Differential regulation of vascular smooth muscle and endothelial cell proliferation in vitro and in vivo by cAMP/PKA-activated p85α\textsuperscript{PI3K}

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DRUG-ELUTING STENTS (DES) have significantly changed interventional cardiology, owing to their remarkable ability to reduce restenosis compared with bare-metal stents (2, 19). However, incomplete reendothelialization after DES deployment seems to drive an increased risk for late thrombosis (24), it has been suggested that DES should ideally have a selective anti-proliferative effect on vascular smooth muscle cells (VSMCs), but be inert toward endothelial cells (ECs) or, greater still, promote their proliferation (34). The latter could be achieved by the identification of drugs/intracellular molecules that play differential roles on VSMC and EC growth.

In the last few years, our laboratory has shown that cAMP activates a PKA-dependent molecular pathway, culminating in VSMC growth inhibition in vitro and in vivo (15, 17, 20). Elevation of cAMP in vascular ECs has been shown to inhibit proliferation (11). However, while it reduces the proliferation of most cell types, cAMP has also been shown to promote the growth of few cell lineages, such as Swiss 3T3 fibroblasts and thyroids (1, 12, 23). Indeed, cAMP exerts its multiple cellular effects, triggering different, sometimes opposing, molecular cascades (5). In many, but not all, cell types, cAMP/PKA inhibits MAPK/ERK cascade (5). In thyroid cells and NIH3T3 fibroblasts, cAMP phosphorylates p85α, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (p85α\textsuperscript{PI3K}), on serine 83 (Ser83) (6, 8), inducing cell proliferation (6), while inhibiting cell growth in NIH3T3 fibroblasts (8). cAMP/PKA-modified p85α promotes p21\textsuperscript{ras}, p13\textsuperscript{K} complex, amplying PI3K signaling while reducing ERK1/2 activation (8). However, how cAMP/PKA-modified p85α affects VSMC and EC growth is unknown.

Here we show that cAMP inhibits VSMC proliferation through the phosphorylation of (Ser83) p85α, which forms an inhibitory complex with p21\textsuperscript{ras}, preventing ERK1/2 activation. On the other hand, cAMP-induced cell cycle inhibition of ECs is independent from cAMP/PKA modification of p85α. In agreement with these differential effects of p85α on VSMCs and ECs in vitro, PKA-modified p85α gene transfer reduces neointimal formation without affecting endothelial regeneration in vivo after balloon injury in rats.

MATERIALS AND METHODS

See online supplemental data for expanded MATERIALS AND METHODS.

Plasmid transfection and cell cultures. Plasmids carrying FLAG-tagged p85α-wild-type or its Ser83 mutated forms were obtained, as previously described (6, 8) (Supplemental Table 1). Plasmids’ transfection was performed using FuGENE 6 Transfection Reagent (Roche). Rat aortic VSMCs (A-10) and rat aortic ECs were from ATCC and Cell Applications, respectively. Cell proliferation was
evaluated by a bromodeoxyuridine (BrdU) assay, while cell apoptosis was detected using the TdT assay (Roche) (10, 31).

**Animal study design and experimental balloon injury.** The animal study protocol was approved by the animal use committee of Magna Graecia University, Catanzaro, Italy. Balloon injury of the right carotid artery and plasmid transfections were performed in male Wistar rats, as described (16, 18). VSMC proliferation, neointimal hyperplasia, and endothelial regeneration were carried out 14 days after balloon injury, as previously reported (18, 21).

**Statistical analysis.** Data were analyzed using ANOVA, SPSS version 10.0. When a significant effect was detected, Bonferroni test was used to compare mean values. A P value of <0.05 was considered significant.

**RESULTS**

cAMP inhibits VSMC proliferation in vitro through PKA modification of p85α. cAMP phosphorylates p85α[superscript*]PKA at Ser83 in VSMCs (Fig. 1A). Then we transfected rat VSMCs with FLAG-tagged plasmids carrying p85α[superscript*]WT, p85α[superscript*]Ser83-mutated with aspartic acid (to mimic this site PKA-dependent phosphorylation; p85α[superscript*]PKA-activated), or with alanine (to prevent this site phosphorylation; p85α[superscript*]PKA-inactive) (Supplemental Table 1). In addition, we silenced p85α[superscript*]PI3K with specific green fluorescence protein (GFP)-tagged small hairpin RNA (p85α[superscript*]shRNA). Control (Con) cells were transfected with GFP plasmid. Expression of p85α[superscript*]WT did not have an effect per se on VSMC proliferation; however, it did exacerbate the cAMP growth-inhibitory response (Fig. 1D). Expression of p85α[superscript*]WT did not have an effect per se on VSMC proliferation; however, it did exacerbate the cAMP growth-inhibitory response (Fig. 1D). Importantly, p85α[superscript*]PKA-activated decreased VSMC proliferation in the absence of cAMP, and cell proliferation in p85α[superscript*]PKA-activated transfected cells was further reduced in the presence of cAMP (1D). p85α[superscript*]shRNA had no effect per se on VSMC proliferation, but it did abolish the cAMP-dependent response (Fig. 1E).

Fig. 1. cAMP inhibits vascular smooth muscle cell (VSMC) proliferation in vitro through PKA modification of p85α. A: cAMP serine (Ser) phosphylates p85α in VSMCs, as shown in the representative Western blot. B, left: similar transfection efficiency of the wild-type (WT) and the Ser83-modified constructs of p85α gene in VSMCs. B, right: representative Western blot for p85α after immunoprecipitation (IP) with FLAG antibody, confirming the efficient transfection of the p85α plasmid constructs in VSMCs. p85α[superscript*]WT, p85α[superscript*]WT, p85α[superscript*]PI3K Ser83-mutated with aspartic acid (to mimic this site PI3K at Ser83 phosphorylation; p85α[superscript*]PI3K Ser83-mutated aspartic acid), or with alanine (to prevent this site phosphorylation; p85α[superscript*]PI3K Ser83-mutated alanine). C: FACS analysis showing that a high percentage of VSMCs express the green fluorescence protein (GFP) tag of the transfected p85αshRNA construct, p85αshRNA significantly reduced p85α expression in VSMCs, as shown in the Western blot (bottom). Scrambled small hairpin RNA (shRNA) (scrambled shRNA, negative control) did not affect p85α expression. Actin was used to normalize for protein loading. D: cumulative data of the effects of the different p85α plasmids with and without cAMP on VSMC proliferation. *P < 0.05 vs. Base; †P < 0.05 vs. no cAMP; ‡P < 0.05 vs. control (Con; cAMP); ▲P < 0.05 vs. Con, p85α[superscript*]shRNA, and p85α[superscript*]PKA-activated (no cAMP). E: compared with Con, p85αshRNA did not affect VSMC proliferation per se, while it attenuated cAMP-induced growth inhibition. *P < 0.05 vs. Base; †P < 0.05 vs. no cAMP. BrdU, bromodeoxyuridine.
As the p85α constructs were generated from bovine p85α cDNA, which shares a high rate of homology with human and porcine coronary smooth muscle cells and observed similar results (data not shown).

These results demonstrate that cAMP/PKA reduce VSMC proliferation through p85α phosphorylation. Also, p85PKA-activated gene transfer in vitro is able to reduce VSMC growth per se.

cAMP phosphorylates p85α, which binds p21ras and inhibits ERK1/2 phosphorylation in VSMCs. As p21ras/ERK1/2 cascade is pivotal in inducing VSMC proliferation in vitro and in vivo (19), we set to evaluate how PKA-mediated p85α (Ser83) phosphorylation regulates p21ras-ERK1/2 cascade in VSMCs.

Forty-eight hours after plasmid transfection, p85α plasmid transfected VSMCs were serum starved for 24 h and then stimulated for 20 min with IGF-I (100 nM) or 10% fetal bovine serum with or without cAMP. IGF-I is a classical mitogen, which activates the p21ras/ERK cascade (3).

Stimulation of VSMCs with IGF-I did not induce p21ras-p85α association and resulted in ERK1/2 phosphorylation in Con (GFP transfected), p85WT, and p85PKA-inactive transfected cells (Fig. 2, A and B). On the other hand, transfected p85PKA-activated coupled with p21ras, and it reduced IGF-I-induced ERK1/2 phosphorylation (Fig. 2, A and B).

cAMP promoted p21ras-p85α PI3K complex, reducing ERK1/2 phosphorylation in serum-stimulated Con cells (Fig. 2, C and D). Overexpression of p85WT did not affect p21ras-p85α PI3K complex and ERK phosphorylation in serum stimulated conditions. However, on cAMP treatment, p21ras-p85α PI3K complex increased and ERK inhibition was exacerbated in p85WT transfected compared with Con cells (Fig. 2, C and D).

Transfected p85PKA-inactive neither targeted p21ras nor affected serum-induced ERK1/2 phosphorylation, but it attenuated cAMP-induced p21ras-p85αPI3K complex and inhibition of ERK1/2 phosphorylation (Fig. 2, C and D). On the other hand, in serum-stimulated conditions, transfected p85PKA-activated coupled with p21ras and reduced ERK1/2 phosphorylation, compared with Con cells. cAMP further increased p21ras-p85αPI3K complex and decreased the ERK1/2 phosphorylation in p85PKA-activated transfected cells (Fig. 2, C and D).

These results show that p85α PI3K mediates cAMP-dependent inhibition of p21ras-ERK1/2 cascade in VSMCs in vitro. Hence, p85PKA-activated reduces p21ras-ERK1/2 activation per se.

cAMP/PI3K-modified p85α activates Akt and improves VSMC survival in vitro. We then tested the hypothesis that cAMP phosphorylation of (Ser83) p85α PI3K diverges p21ras from ERK1/2 activation to PI3K signaling in VSMCs. p85α plasmid transfected VSMCs, after a 24-h starvation period, were stimulated for 20 min with IGF-I or cAMP, and Akt phosphorylation was measured. Accordingly, IGF-I is also a well-known stimulator of PI3K/Akt pathway (3).

IGF-I resulted in Akt phosphorylation in Con VSMCs (Fig. 3, A and B). Expression of p85WT and p85PKA-inactive did not affect Akt phosphorylation upon IGF-I stimulation (Fig. 3, A and B). On the other hand, the expression of p85PKA-activated induced Akt phosphorylation independently from the IGF-I stimulation (Fig. 3, A and B). IGF-I stimulation further in-

![Fig. 2. cAMP phosphorylates p85α, which binds p21ras and inhibits ERK1/2 phosphorylation in VSMCs. A. IGF-I effects on p21ras-p85α association (assessed by IP with p21ras antibody and Western blot for p85α) and ERK1/2 phosphorylation in Con (GFP transfected) and p85α-plasmids-transfected VSMCs. B: cumulative semiquantitative data represented by the immunoblots in A. •P < 0.05 vs. no IGF-I; †P < 0.05 vs. IGF-I; ‡P < 0.05 vs. Con, p85WT, and p85PKA-inactive. C: immunoblots showing cAMP effects on p21ras-p85α association and ERK1/2 phosphorylation in serum-stimulated Con and p85α-plasmids-transfected VSMCs. D: cumulative semiquantitative data represented by the immunoblots in C. •P < 0.05 vs. no cAMP; †P < 0.05 vs. Con (cAMP); ‡P < 0.05 vs. Con, p85WT, and p85PKA-inactive (no cAMP).]
creased Akt phosphorylation in p85PKA-activated-transfected VSMCs compared with Con (Fig. 3, A and B).

Treatment with cAMP increased Akt phosphorylation in Con VSMCs (Fig. 3, C and D). Importantly, p85PKA-activated overexpression reduced cAMP-mediated Akt phosphorylation (Fig. 3, C and D). cAMP treatment increased Akt phosphorylation in p85PKA-activated VSMCs, which was greater than in Con (Fig. 3, C and D).

These results show that p85PKA-activated mediates cAMP-dependent activation of PI3K/Akt pathway in VSMCs. Also, p85PKA-activated promotes Akt activation per se.

As PI3K/Akt is a well-known cascade regulating cell survival, and VSMC apoptosis contributes to vascular response to injury (26), we evaluated the role of cAMP/PI3K/p85α signaling on H2O2-induced VSMC apoptosis in vitro.

cAMP reduced VSMC apoptosis in Con cells (Fig. 3E). This cAMP cytoprotection was abolished by wortmannin, a PI3K/ Akt inhibitor (data not shown). Overexpression of p85WT did not have any effect on H2O2-induced VSMC apoptosis (Fig. 3E). However, cAMP-induced survival was improved by p85WT transfection, compared with Con (Fig. 3E). On the other hand, p85PKA-inactive overexpression did not affect VSMC death, but it eliminated cAMP-mediated cell protection (Fig. 3E). Importantly, p85PKA-activated decreased VSMC apoptosis in the absence of cAMP, and cell death in p85PKA-activated transfected VSMCs was further reduced by cAMP, compared with Con (Fig. 3E).

These data indicate that Ser83 phosphorylation of p85αPI3K mediates cAMP inhibition of VSMC death and that p85PKA-activated has vascular protective effects per se in preventing VSMC apoptosis in vitro.

cAMP inhibits EC proliferation in vitro independently from PI3K and ERK1/2 inhibition. In ECs (Fig. 4A), we assessed the effects of cAMP/PI3K modification of p85αPI3K on rat aortic ECs in vitro. p85WT, p85PKA-activated and GFP plasmids were efficiently transfected in ECs (Fig. 4B and C). We then assessed cell proliferation in ECs transfected with p85WT, p85PKA-activated and p85PKA-inactive or GFP (Con) (Table 1). After 36-h starvation (no serum), BrdU incorporation was similar across all plasmid transfections (data not shown), and we pooled the data together (base, Fig. 4D). Twenty-four hours after serum stimulation, cAMP treatment reduced proliferation in Con ECs (Fig. 4D). p85WT and p85PKA-inactive transfection did not affect EC proliferation, neither did it modify the cAMP response (Fig. 4D). Similar results were obtained in human umbilical vein ECs (data not shown).
These results demonstrate that cAMP inhibits EC proliferation in vitro independently from PKA modification of p85α and ERK1/2 inhibition. Accordingly, PKA-activated p85α does not affect EC growth in vitro. Then, similar to VSMCs, we determined the effects of cAMP and the different p85α plasmids on p21ras-p85αPI3K complex and ERK1/2 activation in ECs. cAMP promoted p21ras-p85αPI3K complex, but this complex did not affect ERK1/2 phosphorylation in serum-stimulated Con ECs (Fig. 4, E and F). Overexpression of p85WT did not affect p21ras-p85αPI3K complex and ERK phosphorylation in serum-stimulated conditions. Upon cAMP treatment, p21ras-p85αPI3K complex increased, but ERK activation occurred normally in p85WT-transfected compared with Con ECs (Fig. 4, E and F). Transfected p85PKA-inactive neither targeted p21ras, nor affected serum-induced ERK1/2 phosphorylation. While p85PKA-inactive overexpression did attenuate cAMP-induced p21ras-p85αPI3K

Table 1. Morphological results 14 days after balloon injury in the rat groups included in the in vivo study

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Gel</th>
<th>p85WT</th>
<th>p85PKA-inactive</th>
<th>p85PKA-activated</th>
</tr>
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<tbody>
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<td>10</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>EEL CSA</td>
<td>0.45±0.03</td>
<td>0.43±0.03</td>
<td>0.45±0.05</td>
<td>0.46±0.07</td>
<td>0.47±0.04</td>
</tr>
<tr>
<td>Neointimal CSA</td>
<td>0.16±0.04</td>
<td>0.14±0.04</td>
<td>0.15±0.04</td>
<td>0.18±0.3</td>
<td>0.08±0.02*</td>
</tr>
<tr>
<td>N/M ratio</td>
<td>1.28±0.33</td>
<td>1.20±0.32</td>
<td>1.18±0.20</td>
<td>1.33±0.16</td>
<td>0.49±0.14*</td>
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Values are means ± SE; n, no. of rats. Con, control; WT, wild type; EEL, external elastic lamina; CSA, cross-sectional area (mm²); N/M ratio, neointima-to-media ratio. *P < 0.05 vs. all.
cAMP inhibits EC apoptosis in vitro through PKA modification of p85α and Akt activation. A: cAMP effects on Akt phosphorylation in p85α-plasmids-transfected ECs, compared with Con cells. B: cumulative semiquantitative data as shown by the representative experiment in A. *P < 0.05 vs. no cAMP; †P < 0.05 vs. Con (cAMP); ‡P < 0.05 vs. Con, p85WT, and p85PKA-inactive (no cAMP). C: apoptotic death in vitro in p85α-plasmids-transfected ECs, compared with Con ECs in the presence or absence of cAMP. *P < 0.05 vs. Base; †P < 0.05 vs. no cAMP; ‡P < 0.05 vs. Con and p85PKA-inactive (cAMP); *P < 0.05 vs. Con, p85WT, and p85PKA-inactive (no cAMP).

Importantly, the expression of p85PKA-activated increased Akt phosphorylation (Fig. 4, A and F). Accordingly, transfected p85PKA-activated coupled with p21ras, but it did not reduce ERK1/2 phosphorylation, compared with Con ECs. cAMP further increased p21ras-p85PKA-inactive complex, but this molecular switch had no consequences on ERK1/2 phosphorylation in p85PKA-activated transfected ECs (Fig. 4, A and F).

Additionally, expression of p85WT, p85PKA-inactive and p85PKA-active did not affect ERK1/2 phosphorylation upon IGF-I stimulation, compared with Con ECs (Supplemental Fig. 1).

These results show that cAMP-dependent growth inhibition of ECs is independent from p85α modification with its binding to p21ras and ERK1/2 modulation. Accordingly, PKA-activated p85α does not inhibit ERK1/2 activation and cell proliferation in ECs in vitro.

cAMP inhibits EC apoptosis in vitro through PKA modification of p85α and Akt activation. Similar to how experiments were carried out with VSMCs, cAMP increased Akt phosphorylation also in Con ECs (Fig. 5, A and B). In response to cAMP, p85WT-transfected ECs showed an increased Akt phosphorylation compared with Con (Fig. 5, A and B). In ECs transfected with p85PKA-inactive, cAMP-induced Akt phosphorylation was reduced compared with Con (Fig. 5, A and B). Importantly, the expression of p85PKA-activated increased Akt phosphorylation in the absence of cAMP, and then, in the presence of cAMP, Akt phosphorylation increased further (Fig. 5, A and B). Thus p85PKA-activated mediates cAMP-dependent phosphorylation of Akt and p85PKA-activated promotes Akt activation per se in ECs.

Then we evaluated the role of cAMP/PKA/p85α signaling on H2O2-induced apoptosis in ECs. Treatment with cAMP reduced EC apoptosis in Con (Fig. 5C). cAMP cytoprotection was abolished by wortmannin, a PI3K/Akt inhibitor (data not shown). Expression of p85WT did not have any effect on EC apoptosis (Fig. 5C). However, cAMP treatment reduced apoptotic death in p85WT-transfected ECs, compared with Con (Fig. 5C). p85PKA-inactive did not influence EC death; however, p85PKA-inactive blunted cAMP-dependent cell protection (Fig. 5B). Notably, p85PKA-activated decreased EC apoptosis in the absence of cAMP, and this was further decreased in the presence of cAMP, compared with Con (Fig. 5C).

Therefore, cAMP-induced EC survival is mediated by (Ser83) phosphorylation of p85αPI3K, and p85PKA-activated overexpression reduces EC death per se.

Local delivery of p85PKA-activated prevents VSMC proliferation and neointimal hyperplasia after experimental balloon injury. We determined the effects of the overexpression of the p85αPI3K plasmids in rat carotid arteries subjected to balloon injury. Using pluronic F127 gel, a sustained release polymer (16), rat carotid arteries were locally transfected with FLAG-tagged plasmid vectors expressing p85WT, p85PKA-inactive, and p85PKA-activated immediately after balloon injury. Carotid arteries of Con rats were transfected with a GFP plasmid construct (Con). The latter has no effect per se on neointimal formation after balloon injury (data not shown). As additional controls, only saline was dissolved in the pluronic gel (Gel). Neointimal formation was analyzed 14 days after balloon injury (Table 1).

Forty-eight hours after balloon injury, the expression of the exogenous plasmids in the carotid arteries was verified by immunofluorescence staining, immunoprecipitation, and Western blot analysis in five additional rats per group (Fig. 6, A and B). At this time point [when VSMC proliferation reaches its peak after balloon injury (31)] in the carotid arteries treated with local transfection of p85PKA-activated plasmid, a significant lower ratio of Ki-67-positive nuclei to total cells [proliferation...
PKA-ACTIVATED p85α AND RESTENOSIS

A

B

C

D

E

F

G

Neointima Hyperplasia 14 Days After Balloon Injury

No INJURY

CON

p85WT

p85PKA-inact

p85PKA-act

Downloaded from http://ajpheart.physiology.org/ by 10.220.32.247 on October 23, 2017
index (PI) = 4 ± 2%] was observed, compared with carotid vessels with Con GFP plasmid (Con, PI = 14 ± 3%; P < 0.05) (Fig. 6, C and D). No effects on VSMC proliferation were observed by the transfection of p85WT or p85PKA-inactive constructs (PI = 15 ± 4 and 14 ± 4%, respectively) (Fig. 6C). p85PKA-activated expression significantly reduced neointimal hyperplasia 14 days after balloon injury, and neointimal tissue was reduced by ~55%, compared with the other groups (Table 1 and Fig. 6, E–G). Neither p85WT or p85PKA-inactive had an effect on neointimal formation (Fig. 6, E–G).

p85PKA-activated local delivery does not affect endothelial regeneration in rat balloon-injured arteries. Reendothelialization was assessed through quantitative immunohistochemistry with von Willebrand factor (vWF) staining and by planimetry analysis after Evans blue injection in vivo (21, 32). At 14 days after balloon injury in Con, reendothelialization was minimal in the middle segment (%vWF stained, 24.5 ± 5%), yet more complete at the proximal and distal segments (proximally, 74 ± 3%; distally, 80 ± 4%) (Fig. 7A). This was expected due to the higher injury produced at middle arterial segment by the Fogarty-balloon dilation and back-and-forth passage (25). Reendothelialization was similar to Con at these three vascular segments in the p85PKA-activated transfected carotid arteries (%vWF stained, middle = 28.5 ± 4%, proximal = 79.5 ± 5%; distal = 85 ± 5%; P = nonsignificant) (Fig. 7A). These data were confirmed by Evans blue planimetry analysis performed in an additional four rats per group (Fig. 7, D and E, and Supplemental Fig. 2).

**DISCUSSION**

The major findings of the present study are as follows. 1) cAMP inhibits VSMC proliferation through p85α (Ser83) phosphorylation. p85PKA-activated gene transfer inhibits VSMC proliferation in vitro, through coupling with p21ras and inhibition of ERK1/2 phosphorylation. 2) cAMP inhibits EC proliferation independently from p85α (Ser83) phosphorylation and ERK1/2 inhibition. Accordingly, p85PKA-activated does not affect ERK1/2 activation and cell proliferation of ECs in vitro. 3) cAMP-induced cytoprotection on VSMCs and ECs depends on p85α (Ser83) phosphorylation. Hence, p85PKA-activated attenuates apoptosis in vitro by amplifying Akt pathway activation in both vascular cell types. 4) Local gene transfer of p85PKA-activated inhibits neointimal hyperplasia and does not hinder normal endothelial regeneration after balloon injury in rats.

The cellular and molecular mechanisms regulating restenosis after balloon dilation and/or stent deployment have been deeply investigated in the last decades (19). VSMC proliferation is the main cellular event responsible for restenosis after PCI with stenting (2, 9, 19, 26). The p21ras/MAPKs cascade plays a central role in VSMC proliferation and p21ras, as well as MAPKs inhibition prevents VSMC proliferation and neointimal formation after balloon injury (16, 19, 34). In sharp contrast, increased cAMP levels inhibit VSMC proliferation in vitro and reduce neointimal formation after balloon injury in vivo (15, 17, 20, 29). In most cell types, increasing intracellular cAMP concentration strongly inhibits cell growth and division (30). The main mediator of the cAMP-dependent inhibitory effect on cell proliferation is PKA (19, 23, 35), which has been reported to inhibit the p21ras/ERK1/2 signaling pathway in several cell types (7, 25). In striking contrast, PKA can also activate the ERK1/2 signaling pathway and still inhibit cell proliferation (4). Surprisingly, we have shown that cAMP-activated PKA stimulates cell proliferation by inhibiting ERK1/2, thereby favoring activation of the PI3K signaling pathway in thyroid cells (6). To complicate the matter, cAMP can also inhibit cell proliferation through the inhibition of ERK1/2 in a PI3K-independent manner (13). With regards to ECs, it is generally accepted that an increasing level of cAMP inhibits EC growth (11); also, cAMP differentially regulates EC function, depending on its intracellular compartmentalization (27). However, the mechanisms underlying this cellular events are largely unknown.

PI3K enzyme is a heterodimeric protein, composed of separate regulatory (p85) and catalytic (p110) subunits (33). The p85 regulatory subunit exists in different isoforms, namely p85α, p85β, and p50α/p55α, of which p85α is, by large, the most abundant. The p85α mediates the binding, activation, and localization of the PI3K enzyme (33). Furthermore, recent evidence suggested that, in addition to being a regulatory subunit for p110-PI3K, p85 can independently stimulate signaling pathways (33). In the present study, we found that CAMP/PKA-activated p85α stabilizes p21ras in a molecular complex inhibiting mitogen-dependent ERK1/2 activation, which reduces VSMC proliferation in vivo and in vitro. On the other hand, overexpressing a mutated form of p85α, PKA unphosphorylable (p85PKA-inactive), or p85α deletion by specific siRNA transfection abolished CAMP-induced growth inhibition in VSMCs. These data strongly support the view that p85α is a key modulator of cAMP/PKA molecular and cellular effects in VSMCs.

In contrast with the effects on VSMCs, we show that CAMP reduces EC proliferation in a p85α- and ERK1/2-independent manner in vitro. Indeed, ERK1/2 was normally phosphorylated by serum stimulation, despite CAMP administration and the formation of the p85α-p21ras complex. Accordingly, p85PKA-activated overexpression did not interfere with ERK1/2 activation on serum stimulation in ECs, and cell proliferation occurred normally in ECs overexpressing p85PKA-activated. What is the key molecular target of CAMP mediating its cell proliferation inhibition in ECs remains unknown by now.

We also show that CAMP has cytoprotective effects on both VSMCs and ECs. CAMP reduced VSMC and EC apoptosis in association with Akt activation. Importantly, these CAMP effects were mediated through p85α (Ser83) phosphorylation in both vascular types.

Several studies have uncovered the existence of a molecular cross talk between PI3K/Akt and p21ras/ERK signaling in modulating cell differentiation, proliferation, and survival (14). More specifically to VSMCs, the cross talk entails an inhibitory effect of Akt on ERK1/2 through Raf modulation (28). Thus, in VSMCs, PKA-activated p85α could inhibit ERK activation at two levels: by sequestering p21ras and by activating Akt. However, in specific cell contexts, the cross talk between PI3K/Akt and GTPase Ras/ERK becomes synergistic in a molecular framework involving the activity of phospholipase C (PLC) (14). Incidentally, PLC-ε (one of the PLC isoenzyme) is also a target of PKA-independent CAMP activity (12). Thus it could be hypothesized that another kinase (like PLC) is effective in ECs, as opposed to VSMCs, in preserving ERK1/2 activation upon extracellular mitogen stimuli, despite p85α activation by CAMP/PKA. Nevertheless, it remains
Fig. 7. p85PKA-activated local delivery does not affect endothelial regeneration in rat balloon-injured arteries. A: percentage of endothelial coverage of neointimal area 14 days after BI in p85PKA-activated-transfected and Con rats. *P < 0.05 vs. Con. B: von Willebrand factor (vWF) immunohistochemical staining (diaminobenzidine, black inner layer) showing equal endothelial healing of p85PKA-activated-transfected and Con carotid cross sections, 14 days after BI. C: high-magnification confocal immunofluorescence image showing endothelial healing (vWF staining, red; SMA, green; DAPI, blue) in p85PKA-activated-transfected and Con carotid arteries 14 days after BI. D: Evans blue staining of Con and p85PKA-activated common right carotid arteries showing similar areas of deendothelialized (blue) and reendothelialized (white) areas 14 days after BI. Contralateral left uninjured carotid arteries are included. E: representative Evans blue staining showing the complete endothelium denudation (blue area) 30 min after Fogarty catheter balloon damage in right injured common carotid artery, as opposed to the normal endothelial layer (white area) shown in the contralateral left uninjured common carotid artery.
somewhat puzzling that VSMCs and ECs have an increased Akt activation when overexpressing p85PKA-activated, however, they still show a reduced or normal cell growth response, respectively. It could be that Akt activation levels obtained by p85PKA-activated overexpression are sufficient to complete the Akt-dependent survival pathway, but are not enough to trigger the Akt-dependent cell cycle machinery. These mere speculations, of course, warrant further investigations.

Finally, we show that p85PKA-activated transfection in the vascular wall significantly reduced VSMC proliferation in the tunica media upon balloon injury. This, in turn, resulted in a decreased neointimal formation at 14 days after balloon injury compared with Con rats. Also, p85
toverexpression did not affect VSMC proliferation and neointima formation after balloon injury. The latter is most likely secondary to the reduced cAMP levels and the consequent PKA hypo-phosphorylation in proliferating VSMCs in response to vascular injury (15, 17). Importantly, it was of interest to show that, in agreement with the in vitro experiments, p85PKA-activated in vivo transfection did not adversely affect the endothelial regeneration in the balloon-injured common carotid arteries.

In conclusion, the data of the present study demonstrate that PKA-activated p85Δ has differential effects on VSMC and EC growth in vitro, and, when locally delivered, it inhibits neointimal formation without affecting endothelial repair after vascular injury in vivo. These findings could be of relevance to eventually translate PKA activation of p85Δ as a target for, or possibly a cDNA coding for PKA-activated p85Δ to be released by, DES to prevent restenosis without adding the hazard of lack of reendothelialization.

REFERENCES
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No conflicts of interest are declared by the author(s).

DISCLOSURES


