3-Deazaadenosine Prevents Smooth Muscle Cell Proliferation and Neointima Formation by Interfering With Ras Signaling

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Abstract—3-Deazaadenosine (c3Ado) is a potent inhibitor of S-adenosylhomocysteine hydrolase, which regulates cellular methyltransferase activity. In the present study, we sought to determine the effect of c3Ado on vascular smooth muscle cell (VSMC) function and neointima formation in vivo. c3Ado dose-dependently prevented the proliferation and migration of human coronary VSMCs in vitro. This was accompanied by an increased expression of the cyclin-dependent kinase inhibitors p21\(^{WAF1/Cip1}\), p27\(^{Kip1}\), a decreased expression of G1/S phase cyclins, and a lack of retinoblastoma protein hyperphosphorylation. In accordance with these findings, fluorescence-activated cell-sorting analysis of propidium iodide–stained cells indicated a cell cycle arrest in the G0/G1 phase. Importantly, c3Ado did not affect the number of viable (trypan blue exclusion) or apoptotic cells (TUNEL). Mechanistically, c3Ado prevented FCS-induced Ras carboxyl methylation and membrane translocation and activity by inhibiting isoprenylcysteine carboxyl methyltransferase and reduced FCS-induced extracellular signal-regulated kinase (ERK)1/2 and Akt phosphorylation in a dose-dependent manner. Conversely, rescuing signal transduction by overexpression of a constitutive active Ras mutant abrogated c3Ado’s effect on proliferation. For in vivo studies, the femoral artery of C57BL/6 mice was dilated and mice were fed a diet containing 150 μg of c3Ado per day. c3Ado prevented dilation-induced Ras activation, as well as ERK1/2 and Akt phosphorylation in vivo. At day 21, VSMC proliferation (proliferating-cell nuclear antigen [PCNA]-positive cells), as well as the neointima/media ratio (0.7 ± 0.2 versus 1.6 ± 0.4; \(P<0.05\)) were significantly reduced, without any changes in the number of apoptotic cells. Our data indicate that c3Ado interferes with Ras methylation and function and thereby with mitogenic activation of ERK1/2 and Akt, preventing VSMC cell cycle entry and proliferation and neointima formation in vivo. Thus, therapeutic inhibition of S-adenosylhomocysteine hydrolase by c3Ado may represent a safe and effective novel approach to prevent vascular proliferative disease. (Circ Res. 2009;104:1192-1200.)

Key Words: neointima formation | restenosis | signal transduction | smooth muscle cells | vascular smooth muscle cell proliferation

Vascular smooth muscle cell (VSMC) migration, proliferation, and hypertrophy triggered by inflammatory responses of the vessel wall are considered to be key events in the development of atherosclerosis, postangioplasty restenosis, and venous bypass graft failure.1,2 Consequently, antiinflammatory and antiproliferative strategies have been demonstrated to successfully prevent the development of vascular proliferative disease. Therefore, the identification of novel compounds with combined antiinflammatory/antiproliferative properties holds promise to improve existing therapeutic strategies by limiting late cardiovascular complications like in-stent restenosis or bypass graft failure.

3-Deazaadenosine (c3Ado), a structural analog of adenosine without a adenosine–receptor interaction, is a potent inhibitor of S-adenosylhomocysteine (SAH)-hydrolase, which regulates cellular methyltransferase activity. c3Ado has previously been shown to inhibit a variety of cellular functions, which could be critical for the development of atherosclerosis and restenosis. These include thrombin-stimulated production of platelet-derived growth factor and the expression of endothelial leukocyte adhesion molecule-13 as well as cellular arachidonic acid and ROS production.4,5 Moreover, c3Ado prevents tumor necrosis factor (TNF)-α production, reduces TNF-α-induced macrophage adhesion to endothelial cells in vitro via the inhibition of ICAM-1 synthesis, and promotes monocyte apoptosis.6 We recently demonstrated that c3Ado inhibits endothelial expression of ICAM-1 and VCAM-1 in vivo and prevents diet-induced...
plaque formation in apolipoprotein (apo)E−/− mice. However, the underlying molecular mechanism of c3Ado’s potent antiinflammatory properties is not well understood.

Besides inflammatory responses, the proliferation of VSMCs comprises a major determinant in the development of atherosclerosis and restenosis. It is, however, unclear whether c3Ado, in addition to its antiinflammatory properties, has a direct effect on cell cycle progression and proliferation of VSMCs and thus might be a suitable compound to even prevent highly proliferative vascular responses like postangioplasty restenosis.

Consequently, we aimed to analyze the effect of c3Ado on VSMC proliferation and migration and its impact on neointima formation in vivo. On a molecular basis, c3Ado regulates cellular SAH levels by inhibiting SAH-hydrolase. Because elevated intracellular concentration of SAH results in product inhibition of S-adenosylmethionine–dependent methytransferases, we hypothesized that inhibition of methyltransferase activity could mediate the c3Ado-dependent modulation of VSMC function. Because posttranslational methylation is an important step in the activation of CAAX sequence–containing signaling proteins, such as the Ras superfamily of GTPases, we hypothesized that c3Ado may interfere with Ras function and subsequent downstream signaling regulating VSMC cell cycle progression and proliferation.

In the present study, we demonstrate that inhibition of SAH by c3Ado prevents Ras GTPase carboxyl methylation and membrane translocation and activity, resulting in diminished activation of the downstream signaling molecules extracellular signal-regulated kinase (ERK) and Akt. Diminished activation of these pathways results in an insufficient regulation of cell cycle proteins which control G0/G1/S phase progression of VSMCs, resulting in an inhibition of VSMC proliferation. Finally, we demonstrate that oral administration of c3Ado in a dose-dependent manner prevents VSMC proliferation and neointima formation in a mouse model of vascular injury.

Taken together, our data demonstrate that by exerting potent anti proliferative and antiinflammatory properties, therapeutic inhibition of increased signaling protein methylation in activated VSMCs by c3Ado may represent a novel approach to prevent vascular proliferative disease.

Materials and Methods

Quantification of VSMC Proliferation

Human coronary artery smooth muscle cells (VSMCs; Clonetics, Verviers, Belgium) were grown in 500 μL of supplemented growth medium containing 5% FCS (SmGM-2; Clonetics, Verviers, Belgium) in 24-well culture dishes. Different concentrations of c3Ado were added to the growth medium. After 48 hours of incubation at 37°C/5% CO2, cells were trypsinized and counted using a hemocytometer. Alternatively, 5-bromodeoxyuridine (BrdUrd) incorporation was determined according to the instructions of the supplier (Cell Proliferation ELISA, Roche, Mannheim, Germany).

Preparation of Cellular Lysates and Immunoblot Analysis

Semiquantitative analysis of proteins in cell lysates was performed by western blotting and antibody detection as previously described. Briefly, the cleared supernatant from lysates was run on polyacrylamide gel and blotted onto nitrocellulose (Hybond-ECL; Amersham, Freiburg, Germany) by wet electroblotting. After blocking, blots were incubated with primary antibody for 1 hour at room temperature. (For a detailed description of the antibodies used, refer to the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.) Proteins were then detected by enhanced chemiluminescence (ECL+; Amersham) after labeling with horseradish peroxidase–labeled secondary antibody (1:2000 for 1 hour) according to the instructions of the manufacturer.

Mouse Femoral Artery Angioplasty

Male C57/B1L6 mice (Charles River, Quebec, Canada) were anesthetized with 150 mg/kg body weight ketamine hydrochloride (Ketanest; Pharmacia/Pfizer, Mannheim, Germany) and 0.1 mg/kg body weight xylazine hydrochloride (Rompun 2%; Bayer) underwent transluminal mechanical injury of the left femoral artery by insertion of a straight spring wire (0.38 mm in diameter; Cook, Bloomington, Ind) for >5 mm toward the iliac artery, as described previously. All procedures involving experimental animals were approved by the institutional committee for animal research of the Giessen University and complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1985).

Statistical Analyses

Data were stored and analyzed on personal computers using Excel 2000 (Microsoft) and Sigma Stat 2.03 (Systat, Erkrath, Germany). Data between the study groups were analyzed by 1-way ANOVA, followed by pairwise multicomparison using the Holm–Sidak method. All data are represented as means ± SEM. A probability value of <0.05 was considered statistically significant for all comparisons.

For further descriptions, refer to the expanded Materials and Methods section in the online data supplement.

Results

Effect of c3Ado on VSMC Proliferation and Migration

To evaluate the effect of c3Ado on VSMC proliferation and migration, both known to contribute to vascular lesion formation, VSMCs were grown in growth medium containing 5% FCS in the absence or presence of different concentrations of c3Ado. c3Ado prevented the increase in VSMC numbers in a dose-dependent manner (51.6% versus 73.8% increase in cell number after 48 hour; n=4; *P<0.05 at 50 μmol/L c3Ado; Figure 1A). The reduced increase in cell numbers was attributable to an inhibition of VSMC proliferation. Figure 2B illustrates results from a BrdUrd incorporation assay and shows that DNA replication is inhibited by 60% in VSMCs treated with 50 μmol/L c3Ado compared to controls (n=4; *P<0.05). Furthermore, c3Ado dose-dependently prevented VSMC migration (28.8±2.3 versus 73.8±9.6 cells/high power field; n=4; *P<0.05 at 50 μmol/L c3Ado; Figure 1C).

Effect of c3Ado on VSMC Apoptosis and Necrosis

To exclude the lack of increase in cell numbers as also attributable to an apoptotic effect of c3Ado, we determined the number of apoptotic cells in the absence or presence of c3Ado. As shown in Figure 2A, c3Ado did not induce apoptosis (TUNEL–positive cells), even at concentrations of...
Figure 1. Effect of c3Ado on VSMC proliferation and migration. A, VSMCs were incubated in growth medium in the absence or presence of different concentrations of c3Ado, and total cell number was evaluated after 48 hours (*P<0.05; n=4). B, VSMCs were incubated in growth medium in the absence or presence of different concentrations of c3Ado for 24 hours in the presence of BrdUrd. VSMC proliferation is expressed as mean OD450 ± SEM, as determined by anti-BrdUrd ELISA (*P<0.05; n=4). C, VSMCs were added to the upper side of gelatin-coated tissue culture inserts and allowed to migrate for 6 hours in the presence or absence of c3Ado (100 µmol/L) or different concentrations of c3Ado. After microscopic evaluation of inserts, the number of migrated cells was expressed as cells per high-power field (HPF) (*P<0.05; n=4).

100 µmol/L (1.6±0.6% versus 2.2±0.8% of total cells; n=4; *P=NS at 100 µmol/L c3Ado). There was also no effect of c3Ado on cell necrosis as determined by trypan blue exclusion (80.9±8.8% versus 81.4±6.2% viable cells; n=4; *P=NS at 100 µmol/L c3Ado; Figure 2B). Furthermore, the antiproliferative effect of c3Ado was reversible: cells were serum-starved and synchronized in the presence or absence of c3Ado for 24 hours. After 3 washing steps, cells were incubated in basal medium for 12 hour and then in growth medium containing BrdUrd for 24 hours, and BrdUrd incorporation was quantified. Cells pretreated with c3Ado (50 µmol/L) showed an almost similar proliferation rate as cells pretreated with a control buffer only. In contrast, cells pretreated with actinomycin D did not proliferate in the presence of growth medium (n=4; Figure 2C). These data indicate that there is no toxic or apoptotic effect of c3Ado in the tested concentrations.

c3Ado Prevents Cell Cycle Entry/Progression in the G0/G1 Phase
To elucidate the mechanisms responsible for the antiproliferative effect of c3Ado, we determined the cell cycle progression and expression of cell cycle regulating proteins in the absence or presence of c3Ado. As determined by fluorescence-activated cell-sorting analysis, c3Ado dose-dependently prevented the cell cycle entry/progression in the G0/G1 phase, indicating that c3Ado interferes with very early processes of cell cycle progression (Figure 3A and 3B). c3Ado dose-dependently prevented the down-regulation of the cyclin-dependent kinase inhibitors p21cip1 and p27kip1, as well as the upregulation of cyclin A, cyclin B, cyclin D, and cyclin E, which are essential for the progression through the G1 phase. A block in G0/G1 phase was also confirmed by the lacking hyperphosphorylation of the retinoblastoma gene product (RB) (Figure 3C). However, there was no specific regulation of one of these molecules, suggesting that c3Ado may not affect the expression or function of a single cell cycle regulatory protein but rather may interfere with upstream signaling mechanisms that regulate early cell cycle entry. Moreover, in conclusion with the TUNEL assay results, no activation of caspase-3 was observed.
Effect of c3Ado on Akt and Erk Activation

We previously demonstrated that the mitogen-activated protein kinase pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway are essential triggers for early cell cycle events such as the expression of the cyclin-dependent kinase inhibitors p21cip/waf and p27Kip1 or the expression of cyclins. Therefore, we evaluated the effect of c3Ado on the serum-induced activation of these pathways. Surprisingly, c3Ado did interfere with activation of both pathways. c3Ado dose-dependently prevented the phosphorylation of Raf and ERK (Figure 4A) but also of the protein-dependent kinase 1, of the protein kinase B (Akt), and of the forkhead transcription factor FoxO1a, known to regulate the transactivation of p27kip1 and D-type cyclins (Figure 4B). These data indicate that c3Ado may interfere with processes upstream of the serum-induced activation of these signal transduction pathways.

C3Ado Prevents VSMC Proliferation by Interfering With Ras Signaling

The GTPase Ras has been described to transduce growth factor–induced tyrosine kinase signaling toward ERK and Akt activation. Ras translocation to the membrane and activation is dependent on its carboxyl methylation by methyltransferases, which were shown to be inhibited by c3Ado. Therefore, we sought to determine whether c3Ado may prevent growth factor–induced ERK and Akt activation by interfering with Ras signaling. Our data indicate that c3Ado prevents Ras translocation from the cytosol to the cell membrane, a prerequisite for Ras signaling, as determined by immunohistochemistry (Figure 5A). As shown by membrane fractionation from whole cell lysates and immunoblotting using specific antibodies, the growth factor–induced localization of Ras in the membrane fraction is prevented by c3Ado in a dose-dependent manner (Figure 5B). In accordance with these data, the growth factor–induced Ras activation (Ras-GTP) was also reduced by c3Ado in a dose-dependent manner (Figure 5C). To test whether the inhibitory effect on Ras activity is responsible for the antiproliferative effect of c3Ado, a constitutively active mutant of Ras (RasH110Q) was overexpressed in VSMCs. (Note that only positively transfected cells were sorted and evaluated as described in Materials and Methods.) Cells overexpressing the constitutive active form of Ras were resistant to the c3Ado-induced antiproliferative effect, indicating that c3Ado interferes with VSMC proliferation mainly by preventing Ras-dependent signal transduction (Figure 5D).

c3Ado-Dependent Inhibition of Ras Carboxyl Methylation, Activation, and Signaling Is Dependent on ICMT

We hypothesized that inhibition of isoprenylcysteine carboxyl methyltransferase (ICMT) by c3Ado would inhibit Ras
activation by preventing the methylation of its carboxyl-terminal CAAX motif. We therefore assessed Ras carboxyl methylation and Ras activity in cells treated with c3Ado, the ICMT inhibitor AGGC (20 μmol/L), or the inactive analog AGC (20 μmol/L). Ras carboxyl methylation and Ras activity was significantly decreased after incubation of VSMCs with AGGC as it was after incubation with c3Ado (Figures 5E and 6A). Consistently, treatment with the ICMT inhibitor AGGC significantly prevented VSMC proliferation to the same extent as c3Ado compared with the inactive analog AGC (Figure 6C). Overexpression of ICMT but not of a control plasmid (green fluorescent protein [GFP]) prevented the c3Ado-dependent inhibition of Ras carboxyl methylation (Figure 5E) and Ras activity (Figure 6B), indicating that c3Ado interferes with Ras activity by inhibiting ICMT methyltransferase activity and thereby Ras carboxyl methylation. Conclusively, overexpression of ICMT also prevented the c3Ado-dependent inhibition of VSMC proliferation (Figure 6D). Furthermore, the AGGC-mediated inhibition of proliferation was abrogated when Ras activity was reconstituted by overexpression of a constitutively active Ras mutant that does not require methylation (Figure 6E). Taken together, these data demonstrate that c3Ado inhibits Ras...
methyltransferase activity and that inhibition of Ras represents a key mechanism of c3Ado’s antiproliferative effect.

c3Ado Prevents Neointima Formation In Vivo

To test the effect of c3Ado in vivo, mice were fed with the indicated concentrations of c3Ado for 2 days as previously described.

Ras activity and Akt/ERK phosphorylation were determined in arteries 20 minutes after dilation. As indicated in Figure 7A and 7B, pretreatment of mice with c3Ado reduced dilation-induced Ras activation and Akt and ERK phosphorylation. To evaluate the effect of c3Ado on neointima formation, mouse femoral arteries were excised 21 days after dilation, and morphometric analysis was performed. A significant concentric neointima is evident, with the media clearly defined by the internal and external elastic laminae. The middle and right images in Figure 7C show injured femoral arteries from mice treated with 75 μg of c3Ado per day, or a diet containing 150 μg of c3Ado per day, respectively. Intima/media ratio and neointimal area were significantly reduced in c3Ado-treated femoral arteries compared to control vessels (neointima/media ratio: 0.7 ± 0.2 [150 μg of c3Ado per day] versus 1.6 ± 0.4 [control diet]; n = 6; *P < 0.05; Figure 7D). Medial wall area was not significantly reduced in the c3Ado-treated groups (Figure 7D), which is in accordance to the nonapoptotic/nontoxic effect of c3Ado seen in vitro.

Effect of c3Ado on VSMC Proliferation, Apoptosis, and Reendothelialization In Vivo

Further immunohistochemical evaluation of neointimal tissues revealed that the potency of c3Ado to prevent neointima formation results from an antiproliferative effect, as determined by quantification of PCNA-positive cells 21 days following injury (6.7 ± 2% versus 10.8 ± 0.8% PCNA-positive cells in arteries from mice treated with 150 μg of c3Ado per day; *P < 0.05; Figure 8A). Complementing the in vitro data, c3Ado did not augment apoptosis of VSMCs as determined by quantification of TUNEL-positive cells (1.8 ± 0.6% versus 1.6 ± 0.4% TUNEL-positive cells in arteries from mice treated with 150 μg of c3Ado per day; *P = NS; Figure 8B). Furthermore, treatment of mice with c3Ado at different concentrations had no effect on reendothelialization of denuded arteries 21 days after dilation (n = 6; *P = NS; Figure 8C).

Discussion

VSMCs play a prominent role in the pathogenesis of vascular proliferative disorders such as atherosclerosis, postangioplasty restenosis, bypass vein graft failure, and cardiac allograft vasculopathy. Identification of the key mechanisms involved in VSMC function will help to understand cellular responses to vascular injury. Moreover, the identification and characterization of small molecules interfering with these pathways will help to develop safe and efficient therapeutic strategies for the prevention of vascular proliferative disease.

In the present study we demonstrate that inhibition of SAH-hydrolase by c3Ado, a structural analog of adenosine which lacks adenosine-receptor interaction, prevents VSMC proliferation and the development of postangioplasty restenosis. We provide evidence that c3Ado inhibits Ras methylation and thereby interferes with Ras downstream signaling in response to mitogenic stimuli. c3Ado thereby prevents the activation of the Akt as well as the ERK pathway, both known to be essential for the induction of cell cycle entry. Indeed, treatment with c3Ado resulted in a stabilization of the cyclin-dependent kinase inhibitors p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> while preventing the transcriptional activation of G<sub>1</sub> phase cyclins. Subsequently, c3Ado prevented G<sub>1</sub>/S phase transition and proliferation of VSMCs. Furthermore, c3Ado inhibited VSMC migration, another important component of vascular lesion formation, without promoting apoptosis of VSMCs. The efficiency of c3Ado was also observed in vivo, because oral administration of c3Ado effectively prevented neointima formation in a murine model of wire-induced vascular injury.

In initial experiments, we observed that c3Ado dose-dependently inhibited VSMC proliferation. This is in accordance with previous reports that described a potent antiproliferative effect of SAH-hydrolase inhibition on vascular cells, indicating that inhibition of methyltransferase activity represents a potentially interesting target for antiproliferative strategies. Our results indicated that c3Ado inhibits cell cycle progression very early in G<sub>1</sub>/G<sub>0</sub> phase; however, c3Ado did not seem to specifically regulate the expression of the tested
G0/G1 phase proteins. We therefore hypothesized that c3Ado interferes with upstream signaling regulating G0/G1 phase progression.

We and others previously demonstrated that the mitogen-activated protein kinase ERK and PI3K/Akt pathways are essential for proper cell cycle entry and progression through G0/G1 phase and proliferation of VSMCs.12 We therefore tested whether c3Ado might interfere with Akt or ERK signaling or other signaling pathways (data not shown) regulating G0/G1 progression. Our experiments indicate that c3Ado dose-dependently inhibited the phosphorylation and activation of both, the PI3K/Akt and ERK pathway, suggesting that c3Ado might interfere with methylation-dependent signaling processes upstream of Akt and ERK.

The activation of ERK and Akt is strongly dependent on the GTPase Ras, which regulates a wide variety of cellular functions, including growth, differentiation, and apoptosis. To gain activity, Ras GTPase proteins must associate with cellular membranes.13,14 However, Ras plasma membrane association requires a series of posttranslational modifications of its carboxyl terminus including farnesylation, prenylation, and methylation of its CAAX sequence by an ICMT.15,16 Methylation of CAAX proteins has been shown to be stimulated by various inflammatory and mitogenic stimuli such as TNF-α17 or high glucose.18 Thus, methylation appears to be a common mechanism by which various activators stimulate the activity of signaling proteins. Moreover, the importance of ICMT-dependent methylation was recently demonstrated by the finding that ICMT-deficient mice did not survive beyond mid-gestation.19

ICMT requires S-adenosylmethionine as a methyl group donor to produce methylated Ras, resulting in the accumulation of SAH. SAH is broken down by SAH-hydrolase into homocysteine and homocysteine. However, inhibition of the SAH-hydrolase results in the accumulation of SAH, which then, by product inhibition, acts as a potent inhibitor of S-adenosylmethionine–dependent ICMT activation and subsequently of Ras function.20 Therefore, by inhibiting SAH-hydrolase, c3Ado may prevent the increased methylation and activation of signaling molecules in activated VSMCs.

Our data demonstrate that c3Ado dose-dependently inhibited growth factor–induced Ras membrane association and activity. Furthermore, we provide evidence that inhibition of Ras signaling is one of the key mechanisms of the antiproliferative effect of c3Ado in VSMCs, because overexpression of a constitutive active Ras mutant almost completely rescued VSMCs from the antiproliferative effect of c3Ado. Furthermore, our data indicate that c3Ado regulates Ras methylation via the inhibition of ICMT. Because ICMT inhibitors prevented Ras activation and proliferation to the same extent as c3Ado, the overexpression of ICMT restored serum-induced Ras activation in the presence of c3Ado. These data are in accordance with results previously described by Wang et al,11 who demonstrated that coincubation of endothelial cells with homocysteine and an adenosine deaminase inhibitor, a strategy that inhibits SAH-hydrolase, and thereby ICMT activity, decreased the level of carboxyl methylation and plasma membrane localization of v-H-Ras, as well as endothelial cell proliferation.

Recently, a role for Ras-GTPase in vascular homeostasis and disease development has been suggested: Dependency of Ras-GTPase activities on the activation of signaling molecules in activated VSMCs.21 Moreover, the local delivery of H-ras dominant negative mutant (N17 and L61, S186) plasmid constructs,22 adenovirus-mediated transfer of dominant negative H-ras,23 or prevention of posttranslational modification by local delivery of a Ras farnesyl transferase inhibitor24 have been shown to prevent ERK activation and to...
significantly reduce neointima formation. Recent findings, however, indicate that the inhibition of the post–prenylation-processing steps, particularly that of ICMT-catalyzed methylation of Ras might provide the most effective approach to control cell proliferation. Accordingly, our data indicate that c3Ado effectively inhibited Ras activation, VSMC proliferation, and neointima formation in vivo.

However, in addition to the antiproliferative effect of c3Ado on VSMCs, the previously described inhibitory effects of c3Ado on ROS production, adhesion molecule expression, leukocyte recruitment, and monocyte and T-cell activation may further contribute to the effective reduction of vascular lesion formation.

Previous studies reported that inhibition of ICMT and subsequent Ras signaling may cause apoptosis of pulmonary artery endothelial cells. Furthermore, the antipoptotic effect of Ras-dependent Akt activation was described in a wide variety of cell types. Given the concerns that c3Ado might also modulate endothelial cell apoptosis and proliferation, we assessed the re-endothelialization of denuded vessels. However, we did not observe any differences in reendothelialization 4 weeks after injury, suggesting that therapeutic application of c3Ado is not accompanied by an increased risk of target vessel thrombosis. Moreover, our data indicate that c3Ado does not induce apoptosis of VSMCs in vitro or in vivo even at the highest concentrations tested. Furthermore, there was no toxic effect of c3Ado, as demonstrated by trypan blue exclusion and the reversibility of the antiproliferative action of c3Ado in vitro, as well as in the in vivo findings showing no thinning of the medial wall. These findings are in accordance with previous reports showing that a specific, reversible type III SAH-hydrolase inhibitor (DZ2002) did not exert any toxic effects despite its potent inhibitory potential and that c3Ado prevented thrombin-dependent endothelial cell activation without inducing cytotoxic effects.

Therefore, with regard to toxicity and safety of a potential therapeutic application, SAH-hydrolase inhibition by c3Ado might be superior to existing Ras targeting approaches. Importantly, our data suggest that by interfering with VSMC proliferation in acute or early lesions and by interfering with inflammatory responses without inducing apoptosis of vascular cells in stable lesions, c3Ado may represent an attractive molecule for the prevention of acute as well as chronic vascular disease, even though its safety especially during long term systemic application needs further evaluation.

In conclusion, our data indicate that the prevention of increased methylation of signaling proteins by c3Ado, which inhibits SAH-hydrolase, ICMT, and subsequent Ras signaling, may represent an attractive therapeutic strategy for the prevention of vascular proliferative disease.

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Disclosures

None.

References


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Expanded Materials and Methods

Cells and Reagents

Human coronary artery smooth muscle cells (VSMC) were cultured in SmGM-2-Smooth Muscle Medium 2 with or without the addition of a growth factor cocktail and 5% FBS (Cambrex, Verviers, Belgium). The following antibodies were used: mouse anti-Ras (Ras10), rabbit anti-Cyclin D1 (Upstate Charlottesville, Virginia, USA), rabbit anti-p21, mouse anti-p27, rabbit anti-Cyclin B1, rabbit anti-Cyclin E and rabbit anti-Cdk4, goat anti-actin (Santa Cruz Biotechnology, Santa Cruz, California, USA), mouse anti-Cyclin A (BD Biosciences, Erembodegem, Belgium), rabbit anti-pRaf, rabbit anti-pRb, rabbit anti-phosphoinositide-dependent kinase-1 (pPDK1), mouse anti-phospho-p44/42 MAPK (pERK), rabbit anti-phospho-Akt (ser473, pAkt), rabbit anti-pFKHR, rabbit anti-cleaved caspase-3 (Cell Signalling, Beverly, Massachusetts, USA), secondary antibodies were goat anti-rabbit IgG and goat anti-mouse IgG, the latter two antibodies being linked to horseradish peroxidase (Santa Cruz Biotechnology Inc. Santa Cruz, California).

N-Acetyl-S-geranyl-L-cysteine (AGC) and N-Acetyl-S-geranylgernayl-L-cysteine (AGGC) were from Biomol, Hamburg, Germany. C3Ado was from Southern Research, Birmingham, Alabama, USA.

Quantification of VSMC Migration

VSMC were detached using 1 mmol/L Trypsin (Gibco), harvested by centrifugation, resuspended in 500 µL of SmGM-2 and counted. 25x10³ cells were seeded in the upper chamber of a modified Boyden chamber (8 µm pore size) and were allowed to attach. Chambers were placed in a 24-well culture dish containing SmGM-2 and human recombinant PDGF (20 ng/mL) and were allowed to migrate in the absence or presence of different
concentrations of c3Ado. After 6 hours of incubation at 37°C, 5% CO₂, the remaining cells in the upper chamber were removed with a cotton wool swab. The lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with trypan blue. Migrating cells into the lower chamber were counted in three high power fields.

**Quantification of VSMC Apoptosis**

VSMC were cultured in the absence or presence of different concentrations of c3Ado for 24 h. VSMC in the supernatant were collected and the remaining, attached VSMC were trypsinized. Both fractions were mixed, uniformly distributed on glass slides using a cytocentrifuge, fixed using 2% paraformaldehyde for 10 min, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) was performed according to the supplier’s instructions (In situ cell death detection kit, Roche, Mannheim, Germany). After staining all nuclei with DAPI, samples were viewed with an inverted fluorescence microscope (Leica, Mannheim, Germany) and two independent investigators, blinded to the treatment, evaluated the relative number of apoptotic cells per well by counting 4 randomly selected high-power fields.

**Trypan blue staining**

Trypan blue exclusion was used to determine the viability of VSMC. After addition of different concentrations of c3Ado for 24h, VSMC in the supernatant were collected and the remaining, attached VSMC were trypsinized. Both fractions were mixed and incubated with 0.4% trypan blue dye for 2 min. VSMC were observed with the use of a hemocytometer under a light microscope. Cells that were able to exclude the stain were considered viable and the percentage of non-blue cells over total cell number was used as an index of viability.
Flow Cytometry (Cell Cycle Distribution)

Cells were harvested by trypsinization, fixed overnight with 75 % methanol, washed, and incubated with 100 μg/ml RNase (Oncogene Research Products, Cambridge, Massachusetts) and 10 μg/ml propidium iodide in PBS for 1 h at 37 °C. Samples were analyzed for DNA content using a high speed cell sorter (EPICS Altra, Beckman Coulter, Miami, Florida). Data were computer-analyzed with a commercially available software (Multicycle, Phoenix Flow Systems, San Diego, California).

Transient Transfection and Magnetic-Activated Cell Sorting (MACS)

For MACS, cells were co-transfected with equimolar amounts of pMACS.Kk-II plasmid and the respective expression plasmid in a 1:3 ratio. pICMT (clone IRATp970D0250D coding for the full length human ICMT was from the German Resource Center for Genomic Research, RZPD, Berlin, Germany. Transfection was done using Fugene (Roche). For a 35-mm dish, 6 μl liposomes were added to 100 μl Opti-MEM (GIBCO BRL) and mixed with 2 μg vector-DNA before adding the mixture to the cells in a serum free medium. After 4 hours, medium was withdrawn and fresh medium containing 10% FCS was added. Transfection efficiency was 20-30%. After 24 hours, transduced cells were trypsinized and magnetically labelled using MACSelect Kk-II MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Co-transfected (Kk-II positive) cells were then separated on MS+/RS+ separation columns (Miltenyi Biotec) and subsequently replated. Selected cells were >85% positive for green fluorescent protein (GFP) when co-transfected with a GFP expressing plasmid.

Detection of Ras-Protein methylation.

VSMC were cultured in the absence or presence of different concentrations of c3Ado in methionine-free medium containing tracer amounts (200 mCi/ml) of L-[methyl-3H]methionine (70 Ci/mmol, NEN Life Science Products), and the cells were allowed to
incubate for 24 h to allow the methyl donor, $S$-adenosyl-L-methionine, to reach labeling equilibrium. After 24 h, cells were harvested, resuspended in lysis buffer (10 mM Tris-HCl, pH 7.7, 1 mM MgCl$_2$, 0.5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µM DTT 0.2%). Ras was immunoprecipitated from 500 µg total protein with 5 µg of magnetic bead-linked (Dynabeads ProteinA; Invitrogen) mouse anti-Ras antibody (Ras10; Upstate) and resolved by 15% SDS-PAGE. Ras protein was identified by protein size markers and excised from the gel. Gel slices were placed in top-free Eppendorf tubes containing 1 M NaOH, and the tubes were placed upright into scintillation vials containing 5 ml of the aqueous scintillation mixture Econo-safe (Research Products International Corp). The vials were sealed and incubated overnight at 37°C, and the base labile counts were determined. Counts were corrected to total Ras levels as determined by immunoblotting using mouse anti-Ras (Ras10; Upstate).

**Ras Activity Assay**

VSMC were grown to near confluence, serum-starved and incubated with c3Ado at the indicated concentrations for 24 h, and stimulated with VSMC growth medium for 10 min, all at 37 °C, 5% CO$_2$. Cells were lysed and centrifuged, and equivalent amounts (0.5 mg/experiment) of supernatant protein from each condition were assayed for active Ras (Ras-GTP) according to the supplier’s instructions (Upstate, Lake Placid, NY). Alternatively, and for in vivo experiments, a more sensitive ELISA-based Ras activity assay, requiring less amounts of protein, was performed according to the supplier’s instructions (Ras GTPase Chemi ELISA kit, Active Motif, Rixensart, Belgium).

**siRNA transfection**

On-Target plus SMARTpool anti human HRas siRNA were obtained from Dharmacon, Lafayette, Co, USA. Sequences will be available on request due to the policy of the company.
VSMC were grown to 50% confluency and transfected using Lipofectamine® in Opti-MEM I® Reduced Serum Medium transfection reagent (Invitrogen, Carlsbad, USA) according to the information of the supplier.

**Real-Time Quantitative Reverse Transcription-PCR.**

Real-time quantitative RT-PCR was performed on the Stratagene MX300 quantitative PCR System (Stratagene, La Jolla, CA) by monitoring the increase of fluorescence by the binding of SYBR Green to double-stranded DNA. The reaction was run at the default setting program [95°C (15 s), 60°C (1 min), 40 cycles]. For H-Ras primers were as follows: forward, tac att gga aca tca gcc aag; reverse, cag gag aca cat ttg ca. For quantification of gene expression changes, the ΔΔCt method was used to calculate relative – fold changes normalized against the glyceraldehydes-3-phosphate dehydrogenase.

**Morphometric Analysis**

Samples were sectioned on a Leica cryostat (6 µm) and placed on poly-L-lysine (Sigma)–coated slides for immunohistochemical analysis. For morphometric analyses, hematoxylin and eosin staining was performed according to standard protocols. All sections were examined under a Leica DMRB microscope. For morphometric analyses, KS300 software (Carl Zeiss, Hallbergmoos, Germany) was used to measure external elastic lamina, internal elastic lamina, and lumen circumference, as well as medial and neointimal area of 6 sections per animal.

Endothelial cell coverage of the luminal surface was assessed by X400 microscopic examination of sections with a nuclear DAPI counterstain and staining for CD31 when both an endothelial cell nucleus and immunostaining were present.
**Immunohistochemistry**

Cross sections of mouse femoral arteries were fixed for 10 min in freshly prepared, buffered 4% paraformaldehyde. Sections were covered for 20 min with 10% normal goat serum, followed by incubation with monoclonal rat anti mouse CD31 (1:500, BD Biosciences, Erembodegem, Belgium) and PE conjugated mouse monoclonal anti-smooth muscle actin (sma, 1:500, Sigma) for 1 h in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. After two washing steps (10 min in PBS), cross sections were incubated with a secondary antibody for 40 min: donkey anti-mouse conjugated to Alexa Fluor® 488 (1:200, Molecular Probes, Leyden, The Netherlands). After washing, slides were mounted in Vectashield® mounting medium H-1000 containing 4′,6-Diamidino-2-phenylindole, 2HCl (DAPI, 5 µg/ml, Linaris, Wertheim, Germany) and evaluated using an epifluorescence microscope (DMRB, Leica, Wetzlar, Germany). Negative controls were performed using an unspecific primary antibody or the secondary antibody only. For the detection of VSMC proliferation in vivo, PCNA staining was performed according to the supplier’s instructions (PCNA staining kit, Zymed - Invitrogen, Karlsruhe, Germany). The number of apoptotic VSMC was quantified by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) according to the supplier’s instructions (In situ cell death detection kit, Roche, Mannheim, Germany).
Online Figure II

![Bar graph showing VSMC proliferation (OD 450 nm) with FCS and c3Ado treatments.](image-url)

- FCS: - - + + +
- c3Ado (µM): - - 10 50 100
- VSMC proliferation (OD 450 nm): 0.0 - 1.2

* indicates statistical significance.
Online Figure III

A

Relative Ras-mRNA expression (x-fold expression)

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B

Ras

Tubulin

C

VSMC proliferation (OD 450 nm)

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A

Online Figure IV

\[ \text{pAkt} \rightarrow \]
\[ \text{pERK} \rightarrow \]
\[ \text{tubulin} \rightarrow \]

<table>
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B

\[ [3H]-\text{methylation (base-labile CPM)} \times 10^3 \]

\[ \mu \text{M} \]

\[ \text{AGC} 2 \] \hspace{1cm} \[ \text{AGC} 10 \] \hspace{1cm} \[ \text{AGC} 20 \] \hspace{1cm} \[ \text{AGGC} 2 \] \hspace{1cm} \[ \text{AGGC} 10 \] \hspace{1cm} \[ \text{AGGC} 20 \]

C

\[ \text{Ras activity (RLU} \times 10^4) \]

\[ \mu \text{M} \]

\[ \text{AGC} 2 \] \hspace{1cm} \[ \text{AGC} 10 \] \hspace{1cm} \[ \text{AGC} 20 \] \hspace{1cm} \[ \text{AGGC} 2 \] \hspace{1cm} \[ \text{AGGC} 10 \] \hspace{1cm} \[ \text{AGGC} 20 \]

D

\[ \text{pAkt} \rightarrow \]
\[ \text{tubulin} \rightarrow \]

\[ \text{AGC} \] \hspace{1cm} \[ \text{AGGC} \]
Figure legends for online figures:

**Online Figure I.** Effect of c3Ado on VSMC viability, caspase 3/7 activation and apoptosis. 

A, VSMC were incubated in growth medium in the absence or presence of different concentrations of c3Ado for 48 h and cell viability was evaluated after trypsinisation by counting the relative number of cells that excluded the trypan blue dye ($P=n.s.$, $n=4$).

B, VSMC were incubated in growth medium in the absence or presence of different concentrations of c3Ado for 48 h. Induction of apoptotic signaling was assessed by measuring caspase-3/7 activities using an Apo-ONETM homogeneous caspase-3/7 assay kit (Promega). Briefly, cells were lysed using bifunctional cell lysis/activity buffer, which contained a profluorescent caspase-3/7 consensus substrate, rhodamine 110 bis-N-benzyloxy carbonyl-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide (Z-DEVD-R110). After incubation at room temperature for 1 h, aliquots (150 µl) were transferred to a 96-well clear bottom plate. Fluorescence was measured at an excitation wavelength of 485nm and an emission wavelength of 535nm (*$P<0.05$, $n=4$).

C, VSMC were incubated in growth medium in the absence or presence of different concentrations of c3Ado or actinomycin D (AmD; serving as positive control) for 48 h and the relative number of apoptotic cells was evaluated at the indicated time points by TUNEL staining ($P=n.s.$, $n=4$).

**Online Figure II.** Effect of c3Ado on VSMC proliferation at higher cell density. 

A, VSMC were seeded at a density of 5000 cells/96-well and were incubated in growth medium in the absence or presence of different concentrations of c3Ado for 24 h in the presence of BrdU. VSMC proliferation is expressed as mean OD450 ± SEM as determined by anti-BrdU ELISA (*$P<0.05$, $n=4$).
**Online Figure III.** Effect of Ras on VSMC proliferation. **A,** VSMC were transduced with Ras-targeting or control siRNA (40nM) and Ras mRNA expression was determined by real-time PCR at the indicated timepoints. Shown is the x-fold change in mRNA expression (n=3, *P*<0.05). **B,** VSMC were transduced with different concentrations of Ras-targeting or control siRNA (100nM) and Ras total protein expression was determined by immunoblotting. **C,** VSMC were transduced with Ras-targeting or control siRNA (40nM) for 24h. Then, VSMC were incubated in growth medium in the absence or presence of different concentrations of c3Ado for 24 h in the presence of BrdU. VSMC proliferation is expressed as mean OD450 ± SEM as determined by anti-BrdU ELISA (*P*<0.05, n=4).

**Online Figure IV.** **A,** Effect of Ras depletion on Akt- and Erk-activation. VSMC were transduced with Ras-targeting or control siRNA (40nM) for 48h. Then, phosphorylation of Erk and Akt was determined by immunoblotting 10 min after addition of growth medium using specific antibodies. Tubulin served as control for equal protein loading. **B,** VSMC were incubated for 24 h in the presence or absence of the indicated substances and L-[methyl-3H]methionine. After Ras had been immunoprecipitated and resolved on a SDS/polyacrylamide gel, carboxyl methylation was measured by alkaline hydrolysis of protein methyl esters in a vapor phase assay. The values represent the means ± S.D. from three independent experiments (*P*<0.005, n=3). **C,** VSMC were incubated for 24 h in the presence or absence of the indicated substances and Ras activity was determined by an ELISA-based activity assay and results are expressed as relative light units (RLU;*P*<0.05, n=4). **D,** VSMC were incubated for 24 h in basal medium in the presence or absence of the indicated substances and Akt phosphorylation was assessed following serum stimulation for 10 min. By immunoblotting using specific antibodies.