Experience of falls in the past year is an established and powerful tool for assessing fall risk,[2] and was reported by the patient and/or his or her family members. Duration time of one-leg standing test, which can be carried out in a narrow limited space of the outpatient office, was measured using the leg with the eyes open, until the raised leg was put down on the floor. We examined both right and left legs once for each, and the longer of the two measurements was used for statistical analysis.[12]

Data analysis and statistical methods

Values are expressed as means ± standard deviation. In order to analyze the relationship between each fall risk index and comorbidities or drugs, variables were compared using Student's t-test or the χ^2-test as appropriate. The correlations between the two continuous variables were analyzed using Pearson's r coefficient. In multivariate analysis, logistic regression analysis was performed for history of falls and multiple regression analysis for the remaining three indices, to determine the association of fall risk with the variables. Differences between the groups of number of drugs and three indices of fall tendency were analyzed using one-factor ANOVA followed by Tukey–Kramer test. Data were analyzed using JMP version 8.0.1.

Results

The characteristics of the study subjects are shown in Table 1. Calcium channel blockers, angiotensin-II receptor blockers (ARB), statins and aspirins were prescribed in more than 20% of all the patients. Calcium channel blockers prescribed in this study were all long-acting agents, and aspirin dosage prescribed were all 100 mg. Less than 10 patients received insulin therapy, took non-steroidal anti-inflammatory drugs or anticoagulants. No patients were taking neuroleptics, nor antiparkinsonian drugs. Patients prescribed five drugs or more were 36.3%.

On univariate analyses, the number of drugs was the only factor which was significantly associated with history of falls in the past year (no/yes 3.2 ± 2.6/4.0 ± 3.1 drugs, $P < 0.05$). Older age, female, hypertension, osteoporosis, history of stroke, the number of comorbidities, use of ARB, aspirin, bisphosphonates, hypnotics and number of prescribed drugs were significantly associated with either one of three indices of fall risk (Table 2). Number of drugs was significantly correlated with three scores excluding the

Table 2 Univariate analysis of association between risk factor variables and three fall indices: fall-predicting score, simple screening test, one-leg standing test

		Fall risk index (points)	Simple screening test (points)	One-leg standing test (seconds)
Age		0.23***	0.23***	–0.46***
Female	No/Yes	7.0 ± 3.1/8.4 ± 4.0**	3.8 ± 3.3/4.7 ± 3.6*	19.7 ± 11.7/16.2 ± 11.7*
Hypertension	No/Yes	7.2 ± 3.6/8.4 ± 3.8*	3.7 ± 3.3/4.8 ± 3.5*	18.9 ± 11.1/16.2 ± 12.1
Osteoporosis	No/Yes	7.6 ± 3.7/8.9 ± 4.0*	4.3 ± 3.6/4.8 ± 3.1	17.9 ± 11.7/15.6 ± 11.9
History of stroke	No/Yes	7.8 ± 3.7/9.7 ± 4.1*	4.3 ± 3.4/5.6 ± 4.1	17.9 ± 11.8/8.5 ± 8.7**
Number of comorbidies		0.27***	0.17*	–0.24***
Antihypertensives	No/Yes	7.3 ± 3.6/8.5 ± 3.8*	3.7 ± 3.3/4.9 ± 3.5*	18.8 ± 11.4/15.9 ± 12.0
Angiotensin-II receptor blockers	No/Yes	7.6 ± 3.7/8.7 ± 3.8*	3.9 ± 3.4/5.2 ± 3.5**	17.6 ± 11.5/16.3 ± 12.2
Calcium channel blockers	No/Yes	7.6 ± 3.7/8.5 ± 3.7	4.1 ± 3.5/4.8 ± 3.5	18.8 ± 11.6/14.3 ± 11.6**
Aspirin	No/Yes	7.7 ± 3.8/8.9 ± 3.8*	4.1 ± 3.5/5.5 ± 3.7*	18.0 ± 11.8/13.5 ± 11.5*
Bisphosphonates	No/Yes	7.8 ± 3.8/9.9 ± 2.5*	4.3 ± 3.5/6.5 ± 2.7*	17.3 ± 11.8/14.9 ± 11.7
Hypnotics	No/Yes	7.6 ± 3.6/9.7 ± 4.1***	4.2 ± 3.6/5.2 ± 3.1	17.6 ± 11.9/15.2 ± 11.3
Number of drugs		0.30***†	0.27***†	–0.35***

*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$, compared to "No" by simple Student's t-test. For age, number of comorbidities and number of drugs, Pearson's correlation coefficient between each indices of fall tendency are shown. †For analysis of number of drugs, a questionnaire asking "whether taking five or more drugs" were excluded for analysis. Therefore, fall risk index was analyzed by a total of 21 items, and a simple screening test by a total of 11 points. For other risk factor variables shown in the table, mean ± standard deviations are expressed. Other risk factor variables not shown in this table showed no statistically significant relationship with either one of three indices.

[Table 2 amended after online publication date September 27, 2011] question on polypharmacy. Number of comorbidities was significantly associated with age ($r = 0.32$, $P < 0.0001$) and with the number of drugs ($r = 0.62$, $P < 0.0001$).

Next, on multivariate analyses, the questionnaire asking "whether taking five or more drugs" were excluded from the fall risk index and the simple screening test. Therefore, the fall risk index was analyzed by a total of 21 items and the simple screening test by a total of 11 points in this analysis. To evaluate the association of four fall risk indices with comorbidities and drugs, all the variables that were significantly associated in either one of four univariate analyses were entered into the model. As shown in Table 3, the number of drugs was the only factor which was significantly associated with all four indices, independent of age, sex and other variables. Because each disease variable or drug variable might have affected the number of comorbidities or the number of drugs in this analysis, we just compared the number of comorbidities and the number of drugs to exclude the double count in next analysis. As shown in Table 4, the number of drugs was significantly associated with all of the four fall risk indices independent of age, sex and the number of comorbidities, while the number of comorbidies was inversely associated with history of falls and simple screening test. As shown in Figure 1, the association of the number of drugs with

Table 3 Multivariate analysis of association between risk factor variables and four fall indices: history of falls in a year, fall risk index, simple screening test, one leg standing test

		History of fall in a year (No = 0/Yes = 1) Odds ratio (95% CI)	Fall risk index (21 items)† β	Simple screening test (11 points)† β	One-leg standing test (s) β
Age		1.00 (0.96–1.05)	0.073	0.127	–0.370***
Female	(No = 0/Yes = 1)	2.36 (1.12–5.00)*	0.199**	0.197**	–0.149*
Hypertension	(No = 0/Yes = 1)	1.87 (0.61–5.76)	0.166	0.218*	–0.110
Osteoporosis	(No = 0/Yes = 1)	0.67 (0.28–1.60)	0.093	0.027	0.023
History of stroke	(No = 0/Yes = 1)	1.43 (0.38–5.45)	0.080	0.032	–0.083
Number of comorbities		0.60 (0.38–0.95)*	–0.062	–0.237*	–0.024
Antihypertensives	(No = 0/Yes = 1)	0.52 (0.18–1.54)	–0.141	–0.158	0.142
Aspirin	(No = 0/Yes = 1)	1.59 (0.72–3.50)	0.053	0.046	0.002
Bisphosphonates	(No = 0/Yes = 1)	2.27 (0.73–7.07)	0.055	0.105	0.033
Hypnotics	(No = 0/Yes = 1)	0.84 (0.33–2.15)	0.094	–0.018	0.084
Number of drugs		1.24 (1.07–1.45)*	0.247**	0.335***	–0.250**

*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$. Logistic regression analysis was performed for history of fall in a year, and multiple regression analysis for the remaining three. The risk factor variables used in these multivariate analyses were those associated in either of the four univariate analysis significantly. †The questionnaire asking "whether taking five or more drugs" were excluded from the scores in this analysis. Therefore, fall risk index were analyzed by a total of 21 items and simple screening test by a total of 11 points. CI, confidence interval; β, standardized regression coefficient.
[Table 3 amended after online publication date September 27, 2011]

Table 4 Multivariate analysis of association between number of comorbidities and drugs with four fall indices: history of falls in a year, fall risk index, simple screening test, one-leg standing test

	History of fall in a year (No = 0/Yes = 1) Odds ratio (95% CI)	Fall-risk index (21 items)† β	Simple screening test (11 points)† β	One-leg standing test (s) β
Age	1.00 (0.96–1.05)	0.101	0.115	–0.376***
Female (No = 0/Yes = 1)	1.73 (0.90–3.34)	0.207**	0.191**	–0.110
Number of comorbidities	0.63 (0.45–0.89)*	0.073	–0.137	–0.034
Number of drugs	1.23 (1.08–1.41)*	0.223*	0.316***	–0.233**

*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$. Logistic regression analysis was performed for history of fall in a year, and multiple regression analysis for the remaining three. †The questionnaire asking "whether taking five or more drugs" were excluded from the scores in this analysis. Therefore, fall risk index was analyzed by a total of 21 items and simple screening test by a total of 11 points. CI, confidence interval; β, standardized regression coefficient.

fall predicting score, simple screening test and duration time of one-leg standing test was stepwise.

Discussion

Epidemiological studies have assessed the risk of falls in community-dwelling people, but not in geriatric outpatients, who are likely to fall and need special consideration for the treatment of their illness. This cross-sectional study investigated the association between comorbidities, medications and fall risks in Japanese elderly outpatients and found that all four indices were significantly associated with the number of drugs. Because polypharmacy is frequently seen in patients with multiple comorbidities, this study compared the impact of the number of drugs with that of the number of comorbidities on fall risk, and found the significance of polypharmacy as fall risk in elderly outpatients.

In the present study, the number of comorbidities was inversely associated with the history of fall in the past year and with an 11-point simple screening test in the multivariate analysis. The reason is unclear; however, there are some speculations about this. None of the patients with four or more comorbidities ($n = 19$, 79.4 ± 5.2 years old) had history of fall in the past year. This accounts for the lower points of the simple screening test in these patients, because the history of fall consists of 5 points out of a total of 11 points in the simple screening test. So the question is why they had lower frequency of falling experiences, although they are at higher risk of falls according to fall risk index and one-leg standing test (9.6 ± 3.8 items and 8.6 ± 9.4 s, respectively). These patients may take care not to fall in their daily lives because of their consciousness of fall risk or frailty, or maybe due to elevated vigilance of caregivers and their constant physical assistances. They might have simply forgotten their fall experiences due to subclinical cognitive impairment, although demented patients were not included in this study. It is also possible that the patients who had more comorbidities and had fallen did not meet our inclusion criteria because of their recent injurious falls or their severe conditions.

Several medications and comorbidities have been reported as risks of fall.[6,7,13–19] Among these, diabetes,[9,10] imsonia,[13] hypnotics[13–15] and antihypertensive use[8] were not significantly associated with fall risk in our study. Only 20 patients (40.8% of diabetic patients) were prescribed hypoglycemic agents such as sulfonylurea ($n = 17$) or insulin ($n = 3$) in this study. Because hypoglycemia is considered to be the main cause of accidental falls in diabetic patients, relatively less prescription of hypoglycemic agents might have affected our result. The patients who were prescribed hypnotics tended to be at higher risk of falls in univariate analysis, which did show statistical significance. Also, antihypertensives such as diuretics are reported to increase the fall risk.[8] No

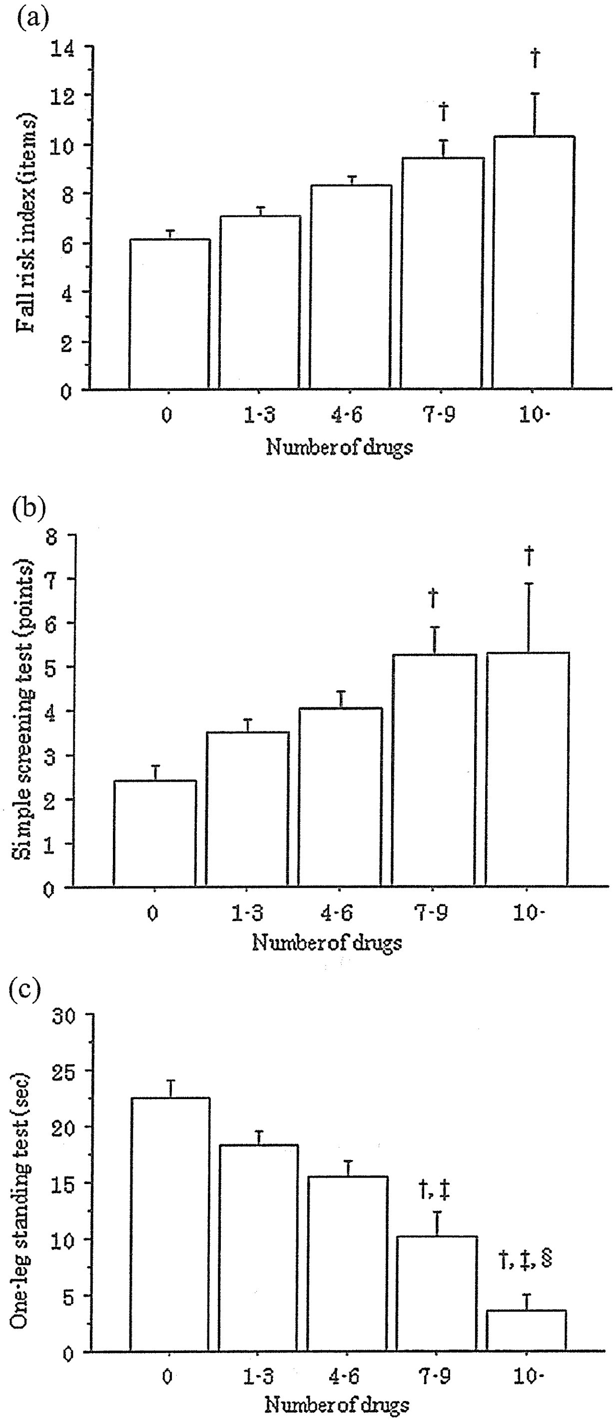

Figure 1 Averages of fall risk according to the number of drugs. (a) Fall risk index excluding the questionnaire concerning polypharmacy. (b) Simple screening test excluding the questionnaire concerning polypharmacy. (c) Duration time of one-leg standing test. The differences between the number of the drugs were compared through ANOVA, $P < 0.0001$ for (a), $P < 0.005$ for (b), $P < 0.0001$ for (c). For post-hoc analysis, $^{\dagger}P < 0.05$ vs 0 drug; $^{\ddagger}P < 0.05$ vs 1–3 drugs; $^{\S}P < 0.05$ vs 4–6 drugs. Values are expressed as mean ± standard error.

association between these drugs and fall risk in our study might be due to the small sample size. Other drugs such as major tranquillizers,[14] antidepressants[17,18] and antiparkinsonians[19] might increase fall risk; however, very few patients used these drugs in this study.

There are some other limitations. First, the causal relationship of the associations observed in this study is unknown because of the cross-sectional design. Polypharmacy has been regarded as a risk in several aspects in elderly patients. Previous studies have shown that adverse drug events were seen more frequently in the polypharmacy patients during their stay in the geriatric inpatient ward,[20] and polypharmacy was one of the important predictors for postdischarge mortality in elderly patients after emergent hospitalization.[21] Because patients with multiple diseases and in severer conditions are likely to take more medications, we used the number of comorbidities in analysis as fall risk variables. However, it is still unclear whether polypharmacy is a risk of falls independent of severity of each comorbidity. Interventional studies to reduce the number of drugs are needed to clarify the causal relationship between polypharmacy and fall risk. Second, this study did not evaluate the fall itself. The validity of four indices used in this study is well established as fall risk markers. However, prospective studies which evaluate the incidence of fall should be carried out in the future. Third, although the included subjects were receiving the same prescriptions for more than 1 month, the exact duration of each drug use or polypharmacy was not assessed in this study. Consequently, the long-term adverse effects over months or years seen in elderly patients should be more precisely investigated.

In summary, this study demonstrated that geriatric outpatients with polypharmacy were at higher risk of falls, consistent with the previous studies conducted in community-dwelling elderly. Our finding may add new information on pharmacotherapy in elderly patients with chronic diseases. Prospective studies and intervention studies examining the effect of drug reduction are needed in the future.

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Appendix I. 22 items of fall-predicting score (questionnaire)

Q1. Have you fallen during the last 12 months?	Yes, 1; No, 0.
Q2. Have you tripped during the last 12 months?	Yes, 1; No, 0.
Q3. Can you climb stairs without help?	Yes, 0; No, 1.
Q4. Do you feel your walking speed has declined recently?	Yes, 1; No, 0.
Q5. Can you cross a road within the green signal interval?	Yes, 0; No, 1.
Q6. Can you walk 1 km without stopping?	Yes, 0; No, 1.
Q7. Can you stand on one foot for about five seconds?	Yes, 0; No, 1.
Q8. Do you use a stick when you walk?	Yes, 1; No, 0.
Q9. Can you squeeze a towel tightly?	Yes, 0; No, 1.
Q10. Do you feel dizzy at times?	Yes, 1; No, 0.
Q11. Is your back bent?	Yes, 1; No, 0.
Q12. Do you have knee pain?	Yes, 1; No, 0.
Q13. Do you have a problem with your vision?	Yes, 1; No, 0.
Q14. Do you have a hearing problem?	Yes, 1; No, 0.
Q15. Do you think you are forgetful?	Yes, 1; No, 0.
Q16. Do you feel anxious about falling when you walk?	Yes, 1; No, 0.
Q17. Do you take five or more prescribed medicines?	Yes, 1; No, 0.
Q18. Do you feel unsafe because your home is dark?	Yes, 1; No, 0.
Q19. Are there any obstacles in your house?	Yes, 1; No, 0.
Q20. Is there any difference in level within your home?	Yes, 1; No, 0.
Q21. Do you have to use stairs in daily living?	Yes, 1; No, 0.
Q22. Do you have to walk on a steep slope around your house?	Yes, 1; No, 0.

Appendix II. Simple screening test for risk of falls

Q1. Have you fallen during the last 12 months?	Yes, 5 points; No, 0.
Q2. Do you feel your walking speed has declined recently?	Yes, 2 points; No, 0.
Q3. Do you use a cane when you walk?	Yes, 2 points; No, 0.
Q4. Is your back bent?	Yes, 2 points; No, 0.
Q5. Do you take five or more prescribed medicines?	Yes, 2 points; No, 0.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Sirtuin 1 Retards Hyperphosphatemia-Induced Calcification of Vascular Smooth Muscle Cells

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Sirtuin 1 Retards Hyperphosphatemia-Induced Calcification of Vascular Smooth Muscle Cells

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Objective—Arterial calcification is associated with cardiovascular disease as a complication of advanced atherosclerosis. Aged vascular cells manifest some morphological features of a senescent phenotype. Recent studies have demonstrated that mammalian sirtuin 1 (SIRT1), a histone deacetylase, is an exciting target for cardiovascular disease management. Here, we investigated the role of SIRT1 in a calcification model of vascular smooth muscle cells (SMCs).

Methods and Results—In adenine-induced renal failure rats with hyperphosphatemia, massive calcification was induced in the aortic media. Senescence-associated β-galactosidase (SAβ-gal) activity, a marker of cellular senescence, in medial SMCs was significantly increased, and its induction was positively associated with the degree of calcification. In cultured SMCs, inorganic phosphate (Pi) stimulation dose-dependently increased SAβ-gal-positive cells, and Pi-induced senescence was associated with downregulation of SIRT1 expression, leading to p21 activation. The activation via SIRT1 downregulation was blunted by inhibition of Pi cotransporter. Activation of SIRT1 by resveratrol significantly reduced the senescence-associated calcification. Conversely, SIRT1 knockdown by small interfering RNA accelerated the Pi-induced SMC senescence and subsequent calcification. In addition, SIRT1 knockdown induced phenotypic change from a differentiated state to osteoblast-like cells. The senescence-related SMC calcification was completely prevented by p21 knockdown. In addition to Pi-induced premature senescence, SMCs with replicative senescence were also more sensitive to Pi-induced calcification compared with young SMCs, and this finding was attributable to augmented p21 expression.

Conclusion—SIRT1 plays an essential role in preventing hyperphosphatemia-induced arterial calcification via inhibition of osteoblastic transdifferentiation. In addition, Pi-induced SMC calcification may be associated with both premature and replicative cellular senescence. **(*Arterioscler Thromb Vasc Biol.* 2011;31:2054-2062.)**

Key Words: cellular senescence ■ hyperphosphatemia ■ longevity gene SIRT1 ■ vascular calcification ■ vascular smooth muscle cell

Atherosclerotic vascular damage associated with aging manifests several features, namely atherosis, sclerosis, and calcific change, finally leading to cardiovascular events. These pathological changes result in arterial wall thickening (localized morphological changes) and arterial stiffening (functional changes).[1] Arterial calcification makes the management of hemodynamics more difficult in the elderly, because ectopic calcium deposition in the aorta and arteries contributes to vessel wall stiffening and loss of elastic recoil.[2] These pathological conditions result in unstable hemodynamic consequences, finally leading to a decline in end-organ perfusion and subsequent ischemic events. Recently, several reports have demonstrated that aortic calcification detectable on chest X-ray examination is a strong predictor of future cardiovascular events beyond traditional risk factors.[3]

Arterial calcification is anatomically separated into two types, intimal and medial calcification.[4] Intimal calcification, which is seen as patchy scattered deposits only occurring within atherosclerotic plaques, is shown to be associated with plaque vulnerability.[5] On the other hand, medial calcification, which is frequently seen in the elderly and in diabetes and chronic renal failure, is observed as continuous linear deposits along the internal elastic lamina.[6] Advanced atherosclerosis with both types of calcified lesions is the consequence of overlapping pathological mechanisms.

Ectopic calcification in the vasculature has been shown to result from passive precipitation of calcium with aging and osteoporosis, the so-called calcium shift theory, as a previous hypothesis.[7] However, accumulating recent evidence has shown it to be attributable to an active "cell-mediated process" resembling osteogenesis in bone rather than passive mineral precipitation in vascular smooth muscle cells (SMCs).[8,9]

Silent information regulator-2 (Sir2), an NAD$^+$-dependent HDAC, is highly conserved in organisms ranging from Archaea

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to humans.[10] In yeast, Sir2 has been shown to play critical roles in DNA repair, stress resistance, and longevity. Mammalian sirtuin 1 (SIRT1), the closest homolog of Sir2, regulates the cell cycle, apoptosis, and metabolism by interacting with a number of molecules, including p53, promyelocytic leukemia protein, Foxo, Ku70, and peroxisome proliferator-activated receptor-γ.[11] A previous study has shown that SIRT1 antagonizes p53-mediated premature senescence in mouse embryo fibroblasts.[12] In addition, we have recently demonstrated that SIRT1 inhibits oxidative stress-induced premature senescence in vascular endothelial cells.[13] However, the detailed mechanism of how SIRT1 affects vascular SMC senescence and arterial calcification remains unclear.

In this study, we hypothesized that SIRT1 plays an important role in preventing arterial calcification due to renal failure, in association with modulation of cellular senescence. Here, we demonstrated the protective potential of SIRT1 against hyperphosphatemia-induced premature and replicative senescence and subsequent calcification in SMCs.

Methods

Aortic Calcification in Renal Failure Rats

Renal failure was induced in rats by a 0.75% adenine-containing diet as previously described.[14] All procedures and animal care were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo. Detailed methods are described in the supplemental materials, available online at http://atvb.ahajournals.org.

Induction of SMC Calcification

Primary human aortic SMCs (HASMCs) were treated with a pathological concentration of inorganic phosphate (Pi) up to 3.2 mmol/L in culture medium as previously described.[29] To quantitatively measure Pi-induced calcification, two distinct experiments were performed as previously described[14]: (1) intracellular calcium deposition as determined by *o*-cresolphthalein complexone method, and (2) visualization of mineralization as determined by von Kossa staining, Detailed methods are described in the supplemental materials.

Senescence-Associated *β*-Galactosidase Staining

To assess senescent changes in the phenotype of cultured HASMCs or aortic medial cells of rats, staining for senescence-associated *β*-galactosidase (SA*β*-gal), a well-established biomarker of cellular senescence, was performed. Detailed methods are described in the supplemental materials.

Knockdown of SIRT1 or p21 by Small Interfering RNA

HASMCs were transfected with 200 pmol/L small interfering RNA (siRNA) for SIRT1, $p21^{WAF1/CIP1}$, or both. Detailed methods are described in the supplemental materials.

Real-Time Polymerase Chain Reaction Analysis: Osteoblastic Markers

To examine whether Pi stimulation induces change to an osteoblastic phenotype, the expression of Runx-2/Cbfa-1 and alkaline phosphatase, which are well known to be representative osteoblastic markers, was checked using real time-polymerase chain reaction analysis. In addition, the effect of knockdown of SIRT1, p21, or both by siRNA on the osteoblastic phenotypic change in HASMCs was examined. Primer sequences are shown in Supplemental Figure I.

Results

Association of Senescent Vascular Cells With Aortic Medial Calcification in Renal Failure Rats

The adenine-fed rats had severe renal failure, with a huge increase in serum creatinine (3.0±0.9 mg/dL in renal failure rats versus 0.3±0.0 mg/dL in control rats), similar to a previous report.[14] The renal failure rats showed an approximately 2.0-fold increase in serum phosphorus (18.9±4.7 mg/dL) compared with control rats (9.8±0.9 mg/dL). Histological assessment using von Kossa staining showed that the aorta in renal failure rats had extensive linear calcification, which was localized in the aortic media, resembling the typical Mönckeberg's pattern (Figure 1A). Numerous SA*β*-gal positive cells were found in the aortic media of renal failure rats, whereas the aortic wall in control rats did not contain senescent cells (Figure 1B). The senescent cells were mainly localized to the calcified area and its surrounding area, which was defined as the area not stained black by von Kossa staining. Quantitative assessment showed that the number of senescent cells with high SA*β*-gal activity was positively correlated with the calcified area in the aortic media (Figure 1C).

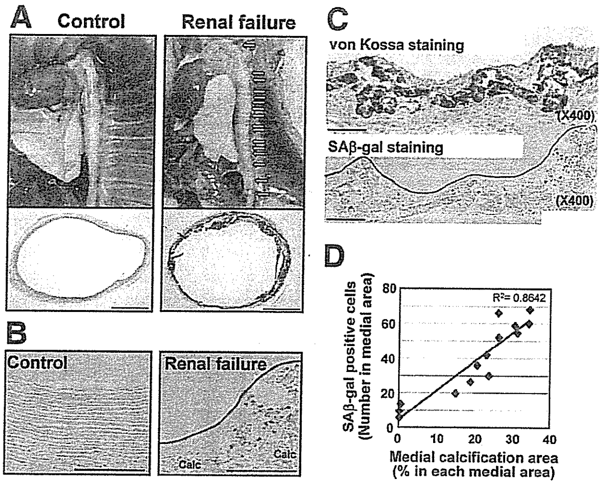

Figure 1. Presence of senescent vascular cells colocalized with calcification in aortic media of renal failure rats. A, Rats with severe renal failure had massive calcification throughout the aorta (right) compared with control rats (left) (n=5). Yellow arrows indicate calcified area. Morphological assessment by von Kossa staining showed extensive calcification in the aortic media of renal failure rats. Scale bar=500 *μ*m. B, Senescent vascular cells (senescence-associated *β*-galactosidase [SA*β*-gal]-positive: blue) were significantly detected throughout the calcified area (Calc) in renal failure rats, whereas these senescent cells were not present in control rats. Scale bar=100 *μ*m. C, Localized association between calcification and senescent cells is shown in renal failure rats. SA*β*-gal-positive cells were frequently found in areas with marked calcification. D, The association of the number of SA*β*-gal-positive cells with the calcified area in each photograph was evaluated. The senescent cell number was linearly correlated with the area of calcification in the aortic media of renal failure rats (calcified area in media: percentage).

Pi Induces Cellular Senescence in Cultured SMCs

On the basis of our results obtained from animal experiments, we hypothesized that senescent SMCs in the aortic media are strongly associated with the development of arterial calcification. Therefore, the effect of excessive Pi stimulation (2.6 mmol/L) on cellular senescence in cultured SMCs was examined. SA*β*-gal-positive senescent HASMCs were significantly induced by not only angiotensin II (Ang II) but also Pi

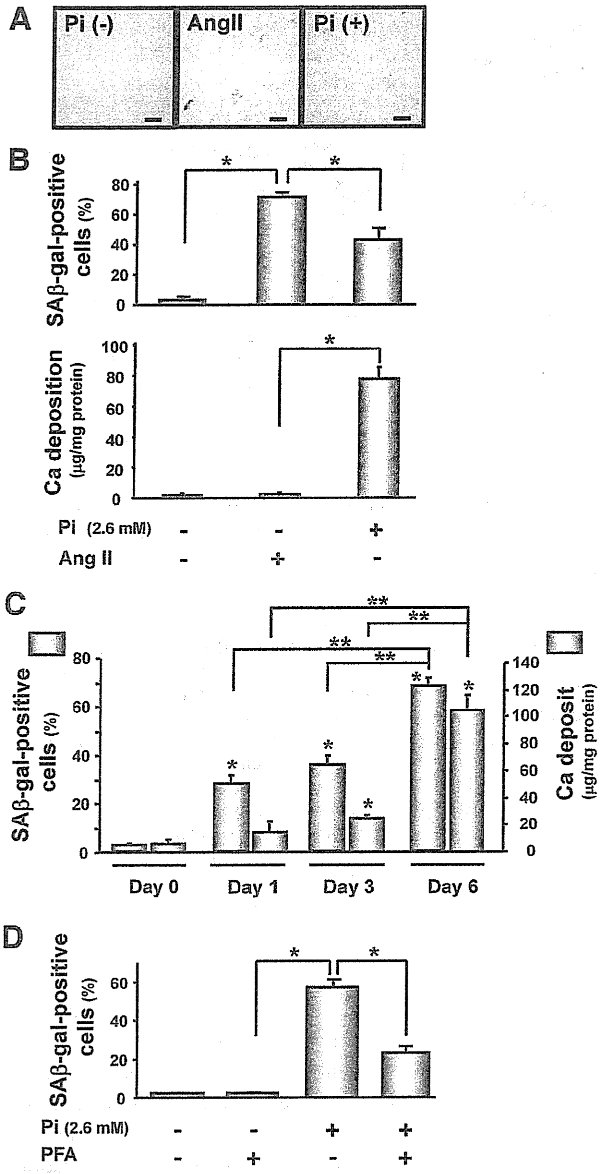

Figure 2. Inorganic phosphate (Pi) stimulation induces cellular senescence in vascular smooth muscle cells (SMCs) via its cotransporter. A, The effect of Pi on senescent transition in human aortic SMCs (HASMCs) was examined. Representative photographs showed that senescence-associated β-galactosidase (SAβ-gal) activity (blue) in cells was significantly induced by not only angiotensin II (Ang II: 10 pmol/L, as a positive control) but also Pi stimulation (2.6 mmol/L). B, The number of senescent cells was significantly increased by not only Ang II but also Pi. Calcium deposition was significantly increased by Pi; however, calcification in HASMCs was not induced by Ang II alone in the absence of Pi. C, Senescent cells were significantly increased by Pi stimulation even on day 1; however, a statistically significant increase in calcium deposition was found from day 3 and later. D, Inhibition of the phosphate cotransporter Na-dependent phosphate cotransporter by the inhibitor phosphonoformic acid (PFA) (100 μmol/L) reduced SAβ-gal activity, which was increased by Pi (2.6 mmol/L) in HASMCs. Each experiment was performed al least 3 times.

stimulation (Figure 2A). Notably, Pi stimulation increased calcium deposition; however, Ang II alone did not (Figure 2B). It suggests that high-dose Pi condition, but not stress by Ang II alone, is indispensable to induce SMC calcification. These findings also suggest that intracellular Pi influx at least is essential to induce this SMC calcification model.

In addition, to determine how many days after the initiation of Pi stimulation the cells showed a senescent phenotype and subsequent calcification, the time-dependent effects of Pi stimulation on both SAβ-gal activity and calcium deposition were examined. As shown in Figure 2C, SAβ-gal-positive cells were significantly increased by Pi stimulation even on day 1, although calcium deposition was not markedly increased at the same time point. A statistically significant increase in calcium deposition was found from day 3 and later. Cotreatment with phosphonoformic acid, an inhibitor of Na-dependent phosphate cotransporter (NPC), showed significant inhibition of Pi-induced senescence (Figure 2D). Our previous report showed that treatment with PFA completely inhibited Pi-induced SMC calcification,[15] suggesting the importance of increased intracellular influx of phosphate in Pi-induced SMC senescence.

Downregulation of SIRT1 by Pi

Treatment of HASMCs with Pi caused downregulation of SIRT1 expression in a time-dependent manner (Figure 3A). The decline was dependent on Pi concentration (data not shown). An increase in acetylation of both substrates of SIRT1, histone-3 and p53 (a nonhistone substrate), was found according to the decline in SIRT1 deacetylase activity. In addition, expression of p21, a downstream molecule of p53, was significantly induced by Pi as well. Quantitative assessment showed that an increase in these expression levels of acetylated (Ac)-p53 and p21 on day 3 and day 6 was statistically significant compared with the pretreatment levels, suggesting that downregulation of SIRT1 activity may mediate the subsequent increase in Ac-p53 and p21 expression.

To address whether SIRT1 downregulation-related SMC senescence and calcification are reversible or not, the effects of continuation or termination of high-dose Pi were examined. As shown in Figure 3B, the continuation of Pi up to day 10 was associated with SIRT1 downregulation and subsequent upregulation of Ac-p53 and p21, leading to induction of senescence-related calcification. However, the slight increase in senescent cells was not statistically significant, although calcification was significantly induced. Of note, the Pi-induced downregulation of SIRT1 was almost completely reversed by withdrawal (termination) of Pi stimulation (exchange of Pi from 2.6 mmol/L to 1.4 mmol/L as a normal level on day 6) as shown in Figure 3B. According to the restoration of SIRT1, levels of both Ac-p53 and p21 were also decreased without more progression. In addition, termination of Pi showed no progression of senescence-related calcification; however, preexisting senescent cells and calcification on day 6 continued without regression.

Next, NPC inhibition by PFA completely blunted Pi-induced SIRT1 downregulation and subsequent activation of its downstream p53/p21 pathway (Figure 3C).

Regulation of SIRT1 Modulates Pi-Induced SMC Senescence and Calcification

The effects of modulation of SIRT1 activity on Pi-induced cellular senescence were investigated. First, sirtinol, a chem-

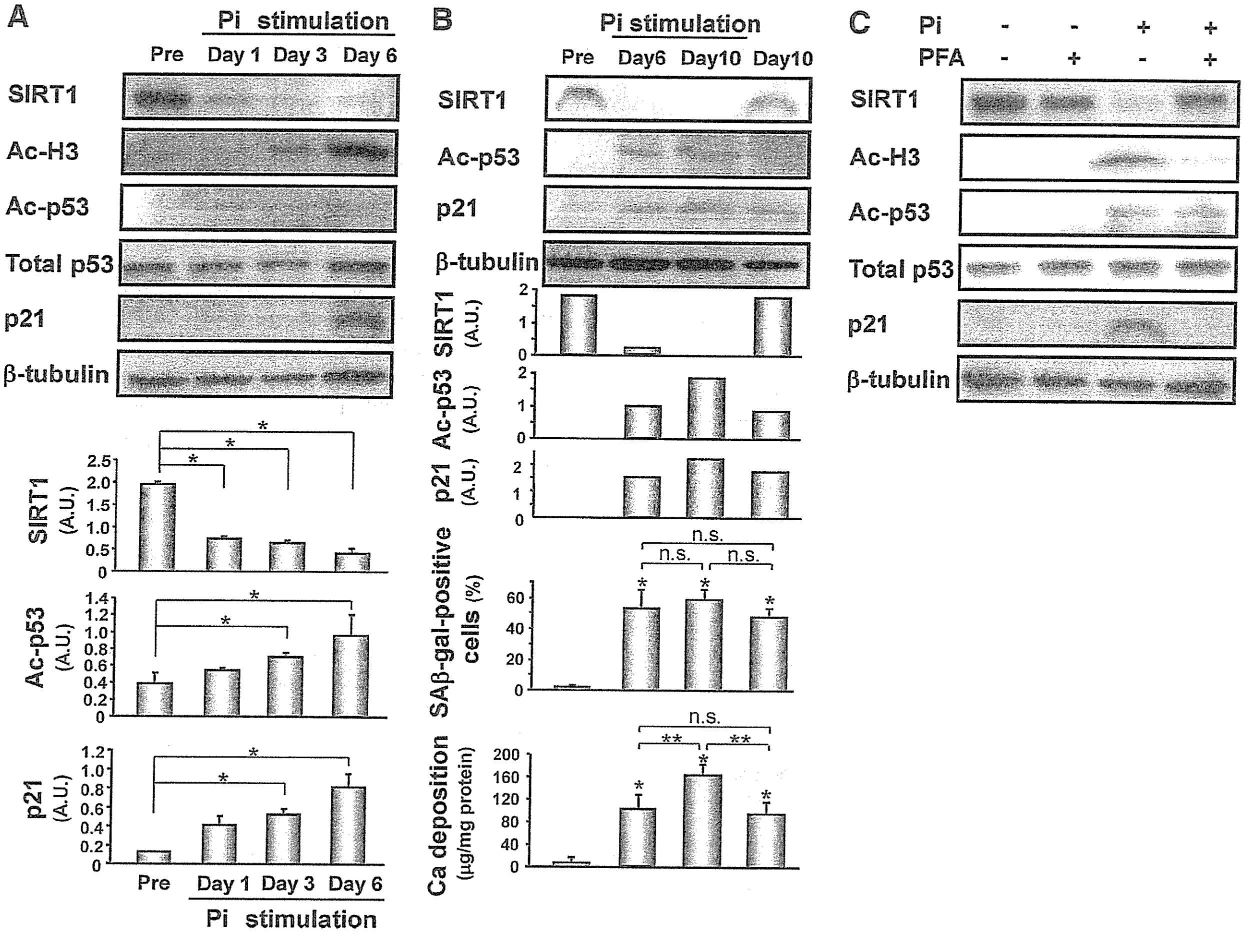

Figure 3. Inorganic phosphate (Pi) stimulation leads to sirtuin 1 (SIRT1) downregulation and subsequent p21 activation. A, The effect of Pi on SIRT1 expression and its downstream pathway was examined. Treatment of human aortic SMCs (HASMCs) with Pi (2.6 mmol/L) showed downregulation of SIRT1 expression, leading to an increase in acetylation of its substrates (acetylated [Ac]-H3 and Ac-p53) and p21 expression. Bottom: Quantitative analysis showed that Pi gradually induced not only SIRT1 downregulation but also upregulation of Ac-p53 and p21. B, To address whether SIRT1 downregulation-related senescence and subsequent calcification are reversible, the effects of continuation or termination of high-dose Pi were examined. As shown in 4th lane from left, termination (on day 6) of Pi showed no progression of senescence-related calcification in association with restoration of SIRT1, whereas continuation (up to day 10, 3rd lane from left) of Pi stimulation showed further progression of calcification. C, Treatment with phosphonoformic acid (PFA), a Na-dependent phosphate cotransporter inhibitor, completely reversed Pi-induced SIRT1 downregulation. A decline in Ac-H3 and Ac-p53 reflected the restoration of SIRT1 deacetylase activity. Pi-induced p21 activation was significantly inhibited by inhibition of Pi transport.

ical inhibitor of SIRT1, induced an increase in SAβ-gal-positive cells even under a normal Pi (1.4 mmol/L), and the increased number of senescent cells induced by Pi was significantly augmented by sirtinol (Figure 4A). Sirtinol dose-dependently augmented Pi-induced calcification, although no augmentation was found under a normal Pi (Figure 4B and 4C). Conversely, treatment with resveratrol, an activator of SIRT1, significantly reduced both Pi-induced senescent transition and calcification in a dose-dependent manner (Figure 4D to 4F).

Second, complete knockdown of SIRT1 by siRNA caused a significant increase in acetylation of both substrates (histone-3 and p53) and p21 expression (Figure 5A). Similarly to sirtinol, SIRT1 inhibition by siRNA also augmented not only senescent transition (Figure 5A, bottom) but also calcium deposition (Figure 5C, top).

Although stimulation with Ang II alone could increase the number of SAβ-gal-positive cells, it did not increase calcium deposition. To understand the mechanism of these discrepant phenomena, the effect of Ang II alone on osteoblastic phenotypic change was examined. Ang II alone did not increase the expression of Runx2 in the absence of Pi stimulation, unlike Pi stimulation (Figure 5B).

To understand the detailed mechanism by which SIRT1 modulates senescence-related calcification, the effect of SIRT1 on phenotypic change in HASMCs was examined. Pi inhibited the expression of caldesmon, a differentiated SMC lineage marker, and complete knockdown of SIRT1 augmented the Pi-induced partial downregulation of caldesmon (Figure 5C, middle). In contrast, real-time polymerase chain reaction analysis showed that Pi induced the expression of two representative osteoblastic markers, Runx-2/Cbfa-1 and alkaline phosphatase (Figure 5C, bottom) with statistical significance. In addition, complete knockdown of SIRT1 using siRNA significantly accelerated the Pi-induced os-

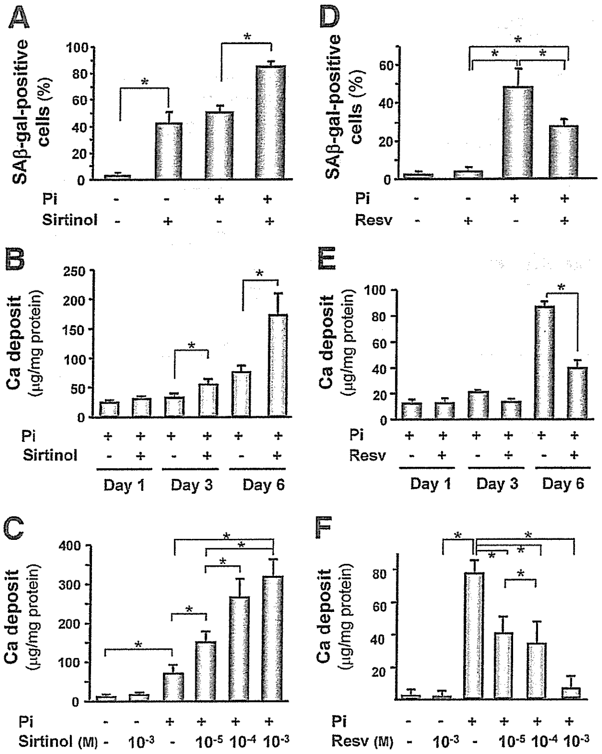

Figure 4. Modulation of sirtuin 1 (SIRT1) affects inorganic phosphate (Pi)–induced senescent phenotypic change and calcification in smooth muscle cells (SMCs). The effects of sirtinol (a chemical inhibitor of SIRT1 activity; A to C) and resveratrol (an activator of SIRT1; D to F) on Pi-induced senescent phenotypic change and calcification were examined ($n=6$). A, SIRT1 inhibition by sirtinol (10 μmol/L) showed an increase in the number of senescence-associated β-galactosidase (SAβ-gal)-positive cells even without Pi stimulation. The increase in Pi-induced senescence was significantly augmented by sirtinol. Sirtinol augmented Pi-induced calcium deposition in human aortic SMCs (HASMCs) in a time-dependent (B) and dose-dependent manner on day 6 (C). Conversely, treatment with resveratrol (Resv; 10 μmol/L) showed a reduction of the Pi-induced senescent phenotype (D) and calcification (E). The inhibitory effect of resveratrol on calcification was dose dependent (F).

teoblastic phenotypic change, suggesting that modulation of SIRT1 is associated with osteoblastic phenotypic change in SMCs.

Inhibition of Senescence-Related Calcification in SMCs by p21 Knockdown

To address the association of p21 with senescence-related calcification, knockdown of p21 using siRNA was performed. Treatment of p21 siRNA (up to 200 pmol/L) completely inhibited p21 (Figure 5D). p21 knockdown completely inhibited Pi-induced senescence and subsequent calcification (Figure 5E).

Regulation of NPC-Mediated Runx2 Expression by SIRT1/p21 Pathway

As the next step, the role of SIRT1 in NPC-mediated Runx2/Cbfa1 expression was examined. First, complete knockdown of SIRT1 did not show any change in both osteoblastic markers, Runx2 and alkaline phosphatase, in a normal Pi (Supplemental Figure I). As shown in Figure 5F, Pi-induced Runx2 was significantly blunted by PFA, an NPC inhibitor. SIRT1 activation by resveratrol inhibited Pi-induced Runx2 activation. The Runx2 induction was augmented by knockdown of SIRT1 by siRNA, and the activation was completely inhibited by PFA. Surprisingly, Runx2 activation was strongly inhibited by knockdown of p21 alone. In addition, the inhibition of Runx2 induction by double knockdown of SIRT1 and p21 was less than that by single knockdown of SIRT1.

To address a difference in senescent induction by Pi or Ang II, immunohistological assessment of SIRT1 in HASMCs was examined (Supplemental Figure II). Although SIRT1 was predominantly localized in nucleus without Pi, the translocation of SIRT1 to cytoplasm was observed after Pi stimulation for 24 hours, and its expression disappeared in both areas on day 6. In contrast, Ang II stimulation did not show the dynamic translocation.

High Sensitivity of SMCs With Replicative Senescence to Pi-Induced Calcification

Not only Pi-induced "premature senescence" in HASMCs but also the effects of Pi on "replicative senescence" were evaluated. Senescent cells (passage 18) were more sensitive to Pi-induced calcification compared with young cells (passage 7) (Figure 6A). SIRT1 expression was downregulated in senescent cells compared with young cells, and the downregulation was significantly augmented by Pi stimulation (Figure 6B, top). In parallel with this finding, senescent cells showed an increase in Ac-p53 and p21 expression. Statistical analyses using densitometric measurement showed that (1) downregulation of SIRT1 and upregulation of Ac-53 and p21 were augmented by replicative senescence, and (2) Pi inhibited the SIRT1-p21 pathway even in cells with replicative senescence (passage 18) (Figure 6B, bottom).

Discussion

Vascular aging, leading to cardiovascular disease, manifests complex and diverse vascular changes (eg, impairment of distensibility due to loss of arterial elasticity).[1,16] Arterial wall stiffness resulting from ectopic calcification is a complication of advanced atherosclerosis and makes the management of hemodynamics more difficult in the elderly. Few reports have addressed whether cellular senescence is associated with SMC calcification. This study showed the importance of SIRT1, a longevity gene, in arterial calcification in association with cellular senescence.

First, our data obtained from animal experiments clearly showed the association of senescent SMCs with aortic medial calcification in the renal failure rats with hyperphosphatemia. Senescent cells showed significant colocalization with calcium deposition. Intriguingly, numerous senescent cells could be detected before microscopic calcification occurred at 4 weeks after the start of renal failure induction (data not shown), suggesting that the transition to a senescent phenotype in medial SMCs may be associated with the initiation and progression of calcification. Therefore, hyperphosphatemia, a potent uremic factor, may be a stimulator to induce senescent phenotypic transition of medial SMCs.

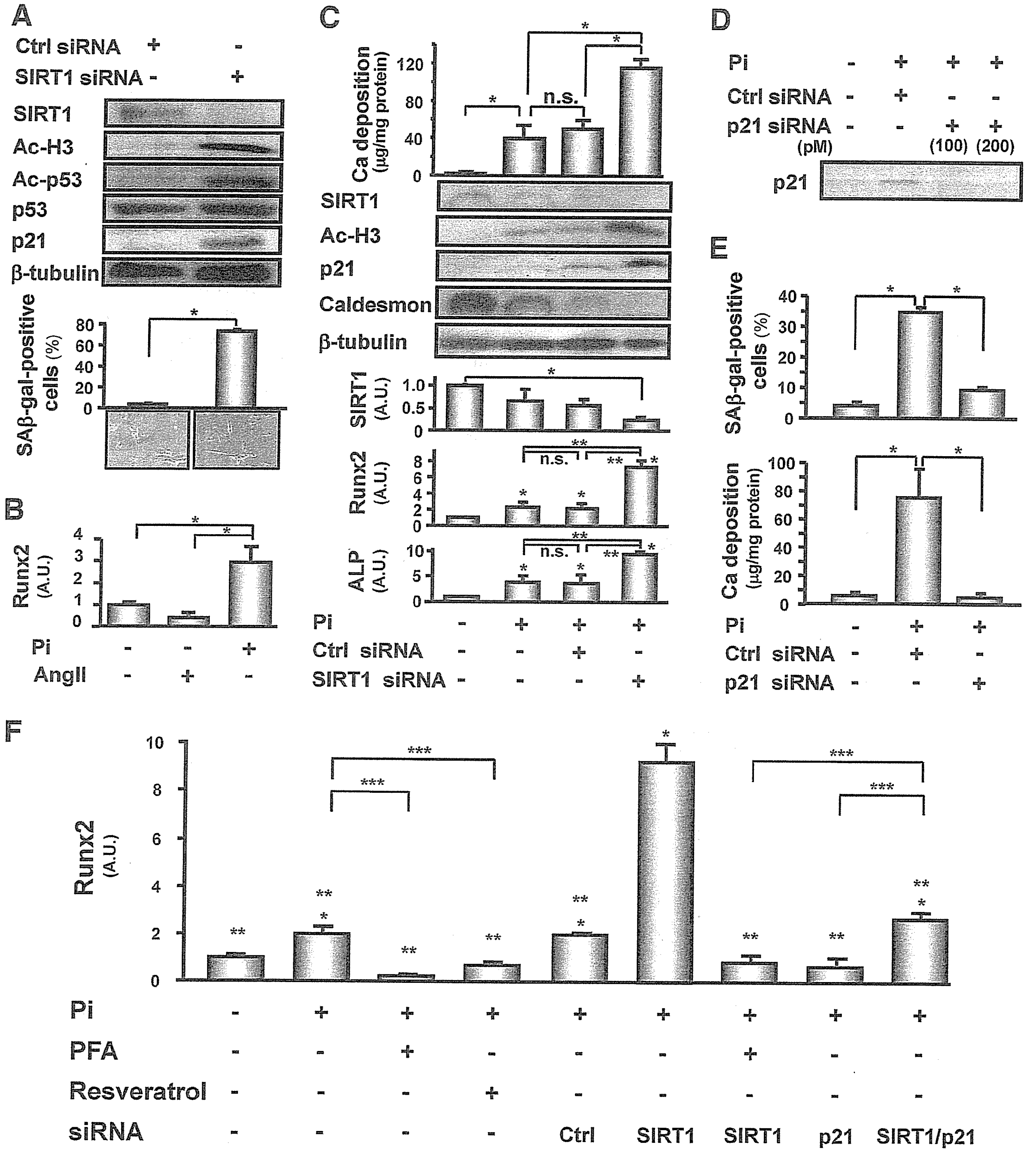

Figure 5. Augmentation of senescence-related smooth muscle cell (SMC) calcification by sirtuin 1 (SIRT1) knockdown in association with osteoblastic phenotypic change and prevention of inorganic phosphate (Pi)–induced changes by p21 knockdown. A, To achieve SIRT1 knockdown in human aortic SMCs (HASMCs), small interfering RNA (siRNA) was simultaneously administered at the start of Pi stimulation (2.6 mmol/L). Complete inhibition of SIRT1 showed a significant increase in acetylation of both substrates (acetylated [Ac]-H3 and Ac-p53), p21 expression and senescence-associated β-galactosidase (SAβ-gal)-positive cells. B, Angiotensin II (Ang II) alone (10 pmol/L) did not increase the expression of Runx2 in the absence of Pi stimulation, unlike Pi stimulation. C, top: SIRT1 knockdown by siRNA significantly accelerated Pi-induced calcification (n=6), whereas control (Ctrl) siRNA did not. C, middle and bottom: Western blots showed that Pi partially inhibited the expression of a differentiated SMC marker, caldesmon, and complete knockdown of SIRT1 by siRNA augmented its downregulation. Real-time polymerase chain reaction analysis showed that Pi induced the expression of Runx-2 and alkaline phosphatase (ALP). Complete knockdown of SIRT1 significantly accelerated the Pi-induced osteoblastic markers. A.U. indicates arbitrary units. *$P<0.05$. D and E, Knockdown of p21 by siRNA (200 pmol/L) significantly reduced the senescent phenotypic change and subsequent calcification (n=6). F, The role of SIRT1/p21 axis in Na-dependent phosphate cotransporter-mediated Runx2 expression was evaluated. Augmentation of Pi-induced Runx2 expression by SIRT1 knockdown was significantly inhibited by double knockdown of SIRT1 and p21. *$P<0.05$ vs control without Pi stimulation (left column), **$P<0.05$ vs Pi-stimulated cells with SIRT1 siRNA (sixth column from left).

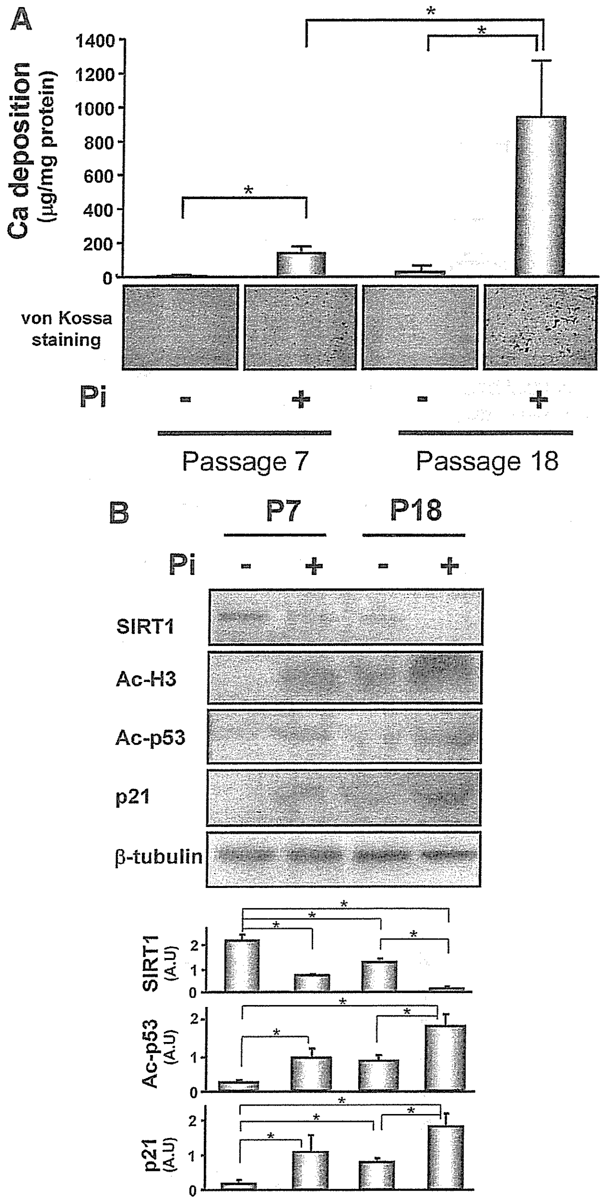

Figure 6. High sensitivity of smooth muscle cells (SMCs) with replicative senescence to inorganic phosphate (Pi)–induced calcification. The effects of replicative senescence in human aortic SMCs (HASMCs) on Pi-induced calcification (A) and sirtuin 1 (SIRT1)-related molecules (B) were also evaluated. A, Senescent cells (passage 18 [P18]) were more sensitive to Pi-induced calcification compared with young cells (passage 7 [P7]) (n=6). Representative photographs of von Kossa staining (bottom) show strong induction of calcium deposition by Pi (2.6 mmol/L). B, Senescent HASMCs (P18) showed a decline in SIRT1 expression and an increase in p21 expression compared with young cells (P7). Pi stimulation of senescent cells significantly inhibited SIRT1 expression and accelerated the increase in p21 and acetylated (Ac)-p53. Densitometric analysis confirmed these more sensitive responses in senescent cells. A.U. indicates arbitrary units. *$P<0.05$.

Second, we also confirmed the association of Pi-induced SMC senescence with calcification in in vitro experiments. Senescent SMCs were significantly increased by Pi even on day 1, although calcium deposition was not markedly increased at the same time point. A statistically significant increase in calcium deposition was found from day 3 and later. Considering these data, we hypothesize that (1) calcium deposition may be more readily induced in senescent cells compared with nonsenescent cells, and (2) Pi-induced senescent change is observed earlier than calcium deposition. In other words, senescent transition associated with Runx2 induction may lead to progressive calcification.

Senescent SMCs were associated with the SIRT1-related p53/p21 pathway, based on the findings that SIRT1 knockdown augmented not only cellular senescence but also calcification. In addition, p21 knockdown completely inhibited senescence-related calcification induced by Pi. This raises the question of how cellular senescence in SMCs is associated with calcification. Our experiments to understand the detailed mechanisms by which SIRT1 modulates senescence-related calcification showed that Pi-induced SIRT1 downregulation led to the phenotypic change from a differentiated state to osteoblast-like cells in SMCs. It has been reported that Pi induces osteoblastic change, in which NPC plays a role in inducing Runx2/Cbfa-1 expression, in SMCs.[17] As the next step, to determine how SIRT1 regulates NPC-mediated Runx2 expression, we examined the effects of knockdown of SIRT1, p21, or both by siRNA on Pi-induced Runx2 expression. Our data shown in Figure 5F suggested that (1) NPC plays an essential role in Pi-induced Runx2 expression, (2) SIRT1 has an inhibitory effect on NPC-mediated Runx2 expression, (3) knockdown of p21 alone ameliorates Runx2 induction, and (4) p21-related osteoblastic change is at least in part dependent on SIRT1.

There is now the new question of how SIRT1 regulates Runx2 regulation. A report by Jeon[18] has shown that acetylation of Runx2 itself is important in osteoblast differentiation, and it is downregulated by HDAC activities. Based on this evidence, SIRT1, 1 of the HDACs, may be able to deacetylate Runx2, leading to inhibition of Runx2-related osteoblastic transition in SMCs. Therefore, the inhibition of SIRT1 by hyperphosphatemia may lead to Runx2 activation via its hyperacetylation. Further investigation of the detailed mechanism of the SIRT1/p21/osteoblastic gene axis is needed. These data clearly suggest that SIRT1 activation may inhibit the hyperphosphatemia-induced osteoblastic phenotypic change of SMCs, and the degree of change may be dependent on SIRT1 expression level. It is possible that the inhibition of SIRT1 expression by Pi alone is "partial," because complete downregulation of SIRT1 by siRNA worsened the dynamic phenotypic change compared with Pi only. We have already shown that tumor necrosis factor-α, a potent atherogenic cytokine, augmented Pi-induced SMC calcification, as previously described.[19] In addition, tumor necrosis factor-α significantly decreased Pi-induced SIRT1 downregulation further (data not shown). According to these results, we currently hypothesize that hyperphosphatemia induces SIRT1 downregulation and subsequent osteoblastic phenotypic change in SMCs, leading to calcification, and these changes are worsened by some harmful atherogenic factors, which decrease SIRT1 expression/activity further. These results provide a new insight, showing that SIRT1 plays an essential role in the prevention of arterial calcification and that the beneficial effect may be associated with an inhibition in Pi-induced SMC senescent transition.

In addition, Ang II did not increase calcium deposition, although the stimulation increased the number of senescent cells. Of note, Ang II alone did not increase Runx2 expression in the absence of Pi (Figure 5B). This result suggests that SMC senescence shows two different features: one is SAβ-gal-positive cells with an increase in Runx2 and the other is SAβ-gal-positive cells without. First, it has recently been reported that SMCs with replicative senescence, rather than the cells without senescence, show hypersensitivity in response to induction of calcification with the more induction of osteoblastic markers,[20] suggesting that the induction of osteoblastic transdifferentiation is strongly associated with the senescent change in SMCs. In addition, the translocation of SIRT1 to cytoplasm was observed after Pi stimulation for 24 hours, although SIRT1 predominantly localized in nucleus without Pi. In contrast, Ang II did not show the dynamic translocation. Thinking about the mechanism for regulating the activity of HDACs, including SIRT1, recent several reports show the importance of their coordinated shuttling between nucleus and cytoplasm. A report demonstrates that HDAC7, an HDAC, represses the transcriptional activity of Runx2 and that osteogenic stimuli induce export of HDAC7 from nucleus, leading to a decline in the repressive potentials of HDAC7 for Runx2.[21] On the basis of our findings and a previous report, the reason that stimulation with Ang II alone did not induce Runx2 expression and subsequently SMC calcification may in part depend on the difference of SIRT1 translocation after stimulation. Therefore, we strongly hypothesize that in the senescent SMCs with upregulation of p21, Pi stimulation, but not Ang II stimulation, may activate Runx2 via at least two phenomena, the hyperacetylation of Runx2 by SIRT1 downregulation and the dynamic SIRT1 translocation, leading to marked osteoblastic transdifferentiation and subsequent calcification. In addition, we have another hypothesis. In general, it has been shown that high-dose Pi navigates release of matrix vesicles from SMCs in parallel with osteoblastic transdifferentiation. The vesicles play an essential role in the initiation of hydroxyapatite aggregation, so-called nucleation. Accumulating recent reports show that the nanocrystal formation as an initial step under hyperphosphatemia accelerates the harmful cascade of osteoblastic transdifferentiation in SMCs via endocytosis.[22,23] Maybe Ang II alone does not induce the nanocrystal formation and the cascade of osteoblastic change. Therefore, we explain that the difference of senescent phenotypic changes in SMCs between both stimulations, Pi and Ang II alone, may depend on (1) SIRT1 translocation and (2) nanocrystal formation to accelerate calcification. Further investigation to address the detailed mechanisms by which SIRT1 regulates osteoblastic transdifferentiation in SMCs under the cellular senescence is needed.

Are SIRT1 downregulation-related SMC senescence and subsequent calcification reversible or not? To answer this question, the effects of continuation or termination of high-dose Pi were examined. As shown in Figure 3B, termination (on day 6) of Pi showed no progression of senescence-related calcification in association with the restoration of SIRT1, whereas continuation (up to day 10) of Pi stimulation showed further progression of calcification. It is suggested that a therapeutic strategy to manage hyperphosphatemia to the normal range of serum phosphate concentration may lead to at least termination of progressive calcification via reversal of SIRT1 activity.

Cellular senescence has been shown to have two features: not only stress-induced premature senescence but also replicative senescence, indicating a limited number of divisions in culture.[24] In fact, both endothelial cells and SMCs derived from human atherosclerotic plaques show a senescent phenotype earlier than do cells from normal vessels.[25] Notably, we found that senescent HASMCs were significantly more sensitive to Pi-induced calcification compared with young cells. These results suggest that calcium deposition may be more readily induced in arterial medial SMCs with replicative senescence. This insight may explain the mechanisms by which arterial calcification occurs in the elderly more frequently than in the young population. Therefore, these observations support our hypothesis that arterial calcification is accelerated by both senescent types (premature and replicative senescence) in SMCs. To explore new therapeutic strategies against arterial calcification, it is essential to investigate how to maintain a higher SIRT1 level in the vasculature, leading to prevention of medial SMC senescence and which drug is capable of achieving it.

How does SIRT1 exert protective effects against SMC calcification? This study clearly showed that inhibition of SIRT1 was associated with increases in both Ac-p53 and p21 expression. These findings were significantly induced by not only replicative senescence but also Pi-induced premature senescence. SIRT1-mediated deacetylation of p53 inhibits p53-dependent transactivation of target genes, including p21. A report showed that a decline in cellular deacetylase activity increases the half-life of endogenous p53,[26] suggesting that p53 acetylation is also associated with p53 stabilization. Therefore, the increased Ac-p53 by Pi-induced SIRT1 downregulation may induce SMC senescence because of a decline in degradation of p53, leading to calcification. In addition, p53 itself can inhibit SIRT1 transcription because the SIRT1 promoter has two response elements to p53.[27] Further investigation to address how the SIRT1-p53 negative regulatory pathway is associated with SMC calcification is needed.

On the other hand, regarding p21 activation, it is reported that inhibition of p21 expression in the vasculature significantly attenuates cellular senescence, leading to prevention of atherosclerosis.[28] This evidence suggests a pivotal role of p21 in the development of atherosclerosis. p21 activation has been shown to be regulated by a pathway that is p53 dependent, p53 independent, or both. Okamoto et al have demonstrated that inhibition of HDAC by trichostatin A showed activation of p21 promoter activity by the Sp1 site even in vascular SMCs, and the induction of p21 was independent of the p53 pathway.[29] The p21 transcriptional activation in response to HDAC inhibitors was mediated by histone hyperacetylation in its promoter region. Based on these findings, Pi-induced p21 activation via SIRT1 downregulation may be in part involved in a p53-independent pathway, leading to a senescent phenotype of SMCs. Further investigation exploring which molecule activates the p21 promoter under hyperphosphatemia is needed.

Conclusion

We showed that SIRT1 exerts a protective role in hyperphosphatemia-based arterial calcification via inhibition of osteoblastic transdifferentiation, in association with cross-talk between calcification and cellular senescence. This ability of SIRT1 may orchestrate an analogous protective/longevity paradigm even in vascular SMCs, leading to maintenance of healthy elasticity of the arterial wall. Strategies to maintain a higher level of SIRT1 activity may provide novel therapeutic opportunities for the prevention of arterial calcification.

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Disclosures

None.

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Supplement Material

Sirtuin SIRT1 retards hyperphosphatemia-induced calcification of vascular smooth muscle cells

Methods

Aortic calcification in renal failure rats

Renal failure was induced in rats by a 0.75% adenine-containing diet as previously described.[28] Twelve-week-old male Wistar rats (Nippon Clea Inc., Japan) were pair-fed standard CE-2 chow (containing 1.2% calcium and 0.6% phosphorus; Nippon Clea Inc.) in the control group or CE-2 chow containing 0.75% adenine (Sigma) in the renal failure group for 4 weeks. Then, the diet was returned to normal chow for an additional 4 weeks. After induction of renal failure for 8 weeks in total, the rats were sacrificed to collect samples. After perfusion with saline at a constant, nonpulsatile pressure of 100 mmHg, the aorta was immediately embedded in OCT compound frozen section and sequentially cut into cross-sections with 5-μm thickness from each part of the aorta. To detect calcification in the aortic wall, each cross-section was subjected to von-Kossa staining to demonstrate mineralization. The calcified area and number of

SAβ-gal-positive cells in the cross-section were measured by image analysis software (ImageJ, Scion Image, Maryland, USA). All procedures and animal care were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo.

Induction of SMC calcification

Primary human aortic SMC (HASMC), derived from the internal thoracic artery (Clonetics), were treated with a pathological concentration of inorganic phosphate (Pi) in culture medium. To set up the calcification medium, a mixed solution of Na_2HPO_4 and NaH_2PO_4 whose pH was adjusted to 7.4 was added to serum-supplemented DMEM to final doses of up to 3.2 mmol/L as previously described.[29] To quantitatively measure Pi-induced calcification, two distinct experiments, (1) intracellular calium (Ca) deposition as determined by *o*-cresolphthalein complexone method and (2) visualization of mineralization as determined by von-Kossa staining, were performed as previously described.[28] We have previously confirmed that excessive Pi stimulation dose- and time-dependently induced calcium deposition in HASMC, whereas a normal Pi dose (1.4 mmol/L), equivalent to the human physiological level of serum phosphate, did not.[29]

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

To assess senescent changes in the phenotype of cultured HASMC or aortic medial cells of rats with/without renal failure, staining for senescence-associated β-galactosidase (SAβ-gal), a well-established biomarker of cellular senescence, was performed at pH 6.0, as opposed to endogenous lysosomal enzyme detected at pH 4.0 in normal cells, as previously described.[30] Numbers of SAβ-gal-positive cells were quantitatively counted in the aortic wall or cultured HASMC. As a positive control in *in vitro* experiments, angiotensin II (AngII) was used to induce transition to a senescent phenotype in HASMC.

Knockdown of SIRT1 or p21 by small interfering RNA

HASMC were transfected with 200 pmol/L small interfering RNA (siRNA) for SIRT1 (GAT GAA GTT GAC CTC CTC A and TGA AGT GCC TCA GAT ATT A, Santa Cruz Biotechnology) or control (Cntl) using silMPORTER (Upstate). In addition, knockdown of $p21^{WAF1/CIP1}$ was performed using 100 to 200 pmol/L siRNA for p21 (CGA CUG UGA UGC GCU AAU G, CCU AAU CCG CCC ACA GGA A, CGU CAG AAC CCA UGC GGC A, and AGA CCA GCA UGA CAG AUU U) by the same method. To inhibit p21 expression effectively and completely, four kinds of sequences of p21 siRNA were used. HASMC were treated simultaneously with these siRNAs at the start of Pi stimulation.

Western blot and SDS-PAGE

Protein expression was assessed by Western blot analysis with chemiluminescence detection. SIRT1 was detected using a rabbit polyclonal anti-SIRT1 antibody (Abcam), and p53, acetylated p53 (Ac-p53; Lys-382), acetylated histone-3 (Ac-H3), p21, caldesmon and β-tubulin were detected with monoclonal antibodies (Santa Cruz Biotechnology). The expression levels of Ac-p53 and Ac-H3 were used to reflect SIRT1 activity as a deacetylase. Caldesmon was used to reflect a lineage marker of SMC differentiation.

Real-time PCR analysis: Osteoblastic markers

Primer sequences were as follows:

ALP; (forward) ACCATTCCCACGTCTTCACATTTG,

(reverse) AGACATTCTCTCGTTCACCGCC,

Runx-2/Cbfa-1; (forward) TCTGGCCTTCCACTCTCAGT, (reverse) GACTGGCGGGGTGTAAGTAA,

SIRT1; (forward) CCTGACTTCAGGTCAAGGGATGGTA,

(reverse) CTGATTAAAAATATCTCCTCGTACAG,

β-actin; (forward) CTGGAACGGTGAAGGTGACA,

(reverse) AAGGGACTTCCTGTAACAATGC.

Materials

Angiotensin II (AngII) was used to induce senescent phenotypic change in cultured HASMC as a positive control. To inhibit activity of Na-dependent phosphate cotransporter (NPC) stimulated by treatment with exogenous Pi, phosphonoformic acid (PFA; SIGMA), a chemical inhibitor, was used. Sirtinol (a chemical inhibitor; Calbiochem) or resveratrol (an activator of SIRT1; WAKO) was used for modulation of SIRT1 activity. Localization of SIRT1 in HASMC was detected using its antibody (Santa Cruz: sc-15404).

Immunohistological staining

To address a difference in senescent induction by Pi or AngII, the localization of SIRT1 in HASMC was compared using immunohistological assessment. SIRT1 specific