Smooth Muscle Cell Pro-angiogenic Phenotype Induced by Cyclopentenyl Cytosine Promotes Endothelial Cell Proliferation and Migration

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Running title: CPEC stimulates VSMC re-differentiation

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Keywords: cyclopentenyl cytosine, smooth muscle, endothelial cell, proliferation, migration, adenosine receptor

ABSTRACT

Vascular smooth muscle cells (SMCs) and endothelial cells (ECs) have a close contact with the blood vessel. SMC phenotypes can be altered during pathological vascular remodeling. However, how SMC phenotypes affect EC properties remain largely unknown. In the present study, we found that platelet-derived growth factor (PDGF)-BB-induced synthetic SMC suppressed EC proliferation and migration while exhibiting an increased expression of anti-angiogenic factors such as endostatin and decreased pro-angiogenic factors including C-X-C motif ligand 1 (CXCL1). Cyclopentenyl cytosine (CPEC), a CTP synthase inhibitor that previously reported to inhibit SMC proliferation and injury-induced neointima formation, induced SMC re-differentiation. Interestingly, CPEC-conditioned SMC culture medium promoted EC proliferation and migration due to an increase in CXCL1 along with a decreased endostatin production in SMC. The addition of recombinant endostatin protein or blockade of CXCL1 with neutralizing antibody suppressed the EC proliferation and migration induced by CPEC-conditioned SMC medium. Mechanistically, CPEC functions as a cytosine derivate to stimulate adenosine receptor A1 and A2a, which further activate the downstream cAMP and Akt signaling, leading to the phosphorylation of cAMP response element binding protein and consequently SMC re-differentiation. These data provided a proof of a novel concept that synthetic SMC exhibits an anti-angiogenic SMC phenotype while contractile SMC shows a pro-angiogenic phenotype. CPEC appears to be a potent stimulator switching the anti-angiogenic SMC phenotype to the pro-angiogenic phenotype, which may be essential for CPEC to accelerate re-endothelialization for vascular repair during injury-induced vascular wall remodeling.

Pathological vascular remodeling is one of the major obstacles limiting the long-term clinical efficacy of cardiovascular intervention including angioplasty, bypass surgery, and transplantation arteriopathy, etc. (1,2). Injury-caused endothelium denudation promotes a series of pro-inflammatory responses (3,4), which further impair endothelial cell (EC) and smooth muscle cell (SMC) functions (5-9). EC dysfunction may alter EC proliferation and migration, causing a delayed endothelium repair with an increased risk of thrombosis (10,11). It is unknown, however, if SMC phenotype affects EC properties during vascular remodeling and endothelium recovery.

SMC is known to exhibit a remarkable phenotypic plasticity (12-14). SMCs within the adult arteries express contractile SMC markers, ion channels, and signaling molecules related to their contractile function (15,16). Injury-induced growth factors such as PDGF-BB suppresses SMC marker expression, leading to a phenotype alteration to synthetic SMC (13,14,17). This phenotypic modulation (18) is an essential event...
in the vascular remodeling/neointima formation (19-21). SMC and EC interact in various physiological and pathological conditions (22-25). During vascular development, e.g., vasculogenesis, EC tube formation recruits supporting cells including SMC to form functional blood vessel (26). Conversely, growth-arrested pericyte or SMC inhibits capillary EC growth in a cell-cell contact-dependent manner in vitro (27). SMC may also interact with EC through β-catenin related pathways and thus impact inflammatory responses of ECs (28). It is unknown, however, if the proliferating SMC seen in pathological conditions affects arterial EC proliferation and migration that are essential for vascular repair following mechanic injury.

Our previous studies have shown that cyclopentenyl cytosine (CPEC), a CTP synthase (CTPS) inhibitor, suppresses neointima formation while promoting re-endothelialization (29). Since CPEC does not induce EC proliferation and migration (29), the underlying mechanisms controlling the CPEC-accelerated re-endothelialization remain to be determined. Also, although CPEC affects SMC proliferation, it is unclear if CPEC affects SMC phenotype. In the present study, we found that cyclopentenyl cytosine (CPEC), a CTP synthase (CTPS) inhibitor, suppresses neointima formation while promoting re-endothelialization (29). Since CPEC does not induce EC proliferation and migration (29), the underlying mechanisms controlling the CPEC-accelerated re-endothelialization remain to be determined. Also, although CPEC affects SMC proliferation, it is unclear if CPEC affects SMC phenotype. In the present study, we found that PDGF-BB-induced synthetic SMC displays an anti-angiogenic phenotype and thus inhibits EC proliferation/migration. However, CPEC induces SMC re-differentiation to a contractile phenotype that shows a pro-angiogenic property as evidenced by an induction of pro-angiogenic factors and an inhibition of anti-angiogenic factors. Of importance, the CPEC-induced SMC stimulates EC proliferation and migration via a pro-angiogenic paracrine effect.

RESULTS

PDGF-BB-induced synthetic SMC suppressed EC proliferation and migration—PDGF-BB is a potent and known SMC mitogen that induces SMC proliferation and migration (29-31). PDGF-BB treatment also results in a synthetic SMC phenotype as shown by the reduction of SMC contractile proteins such as smooth muscle myosin heavy chain (SMMHC), smooth muscle α-actin (αSMA), SM22α, and calponin (CNN1) (Fig 1A-1B) (32). Since SMC has been shown to affect EC proliferation in a co-culture system (27), we sought to determine if synthetic SMC has a pro- or anti-angiogenic property. Therefore, we first detected if synthetic SMC expresses pro-angiogenic or anti-angiogenic factors. As shown in Fig 1C, PDGF-BB-induced synthetic SMC exhibited a decreased expression of several well-characterized pro-angiogenic factors, including CCL2 (33), CXCL1 (34), CRY61 (35), G-CSF (36), IGF-1 (37), IL-1β (38) and IL-6 (39) and an increased expression of anti-angiogenic factors endostatin (EST) (40) and TSP1 (41). Accordingly, synthetic SMC culture medium inhibited the proliferation and migration of ECs (Fig 1D-1F). These data indicate that synthetic SMC exhibits an anti-angiogenic phenotype.

CPEC induced synthetic SMC re-differentiation into a contractile SMC phenotype—Our previous studies showed that CPEC inhibits SMC proliferation (29). Since synthetic SMC displays an anti-angiogenic phenotype, and CPEC promotes re-endothelialization without affecting EC proliferation, we sought to determine if CPEC alters SMC phenotype. As shown in Fig 2A, CPEC induced SMC marker gene expression in both vehicle and PDGF-BB-treated synthetic SMC. In fact, CPEC induced the expression of SMC contractile proteins such as SMMHC and SM22α in PDGF-BB-treated SMC in a dose- (Fig 2B-2C) and time-dependent manner (Fig 2D-2E), suggesting that CPEC induces the re-differentiation of synthetic SMC to a contractile SMC phenotype. Since contractile SMC has a spindle-shaped morphology, we tested if CPEC treatment also alters SMC morphology. As shown in Fig 2F, CPEC induced SMC to become an elongated and spindle-shaped morphology, resembling the SMC phenotype observed in the artery media. To further verify if CPEC induces SMC differentiation, we tested if CPEC induces SMC progenitor cells to express SMMHC. As shown in Fig 2G-2H, CPEC stimulated a dose-dependent expression of SMMHC in human embryonic stem cell-derived mesenchymal stem cells (hMSC). These data indicate that CPEC can induce the contractile phenotype from either synthetic SMC or SMC progenitors.

CPEC-conditioned SMC culture medium promoted EC proliferation and migration—Since synthetic SMC exhibited anti-angiogenic effect (Fig 1C-1F), we sought to determine if CPEC-induced contractile SMC has a pro-angiogenic property. Thus, we tested if...
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CPEC-treated SMC expresses pro- or anti-angiogenic factors. As shown in Fig 3A-3B, although PDGF-BB blocked the expression of pro-angiogenic factors CXCL1, CYR61 and G-CSF and induced anti-angiogenic factors EST and TSP1, CPEC reversed the effect of PDGF-BB as shown by stimulating the expression of the pro-angiogenic factors while inhibiting the expression of anti-angiogenic factors. Importantly, CPEC-conditioned SMC culture medium promoted EC proliferation and migration (Fig 3C-3E). These results suggest that CPEC-induced contractile SMC has a pro-angiogenic property.

CPEC induced the pro-angiogenic effect of SMC via EST and CXCL1—Because CXCL1 and EST exhibited the most dramatic changes in SMC (Fig 1C, and 3A-3B), we tested if CPEC induces the paracrine angiogenic effects of SMC via EST or CXCL1. PDGF-BB induced EST while down-regulated CXCL1 protein expression in synthetic SMCs (Fig 4A-4B), consistent with their mRNA expression (Fig 1C and 3A-3B). CPEC, however, reversed the effect of PDGF-BB as shown by the inhibition of EST and the increase of CXCL1 protein expression in PDGF-BB-treated SMC (Fig 4C-4D). CPEC also regulated EST and CXCL1 protein expression in a time-dependent manner (Fig 4E-4F). To determine if CPEC affects EST and CXCL1 secretion in addition to their expression, we detected the EST and CXCL1 protein levels in SMC culture medium via enzyme-linked immunosorbent assay (ELISA). As shown in Fig 4G-4H, CPEC suppressed EST while increased CXCL1 secretion into SMC culture medium with PDGF-BB treatment.

To test if CXCL1 and EST play roles in the pro-angiogenic effect of CPEC-induced SMC on EC proliferation and migration, we added recombinant EST or CXCL1 neutralizing antibody (to block CXCL1 function,) in CPEC-conditioned SMC culture medium. As shown in Fig 4I-4J, recombinant EST (Fig 4I) or CXCL1 neutralizing antibody (Fig 4J) effectively suppressed the EC proliferation and migration promoted by the paracrine effects of contractile SMC that was induced by CPEC, suggesting that CXCL1 and EST mediated the pro-angiogenic effect of CPEC-induced SMC phenotype.

CPEC promoted SMC re-differentiation through adenosine receptor (ADOR) activation—CPEC is an inhibitor of CTPS. However, blockade of CTPS1 expression by its shRNA did not increase the expression of contractile SMC marker SM22α (Fig 5A-5B), suggesting that CPEC induced SMC re-differentiation through a CTPs-independent mechanism. In addition to inhibiting CTPS, CPEC can deplete intercellular CTP pool. Thus, we tested if CPEC induces SMC re-differentiation by altering the CTP pool. Cytidine feeding has been shown to increase intercellular CTP concentration (42), so we added cytidine to CPEC-treated SMC to counter CPEC effect. Surprisingly, combined treatment of CPEC and cytidine did not attenuate CPEC effect; rather, it led to a higher SM22α expression (Fig 5C-5D). This increased effect due to the addition of cytidine prompted us to hypothesize that CPEC may act as a cytosine derivate to stimulate SMC re-differentiation. In support of this hypothesis, other endogenous cytosine derivates such as cytosine, cytidine, and CMP exhibited similar effects in inducing SM22α expression in SMC (Fig 5E-5F).

Previous studies have shown that adenosine receptors can function as receptors for nucleotide mimics (43). CPEC up-regulated the expression of ADORα1 (ADORα1) and A2a (ADORα2a) in both control and PDGF-BB-treated SMC (Fig 6A). CPEC also enhanced ADORα2a protein expression (Fig 6B-6C). Moreover, the ADORα downstream signal was also activated by CPEC treatment as evidenced by the increased intercellular cAMP level (Fig 6D) and the phosphorylation of cAMP response element binding protein (CREB) (Fig 6E-6F). Importantly, both ADORα1 inhibitor CPDX and ADORα2a inhibitor KW-6002 suppressed CPEC-induced SMC re-differentiation (Fig 6G-6H), indicating that CPEC regulates SMC re-differentiation through ADORα signaling. Notably, KW-6002 displayed a more potent effect than CPDX (Fig 6G-6H), suggesting that ADORα2a may share more responsibility than ADORα1 in mediating CPEC function.

CPEC triggered SMC re-differentiation through ADOR downstream Akt signaling—It is well-established that Smad3 activation is critical for the contractile protein expression and SMC differentiation (44). However, CPEC did not enhance Smad3 expression or phosphorylation (Fig 7A-7B), suggesting that CPEC induces SMC re-differentiation through a Smad-independent mechanism. Indeed, CPEC induced Akt phosphorylation in a dose-dependent manner in both control and PDGF-BB-treated SMC (Fig 7A-7B). CPDX or
KW-6002 suppressed CPEC-induced Akt phosphorylation (Fig 7C-7D), suggesting that CPEC activates Akt signaling via ADORA1 and ADORA2a. Since Akt signaling is involved in CREB activation (45), which further regulates SMC differentiation (46), we test if CPEC induced SMC re-differentiation through ADORA-Akt-CREB axis. Thus, we tested if PI3/Akt signaling mediated CPEC-induced CREB phosphorylation. As shown in Fig 7E-7F, the blockade of PI3K/Akt signaling by its inhibitor LY-294002 attenuated CPEC-enhanced CREB phosphorylation as well as SMC marker SM22α expression (Fig 7G-7H), indicating that CPEC induced SMC re-differentiation through ADORA-Akt-CREB axis.

CPEC induced neointimal SMC re-differentiation, inhibited EST while enhanced CXCL1 expression, and promoted re-endothelialization in vivo—CPEC did not promote EC proliferation and migration in vitro (29), but CPEC promoted EC proliferation/migration via the paracrine effect of CPEC-induced contractile SMC. To test if CPEC-induced contractile SMC produces angiogenic factors under pathological conditions, we used rat carotid artery balloon-injury model to mimic vascular injury in vivo and use an osmotic pump to infuse saline or CPEC into rat undergoing the artery injury. As shown in Fig 8A-8B, infusion of CPEC significantly attenuated neointimal formation while promoted redifferentiation of the neointimal SMC as indicated by the expression of SMC marker SM22α compared to the saline-treated artery. Importantly, neointimal SMC in CPEC-treated artery showed a reduced expression of anti-angiogenic factor EST and a significant increase of pro-angiogenic CXCL1 (Fig 8C-8F). Consequently, CPEC treatment promoted the re-endothelialization as demonstrated by the CD31 staining (Fig 8G-8H), consistent with our previous finding (29). These data suggest that CPEC induces a contractile/pro-angiogenic phenotype in neointimal SMC, which promotes EC proliferation/migration, resulting in the accelerated re-endothelialization.

**DISCUSSION**

EC proliferation and migration are key events during the vascular repair following injury. The mechanisms underlying re-endothelialization are thought to attribute primarily to the intrinsic factors or signaling of ECs. Our present study indicates that SMC phenotypes play critical roles in EC properties. The proliferative or synthetic SMC inhibits EC proliferation and migration while CPEC-induced contractile SMC stimulates the EC proliferation/migration, which is due to the production of pro-angiogenic factors and the blockade of anti-angiogenic factors within the re-differentiated SMC. Therefore, contractile SMC exhibits a pro-angiogenic phenotype while synthetic SMC displays an anti-angiogenic phenotype. This concept is also supported by a previous study showing that serum-treated SMC suppresses EC replication although the mechanism is not determined (47). It is likely that serum-treated SMC acts similarly as the PDGF-BB-treated SMC, i.e., producing anti-angiogenic factors to block EC proliferation.

The pro-angiogenic function of CPEC-induced SMC is likely to be a unique property of the contractile SMC converted from the neointima/proliferative SMC during vascular repair following pathological remodeling. Thus, it may not be relevant to the physiological angiogenesis because mature SMC inhibits excessive EC proliferation and migration during vascular development (27). Accordingly, CPEC-induced SMC may not be identical to the mature SMC in the blood vessel that is formed during the vascular development although CPEC can also induce SMC differentiation from the mesenchymal progenitors.

Interestingly, although CPEC is an inhibitor of CTPS, it does not cause SMC phenotypic alteration by its function on CTPS activity or the depletion of CTP pool. Instead, CPEC plays a new role by acting as a cytosine derivate to induce SMC re-differentiation. Importantly, other cytosine derivates can also induce SMC re-differentiation although their effects are much less comparing to CPEC (Fig 5E). Combined treatment of CPEC and cytidine significantly increases the SMC marker gene expression, suggesting that CPEC and other cytosine derivates have a synergistic effect in inducing SMC re-differentiation. Collectively, it appears that CPEC promotes vascular repair through two independent mechanisms, i.e., blocking SMC proliferation via inhibition of CTPS activity and prompting a contractile/pro-angiogenic SMC phenotype by acting as a cytosine derivate. Both functions appear to be critical for promoting re-endothelialization and vascular repair.

CPEC induces SMC re-differentiation via...
ADOR-PI3K/Akt-CREB axis. ADOR is a class of G protein-coupled purinergic receptors using free nucleotides (mainly adenosine and uridine) as ligands (48). SMC express ADORs. Functionally, these receptors are involved in cell contraction and ion channel activities related to the cAMP signaling (49-51). Our results demonstrate for the first time that ADORA1 and ADORA2a may also serve as receptors for CPEC, which activates both cAMP and Akt; cAMP and Akt further promote CREB phosphorylation, leading to SMC re-differentiation. The results that PI3K/Akt inhibitor blocks CPEC-induced SMC re-differentiation are consistent with previous findings that PI3K/Akt pathway is essential for maintaining the differentiated SMC phenotype (52) and that cAMP-mediated Akt signaling is necessary for SMC contraction (49-51). Therefore, PI3K/Akt signaling may regulate SMC phenotype via both CREB-dependent and CREB-independent mechanisms.

In summary, we have demonstrated that PDGF-BB-induced synthetic SMC exhibits an anti-angiogenic phenotype inhibiting EC proliferation/migration while CPEC-induced contractile SMC shows a pro-angiogenic phenotype promoting EC proliferation/migration. CPEC induces SMC re-differentiation through activating ADORA-Akt-CREB cascade (Fig 8).

EXPERIMENTAL PROCEDURES

Reagents and cell culture—Rat aortic smooth muscle cells (SMCs) and endothelial cells (ECs) were cultured by enzyme digestion method from rat thoracic aorta as described previously (53,54). SMCs were maintained at 37 °C in a humidified 5% CO2 incubator in DMEM containing 10% fetal bovine serum, 4.5g/L glucose, 4.5 g/L sodium pyruvate, 2mM L-Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Primary ECs were maintained at 37 °C in a humidified 5% CO2 incubator in DMEM containing 20% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin, 2 mM L-Glutamine, 10 mM non-essential amino acids, 1 mM sodium pyruvate, 25 mM HEPES, 100 µg/ml heparin, 100 µg/ml endothelial cell growth supplement (ECGS). Cells within passage 3 were used for all experiments in this study. SMC and EC phenotype of the cultured cells were confirmed by the expression of α-SMA and CD31, respectively. The human embryonic stem cell-derived mesenchymal stem cells (hMSC) were obtained from ArunA Biomedical (Athens, GA). hMSC were cultured in α-minimal essential medium (αMEM, Cellgro, Fisher Scientific, Pittsburgh, PA) with 10% mesenchymal stem cell-qualified fetal bovine serum (HyClone) and 2 mM L-glutamine (HyClone).

SMCs were cultured in complete medium with or without treatment of CPEC and/or PDGF-BB. 48 hours after the treatment, 3X10⁶ cells were re-seeded in 6 cm culture dishes. After SMC monolayer was formed, cells were washed with warm PBS for 3 times and incubated with serum-free DMEM medium for 12 hours followed by the collection of conditioned medium that was filtered through a 0.22-µm filter, and stored at −80 °C.

CPEC (compound 375575) was obtained from the Open Chemical Repository of National Cancer Institute Developmental Therapeutics Program. Cytosine, cytidine, CMP, CDP, CTP, CPDX, KW6002 was purchased from Sigma-Aldrich (St. Louis, MO, USA). PDGF-BB was purchased from R&D Systems (Minneapolis, MN, USA). SM22α (ab10135), CNN1 (ab46794), CXCL1 (ab86436) antibodies were purchased from Abcam (Cambridge, MA, USA). Smad3 (9523S), p-Smad3 (9520S), Akt (4691S), pAkt (9271S) antibodies were purchased from Cell Signaling (Danvers, MA, USA). Endostatin (EST, abc60) antibody was purchased from R&D Systems (Minneapolis, MN). EST proteins and CXCL1 neutralizing antibody were purchased from EMD Millipore (Billerica, MA, USA). GAPDH (G8795) antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). EST proteins and CXCL1 neutralizing antibody were purchased from R&D Systems (Minneapolis, MN).

Animals—Male Sprague-Dawley rats weighing 450 to 500 g were purchased from Harlan. All animals were housed under conventional conditions in the animal care facilities and received humane care in compliance with the Principles of Laboratory
Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Animal surgical procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Rat carotid artery injury model and immunohistochemistry staining—Rat carotid artery balloon injury was performed using 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare) as described previously (53). 14 days later, the balloon-injured arteries were perfused with saline, fixed with 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned. For immunohistochemistry (IHC) staining, sections were rehydrated, permeabilized with 0.01% Triton X-100 in PBS, blocked with 10% goat serum and incubated with primary antibodies overnight at 4°C followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. The sections were counterstained with hematoxylin.

Quantitative RT-PCR (qPCR) —Total RNA was extracted from primary cultured SMCs using Trizol reagent (Invitrogen) and reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad). qPCR was performed on a Stratagene Mx3005 qPCR thermocycler (Agilent Technologies, La Jolla, CA) as described previously (55). Primer sequences for qPCR were listed in Supplemental Table S1. Cyclophilin (CYP) was used as an internal control. The primer efficiency was verified by the dissociation curve in qPCR reactions. Fold change for each target gene is calculated as $2^{(-\Delta\Delta Ct)}$.

Western Blot—Western blot was performed as described previously (55).

EC Proliferation assay—ECs were seeded in 24-well plates at 1X10^4/well in EC complete medium. After 24 hours, medium was changed into DMEM containing 20% SMC-conditioned medium, 2% FBS, and 2 mM L-Glutamine and cultured for another 4 days. Fresh medium was changed every other day, and cell numbers were counted every day.

Transwell assay—Transwell assay was carried out according to the manufacturer’s instructions (Corning Inc., Corning, NY, USA). Primary ECs were seeded onto the transwell upper chamber inserts (1x10^5/insert). The inserts were then put back into the receiver plate filled with 80% endothelial culture medium plus 20% SMC-conditioned medium and incubated at 37 °C in a humidified 5% CO2 incubator for 12 h. After incubation, transwell inserts were washed with PBS for three times and fixed with 4% PFA for 10 min at room temperature followed by washing with PBS. Cells were stained with Wright Giemsa for acquiring images or DAPI for cell counting. Images of migrating cells were captured using a dissection microscope (Olympus).

ELISA assay—CXCL1 and EST levels in SMC-conditioned medium were measured with commercial rat CXCL1 (MBS824537) and Endostatin ELISA Kits (MBS730385) from MyBioSource Inc, respectively. SMC-conditioned medium was collected, filtered through a 0.22-μm filter, and stored at −80 °C for later use. Purified rat CXCL1 and EST were serially diluted and served as standards. Intracellular cAMP levels were measured with commercial cAMP ELISA kit (ELA-CAMP-1) from RayBiotech, Inc. ELISA assays were performed according to the manufacturer’s protocol.

Statistical analysis—Each experiment was repeated for at least three times. All values are presented as means ± SEM. Comparisons of parameters among groups were made by one-way analysis of variance, and comparisons of different parameters between each group were made by a post hoc analysis using a Bonferroni test. P values < 0.05 were considered statistically significant.

Acknowledgments: We thank the Developmental Therapeutics Program of National Cancer Institute for providing CPEC.
Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: RT and SYC conceived and coordinated the study. RT designed and performed the experiments in Figures 1 to 8. GZ provided technical assistance and contributed to the experiments in Figures 2, 4, 5 and 6. RT and SYC analyzed the results. RT and SYC wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

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FOOTNOTES

This work was supported in whole or part by National Institute of Health Grants HL123302 and HL119053.

The abbreviations used are: SMC: smooth muscle cell; EC: endothelial cells; EST: endostatin; CXCL1: C-X-C motif ligand 1; CCL2: Chemokine (C-C motif) ligand 2; CRY61: cysteine-rich, angiogenic inducer, 61; G-CSF: Granulocyte colony-stimulating factor; IGF-1: Insulin-like growth factor 1; IL-1β: Interleukin-1 beta; IL-6: Interleukin 6; TSP1: Thrombospondin 1; CPEC: cyclopentenyl cytosine; CPDX: 1,3-dipropyl-8-phenylxanthine; ADORA: adrenoceptor alpha; shRNA: small hairpin RNA; PDGF: platelet-derived growth factor.

FIGURE LEGENDS

Figure 1. PDGF-BB-induced synthetic SMC suppressed EC proliferation and migration. (A) PDGF-BB (20 ng/ml, 24 h) down-regulated SMC marker protein expression. (B) Quantification of protein expression shown in A by normalizing to α-Tubulin. (C) PDGF-BB (20 ng/ml, 12 h) down-regulated the mRNA expression of pro-angiogenic factors and up-regulated the expression of anti-angiogenic factors in cultured SMC. (D) PDGF-BB-conditioned SMC medium suppressed EC proliferation. (E) PDGF-BB-conditioned SMC medium suppressed EC migration. Migrating ECs were indicated by white arrows. (F) Quantification of EC migration showed in E. Ct-SMCS: control SMC culture medium; BB-SMCS: PDGF-BB-conditioned SMC medium. *P<0.05; **P<0.01, n=3.

Figure 2. CPEC induced re-differentiation of synthetic SMC to contractile SMC phenotype. (A) CPEC treatment (100 nM, 12 h) induced SMC marker mRNA expression. Ct: vehicle treatment; BB: PDGF-BB treatment (20 ng/ml); CP: CPEC; BB-CP: PDGF-BB with CPEC. (B) CPEC induced contractile protein expression in PDGF-BB-treated SMC in a dose-dependent manner. The cells were treated with CPEC for 24 h. (C) Quantification of protein expression shown in B by normalizing to GAPDH. (D) CPEC (1 μM) induced SMC contractile protein expression in a time-dependent manner. (E) Quantification of protein expression shown in D by normalizing to α-Tubulin. (F) CPEC (1 μM, 24 h) induced a spindle-shaped contractile SMC morphology. Bar: 25 μm. (G) CPEC (1 μM, 24 h) induced SMMHC expression in human mesenchymal stem cells. (H) Quantification of protein expression shown in G by normalizing to α-tubulin. *P<0.05, **P<0.01, n=3.

Figure 3. CPEC-conditioned SMC culture medium promoted EC proliferation and migration. (A) CPEC treatment (100 nM, 12 h) up-regulated the mRNA expression of pro-angiogenic factors CXCL1, CYR61, G-CSF in PDGF-BB-treated SMC. BB: PDGF-BB; BB-CP: PDGF-BB with CPEC. (B) CPEC (100 nM, 12 h) down-regulated mRNA expression of anti-angiogenic factors EST and TSP-1 in PDGF-BB-treated SMC. (C) CPEC-conditioned SMC medium promoted EC proliferation. The cells were cultured in 24-well plate for 4 days. Ct-SMCS: control SMC medium; CP-SMCS: CPEC-conditioned SMC medium; BB-SMCS: PDGF-BB-conditioned SMC medium; BB-CP-SMCS: both PDGF-BB- and CPEC-conditioned SMC medium. (D) CPEC-conditioned SMC medium promoted EC migration. Migrating ECs were indicated by white arrows. (E) Quantification of EC migration shown in D. **P<0.01, n=3.

Figure 4. CPEC induced a pro-angiogenic paracrine effect of SMC via EST and CXCL1. (A) PDGF-BB (20 ng/ml, 24 h) down-regulated CXCL1 and up-regulated EST protein expression in SMC. (B) Quantification of protein expression shown in A by normalizing to α-Tubulin. (C-F) CPEC up-regulated CXCL1 and down-regulated EST protein expression in PDGF-BB-treated SMC in dose (C-D) (24 h treatment)- and time (100 nM CPEC)-dependent manners (E-F). The protein expression in C and E was normalized to α-Tubulin (D) and GAPDH (F), respectively. (G) CPEC (1 μM, 24 h) suppressed EST secretion in SMC culture medium. EST protein levels were normalized to total cellular proteins (ng/ml/1μg total cellular proteins). (H) CPEC (1 μM, 24 h) promoted CXCL1 secretion in SMC culture medium. CXCL1 protein levels were normalized to total cellular proteins.
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(ng/ml/1μg total cellular proteins). (I-J) Recombinant endostatin protein (EST, 100 ng/ml) or CXCL1 neutralizing antibody (CXCL1nAb, 1 μg/ml) suppressed EC migration (I) and proliferation (J) induced by CPEC-conditioned SMC medium (BB-CP-SMCS). Ct-SMCS: control SMC medium; BB-SMCS: PDGF-BB-conditioned SMC medium. BB-CP-SMCS: both PDGF-BB- and CPEC-conditioned SMC medium. *P<0.05, **P<0.01, n=3.

Figure 5. CPEC prompted the SMC re-differentiation by serving as a cytidine derivate mimics. (A) CTPS1 knockdown by shRNA did not increase SMC marker SM22α expression. (B) Quantification of protein expression shown in A by normalizing to α-Tubulin level. (C) Cytidine (1 μM, 24 h) significantly increased CPEC-induced SMC contractile protein SM22α expression. (D) Quantification of protein expression shown in C by normalizing to α-Tubulin. (E) Cytosine derivates (1 μM each, 24 h) induced SM22α expression. (F) Quantification of protein expression shown in E by normalizing to α-Tubulin. *P<0.05, **P<0.01, n=3.

Figure 6. CPEC triggered the SMC re-differentiation by activating adenosine type A receptor (ADORA) signaling. (A) CPEC (100 nM, 12 h) induced ADORA1 and ADORA2α mRNA expression. (B) CPEC (1 μM, 24 h) induced ADORA2α protein expression. (C) Quantification of protein expression shown in B by normalizing to GAPDH. (D) CPEC (1 μM, 15 min) increased intercellular cAMP level (ng/10E7 cells) in SMCs. (E) CPEC (1 μM) induced CREB phosphorylation in SMCs. (F) Quantification of protein expression shown in E by normalizing to GAPDH. (G) ADORA1 inhibitor CPDX (10 nM) and ADORA2a inhibitor KW-6002 (10 nM) suppressed CPEC (1 μM, 24 h)-induced SMC marker expression. (H) Quantification of protein expression shown in G by normalizing to α-Tubulin. C: control (vehicle treatment); CP: CPEC treatment; CP+DX: CPEC with CPDX; CP+DW: CPEC with KW-6002. *P<0.05, **P<0.01, n=3.

Figure 7. CPEC induced the SMC re-differentiation through ADORA-Akt-CREB axis. (A) CPEC induced Akt phosphorylation in a dose-dependent manner. Note that Smad3 phosphorylation was suppressed in a high concentration CPEC (1 μM) probably due to an inhibition of Smad3 protein expression. (B) Quantification of protein expression shown in A by normalizing to GAPDH level. BB: PDGF-BB; CP: CPEC. (C) Both ADORA1 (CPDX) and ADORA2α (KW-6002) inhibitors suppressed CPEC (1 μM)-induced Akt phosphorylation. (D) Quantification of Akt phosphorylation shown in C by normalizing to the total Akt level. CP: CPEC; CP+DX: CPEC with CPDX; CP+KW: CPEC with KW-6002. (E) PI3K/Akt pathway inhibitor LY294002 (10 nM) suppressed CPEC (1 μM)-induced CREB phosphorylation. (F) Quantification of CREB phosphorylation shown in E by normalizing to the total CREB level. (G) PI3K/Akt pathway inhibitor LY294002 (10 nM) suppressed SM22α protein expression enhanced by CPEC (1 μM). (H) Quantification of protein expression shown in G by normalized to α-Tubulin level. BB: PDGF-BB; BB+CP: PDGF-BB with CPEC; BB+CP+LY: treatment with PDGF-BB, CPEC and LY294002. **P<0.01, n=3.

Figure 8: CPEC promoted re-endothelialization through inducing neointima SMC re-differentiation in vivo. Rat left carotid arteries were injured with 2F Fogarty arterial embolectomy balloon catheter. 1 mg/kg/day of CPEC was infused to rat through osmotic minipump that was implanted on the day of artery injury. 14 days later, the arteries were removed, and IHC staining with antibodies indicated was performed. (A-B) CPEC significantly increased contractile SMC marker SM22α expression in neointimal SMC. (C-D) CPEC suppressed the expression of anti-angiogenic factor EST in neointimal SMC. (E-F) CPEC induced the expression of pro-angiogenic factor CXCL1 expression in neointimal SMC. (G-H) CPEC accelerated re-endothelialization. The endothelium was stained by CD31. The expression of SM22α, EST and CXCL1 were quantified by measuring mean intensity per area in 10 different fields and shown as fold changes. The re-endothelialization was quantified by averaging the CD31 positive cells in 10 different fields and shown as fold changes. **P<0.01, n=10.
Figure 5
Figure 6

A

Fold Change

ADOR1  ADORA2a  ADORA2b  ADORA3

Ct  CPEC  BB  BB-CPEC

B

Ct  CPEC

ADOR2a

GAPDH

C

Fold Change

Ct  CPEC

D

Cellular cAMP

Ct  CPEC

E

PDGF  CPEC

pCREB  CREB  GAPDH

F

Fold Change

pCREB  CREB

Ct  CP  BB  BB-CP

G

CPEC  CPDX  KW-6002

SM22α  αSMA  CNN1  α-Tubulin

H

Fold Change

SM22α  αSMA  CNN1

Ct  CP+DX  CP  CP+KW
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J. Biol. Chem. published online November 7, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.741967

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