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8-Chloro-cAMP Inhibits Smooth Muscle Cell Proliferation In Vitro and Neointima Formation Induced by Balloon Injury In Vivo

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OBJECTIVES The aims of the present study were to assess 1) the effect of 8-Cl-cAMP (cyclic-3′-5′-adenosine monophosphate) on vascular smooth muscle cell (VSMC) proliferation in vitro and 2) the efficacy of systemic administration of 8-Cl-cAMP on neointimal formation after balloon injury in vivo.

BACKGROUND Neointimal formation after vascular injury is responsible for restenosis after arterial stenting. Recently, 8-Cl-cAMP, a cAMP analogue that induces growth arrest, has been safely administered in phase I studies in humans.

METHODS The effect of 8-Cl-cAMP on cell proliferation was first assessed on SMCs in vitro. To study the effects of cAMP in vivo, balloon injury was performed in 67 rats using a 2F Fogarty balloon catheter.

RESULTS The 8-Cl-cAMP markedly inhibited VSMC proliferation in vitro, reduced protein kinase A (PKA) RI_{α} subunit expression, and induced PKA RII_{β} subunit expression. In addition, 8-Cl-cAMP reduced, in a dose-dependent manner, neointimal area and neointima/media ratio after balloon injury. The proliferative activity, assessed by proliferating nuclear cell antigen immunostaining, revealed a reduction of proliferative activity of VSMCs in vivo in the 8-Cl-cAMP group. Moreover, the systemic administration of 8-Cl-cAMP did not affect renal function, blood pressure and heart rate.

CONCLUSIONS We conclude that 8-Cl-cAMP potently inhibits VSMC proliferation in vitro and reduces neointima formation by balloon injury in vivo after systemic administration. These data may have a clinical relevance in designing future strategies to prevent restenosis after arterial stenting and perhaps after percutaneous transluminal coronary angioplasty. (J Am Coll Cardiol 2000;36:288–93) © 2000 by the American College of Cardiology

Although stent deployment has been shown to reduce restenosis rate compared with balloon angioplasty (1,2), in-stent restenosis is a significant and growing clinical problem. It is now well established that vascular smooth muscle cell (VSMC) proliferation plays a major role in the restenotic process after arterial stenting (3,4) and contributes to restenosis after balloon angioplasty (5,6). The intracellular molecular mechanisms responsible for VSMC growth regulation are now well known and are linked with the *ras* pathway (7) and the cAMP–PKA (cyclic-3′-5′-adenosine monophosphate–protein kinase A) intracellular signaling (8). The cAMP signaling regulates an enormous variety of cellular processes (9–14). We have previously demonstrated that local delivery of 8-bromo-cAMP, using pluronic gel as a vehicle and applied at the time of injury on the external surface of the treated vessel, is able to prevent neointima formation after vascular injury (8). However, the method used in our previous study (8) is not clinically feasible in percutaneous balloon dilation procedures and no data are available regarding the effects of systemic cAMP analogue administration on VSMC proliferation rate and neointima formation after balloon injury in vivo.

Recently, 8-Cl-cAMP has been investigated as a new potential anticancer agent in humans (15). The 8-Cl-cAMP is a site-selective cAMP analogue able to modulate cAMP-dependent PKA activity at micromolar concentration (16,17). The PKA is present in eukaryotic cells as two different isoforms, PKAI and PKAII, which have identical catalytic subunits but different regulatory subunits (RI in PKAI and RII in PKAII) (18,19). The 8-Cl-cAMP is able to discriminate between the two cAMP binding sites present on RI and RII, to modulate the intracellular levels of those regulatory subunits at micromolar concentration, and to arrest cell proliferation by causing down-regulation of RI and up-regulation of RII at transcriptional level in several cell types (16,18,20–25). No data are available regarding the effect of 8-Cl-cAMP on VSMC proliferation. For its high selectivity and its pharmacokinetics, 8-Cl-cAMP can be used for systemic administration at low doses. In this regard, it should be pointed out that neointimal growth triggered by interventional coronary or peripheral procedures is spatially and temporally limited. These features may represent an obvious advantage using antiproliferative agents and can

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Abbreviations and Acronyms

ANOVA = analysis of variance
cAMP = cyclic-3′-5′-adenosine monophosphate
DMEM = Dulbecco's modified Eagle's medium
EEL = external elastic membrane
FCS = fetal calf serum
IEL = internal elastic membrane
PCNA = proliferating cell nuclear antigen
PKA = protein kinase A
PTCA = percutaneous transluminal coronary angioplasty
VSMCs = vascular smooth muscle cells

allow the use of an intermittent exposure regimen of 8-Cl-cAMP administration. Accordingly, the aims of the present study were to assess the effects of 8-Cl-cAMP on VSMC proliferation in vitro, RI-α and RII-β expression. The efficacy of 8-Cl-cAMP on neointima formation induced by balloon injury in vivo was also assessed.

METHODS

Cell culture. To study the effect of 8-Cl-cAMP VSMC proliferation in vitro smooth muscle cells (A10, thoracic aorta, rat) were used. Cells were grown in monolayers at 37°C in a humidified atmosphere at 95% air CO_2 in 10% FCS–DMEM (fetal calf serum–Dulbecco's modified Eagle's medium) with 4 mmol/liter L-glutamine, 4.5 g/liter glucose, and 1.0 mmol/liter sodium pyruvate. For growth inhibition experiments, 2×10^4 cells were plated into 35-mm plates and grown in DMEM 10% FCS in the presence of 1 μmol/liter, 5 μmol/liter, and 10 μmol/liter 8-Cl-cAMP, or in the absence of the same (control). Cell number in both conditions was assessed every 48 h for six days.

Western blotting. The VSMCs from rat aorta were used to test the effect of 1 μmol/liter, 5 μmol/liter, and 10 μmol/liter 8-Cl-cAMP on expression of regulatory subunits of PKAI. Western blotting was performed as described previously (23). Mouse monoclonal antibodies raised against anti-RI_α, anti-RII_β, or anti-C catalytic subunits were used.

Animal preparation. The animals in this study were handled according to the animal welfare regulation of the University Federico II of Naples, and the protocol was approved by the animal use committee of this institution. Fifty Wistar rats weighing 350 to 400 g (Charles River, Calco, Italy) were included in the present study. Rats were anesthetized with an intramuscular injection of ketamine 100 mg/kg (Sigma Chimica, Milan, Italy) and xylazine 5 mg/kg (Sigma Chimica). Angioplasty of the common carotid artery was performed using a balloon embolectomy catheter as previously described and well validated in our laboratory (7,8,26–28).

Drug dosage and administration. The 8-Cl-cAMP was randomly administered intraperitoneally in different protocols: Protocol I: three times at the dose of 1.2 mg/kg at the time of the balloon injury, three and six days later (n = 6); Protocol II: three times at the dose of 6 mg/kg at the time of the balloon injury, three and six days later (n = 7); Protocol III: three times at the dose of 12 mg/kg at the time of the balloon injury, three and six days later (n = 9); Protocol IV: two times at the dose of 12 mg/kg at the time of the balloon injury and three days later (n = 9). In a control group (n = 8), a saline solution was administered intraperitoneally. In additional animals, either 8-Cl-cAMP, as in Protocol III (n = 8), or saline solution (n = 9) was administered, but the evaluation of neointimal thickening was performed 28 days after balloon injury.

Toxicity. To study the 8-Cl-cAMP toxicity, laboratory studies were performed at baseline and two weeks after drug administration (Protocol IV) (n = 6). Histological sections of kidney, liver, and gastrointestinal tract were evaluated for inflammation or necrosis.

Hemodynamic measurements. Arterial pressure and heart rate were measured indirectly by a tail-cuff plethysmographic technique (mod. 50-0002, Harvard Apparatus, South Natick, Massachusetts) (29).

Morphology. At the time of final experiment (14 or 28 days later), the animals were anesthetised and the carotid arteries fixed, cut, and stained with hematoxylin-eosin. The cross-sectional areas of EEL (external elastic membrane), IEL (internal elastic membrane), lumen, media, and neointima were measured using a computerized image analysis system, and the ratios between neointima and media were calculated (26). To standardize the arterial dimensions, the data obtained were normalized by the left noninjured carotid dimensions. Furthermore, the ratio between EEL of right injured artery (EEL_{dx}) and EEL of left noninjured artery (EEL_{sx}) was calculated as the arterial remodeling index.

Assessment of VSMC proliferation rate and immunohistochemistry. To assess systemic 8-Cl-cAMP administration effects on VSMC proliferation, in 11 animals vascular balloon injury was performed as described above, and either 8-Cl-cAMP, as in Protocol IV (n = 6), or saline solution (n = 5) was administered intraperitoneally. Seven days after balloon injury, the arteries were removed and immunohistochemistry for proliferating cell nuclear antigen (PCNA) was performed as previously described (30). A PCNA index was defined as the number of PCNA positive cells divided by the sum of PCNA positive and negative cells and expressed as a percentage. In an additional seven rats, the effects of anesthesia and surgical procedure (without the balloon injury) on VSMC proliferation were also assessed.

Statistical analysis. All data are shown as mean ± SEM. Statistical analysis between groups was performed by analysis of variance (ANOVA) and unpaired *t* test using a Systat program (31). The Tukey test was applied to compare single mean values. A p value <0.05 was considered significant.

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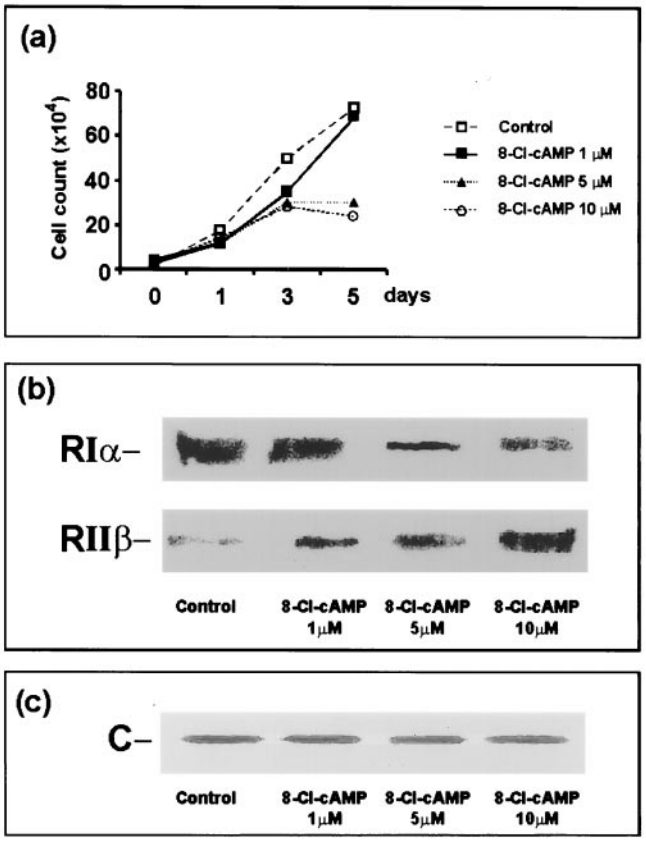

Figure 1. **(a)** Effects of 8-Cl-cAMP (1, 5, and 10 μmol/liter) on the growth inhibition of rat VSMCs. Both 5 and 10 μmol/liter 8-Cl-cAMP induced a significant inhibition of VSMC proliferation at any time. **(b)** Western blot analysis in VSMCs untreated **(control)** or treated with 8-Cl-cAMP (1, 5, and 10 μmol/liter). The 8-Cl-cAMP induced a significant reduction of RI_α level, enhanced level of RII_β, and showed no effect on catalytic subunit **(c).**

RESULTS

The 8-Cl-cAMP induces inhibition of VSMC proliferation in vitro, down-regulation of RI_α and up-regulation of RII_β. Figure 1a shows that cAMP markedly inhibited VSMC proliferation in a dose-dependent manner. The biological effect of the drug was reversible, because its removal from the culture medium resumed cell growth (data not shown). As shown in Figure 1b, the level of RI_α was significantly reduced in VSMCs exposed to 8-Cl-cAMP. Instead, 8-Cl-cAMP induced an enhanced level of RII_β with no effect on the catalitic subunit. These data demonstrated for the first time that 8-Cl-cAMP inhibits cell growth in VSMCs by reducing RI_α and enhancing RII_β levels.

The 8-Cl-cAMP reduced neointima formation in vivo. A reproducible neointima formation was found 14 days after balloon injury in the control group. In the Protocol I group, we did not observe significant differences in neointimal area and the neointima/media ratio compared with control group. In contrast, in animals treated with higher doses of 8-Cl-cAMP (Protocols II, III, and IV) we observed a significant reduction of both neointima and neointima/media ratio (Figs. 2 and 3).

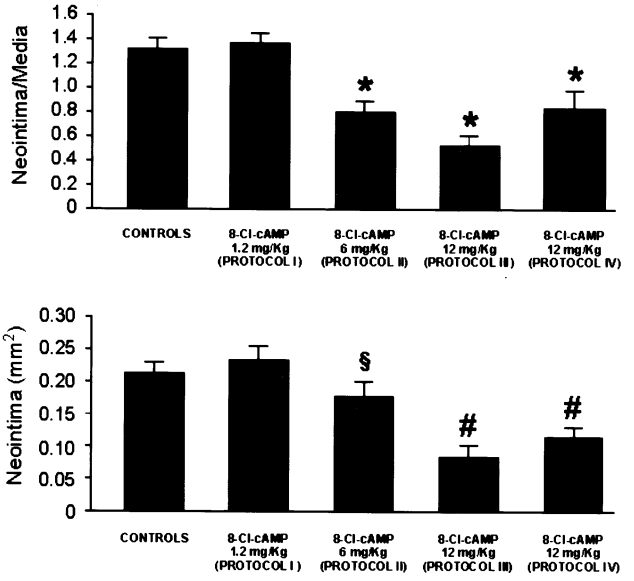

Figure 2. Bar graphs of neointima/media ratio **(top panel)** and neointimal cross-sectional area **(bottom panel)** of common carotid arteries from rats included in the study. The 8-Cl-cAMP and dosages administered in Protocols II, III, and IV significantly reduced both neointimal cross-sectional area and neointima/media ratio. *$p < 0.05$ vs. Controls and Protocol I; #$p < 0.05$ vs. Controls and 8-Cl-cAMP Protocol I; §$p < 0.05$ vs. 8-Cl-cAMP Protocol III.

In the group of animals in which we evaluated neointima thickening 28 days after balloon injury, we observed a significant reduction of neointima/media ratio in rats treated as in the Protocol III (Table 1), and these data obtained at 28 days did not significantly differ from the data obtained at 14 days. Moreover, 8-Cl-cAMP did not induce significant changes in arterial dimension (Table 2).

Systemic effects of 8-Cl-cAMP. No differences in arterial pressure and heart rate were found between sham-operated

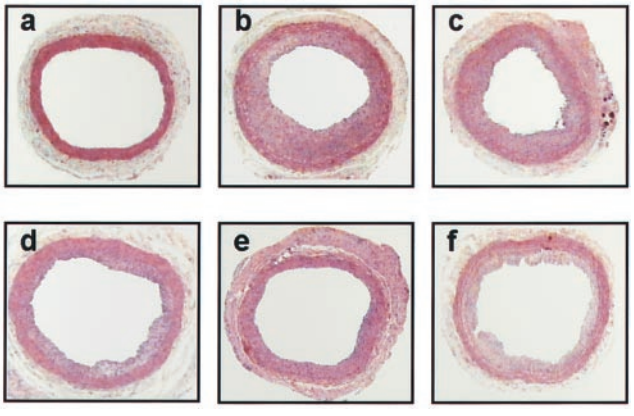

Figure 3. Representative histologic sections stained with hematoxylin-eosin of common carotid arteries, removed 14 days after angioplasty, from **(a)** a normal rat (no balloon injury); **(b)** a control rat subjected to only balloon injury; **(c)** rats included in Protocol I; **(d)** rats included in Protocol II; **(e)** rats included in Protocol III; **(f)** rats included in Protocol IV. The 8-Cl-cAMP reduced significantly the neointima formation after balloon injury **(c, d, e).**

Table 1. Morphologic Characteristic of Common Carotid Artery of the Group of Animals Evaluated 28 Days After Balloon Injury

	Neointima (mm^2)	Neointima/Media
Controls	0.194 ± 0.022	1.387 ± 0.109
8-Cl-cAMP 12 mg/kg (Protocol III)	0.076 ± 0.042*	0.529 ± 0.222*

*$p < 0.005$ vs. controls.

animals and experimental groups. In addition, blood pressure and heart rate were comparable in the different experimental groups (Fig. 4). No renal function alteration was associated with 8-Cl-cAMP administration (Table 3). The 8-Cl-cAMP did not induce any changes of kidney, hepatic, and gastrointestinal histology (data not shown).

DISCUSSION

The major findings of the present study are that 8-Cl-cAMP reduces the VSMC proliferation in vitro and neointima formation induced by balloon injury after systemic administration in vivo. As it has been recently shown that 8-Cl-cAMP can be used and is well tolerated in humans (15), these data might have an important clinical relevance.

Mechanisms of restenosis. It is well known that restenosis limits percutaneous transluminal coronary angioplasty (PTCA) benefit occurring in 30% to 60% of patients (32,33). Using stents has reduced the incidence of restenosis (1,2); unfortunately, stent implantation also markedly induces VSMC proliferation. In fact, neointima formation is the only mechanism responsible for restenosis after stent deployment (3,4), which occurs in about 20% of Benestent-like lesions (1,2) and in about 50% of long lesions and vein grafts (34–36). We have previously shown that local delivery of a transdominant negative H-*ras* gene markedly reduces neointima formation after balloon injury in rats (7). This finding was recently confirmed using adenovirus-mediated gene transfer (37). The inhibition of a protein downstream *ras,* MAPKK, also prevents neointima formation after balloon injury (27). Other investigators have also shown that gene therapy could be useful (38,39); however,

Table 2. Arterial Remodeling Among Groups in the Study

	EEL_{dx}/EEL_{sx}
14 Days' Follow-up	
Controls	1.046 ± 0.085
8-Cl-cAMP 1.2 mg/kg (Protocol I)	1.086 ± 0.074
8-Cl-cAMP 6 mg/kg (Protocol II)	1.171 ± 0.091
8-Cl-cAMP 12 mg/kg (Protocol III)	1.064 ± 0.095
8-Cl-cAMP 12 mg/kg (Protocol IV)	1.066 ± 0.067
28 Days' Follow-up	
Controls	1.089 ± 0.089
8-Cl-cAMP 12 mg/kg (Protocol III)	1.064 ± 0.078

EEL_{dx}/EEL_{sx}: ratio of cross-sectional area of right injuried artery and cross-sectional area of left noninjuried artery.

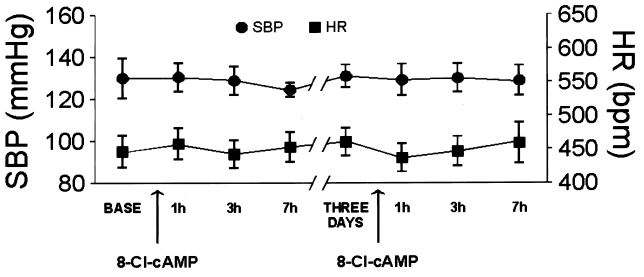

Figure 4. Effects of 8-Cl-cAMP on systolic blood pressure (SBP) and heart rate (HR) in rats treated with 8-Cl-cAMP (Protocol IV). Administration of 8-Cl-cAMP did not induce significant changes in heart rate and blood pressure.

at the present time, cost/benefit analysis and the possibility of plasmid DNA stable integration in VSMC genome leading to unwanted biological effects (13) do not allow the use of a gene therapy approach in the clinical setting to prevent restenosis.

We recently showed that cAMP–PKA signaling activation plays an important role in the regulation of smooth muscle cell proliferation (8). The cAMP pathway stimulation is pharmacologically feasible in the clinical setting, and this may represent a clear advantage over the gene therapy approach. In our previous study, the activation of cAMP–PKA signaling was obtained using 8-Br-cAMP local administration mediated by pluronic gel that, however, is not clinically feasible (8). In addition, the lack of selectivity and the high dose required for 8-Br-cAMP and other cAMP analogues have been the major obstacle to test these drugs in humans (15).

Mechanism of action of 8-Cl-cAMP. In the present study, we used a potent site-selective cAMP analogue, 8-Cl-cAMP, that can be administered systemically (15–17). It is well known that, in mammalian cells, there are two types of cAMP-dependent protein kinases, type I and type II, which differ from R subunits, RI and RII, and that interact with an identical C subunit. It has been shown that differential expression of PKAI and PKAII correlates with cell differentiation and neoplastic transformation (40,41). Preferential expression of PKAII is induced by treatment of tumor cells with cAMP analogues or differentiating agents and is typical of terminally differentiated tumors (41).

In contrast, enhanced levels of PKAI are generally found

Table 3. Effect of 8-Cl-cAMP on Laboratory Studies in Rats After Balloon Angioplasty (Protocol IV)

	Controls	8-Cl-cAMP
BUN (mg/dl)	46.24 ± 2.24	43.50 ± 5.45
CREA (mg/dl)	0.58 ± 0.03	0.63 ± 0.05
K (mmol/liter)	5.92 ± 0.25	6.10 ± 0.25
NA (mmol/liter)	141.2 ± 4.23	139.2 ± 2.50
Red cells (mm^3)	$7.590 \times 10^3 \pm 567 \times 10^3$	$7.962 \times 10^3 \pm 375 \times 10^3$
Leukocyte (mm^3)	13.220 ± 2.955	15.050 ± 3279
Platelets (mm^3)	$579 \times 10^3 \pm 67 \times 10^3$	$597 \times 10^3 \pm 121 \times 10^3$
Hemoglobin (g/dl)	13.88 ± 1.81	15.02 ± 0.47

BUN: blood urea nitrogen; CREA: creatinine levels.

in tumor cells (41). It has been shown that PKAI is involved in mitogenic signaling by several growth factors (22–24). The 8-Cl-cAMP is able to down-regulate PKAI by inducing protein degradation and up-regulate RII subunit expression at the transcriptional level (21). It has been also demonstrated that RI_α down-regulation by 8-Cl-cAMP determines growth arrest and differentiation in a wide variety of human cancer cell lines (16,20–25). Previous studies demonstrated that 8-Cl-cAMP induces growth inhibition of human colon cancer line (20), of HL60-leukemia cells (21) and of human lung carcinoma in athymic mice (24).

In our study we observed that 8-Cl-cAMP induces an inhibition of VSMC proliferation in vitro in a dose dependent manner as we observed in vivo. To study the mechanism by which 8-Cl-cAMP induces inhibition of cultured VSMC proliferation we performed a Western blotting to evaluate the levels of RI_α, RII_β, and catalytic subunit of PKA. The 8-Cl-cAMP induced a reduction of RI_α levels, an enhanced RII_β level, and no effects on catalitic subunits. These data demonstrated for the first time that the inhibition of VSMC proliferation by 8-Cl-cAMP in vitro was associated with down-regulation of RI_α and up-regulation of RII_β regulatory subunits of PKA.

Conclusions and clinical implications. Our data also demonstrate that the systemic administration of 8-Cl-cAMP, at the highest dose used, is able to reduce by approximately 60% the neointimal formation after balloon injury. More interestingly, the dose of 8-Cl-cAMP used in the present study did not affect heart rate, blood pressure, renal function, and histology. The 8-Cl-cAMP effect on neointima formation was dose-dependent, and two administrations (at the time of the vascular injury and three days later) were sufficient to reduce neointima formation after vascular injury in our experimental model. This is an important finding. In fact, our study demonstrates that an agent such as 8-Cl-cAMP, administered systemically only in two or three doses and without chronic treatment, can prevent a very localized phenomenon such as neointimal hyperplasia after vascular injury. Thus, our study demonstrates for the first time that systemic administration of 8-Cl-cAMP, a new cAMP cytostatic analogue, is able to reduce the neointima formation after vascular injury without toxic effects. However, extreme caution should be used to extrapolate the experimental data presented in this study using VSMCs in culture or the rat angioplasty model to the clinical setting. Therefore, further studies should be performed to evaluate the effects of 8-Cl-cAMP on stent restenosis in large animal models and eventually in humans.

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