analysis. The number of senescent PAECs dramatically increased from P1 to P4.
Online Figure V. SIRT1 blocked LKB1-induced cellular senescence in PAECs. After serum starving for 24 hours, cells were transfected with same amounts of expression vectors as in Figure 2 and incubated in normal culture medium for another 48 hours before cell cycle analysis. Representative images generated by the flow cytometer were shown. Note that overexpressing LKB1 arrested the cell cycle and increased the percentage of senescent PAECs, which were blocked by co-transfection with SIRT1. On the other hand, SIRT1(H363Y) mutant promoted cell cycle arrest and senescence in PAECs with or without LKB1 overexpression.
Online Figure VI. The protein and gene expressions of NAMPT were not altered by activation or inactivation of AMPK, and not affected by cellular senescence in PAECs. A, PAECs were infected with adenovirus encoding constitutively active AMPK or dominant negative AMPK, or treated with AICAR, or compound C. The protein levels of SIRT1 and NAMPT were monitored by Western blotting and compared to respective controls. B, the mRNA levels (left) and protein levels (right) of NAMPT were measured in P1 and P3 cells using real time PCR and Western blotting analysis, respectively.
References:


