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ORIGINAL ARTICLE

Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras–MAPK signaling in human cancer cells

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The induction of senescence-like growth arrest has emerged as a putative contributor to the anticancer effects of chemotherapeutic agents. Clinical trials are underway to evaluate the efficacy of inhibitors for class I and II histone deacetylases to treat malignancies. However, a potential antiproliferative effect of inhibitor for Sirt1, which is an NAD^+-dependent deacetylase and belongs to class III histone deacetylases, has not yet been explored. Here, we show that Sirt1 inhibitor, Sirtinol, induced senescence-like growth arrest characterized by induction of senescence-associated β-galactosidase activity and increased expression of plasminogen activator inhibitor 1 in human breast cancer MCF-7 cells and lung cancer H1299 cells. Sirtinol-induced senescence-like growth arrest was accompanied by impaired activation of mitogen-activated protein kinase (MAPK) pathways, namely, extracellular-regulated protein kinase, c-*jun* N-terminal kinase and p38 MAPK, in response to epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I). Active Ras was reduced in Sirtinol-treated senescent cells compared with untreated cells. However, tyrosine phosphorylation of the receptors for EGF and IGF-I and Akt/PKB activation were unaltered by Sirtinol treatment. These results suggest that inhibitors for Sirt1 may have anticancer potential, and that impaired activation of Ras–MAPK pathway might take part in a senescence-like growth arrest program induced by Sirtinol.
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Keywords: Sirt1; Sirtinol; cellular senescence; Ras; MAPK; Akt/PKB

Introduction

Cellular senescence is a state with permanent loss of replicative capability even upon mitogenic stimuli. Cellular senescence is characterized by phenotypic alterations including induction of senescence-associated β-galactosidase (SA-β-gal), a large and flat cell morphology and increased expression of plasminogen activator inhibitor 1 (PAI-1) (Goldstein *et al.*, 1994; Dimri *et al.*, 1995).

Immortalization, an escape from the replicative senescence program, is a necessary step for neoplastic transformation of cells. Hence, transformed cells can bypass the replicative senescence program. However, cancer and leukemia cells still retain the capacity to undergo premature senescence in response to various stimuli. Senescent normal human fibroblasts usually exhibit G1 cell cycle arrest. However, polyploidy and multinucleation are also associated with replicative senescence and premature senescence in various cell types, including human normal endothelial cells (Aviv *et al.*, 2001; Wagner *et al.*, 2001) and breast cancer MCF-7 cells (Kim *et al.*, 2003).

Anticancer chemotherapeutic agents and ionizing radiation have been shown to cause senescence-like growth arrest in human cancer cells *in vitro* and *in vivo* (Han *et al.*, 2002; Schmitt *et al.*, 2002; te Poele *et al.*, 2002; Shay and Roninson, 2004). Induction of SA-β-gal staining by chemotherapy was observed *in vivo* in cancer and lymphoma cells in rodents (Elmore *et al.*, 2002; Roninson, 2002; Schmitt *et al.*, 2002; Christov *et al.*, 2003) and in patients with breast cancer (te Poele *et al.*, 2002). Thus, senescence-like growth arrest has been proposed to be a putative determinant of *in vivo* tumor response to chemotherapeutic agents and ionizing radiation (Mathon and Lloyd, 2001; Wang *et al.*, 2003; Ben-Porath and Weinberg, 2004; Kahlem *et al.*, 2004; Pelicci, 2004; Sharpless and DePinho, 2004; Shay and Roninson, 2004).

Sirt1 is a mammalian NAD^+-dependent deacetylase that belongs to class III histone deacetylases (HDACs) (Imai *et al.*, 2000; Landry *et al.*, 2000; Blander and Guarente, 2004). Sir2, yeast homologue of Sirt1, is involved in a number of cellular processes including gene silencing at telomere and mating loci, DNA repair, recombination and aging. Recent studies demonstrated that Sirt1 plays an important role in the regulation of cell fate and stress response in mammalian cells. Sirt1 promotes cell survival by inhibiting apoptosis or cellular senescence induced by stresses including DNA damage

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and oxidative stress. An increasing number of proteins have been identified as substrates of Sirt1, including p53 (Luo *et al.*, 2001; Vaziri *et al.*, 2001; Langley *et al.*, 2002), FOXO transcription factors (Brunet *et al.*, 2004; Daitoku *et al.*, 2004; Motta *et al.*, 2004; van der Horst *et al.*, 2004), peroxisome proliferator-activated receptor-γ (Picard *et al.*, 2004) and Ku70 (Cohen *et al.*, 2004).

Sirt1 deacetylase, a member of the class III HDAC family, exhibits distinct biochemical characteristics from conventional class I and class II HDACs. Inhibitors for class I and class II HDACs, such as trichostatin A and its derivatives, do not inhibit the deacetylating activity of Sirt1 (Imai *et al.*, 2000). Conversely, specific inhibitors for Sirt1 such as Sirtinol do not inhibit class I and class II HDACs, either (Bedalov *et al.*, 2001; Grozinger *et al.*, 2001).

Class I and class II HDAC inhibitors exhibit antiproliferative effects in human cancer cells (Rosato and Grant, 2004; Vanhaecke *et al.*, 2004; Vigushin and Coombes, 2004). The efficacy of class I and class II HDAC inhibitors for treatment of patients with cancer or leukemia has been examined in phase I and II clinical trials (Piekarz *et al.*, 2001; Sandor *et al.*, 2002; Kelly *et al.*, 2003; McLaughlin and La Thangue, 2004; Piekarz and Bates, 2004). However, the effects of inhibitor for Sirt1 or class III HDACs on cell growth have not yet been investigated. Here, we show that Sirtinol, a specific inhibitor for Sirt1, induced senescence-like growth arrest in human breast cancer MCF-7 and lung cancer H1299 cells, and that Sirtinol-induced senescence-like growth arrest was accompanied by blunted activation of Ras–mitogen-activated protein kinase (MAPK) pathways in response to growth factors.

Results

Sirtinol, Sirt1 inhibitor, induced senescence-like growth arrest in human MCF-7 and H1299 cells

MCF-7 and H1299 cells were exposed to Sirtinol (100 μM) for 24 h; then Sirtinol was removed from the culture media. Treatment with Sirtinol inhibited cell growth in both MCF-7 and H1299 cells (Figure 1a and b). The inhibition of cell growth was persistent and observed up to 9 days after Sirtinol withdrawal. These results suggest that Sirtinol caused a sustained growth arrest. This was supported by reduced incorporation of BrdU in Sirtinol-treated MCF-7 and H1299 cells at 10 days after the addition of Sirtinol, as compared with untreated cells (Figure 1c and d).

We examined the effects of Sirtinol treatment on SA-β-gal activity and the expression of PAI-1, characteristic features of senescence-like growth arrest. Sirtinol treatment increased SA-β-gal-positive cells in a dose-dependent manner 10 days after the addition of Sirtinol in both MCF-7 and H1299 cells (Figures 2 and 3a), but the extent of SA-β-gal induction was relatively smaller in H1299 than in MCF-7 cells. Only a small number of MCF-7 and H1299 cells were SA-β-gal-positive when untreated. Enlarged, flattened morphology was

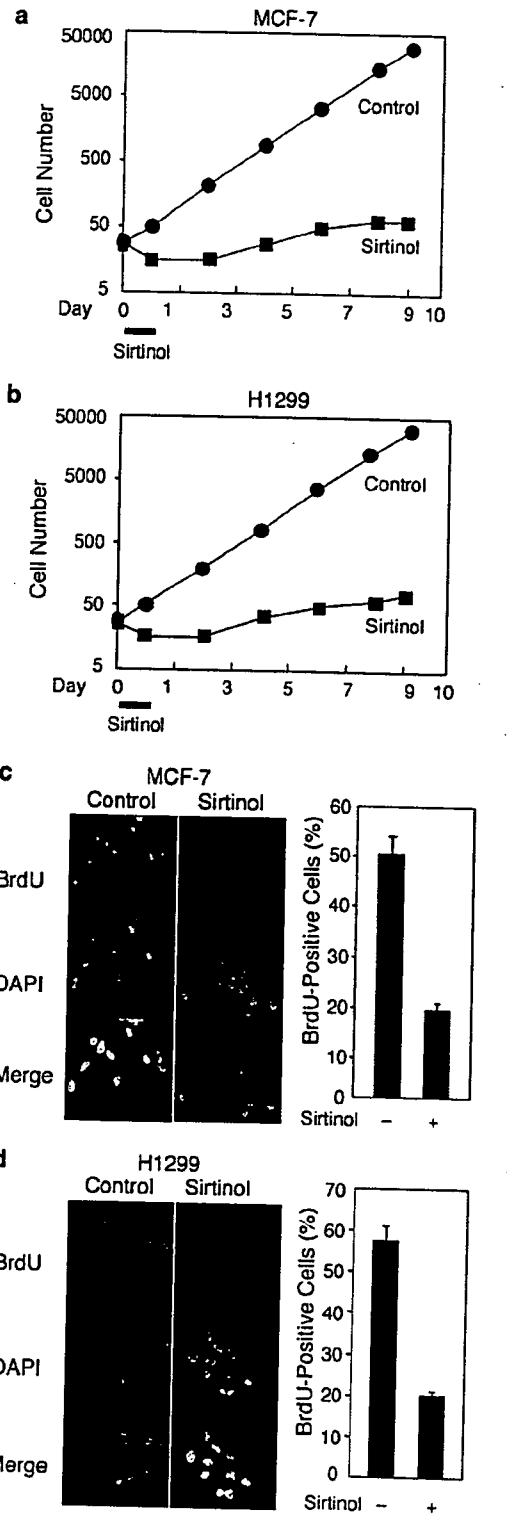

Figure 1 Effects of Sirtinol on cell growth and BrdU incorporation. MCF-7 (a) and H1299 (b) cells were treated with Sirtinol (100 μM) for 24 h. At 24 h after the addition of Sirtinol, Sirtinol was removed from the media, and then the cells were cultured in inhibitor-free complete media. (c, d) BrdU incorporation was evaluated at 10 days after the addition of Sirtinol (100 μM). BrdU incorporation was decreased in Sirtinol-treated MCF-7 (c) and H1299 (d) cells compared with untreated cells (Control).

observed in Sirtinol-treated MCF-7 cells and, to a lesser extent, in Sirtinol-treated H1299 cells, as compared with untreated cells. Sirtinol treatment also resulted in increased expression of PAI-1 in both MCF-7 and H1299 cells (Figure 3b). β-Actin expression, however, was not affected by Sirtinol.

Treatment with Splitomicin, another specific inhibitor for Sirt1, for 24 h also led to the induction of SA-β-gal staining in a dose-dependent manner (Figure 3a). However, greater concentrations of Splitomicin appeared to be required to induce SA-β-gal, as compared with Sirtinol.

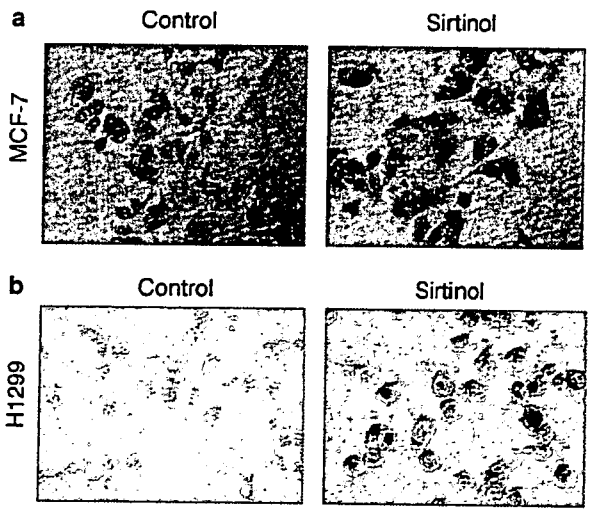

Figure 2 SA-β-gal staining in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), MCF-7 (a) and H1299 (b) cells were stained for SA-β-gal. Sirtinol treatment increased SA-β-gal-positive cells in MCF-7 and H1299 cells. In addition, the number of multinucleated cells was increased in Sirtinol-treated MCF-7 cells, but not in Sirtinol-treated H1299 cells, compared with untreated (Control) cells. Arrowheads denote multinucleated cells in Sirtinol-treated MCF-7 cells.

Colony formation assay also revealed that both Sirtinol and Splitomicin elicited antiproliferative effects in MCF-7 and H1299 cells in a dose-dependent manner (Figure 4). Sirtinol inhibited colony formation at concentrations of 33 μM and higher in MCF-7 and H1299 cells. On the other hand, 33 μM Splitomicin failed to decrease the number of colonies, but Splitomicin at 100 and 333 μM effectively inhibited colony formation in MCF-7 and H1299 cells.

Senescence-like growth arrest by Sirt1 inhibition was further corroborated by experiments using short interfering RNA (siRNA). Gene knockdown of Sirt1 by siRNA resulted in induction of SA-β-gal staining, large and flat cell morphology, decreased BrdU incorporation and increased PAI-1 expression in both MCF-7 and H1299 cells, as compared with control siRNA (Figure 5). Moreover, Sirt1 inhibition by Sirtinol, Splitomicin or siRNA also induced senescence-like phenotype in human diploid fibroblasts, WI-38 and IMR-90 cells, reflected by induction of SA-β-gal staining, and enlarged and flattened cell morphology (Supplementary Figure 1).

In Sirtinol-treated MCF-7 cells, the number of multinucleated cells was increased compared with untreated cells (Figure 2), but multinucleated cells were not found in H1299 cells regardless of whether treated with or without Sirtinol. Consistent with these observations, flow cytometric analysis revealed that the substantial cell population of Sirtinol-treated MCF-7 cells exhibited DNA content over 4*N*, indicative of polyploidy (Figure 6a). Polyploidy fraction estimated by the ratio

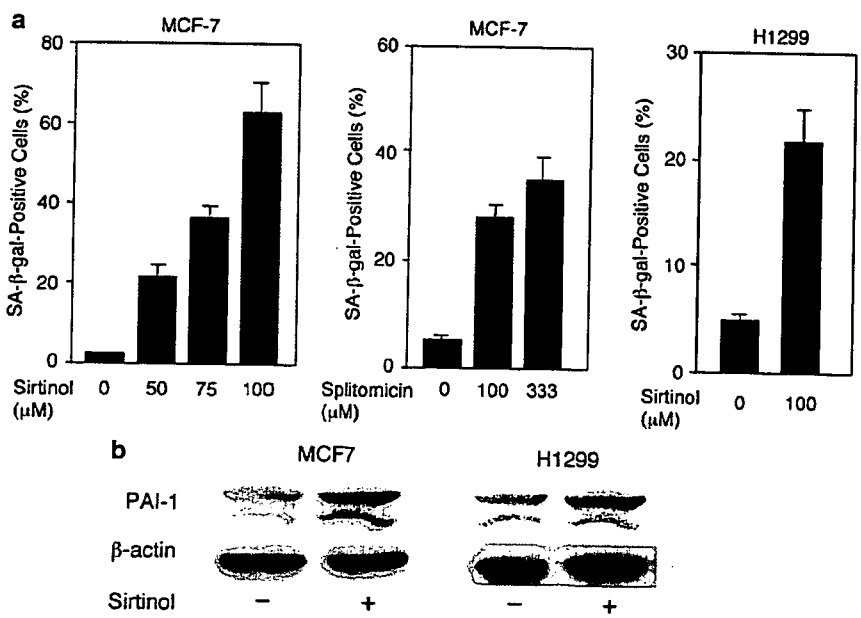

Figure 3 Effects of Sirtinol and Splitomicin on SA-β-gal activity and PAI-1 expression. (a) SA-β-gal-positive cells were counted 10 days after the addition of indicated concentrations of Sirtinol or Splitomicin in MCF-7 and H1299 cells. Treatment with Sirtinol and Splitomicin increased SA-β-gal-positive cells in a dose-dependent manner. (b) Sirtinol treatment resulted in the increased expression of PAI-1 as compared with untreated cells.

of cell population with DNA content of over 4*N* to that with over 2*N* was greater in Sirtinol-treated MCF-7 cells than in untreated cells (Figure 6a, right panel). In contrast, Sirtinol-treated H1299 cells were cell cycle arrested at G1 (Figure 6b). There was little, if any, increase in polyploidy fraction by Sirtinol in H1299 cells (control: 0.6%; Sirtinol: 1.6%).

In cellular senescence, induction of p53, dephosphorylation of Rb and increased expression of cyclin-dependent kinase inhibitors such as p16, p21 and p27 have been shown to be involved (Serrano *et al*., 1997; Collado *et al*., 2000; Alexander and Hinds, 2001; Beausejour *et al*., 2003; Jirawatnotai *et al*., 2003; Mallette *et al*., 2004). We found that phosphorylated Rb was decreased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells, while the protein expression of Rb was unaltered (Figure 7a and b). p27 expression was induced in Sirtinol-treated MCF-7 and H1299 cells (Figure 7f and g), while β-actin expression was unaltered. However, the mRNA level of p27 was not increased by Sirtinol treatment in MCF-7 and H1299 cells at both 3 and 10 days after the addition of Sirtinol, while tamoxifen and serum starvation upregulated the p27 mRNA level in MCF-7 and H1299 cells, respectively (Supplementary Figure 2). In contrast, p21 was not increased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells. p16 was not induced in Sirtinol-treated H1299 cells (Figure 7g). MCF-7 and H1299 cells are deficient in p16 and p53, respectively. On the other hand, treatment with tamoxifen (1 μM) for 24 h increased protein expression of p21 and p27 in MCF-7 cells, and serum starvation for 24 h induced p16, p21 and p27 expression in H1299 cells. Neither expression nor acetylation of p53 was upregulated by Sirtinol

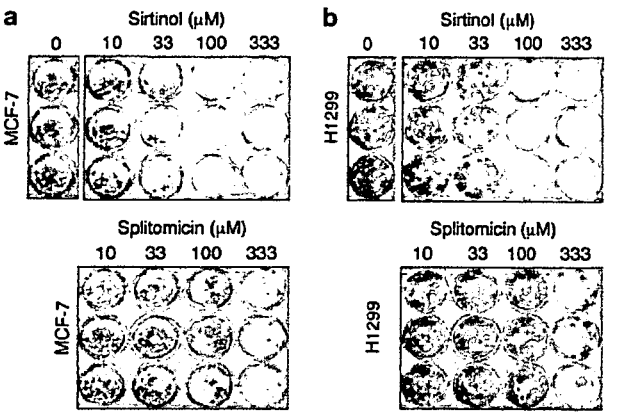

Figure 4 The effects of Sirtinol and Splitomicin on colony formation. MCF-7 (a) and H1299 (b) cells were inoculated onto 12-well plates at the density of 500 cells/well, and treated with the indicated concentrations of Sirtinol or Splitomicin for 24 h. After withdrawal of the Sirt1 inhibitors, the cells were cultured for 14 days. Both Sirtinol and Splitomicin inhibited colony formation in a dose-dependent manner.

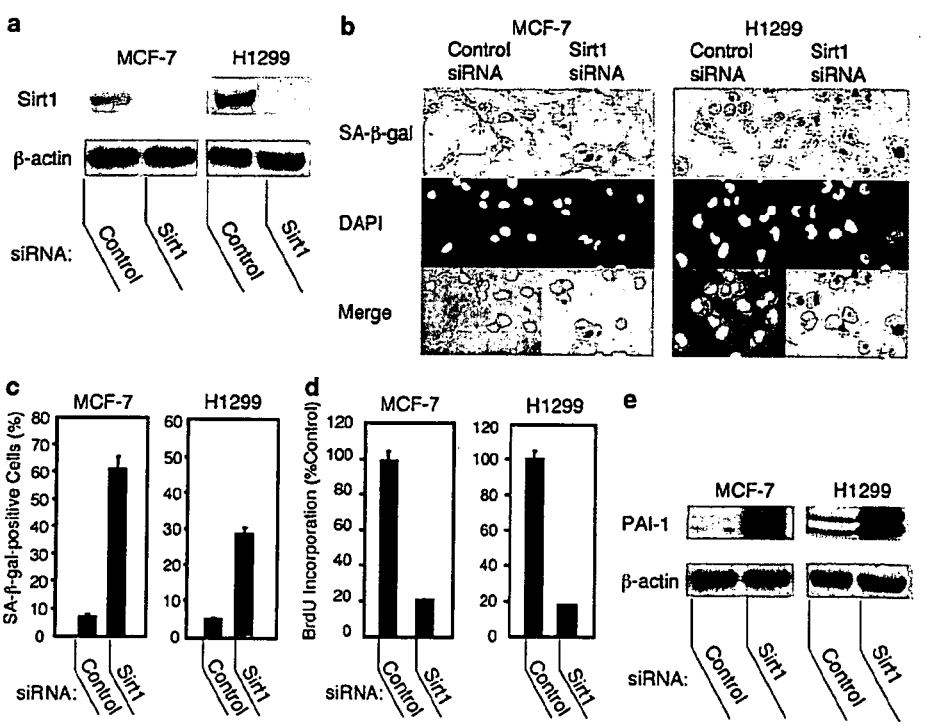

Figure 5 Gene knockdown of Sirt1 by siRNA induced senescence-like phenotype. MCF-7 and H1299 cells were treated with siRNA for Sirt1 or control siRNA. (a) At 3 days after the transfection, immunoblot analysis revealed that Sirt1 siRNA effectively reduced Sirt1 expression in both MCF-7 and H1299 cells. (b, c) At 10 days after the transfection, SA-β-gal-positive cells were significantly increased in Sirt1 siRNA-treated cells compared with control siRNA-treated cells. (d) BrdU incorporation was decreased in Sirt1 siRNA-treated cells compared with control siRNA-treated cells at 10 days after the transfection. (e) Sirt1 siRNA increased PAI-1 protein expression compared with control siRNA at 10 days after the transfection.

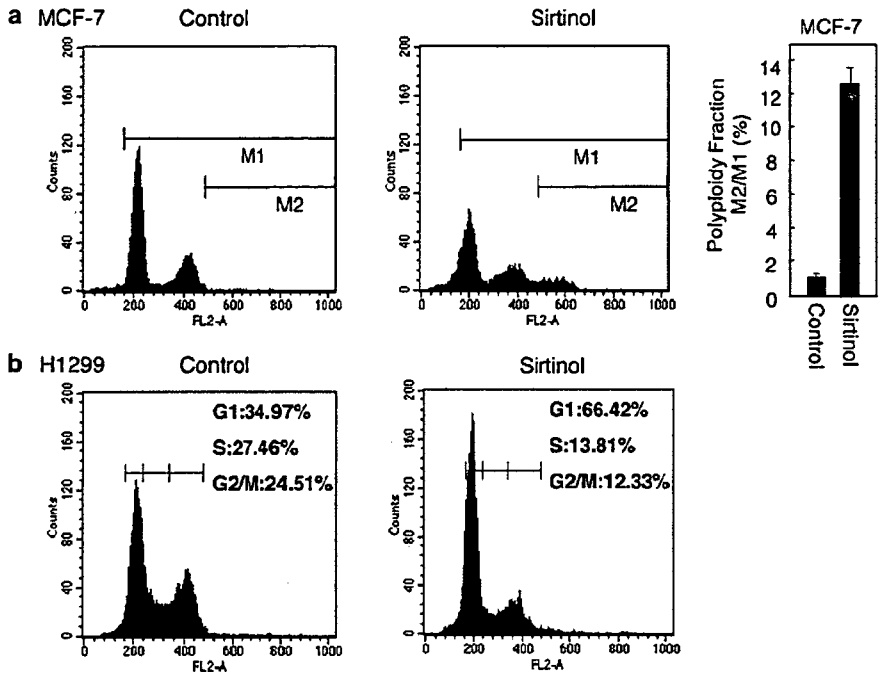

Figure 6 Flow cytometric analysis of Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), the cell cycle of MCF-7 (**a**) and H1299 (**b**) cells was analysed by flow cytometry. In Sirtinol-treated MCF-7 cells, polyploidy fraction (M2/M1; cell population with DNA content of over 4*N* normalized to that with DNA content of over 2*N*) was increased compared with untreated MCF-7 cells (Control). Sirtinol-treated H1299 cells exhibited G1 cell cycle arrest (**b**).

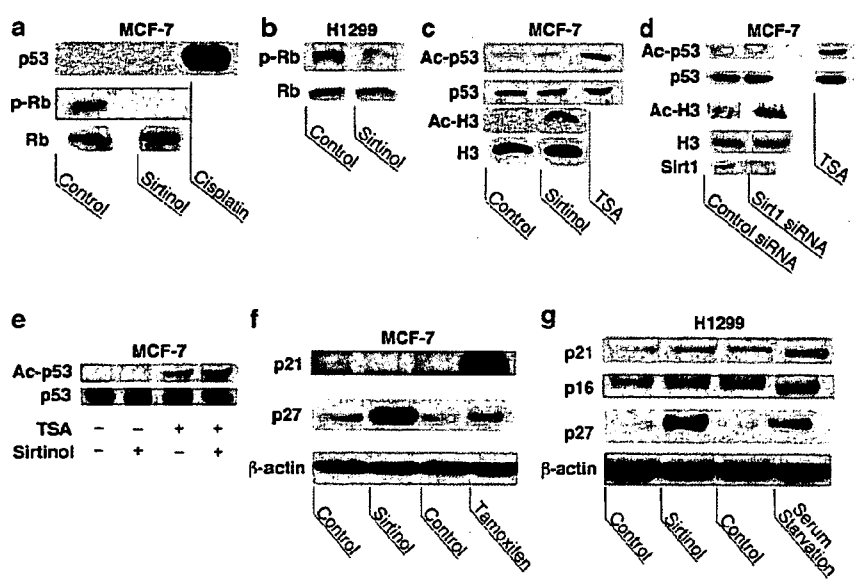

Figure 7 p53, Rb and cyclin-dependent kinase inhibitors in Sirtinol-treated cells. (**a**, **b**) At 10 days after the addition of Sirtinol (100 μM), phosphorylated Rb (p-Rb) was decreased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells. (**a**, **c**, **d**) The expression and acetylation of p53 were not increased by Sirtinol (**a**, **c**) or siRNA for Sirt1 (**d**) in MCF-7 cells. However, a robust increase in expression and acetylation of p53 (Ac-p53) were found in MCF-7 cells when treated with cisplatin (40 μM) for 18 h and trichostatin A (TSA, 5 μM) for 4 h, respectively. In contrast, Sirtinol (100 μM) treatment and siRNA for Sirt1 increased acetylated histone H3 (Ac-H3), while the abundance of histone H3 was unaltered (**c**, **d**). (**e**) MCF-7 cells were treated with or without Sirtinol (100 μM) in the presence or absence of trichostatin A (TSA, 0.5 μM) for 24 h. Sirtinol enhanced trichostatin A-induced acetylation of p53. p53 expression was not altered by Sirtinol or trichostatin A. (**f**, **g**) p21 expression was not increased by Sirtinol treatment in MCF-7 and H1299 cells. p16 expression was not induced by Sirtinol treatment in H1299 cells. In contrast, the expression of p27 was increased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells.

or siRNA for Sirt1 in MCF-7 cells that harbor wild-type p53 (Figure 7c and d), while acetylation of histone H3 was increased by Sirtinol and siRNA for Sirt1. However, cisplatin and trichostatin A, class I and class II HDAC inhibitor, caused robust induction of p53 and acetylation of p53 in MCF-7 cells, respectively (Figure 7a and c–e). Although Sirtinol alone did not increase p53 acetylation, Sirtinol enhanced p53 acetylation in the presence of trichostatin A (Figure 7e). These results are consistent with previous observations that inhibition of Sirt1 by itself did not induce p53 acetylation in the absence of other stimulus, while DNA damage- or oxidative stress-induced p53 acetylation was accentuated by Sirt1 inhibition (Luo *et al.*, 2001; Vaziri *et al.*, 2001; Langley *et al.*, 2002; Cheng *et al.*, 2003).

Senescence-like growth arrest was accompanied by attenuated activation of MAPK pathways in response to growth factors

We examined the activation status of signaling pathways of MAPKs and Akt/PKB in response to growth factors, epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I). When untreated with Sirtinol, upon exposure to EGF or IGF-I, robust phosphorylation of extracellular-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK/SAPK, also termed stress-activated protein kinase) and p38 MAPK was observed in MCF-7 and H1299 cells. By contrast, in Sirtinol-treated senescent MCF-7 and H1299 cells at 10 days after the addition of Sirtinol (100 μM), basal (unstimulated) phosphorylation of ERK, JNK/SAPK and p38 MAPK was reduced compared with untreated cells (Figure 8). In addition, EGF- or IGF-I-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was attenuated in MCF-7 and H1299 cells at 10 days after the addition of Sirtinol, compared to untreated cells.

Reduced activation of ERK, JNK/SAPK and p38 MAPK was corroborated by the phosphorylation status of the endogenous substrates of these MAPKs. Basal (unstimulated) as well as EGF- or IGF-I-stimulated phosphorylation of Elk-1, c-Jun and ATF-2 was also decreased in Sirtinol-treated senescent MCF-7 and H1299 cells at 10 days after the addition of Sirtinol, compared with untreated cells (Figure 9). The protein expression of ERK, JNK/SAPK, p38 MAPK, Elk-1, c-Jun and ATF-2 did not differ between Sirtinol-treated and untreated MCF-7 and H1299 cells.

However, at 3 days after the addition of Sirtinol, unlike at 10 days after the inhibitor addition, EGF-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was not attenuated in MCF-7 cells, compared with untreated cells (Figure 8d). These results suggest that attenuated MAPK pathways may be a consequence, rather than a cause, of Sirtinol-induced commitment of senescence-like growth arrest.

In contrast, tyrosine phosphorylation of the receptors for EGF and IGF-I by their ligands was not altered by Sirtinol treatment in MCF-7 and H1299 cells at 10 days after the addition of Sirtinol (Figure 10). The expression of EGF receptor and IGF-I receptor did not differ between Sirtinol-treated and untreated cells. These findings suggest that the defects responsible for impaired activation of MAPKs may exist at the level(s) of postreceptor signaling cascades.

Ras plays a critical role in growth factor-stimulated activation of MAPK pathways. Active Ras was markedly increased by EGF in untreated MCF-7 and H1299 cells. In Sirtinol-treated senescent MCF-7 and H1299 cells, however, the basal (unstimulated) level of active Ras was reduced compared with untreated cells, and EGF failed to increase active Ras (Figure 11a). Consistent with defective activation of Ras, basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Raf-1, MEK, SEK1/MKK4 and MKK7 was attenuated in Sirtinol-treated cells relative to untreated cells (Figure 11b). However, no difference was found between Sirtinol-treated and untreated MCF-7 and H1299 cells in the protein expression of Ras, Raf-1, MEK, SEK1/MKK4 and MKK7.

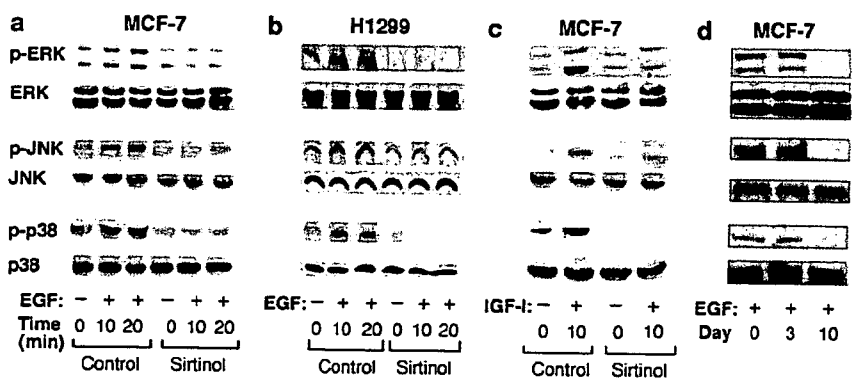

Figure 8 Growth factor-stimulated phosphorylation of MAPKs in Sirtinol-treated cells. (a–c) At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (a, b), or to IGF-I (100 ng/ml) for 10 min (c). In untreated (Control) MCF-7 and H1299 cells, marked phosphorylation of ERK, JNK/SAPK and p38 MAPK was induced by EGF or IGF-I. In Sirtinol-treated MCF-7 and H1299 cells, basal (unstimulated) as well as EGF- and IGF-I-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was decreased compared with untreated cells. (d) At 0, 3 and 10 days after the addition of Sirtinol (100 μM), MCF-7 cells were stimulated with EGF (50 ng/ml) for 20 min. EGF-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was markedly impaired at 10 days, but preserved at 3 days after the addition of Sirtinol.

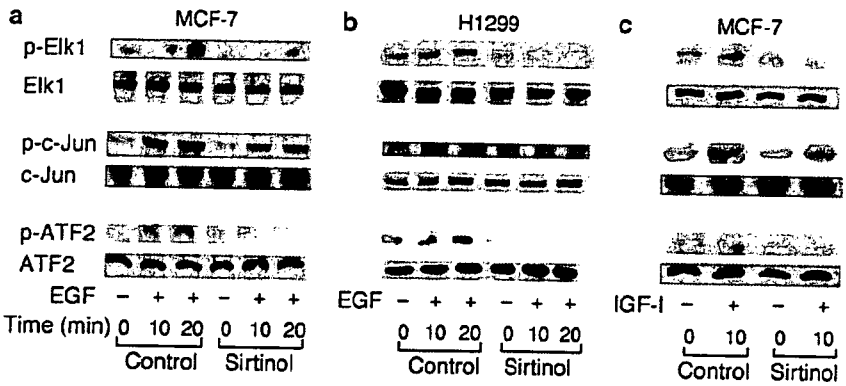

Figure 9 Growth factor-stimulated phosphorylation of Elk1, c-Jun and ATF2. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (**a**, **b**) or to IGF-I (100 ng/ml) for 10 min (**c**). In untreated (Control) MCF-7 and H1299 cells, marked phosphorylation of Elk1, c-Jun and ATF2 was induced by EGF and IGF-I. In Sirtinol-treated MCF-7 and H1299 cells, both basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Elk1, c-Jun and ATF2 were decreased compared with untreated cells.

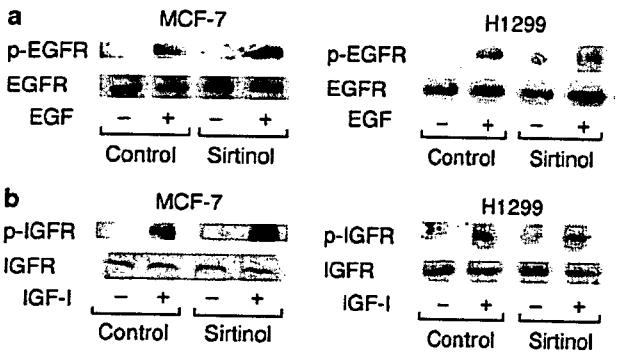

Figure 10 Growth factor-stimulated phosphorylation of EGF receptor and IGF-I receptor in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) (**a**) or IGF-I (100 ng/ml) for 2 min (**b**). There was no difference in tyrosine phosphorylation and protein expression of EGF receptor (EGFR) and IGF-I receptor (IGFR) between Sirtinol-treated and untreated (Control) MCF-7 and H1299 cells.

In contrast to the Ras–MAPK pathway, EGF- or IGF-I-induced as well as basal (unstimulated) phosphorylation of Akt/PKB was not decreased in Sirtinol-treated senescent MCF-7 and H1299 cells compared with untreated cells at 10 days after Sirtinol addition (Figure 12). The expression of Akt/PKB was not altered by Sirtinol treatment, either.

Discussion

We found that Sirt1 inhibition by specific inhibitors, Sirtinol and Splitomicin, and siRNA caused senescence-like growth arrest in human cancer MCF-7 and H1299 cells, as judged by SA-β-gal staining, PAI-1 expression, BrdU incorporation, flattened and enlarged morphology of the cells and flow cytometric analysis (Figures 1–6). Sirtinol-induced senescence-like growth arrest was accompanied by attenuated responses to growth factors in terms of activation of Ras–MAPKs (Figures 8, 9 and 11). By contrast, phosphorylation (activation) of EGF receptor, IGF-I receptor and Akt/PKB by growth factors was not affected in Sirtinol-treated senescent MCF-7 and H1299 cells (Figures 10 and 12).

Consistent with impaired activation of MAPKs, EGF- and IGF-I-stimulated phosphorylation of downstream targets, Elk-1, c-Jun and ATF-2, was also reduced (Figure 9). A hallmark feature of senescent cells is unresponsiveness to mitogenic stimuli in terms of induction of c-*fos* as well as cell proliferation. Previous studies in senescent human diploid fibroblasts showed that induction of c-*fos* (Seshadri and Campisi, 1990) and activation of Elk-1 (Tresini *et al.*, 2001) and MEK–ERK (Torres *et al.*, 2003) in response to growth factors are impaired. Transcriptional activity of Elk-1 regulates the induction of c-*fos*, an immediate early gene. Thus, our results of attenuated activation of Ras–MEK–ERK–Elk-1 in Sirtinol-treated senescent cancer cells are in agreement with previous findings in senescent human fibroblasts.

Our results indicate that the signaling defect in Sirtinol-treated cells is specific for MAPK pathways and that the PI3-K–Akt/PKB pathway is preserved. Ras is a key regulator of MAPK pathways (Lange-Carter and Johnson, 1994). However, Ras does not play a major role in activation of the PI3-K–Akt/PKB pathway (Sakaue *et al.*, 1995; Gnudi *et al.*, 1997; Klesse *et al.*, 1999). Our results showed that active, GTP-bound Ras was reduced in Sirtinol-treated cancer cells compared with untreated cells (Figure 11a). The present data, therefore, suggest that reduced activation of Ras might be involved in a specific attenuation in MAPK pathways in Sirtinol-treated senescent MCF-7 and H1299 cells.

Growth factor-initiated mitogenic signals are conveyed mainly by two major signaling cascades: Ras–ERK and PI3-K–Akt/PKB. Senescent cells remain viable and metabolically active, in spite of irreversible loss of replication capability (Roninson, 2003; Shay and Roninson, 2004). One can reasonably speculate, therefore, that the preserved PI3-K–Akt/PKB pathway might

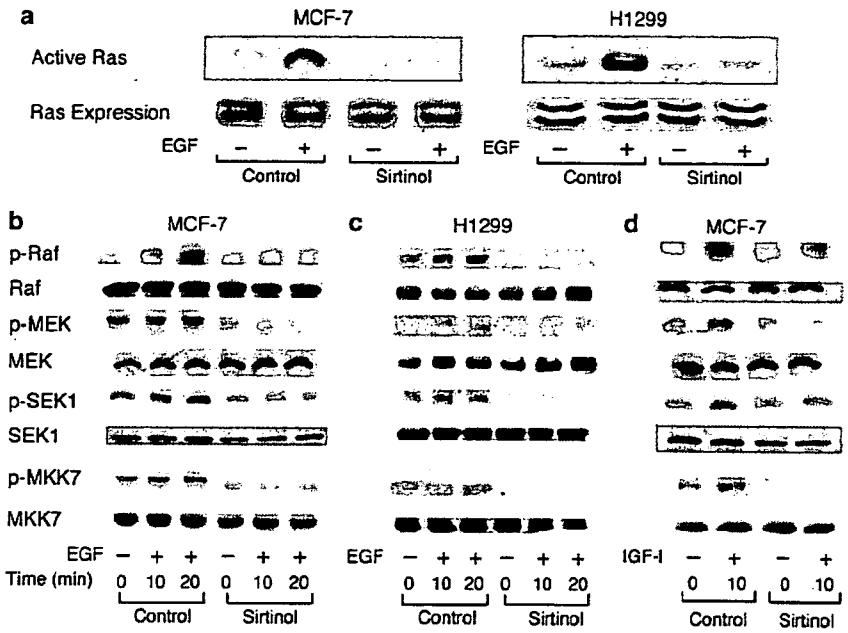

Figure 11 Activation status of Ras and its downstream signaling molecules in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min, or IGF-I (100 ng/ml) for 10 min. (**a**) Active Ras was evaluated as described in Materials and methods. In untreated (Control) MCF-7 and H1299 cells, active Ras was markedly increased by EGF treatment for 20 min. In Sirtinol-treated MCF-7 and H1299 cells, basal (unstimulated) level of active Ras was decreased compared with untreated cells, and EGF failed to increase active Ras. (**b–d**) In untreated (Control) MCF-7 and H1299 cells, EGF and IGF-I induced robust phosphorylation of Raf, MEK, SEK1/MKK4 and MKK7. However, in Sirtinol-treated MCF-7 and H1299 cells, both basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of these molecules were decreased compared with untreated (Control) cells.

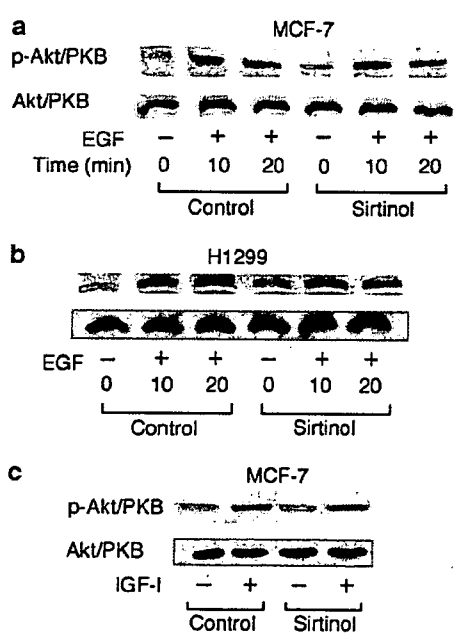

Figure 12 Growth factor-stimulated Akt/PKB phosphorylation in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (**a**, **b**) or to IGF-I (100 ng/ml) for 10 min (**c**). No difference was found in basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Akt/PKB between Sirtinol-treated and untreated (Control) MCF-7 and H1299 cells.

contribute to cell viability and metabolic activities in Sirtinol-treated cells, because the PI3-K–Akt/PKB pathway plays critical roles in cell survival and regulation of metabolism.

p53, Rb and cyclin-dependent kinase inhibitors such as p16, p21 and p27 have been recognized as key mediators of cellular senescence (Serrano *et al.*, 1997; Collado *et al.*, 2000; Alexander and Hinds, 2001; Beausejour *et al.*, 2003; Jirawatnotai *et al.*, 2003; Mallette *et al.*, 2004). Our results showed that hypophosphorylation of Rb and increased expression of p27 were associated with Sirtinol-induced senescence-like growth arrest in MCF-7 and H1299 cells (Figure 7). In addition to regulation at transcriptional level, increased p27 expression may result from reduced protein degradation through a ubiquitin–proteasome system (Carrano *et al.*, 1999). Since Sirt1 is an HDAC, it is possible that Sirt1 inhibition may directly modulate p27 transcription. However, our finding of unaltered p27 mRNA in Sirtinol-treated cells (Supplementary Figure 2) suggests that decreased protein degradation of p27, rather than increased transcription, may contribute to increased p27 protein expression in Sirtinol-treated cells. Thus, our data argue against a direct effect of Sirtinol on transcription of p27.

On the other hand, we did not find increased expression of p53, p21 and p16 in Sirtinol-treated MCF-7 and H1299 cells. It is important to note, however, that previous studies showed that premature senescence can be readily induced independent of p53,

p21 or p16 in cancer cells (Zhu *et al*., 1997; Chang *et al*., 1999a, b; Collado *et al*., 2000; Wainwright *et al*., 2001; Wright and Shay, 2001; te Poele *et al*., 2002; Beausejour *et al*., 2003; Mallette *et al*., 2004; Munro *et al*., 2004). p27 was shown to be required for premature senescence mediated by Rb (Alexander and Hinds, 2001) or PI3-K inhibitor (Collado *et al*., 2000). In accordance with previous findings (Chang *et al*., 1999a; Collado *et al*., 2000; Wainwright *et al*., 2001; Mallette *et al*., 2004), Sirtinol induced senescence-like growth arrest in p16-deficient MCF-7 and p53-deficient H1299 cells. These results indicate that p53 and p16 are not required for Sirtinol-induced senescence-like growth arrest in H1299 and MCF-7 cells, respectively.

Recently, senescence-like growth arrest has been proposed as a new target of cancer therapy. Since p53 and p16 are not expressed or mutated in many types of malignancies, the effectiveness of Sirtinol to induce senescence-like growth arrest in p53- and p16-deficient cancer cells may be of clinical significance for the treatment of patients with malignancy. Taken together, the present study highlights Sirt1 inhibitor as an antitumor drug candidate.

Materials and methods

Materials
Sirtinol, Splitomicin, IGF-I, EGF (Calbiochem, La Jolla, CA, USA), trichostatin A, cisplatin, propidium iodide, RNase, tamoxifen (Sigma, St Louis, MO, USA), anti-phospho-ERK (Thr202/Tyr204), ERK, phospho-JNK/SAPK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), p38, phospho-Raf (Ser259), Raf-1, phospho-MEK1/2 (Ser217/221), MEK1/2, phospho-SEK1/MKK4 (Ser80), SEK1/MKK4, MKK7, phospho-Elk1 (Ser383), Elk1, phospho-ATF2 (Thr71), ATF2, phospho-Rb (Ser795), Rb, phospho-Akt (Ser473), Akt, p53 (Cell Signaling, Beverly, MA, USA), acetylated p53, EGFR, phospho-MKK7 (Thr275/Ser277) (Upstate, Lake Placid, NY, USA), phospho-EGFR (Tyr1173), p16, p21, phospho-c-Jun (Ser63), c-Jun, JNK1 (Santa Cruz, Santa Cruz, CA, USA), p27 (BD Transduction Laboratories, Lexington, KY, USA), PAI-1 (Molecular Innovations, Southfield, MI, USA), acetylated histone H3, histone H3 (Upstate, Charlottesville, VA, USA), phospho-IGF-I receptor (Tyr1162/1163), IGF-I receptor (Biosource, Camarillo, CA, USA) and pan-Ras antibodies (Oncogene, San Diego, CA, USA) were purchased commercially.

Cell culture
Human breast cancer MCF-7 cells and non-small lung cancer H1299 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Sigma), respectively. Logarithmically growing cells were treated with the indicated concentrations of Sirtinol or Splitomicin for 24 h. After exposure to Sirtinol or Splitomicin for 24 h, the cells were washed three times with inhibitor-free medium and cultured for an additional 9 days with the complete media in the absence of inhibitor. Cell viability was determined by the Trypan blue exclusion test, and viable cells were counted. At 9 days after the addition of Sirtinol to the culture media, the cells were serum-deprived for overnight, and then treated with EGF (50 ng/ml) or IGF-I (100 ng/ml) for 2, 10 or 20 min.

SA-β-gal staining
SA-β-gal staining was performed as previously described (Dimri *et al*., 1995) (see Supplementary section).

BrdU incorporation assay
At 10 days after the addition of Sirtinol, BrdU incorporation was assayed as previously described (Takahashi *et al*., 1992). In cells treated with siRNA, at 10 days after the transfection of siRNA, BrdU incorporation was evaluated using a commercial kit (Roche, Indianapolis, IN, USA).

Gene knockdown with siRNA
Cells were plated in six-well plates at 20–30% confluency, and 24 h later transfected with 200 pmol of siRNA for Sirt1 (5′-GAT GAA GTT GAC CTC CTC A-3′ (Picard *et al*., 2004) and 5′-TGA AGT GCC TCA GAT ATT A-3′) or control siRNA (Dharmacom, Chicago, IL, USA), using siIMPORTER (Upstate).

Flow cytometric analysis
At 10 days after the addition of Sirtinol, the cells were fixed with 70% ethanol and treated with 5 μg/ml (RNase) for 30 min. After staining with 50 μM propidium iodide, the cells were subjected to flow cytometric analysis with FACS Calibur and Cell Quest software (Becton-Dickinson, Franklin Lakes, NJ, USA).

Immunoblot analysis
Immunoblot analysis was performed as previously described (Yasukawa *et al*., 2005) (see Supplementary section).

Determination of activation status of Ras
Active, GTP-bound Ras was assayed using the Ras activation assay kit (Upstate) according to the manufacturer's instructions (see Supplementary section).

Colony formation assay
Colony formation assay was performed as previously described (Elegbede *et al*., 2002) (see Supplementary section).

Northern blotting
The mRNA level of p27 was evaluated by Northern blotting as previously described (Sugita *et al*., 2005), using cDNA probe for p27 that was kindly provided by Dr N Fujita (Fujita *et al*., 2002).

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Angiotensin converting enzyme inhibitor attenuates oxidative stress-induced endothelial cell apoptosis via p38 MAP kinase inhibition

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Abstract

Background: The effects of angiotensin converting enzyme (ACE) inhibitors on oxidative stress-induced apoptosis of endothelial cells and the intracellular signaling were investigated.
Methods: Cultured endothelial cells derived from a bovine carotid artery were treated with H_2O_2 or TNF-α to induce apoptosis. Apoptosis was evaluated by DNA fragmentation and cell viability, p38 MAP kinase activity by Western blotting, and oxidative stress by formation of 8-isoprostane. The effects of ACE inhibitors were examined by adding them into the medium throughout the experiments.
Results: Apoptosis was attenuated by ACE inhibitors, temocapril and captopril, in a dose-dependent manner (1–100 μmol/l). H_2O_2 (0.2 mmol/l for 1.5 h) or TNF-α (10 ng/ml for 72 h) treatment stimulated the activities of p38 MAP kinase. Temocapril and captopril decreased the activity of p38 MAP kinase as well as 8-isoprostane formation induced by H_2O_2. A p38 MAP kinase inhibitor, SB203580, partially inhibited the effect of temocapril on apoptosis.
Conclusions: These results suggest that ACE inhibitors protect endothelial cells from oxidative stress-induced apoptosis, and that p38 MAP kinase plays a critical role in the process.

Keywords: Apoptosis; ACE inhibitor; Endothelial cell; p38 MAP kinase

1. Introduction

Stress-induced injury of vascular endothelial cells (ECs) is considered to be an initial event in the development of atherosclerosis [1]. In particular, oxidative stress has been implicated in endothelial injury caused by oxidized LDL and smoking as well as hypertension, diabetes and ischemia-reperfusion [1–3]. This notion is supported by the findings that the production of reactive oxygen species is upregulated in vascular lesions [4,5], and that lesion formation such as endothelial dysfunction is accelerated by superoxide anion [6] and, in contrast, is attenuated by free radical scavengers including vitamin E [7] and superoxide dismutase [8].

Angiotensin converting enzyme (ACE) inhibitors effectively interfere with the renin angiotensin system and exert various beneficial actions on vascular structure and function beyond their blood pressure-lowering effects [9,10]. ACE inhibitors attenuate neointimal formation after vascular injury in animals [11] and endothelial dysfunction in humans [12]. It has been demonstrated that ACE activation induces oxidative stress [13]. However, it has not been elucidated whether ACE inhibitors could attenuate oxidative stress-induced EC apoptosis, an initial and important process in atherosclerosis [14,15].

In this study, we examined the effects of ACE inhibitors, temocapril and captopril, on H_2O_2- and TNF-α-induced EC apoptosis and the pro-apoptotic intracellular signaling, p38 mitogen-activated protein (MAP) kinase, to clarify the underlying mechanism.

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2. Materials and methods

2.1. Induction of EC apoptosis

ECs derived from a bovine carotid artery [16] was cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a 95% air/5% CO_2 atmosphere. ECs of the 5th to 7th passage were used in the experiments. When the cells had grown to 70–80% confluence, ECs were pretreated for 24 h with culture medium containing the reagents that were tested in the experiments. Subsequently, after washing twice with Hank's balanced salt solution (Gibco), the cells were exposed to H_2O_2 (0.1–0.4 mmol/l) diluted in Hank's balanced salt solution for 1.5 h at 37 °C to induce apoptosis. The cells were washed three times with Hank's balanced salt solution, and then cultured in culture medium containing the reagents until assay. Similarly, tumor necrosis factor-α (TNF-α, 5-20 ng/ml; Sigma) was added to the medium until assay after 24-h pretreatment with the reagents tested. EC viability and apoptosis were evaluated at 24 h after H_2O_2 treatment, or at 72 h after TNF-α treatment. The effects of temocapril (1–100 μmol/l) and captopril (1–100 μmol/l) were examined by adding them into the medium throughout the experiments. The effect of a specific p38 MAP kinase inhibitor, SB203580 (10 μmol/l; Calbiochem), was examined by treating ECs with SB203580 for 1 h before H_2O_2 treatment.

2.2. Cell viability

Cell viability was estimated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay [17]. Briefly, 1 mg/ml MTT (final concentration) was added to the well and incubated for 2 h at 37 °C. The medium was removed and cells were lysed with 2-isopropanol containing 0.04 mol/l HCl. The absorbance measured at 595 nm was used to calculate the relative cell viability ratio.

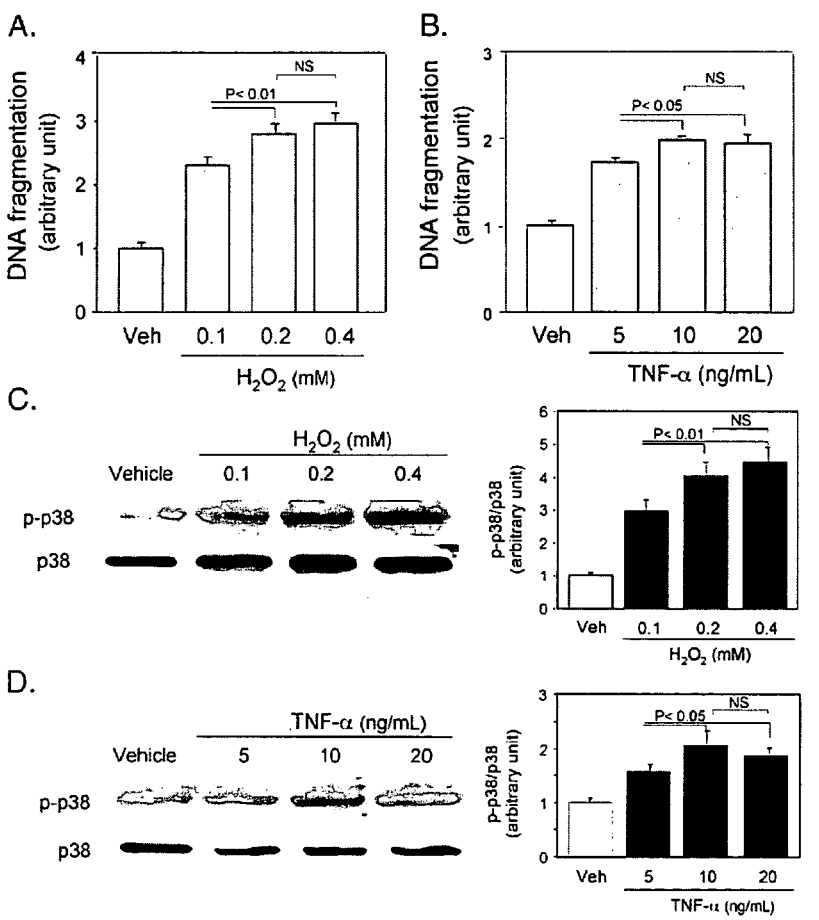

Fig. 1. Dose-dependent effects of H_2O_2 (A, C) and TNF-α (B, D) on EC apoptosis (A, B) and p38 MAP kinase activity (C, D). A and B, apoptosis was evaluated 24 h after H_2O_2 treatment (for 1.5 h) or 72 h after addition of TNF-α by means of DNA fragmentation ($n=3$). C and D, the activity of p38 MAP kinase was evaluated by immunoblotting using the specific antibody against the phosphorylated form of the kinase (p-p38) at 30 min after addition of H_2O_2 or TNF-α. Right panels show the results of densitometric analyses of immunoblotting (mean±SEM, $n=3$). NS, not significant. Values are expressed as mean±SEM ($n=3$).

2.3. Evaluation of EC apoptosis and formation of 8-isoprostane

For quantitative determination, EC apoptosis was measured as DNA fragmentation. DNA fragmentation was evaluated by histone-associated DNA fragments using a photometric enzyme immunoassay (Cell Death Detection ELISA, Roche), according to the manufacturer's instructions. Briefly, attached cells were harvested with trypsin, and the cell suspension was pelleted by centrifugation. Floating and attached cells were lysed. After centrifugation, the supernatant was measured by ELISA.

Formation of 8-isoprostane (8-*iso* prostaglandin $F_{2\alpha}$) was measured using a commercially available EIA kit (Cayman Chemical). Culture supernatants were diluted with EIA buffer when necessary, and were applied to EIA according to the manufacturer's instructions.

2.4. Immunoblotting

The cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer (25 mmol/l Tris/HCl, pH 7.5, 25 mmol/l NaCl, 0.5 mmol/l EGTA, 10 mmol/l NaF, 20 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 1 mmol/l

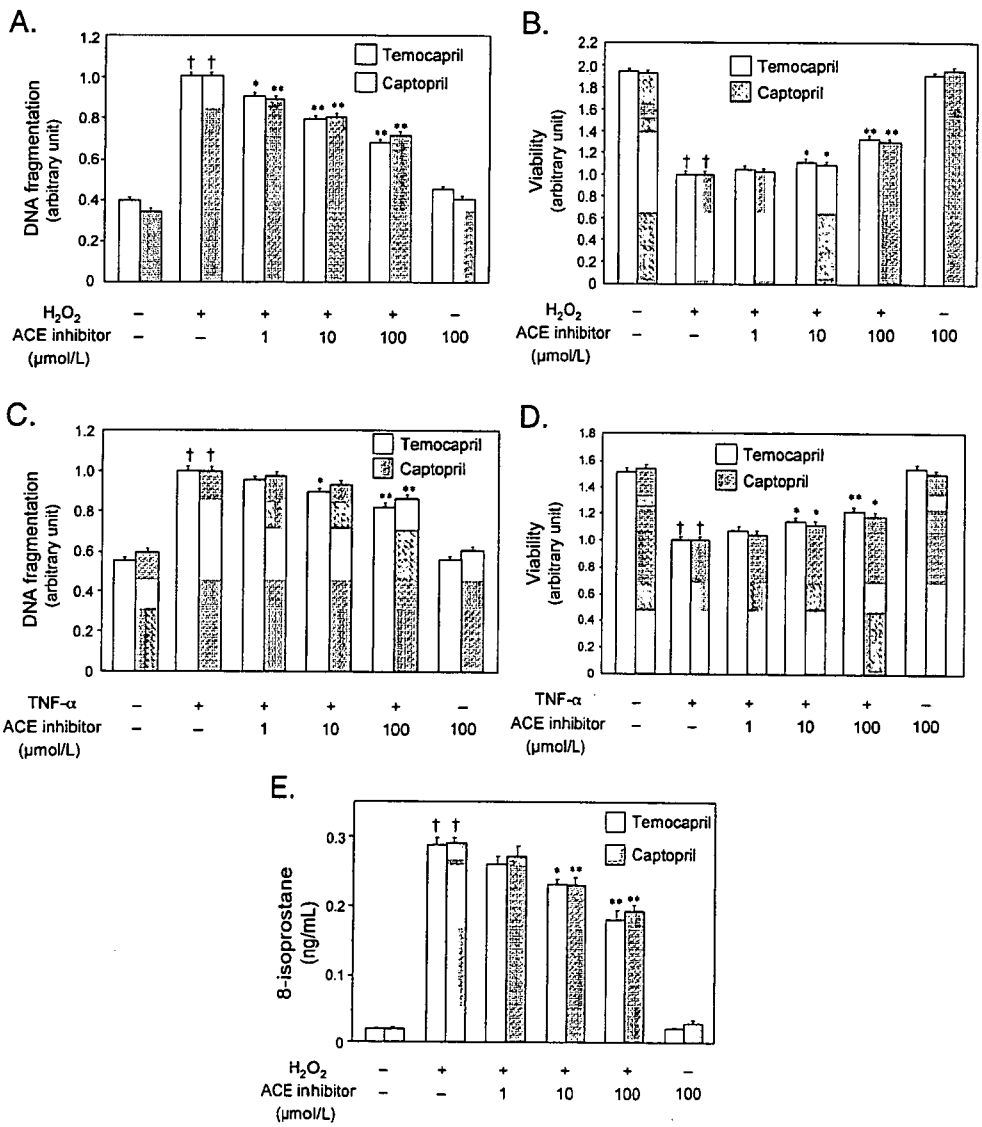

Fig. 2. Effects of ACE inhibitors on H_2O_2-induced (A, B) and TNF-α-induced (C, D) EC apoptosis and the effects of ACE inhibitors on H_2O_2-induced 8-isoprostne formation (E). Temocapril, captopril or their vehicle was added to the culture medium 24 h before H_2O_2 or TNF-α treatment until assay. Apoptosis (A, C) and cell viability (B, D) were evaluated 24 h after H_2O_2 treatment (0.2 mmol/l for 1.5 h) or 72 h after TNF-α treatment (10 ng/ml for 72 h) by means of DNA fragmentation ($n=3$) and MTT assay ($n=8$), respectively. 8-Isoprostane concentration (E; $n=3$) in the culture supernatant was measured 3 h after H_2O_2 treatment. A and B, †$P<0.01$ vs. H_2O_2 (−). *$P<0.05$, **$P<0.01$ vs. H_2O_2 (+)+ACE inhibitor (−). C and D, †$P<0.01$ vs. TNF-α (−). *$P<0.05$, **$P<0.01$ vs. TNF-α (+)+ACE inhibitor (−). Values are expressed as mean±SEM. Similar results were obtained in three independent experiments.

PMSF, and 10 μg/ml aprotinin) at 4 °C. After sonication and centrifugation at 15,000 rpm, the supernatant was used for the following immunoblotting. The lysate (20 μg protein per lane) was separated on 12% SDS-polyacrylamide gel, electroblotted onto nitrocellulose membrane, and immunoblotted with specific primary antibodies, both of which were purchased from Cell Signaling Technology (Beverly, MA). The antibodies used in this study were anti-phospho-p38 MAP kinase (phospho-p38 28B10 #9216) and anti-p38 MAP kinase (#9212). Antibodies were detected by means of a horseradish peroxidase-linked secondary antibody using an enhanced chemiluminescence system (Amersham Pharmacia Biotech). Densitometric analysis was performed using an image scanner and analyzing software (NIH image ver. 1.61). The activity of each kinase was evaluated by calculating the ratio of the amount of the phosphorylated form to that of the total form.

2.5. Data analysis

The values are expressed as mean±SEM in the text and figures. Data were analyzed using one-factor ANOVA. If a statistically significant effect was found, Newman–Keuls' test was performed to isolate the difference between the groups. Differences with a value of $P<0.05$ were considered statistically significant.

3. Results

3.1. Dose-dependent effects of H_2O_2 and TNF-α on EC apoptosis and p38 MAP kinase activity

Increasing concentrations of H_2O_2 and TNF-α were applied to examine the effects on EC apoptosis and p38 MAP kinase activity. Based on the literature [18] and time–response experiments (data not shown), EC apoptosis was evaluated at 24 h after H_2O_2 treatment for 1.5 h, or at 72 h after addition of TNF-α. The activity of p38 MAP kinase, as measured by immunoblotting using the specific antibody against the phosphorylated form of the kinase, was evaluated at 30 min after addition of H_2O_2 or TNF-α, based on time–response experiments (data not shown). As shown in Fig. 1A–D, the effects of H_2O_2 and TNF-α were

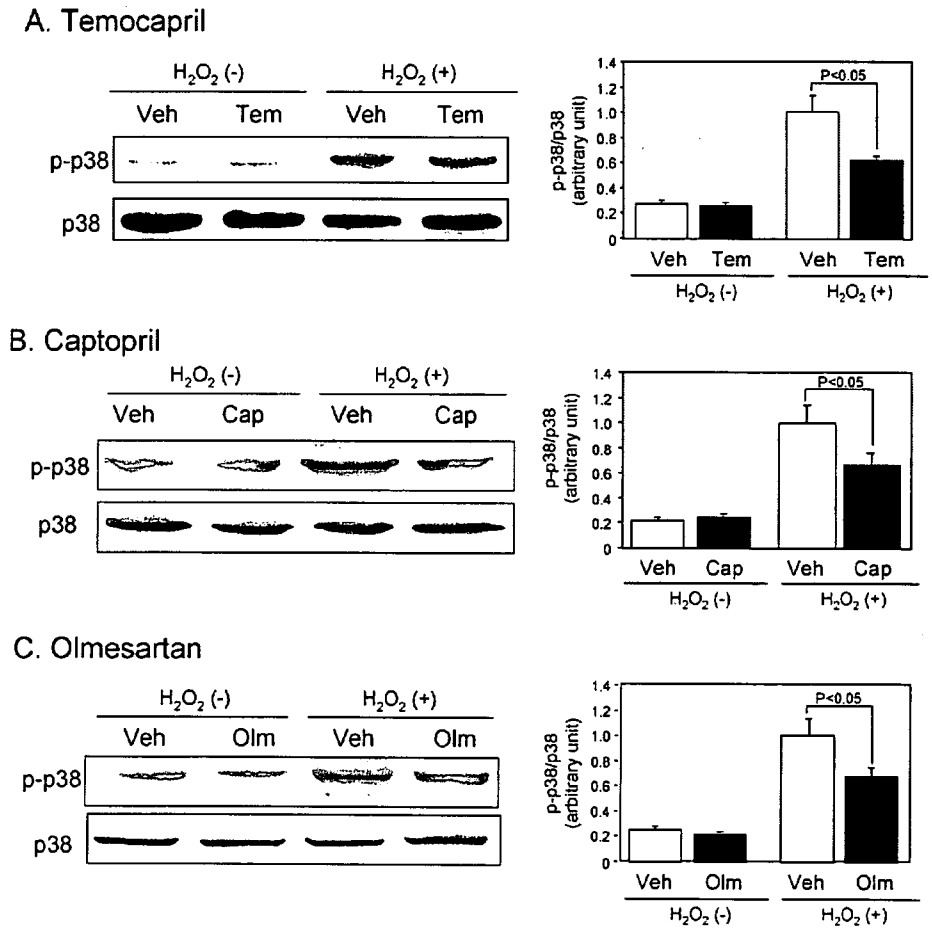

Fig. 3. Effects of temocapril (A), captopril (B) and olmesartan (C) on p38 MAP kinase activity at 30 min after exposure to H_2O_2. Temocapril (100 μmol/l), captopril (100 μmol/l), olmesartan (10 μmol/l) or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. Right panels show the results of densitometric analyses of immunoblotting (mean±SEM, $n=3$).

dose dependent, but there was no significant further increase in EC apoptosis and p38 MAP kinase activity by H_2O_2 of >0.2 mmol/l or by TNF-α of >10 ng/ml. Based on these data, the following experiments were examined using 0.2 mmol/l H_2O_2 or 10 ng/ml TNF-α.

3.2. Effect of ACE inhibitors on EC apoptosis

EC apoptosis, as measured by DNA fragmentation, was significantly attenuated by temocapril and captopril in a dose-dependent manner (Fig. 2A). Reflecting this effect, cell viability was ameliorated by addition of temocapril and captopril in a dose-dependent manner (Fig. 2B).

We also tested using TNF-α whether anti-apoptotic effects of ACE inhibitors would be specific to H_2O_2 or not. As shown in Fig. 2C, both temocapril and captopril effectively inhibited EC apoptosis in a dose-dependent manner. This was associated with the recovery of cell viability by the ACE inhibitors (Fig. 2D). Throughout the experiments, the effects of temocapril were comparable to those of captopril.

To confirm the antioxidant effects of temocapril and captopirl, the formation of 8-isoprostane, a marker of oxidative stress, was measured. Temocapril and captopril restrained 8-isoprostane formation induced by H_2O_2 in a dose-dependent manner (Fig. 2E).

3.3. Effect of ACE inhibitor on p38 MAP kinase activity

Next, the effects of ACE inhibitors on p38 MAP kinase activity were examined because the kinase has been implicated in the cell signaling leading to apoptosis [14,19,20]. As shown in Fig. 3A,B, temocapril and captopril decreased the activity of p38 MAP kinase at 30 min after H_2O_2 treatment by approximately 30–40% without any change in the total protein. An AT1 receptor blocker, olmesartan, showed similar effects on p38 MAP kinase activity (Fig. 3C).

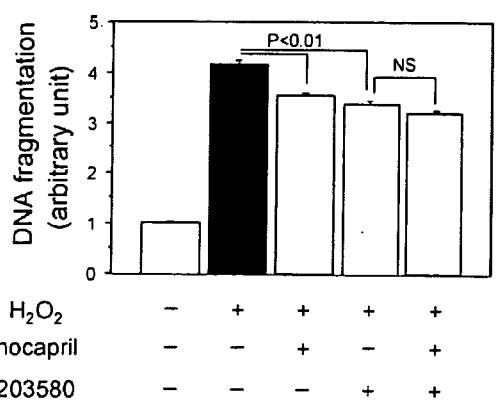

Fig. 4. Effects of temocapril and SB203580 on H_2O_2-induced EC apoptosis. Temocapril (100 μmol/l) or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. SB203580 (10 μmol/l) or its vehicle was added to the culture medium for 1 h before H_2O_2 treatment. EC apoptosis was determined by DNA fragmentation 24 h after H_2O_2 treatment. NS, not significant. Values are expressed as mean±SEM ($n=3$). Similar results were obtained in three independent experiments.

Finally, the effect of a p38 MAP kinase inhibitor, SB203580, was examined. SB203580 reduced H_2O_2-induced EC apoptosis by 20%. More importantly, SB203580 partially but significantly inhibited the effect of temocapril on apoptosis (Fig. 4). Taking these results together with the pro-apoptotic action of p38 MAP kinase, it is suggested that p38 MAP kinase is involved in the effect of temocapril on EC apoptosis.

4. Discussion

A number of investigations have shown that angiotensin II induces oxidative stress in ECs. Angiotensin II stimulates the production of reactive oxygen species in ECs by upregulating the subunits of NAD(P)H oxidase, gp91 phox [21] and p47 phox [22]. It has been reported that the renin angiotensin system contributes to endothelial dysfunction in patients with renovascular hypertension [23]. Conversely, it has been shown experimentally that ACE inhibitors can reduce the production of reactive oxygen species in pathological conditions such as peripheral arteries in rats with chronic heart failure [24], rat diabetic nephropathy [25] and kidney mitochondria in aged rats [26]. In the clinical setting, 4-week treatment with ramipril, in patients with coronary artery disease, diminished the response of endothelium-dependent vasodilation to intracoronary administration of antioxidant vitamin C in parallel with improvement of basal endothelium-dependent vasodilation [27], indicating that ACE inhibitors can improve endothelial function in association with a reduction of oxidative stress.

In the present study, we investigated EC apoptosis, an important process that leads to endothelial dysfunction and atherosclerosis [14,15], and showed that ACE inhibitors, temocapril and captopril, attenuated EC apoptosis induced by H_2O_2 as well as by TNF-α. This result indicates that anti-apoptotic effects of ACE inhibitors are not specific to H_2O_2, but might be attributable to the anti-oxidant action of ACE inhibitors, because reactive oxygen species are known to be involved in TNF-α-induced EC apoptosis [28,29]. Reduction in 8-isoprostane formation by temcapril and captopril further supports the anti-oxidant effects of ACE inhibitors. It is not likely that the anti-apoptotic effects of ACE inhibitors were mediated through nitric oxide production via the inhibition of bradykinin degradation [11], because a nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester, did not influence the effect of temocapril on EC apoptosis (data not shown). Rather, the effects of ACE inhibitors are likely to be mediated through inhibition of angiotensin II production, as was demonstrated by the effect of olmesartan on p38 MAP kinase.

Reactive oxygen species activate many kinds of intracellular signaling, resulting in the transcription of numerous genes and the modulation of cellular function [30]. As

previously reported [31–33], extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and Akt in addition to p38 MAP kinase were activated in ECs by exposure to H_2O_2 (data not shown). Of these serine/threonine kinases, we focused on p38 MAP kinase because p38 MAP kinase is pro-apoptotic signaling, while ERK and Akt are anti-apoptotic, and JNK is anti- or pro-apoptotic depending on conditions [14,19,20]. We found that both temocapril and captopril inhibited the activity of p38 MAP kinase induced by H_2O_2. Although p38 MAP kinase is activated by stress and cytokines and acts on various target proteins, little is known about the downstream signaling [19,20,34]. However, EC apoptosis was effectively blocked in studies using a p38 MAP kinase inhibitor [35,36] and a dominant-negative form of p38 MAP kinase [35], indicating that activation of p38 MAP kinase leads to EC apoptosis. As a matter of fact, a p38 MAP kinase inhibitor, SB203580, partially inhibited H_2O_2-induced EC apoptosis in the present study. More importantly, SB203580 partially but significantly inhibited the effect of temocapril on apoptosis, further implying the role of p38 MAP kinase in the effect of temocapril. However, the partial effects of SB203580 also suggest the role of other pathways than p38 MAP kinase. We should perform future studies to determine the exact mechanism underlying H_2O_2-induced EC apoptosis.

In summary, we found that ACE inhibitors attenuated oxidative stress-induced EC apoptosis in culture. Furthermore, it was suggested that p38 MAP kinase was critical in the inhibitory effect of temocapril on EC apoptosis. These findings provide a mechanistic insight into the effects of ACE inhibitors, which have been used for the treatment of cardiovascular disease.

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Statins Protect Human Aortic Smooth Muscle Cells From Inorganic Phosphate-Induced Calcification by Restoring Gas6-Axl Survival Pathway

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Abstract—Vascular calcification is clinically important in the development of cardiovascular disease. It is reported that hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) inhibited vascular calcification in several clinical trials. However, the mechanism is poorly understood. Recently, it has been suggested that apoptosis is one of the important processes regulating vascular smooth muscle cell (VSMC) calcification. In this study, we investigated the effect of statins on VSMC calcification by testing their effect on apoptosis, focusing in particular on regulation of the survival pathway mediated by growth arrest-specific gene 6 (Gas6), a member of the vitamin K–dependent protein family, and its receptor, Axl. In human aortic smooth muscle cells (HASMC), statins significantly inhibited inorganic phosphate (Pi)-induced calcification in a concentration-dependent manner (reduced by 49% at 0.1 μmol/L atorvastatin). The inhibitory effect of statins was mediated by preventing apoptosis, which was increased by Pi in a concentration-dependent manner, and not by inhibiting sodium-dependent phosphate cotransporter (NPC) activity, another mechanism regulating HASMC calcification. Furthermore, the antiapoptotic effect of statins was dependent on restoration of Gas6, whose expression was downregulated by Pi. Restoration of Gas6 mRNA by statins was mediated by mRNA stabilization, and not by an increase in transcriptional activity. Suppression of Gas6 using small interfering RNA and the Axl-extracellular domain abolished the preventive effect of statins on Pi-induced apoptosis and calcification. These data demonstrate that statins protected HASMC from Pi-induced calcification by inhibiting apoptosis via restoration of the Gas6-Axl pathway. (*Circ Res.* 2006;98:1024-1031.)

Key Words: calcification ■ statins ■ apoptosis ■ Gas6 ■ Axl

Vascular calcification, such as coronary and aortic calcification, is a significant feature of vascular pathology, because this lesion is associated with cardiovascular disease.[1,2] It has been recognized that statins exhibit various protective effects against atherosclerosis, including modification of endothelial function,[3] decreased inflammation,[4] and inhibition of vascular smooth muscle cell (VSMC) proliferation and migration,[5] all of which cannot be accounted for by lipid reduction. One of the interesting pleiotropic effects of statins is the inhibition of vascular calcification. Results from clinical trials suggest an association of statin use with slowed progression of calcific aortic stenosis[6–8] and coronary artery calcification.[9] Statins also inhibited calcification of atherosclerotic plaques in experimental hyperlipidemic animals.[10,11] On the other hand, some recent clinical trials were not able to find such an inhibitory effect.[12,13] To clarify these discrepancies, it is important to identify the detailed regulatory mechanism of vascular calcification and the target of effect of statins.

Based on clinical findings,[14] inorganic phosphate (Pi) has been shown to be an important inducer of VSMC calcification, which is morphologically similar to that observed in calcified human heart valves and the aortic media. Transport of Pi into VSMC has been suggested to play an important role in the initiation of extracellular matrix calcification.[15] Recently, it has been shown that similar structures to matrix vesicles, derived from apoptotic VSMC, have been identified in human calcified arteries.[16] These vesicles have the capacity to concentrate and crystallize Ca, initiating calcification. Pi has been shown to induce apoptosis of hypertrophic chondrocytes, which is associated with cell maturation and extracellular matrix mineralization.[17] However, it is not clear whether or not apoptosis plays a regulatory role in the occurrence of VSMC calcification induced by Pi.

Recently, it was shown that growth arrest-specific gene 6 (Gas6), a member of the vitamin K–dependent protein family, and its receptor, Axl, a membrane receptor tyrosine kinase, are decreased on calcification of vascular pericytes.[18]

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Gas6 is a secreted protein that harbors a γ-carboxylglutamic acid–rich domain and 4 epidermal growth factor–like repeats.[19] Gas6-Axl interaction has been shown to be implicated in the regulation of multiple cellular functions, including growth, survival, adhesion, and chemotaxis.[20–23] In particular, they are known to protect a range of cell types from apoptotic death. However, there is no evidence that Gas6-Axl interaction is involved in Pi-induced apoptosis and calcification of VSMC.

In the present study, we found that statins inhibited Pi-induced calcification by preventing apoptosis in human aortic smooth muscle cells (HASMC). The effect of statins was dependent on restoration of the Gas6-Axl pathway. Furthermore, this beneficial effect was mediated by Gas6 mRNA stabilization, and not by increasing the transcription rate. Our results reveal a novel pathway by which statins regulate Pi-induced calcification in HASMC.

Materials and Methods

Materials

Pravastatin, atorvastatin, and fluvastatin were supplied by Sankyo Co Ltd, Pfizer Inc (New York), and Tanabe Seiyaku Co Ltd, respectively. Recombinant human Gas6 (rhGas6) and Axl-ECD were prepared as described previously.[22,24] All other reagents were of analytical grade.

Cell Culture

HASMC were obtained from Clonetics. They were cultured in DMEM supplemented with 20% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. HASMC were used up to passage 8 for the experiments.

Induction and Quantification of Calcification

For Pi-induced calcification, Pi (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 concentrations of 2.0, 2.6, and 3.2 mmol/L ("calcification medium"). After the indicated incubation period, cells were decalcified with 0.6 mol/L HCl, and Ca content in the supernatant was determined by the *o*-cresolphthalein complexone method (C-Test, WAKO). The remaining cells were solubilized in 0.1 mol/L NaOH/0.1% SDS, and cell protein content was measured by Bio-Rad protein assay. Calcification was visualized by von Kossa's method. Briefly, the cells were fixed with 4% formaldehyde and exposed to 5% aqueous $AgNO_3$.

Induction of Apoptosis

Two different time courses were tested to investigate Pi-induced apoptosis and examine the effect of statins. (1) Short-term condition: Pi was added at final concentrations of 2.0, 2.6, and 3.2 mmol/L for 24 hours at confluence, after the cells were incubated with serum-free DMEM for 48 hours. To test the effect of statins on apoptosis, they were added 24 hours after incubating the cells with serum-free DMEM (12 hours before adding Pi). (2) Long-term condition: at confluence, the medium was switched to calcification medium and cells were cultured for up to 10 days. The medium was changed every 2 days. To test the effect of statins, each was added simultaneously when the medium was switched to the calcification medium.

RNA Extraction, Northern Blot, and mRNA Stability Analysis

The 304-bp product of the Gas6 cDNA probe (forward, 5′-GCGTGGCCAAGAGTGTGAAGT-3′; reverse, 5′-CGCCACTCC-TCAACAGAGAT-3′) was amplified by RT-PCR. For Northern blot analysis, harvested RNA (≈5 to 10 μg) was fractionated on 1.4% formaldehyde-agarose gel and transferred to a nylon filter. The filter was hybridized at 68°C for 2 hours with ^{32}P-labeled Gas6 cDNA and 18S probe in QuickHyb solution (Stratagene) and autoradiographed. To examine Gas6 mRNA stability, serum-starved HASMC were incubated with actinomycin D (Act D, 5 μg/mL) in the presence of 2.6 mmol/L Pi after 12 hours of atorvastatin (0.1 μmol/L) treatment. Total RNA was harvested at 0, 1, 3, and 6 hours for Northern blot analysis. Signal density of the Gas6 mRNA was normalized to that

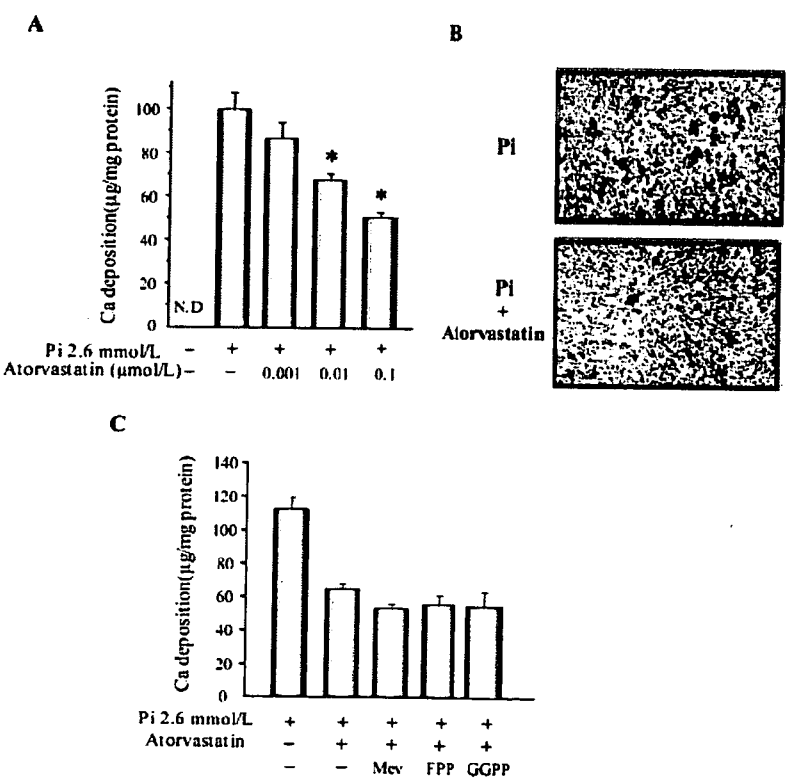

Figure 1. Statins prevent HASMC calcification. A, HASMC were cultured with the indicated concentrations of atorvastatin in the presence of 2.6 mmol/L Pi for 6 days. Ca deposition was measured by *o*-cresolphthalein complexone method and normalized by cell protein content. All values are presented as mean±SEM (n=6). *P<0.05 vs statin (-) by Fisher's test. N.D. indicates not detected. B, On day 6, the inhibitory effect of atorvastatin (0.1 μmol/L) on 2.6 mmol/L Pi-induced Ca deposition was evaluated at the light microscopic level with von Kossa's staining. The arrow points to an area of Ca deposition. C, HASMC were cultured with mevalonate (100 μmol/L), farnesylpyrophosphate (1 μmol/L), or geranylgeranylpyrophosphate (1 μmol/L) in the presence of atorvastatin (0.1 μmol/L) and 2.6 mmol/L Pi for 6 days. All values are presented as mean±SEM (n=6).

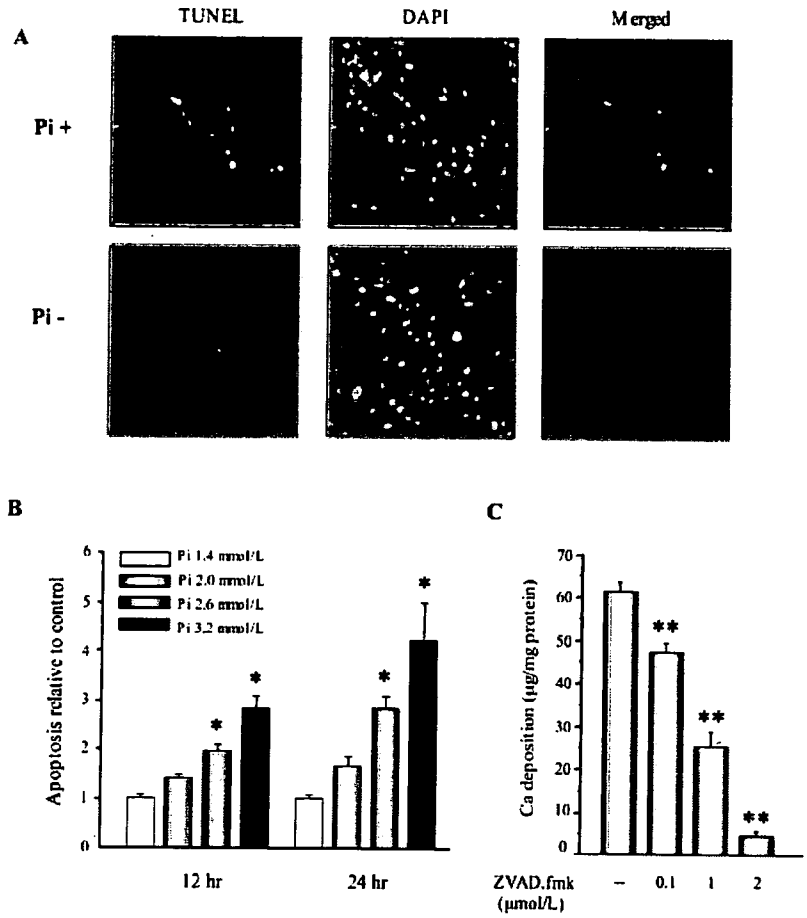

Figure 2. Pi induces apoptosis, and ZVAD.fmk inhibits Pi-induced calcification. A, After incubation with 1.4 (Pi-) and 3.2 mmol/L (Pi+) Pi for 10 days, apoptotic cells were identified by TUNEL staining (green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). B, Serum-starved HASMC were cultured with the indicated concentration of Pi for 24 hours. A quantitative index of apoptosis, determined by ELISA, is presented as the relative value to that with 1.4 mmol/L Pi. All values are presented as mean±SEM (n=3). $^{*}P<0.05$ vs 1.4 mmol/L Pi by Fisher's test. C, HASMC were incubated with the indicated concentration of ZVAD.fmk in the presence of 2.6 mmol/L Pi for 6 days. Ca content was measured and normalized by cell protein content. All values are presented as mean±SEM (n=6). $^{**}P<0.01$ vs 2.6 mmol/L Pi, ZVAD.fmk(-) by Fisher's test. Experiments were performed with at least 3 different cell populations.

of the 18S RNA at each time point, and the half-life was calculated by linear extrapolation.

Preparation of Small Interfering RNA Targeting Gas6 and Transfection

Two small interfering RNAs (siRNAs) were designed to target human Gas6 (accession no. NM_000820) using siRNA design software (Dharmacon). The sequences for Gas6 were 5′-GGACCTGCCAAGACATAGA-3′ and 5′-ACCTCGTGCAGCCT-ATAAA-3′. Nonspecific control siRNA was synthesized using standard templates (Dharmacon). Twenty-four hours after HASMC seeding onto 12-well plates, cells were cultured in serum-free medium for an additional 24 hours, then transfected with Gas6 (100 nmol/L) and control siRNA using transfection reagent (Upstate). To evaluate the effect of Gas6 siRNA on Ca deposition, siRNA was transfected when HASMC had reached 80% to 90% confluence and then transfected every time the medium was changed (every 2 days) up to 6 days. The loss of Gas6 by transfection of siRNA was validated by immunoblotting for Gas6 protein in the cell lysates 48 hours and 6 days after siRNA transfection.

Statistical Analysis

All results are presented as mean±SEM. Statistical comparisons were made by ANOVA, unless otherwise stated. A value of $P<0.05$ was considered to be significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

Results

Statins Inhibit Pi-Induced HASMC Calcification

To induce HASMC calcification, cells were incubated with calcification medium for 10 days. We confirmed that high phosphate (≥2.6 mmol/L) induced Ca deposition in a concentration- and time-dependent manner, whereas 1.4 mmol/L Pi, equivalent to the human physiological serum phosphate level, was not able to induce Ca deposition up to 10 days. To investigate the effect of statins on Pi-induced calcification, HASMC were incubated with atorvastatin in the presence of 2.6 mmol/L Pi. On day 6, Ca deposition was significantly suppressed by atorvastatin in a concentration-dependent manner (51.1±1.9% of control at 0.1 μmol/L) (Figure 1A). An inhibitory effect of the statins on Ca deposition was also found by von Kossa's staining (Figure 1B). Atorvastatin was able to be added at as high a concentration as 0.1 μmol/L without cell damage. The inhibitory effect was also observed with fluvastatin (0.001 to 0.1 μmol/L) and pravastatin (0.01 to 50 μmol/L) (data not shown). The inhibitory effect of statins was not blocked by mevalonate (100 μmol/L), farnesylpyrophosphate (1 μmol/L), or geranylgeranylpyrophosphate (1 μmol/L), suggesting that the effect is not dependent on the mevalonate pathway (Figure 1C).

Inhibitory Effect of Statins on Calcification Is Caused by Preventing Apoptosis, Not by Inhibiting Sodium-Dependent Phosphate Cotransporter Activity

Two different time courses were tested to examine the effect of Pi on HASMC apoptosis: short-term (up to 24 hours) and long-term (up to 10 days; practical time course of calcifica-