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Role of PDE3A in Regulation of Cell Cycle Progression in Mouse Vascular Smooth Muscle Cells and Oocytes: Implications in Cardiovascular Diseases and Infertility

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Abstract

Phosphodiesterase-3 (PDE3) is a major cAMP-hydrolyzing PDE in vascular smooth muscle cells (VSMCs) and oocytes. The exact role and contribution of the two PDE3 isoforms, PDE3A and PDE3B, in VSMC growth regulation and oocyte maturation was examined using PDE3A (3A) and PDE3B (3B) knockout (KO) mouse models. PDE3A-deficient VSMCs exhibit marked reduction in mitogen-induced cell growth due to cell cycle arrest at Go-G1 phase, which resulted from dysregulation of cAMP/Protein kinase A (PKA)- and Mitogen-activated protein kinase (MAPK)-signaling pathways, as well as from alterations in key cell cycle regulatory proteins. Similarly, PDE3A-deficient oocytes exhibit cell cycle arrest at G2/M phase because increased cAMP/PKA signaling in KO oocytes most likely inhibits Cdc25B-catalyzed dephosphorylation/activation of Cdc2 (maturation promoting factor), a key regulator of G2/M transition.

Introduction

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are two very important second messengers involved in intracellular signal transduction in many cell types, including vascular smooth muscle cells (VSMCs [1–2]) and oocytes [3–5]. The biological processes regulated by cAMP and cGMP in VSMCs include cell contractility, proliferation, migration, apoptosis, and inflammatory responses, all of which have been implicated in the regulation of atherogenesis and post angioplasty restenosis [6]. Cyclic nucleotide phosphodiesterases (PDEs) play critical roles in regulating intracellular cyclic nucleotides (cAMP and cGMP) levels and compartmentalization via degradation of cyclic nucleotides [7–8]. PDE3 is the major cAMP-hydrolyzing PDE present in VSMC and oocytes, and its inhibition by nitric oxide-induced accumulation of cGMP results in increased cAMP and protein kinase A (PKA) activity (7, 9–10). Therefore, understanding the exact role of PDE3 isoforms and their regulated cAMP pools in VSMC function, especially in the maintenance of the contractile phenotype and its switch to the synthetic phenotype during atherosclerosis initiation/progression as well as in regulation of oocyte meiosis/maturation and fertility, is critical for the design of pharmacological agents that selectively target the particular PDE3 isoform, without affecting other PDE3 isoforms or other PDE families, and thus reducing side effects.

Analysis of cell cycle and VSMC growth in PDE3A/B KO mouse models

Our laboratory generated PDE3A (3A) and PDE3B (3B) knockout (KO) mouse models to dissect, as well as reconstitute, signaling pathways downstream of cAMP in an effort to elucidate mechanisms which control cell division and mitogenesis in VSMCs, and also to understand the role of PDE3 isoforms in oocyte maturation and control of fertility. Studies with cultured VSMCs, expanded from aortas of 3A- and 3B-KO mice, clearly demonstrate that PDE3A-deficiency differentially inhibits mitogen-induced VSMC proliferation by suppressing cell cycle initiation (G_o-G_1) as well as its progression through $G_1 \rightarrow S$ and $G_2 \rightarrow M$ phases of cell cycle (11). The observed inhibition of cell cycle arrest and VSMC growth is due to a combination of abrogation of PDGF-induced mitogen-activated protein kinase (MAPK kinase) signaling (which is required for cell cycle initiation as well as its progression (12–14)), as well as due to alterations in key cell cycle regulatory proteins such as p53, p21, and Rb. In contrast, the PI3-kinase/Akt arm of PDGF-signaling is preserved in 3A-KO VSMCs. In 3A-KO VSMCs, PKA activity was selectively elevated, and increased cAMP/PKA signaling resulted in increased site-specific inhibitory phosphorylation of Raf-1 at ser259, which causes inhibition of Raf-1 kinase activity (15) and, consequently, impaired MAPK phosphorylation/activation. In addition, in 3A-KO VSMCs, MAPK phosphorylation/activation was reduced due to cAMP/p53-mediated induction of MKP-1, which catalyzes dephosphorylation and inactivation of MAPK (16). More importantly, the observed impairment in MAPK activation and the resultant suppression of the mitogenic response in 3A-KO VSMCs were specific to PDE3A-deficiency, as 3B-KO VSMCs exhibited no elevations in PKA activity, were responsive to PDGF with respect to MAPK activation and DNA synthesis, and did not exhibit increased Raf-1^{ser259} phosphorylation or MKP-1 expression. Cellular depletion of PDE3A in 3B-KO VSMCs by transfection with PDE3A siRNA inhibited PDG–induced DNA synthesis in 3B-KO VSMCs. A recent study by Hewer et al (17) indicated that PKA and Epac (guanine nucleotide exchange protein activated by cAMP) synergistically mediated cAMP-induced growth arrest in VSMCs via a Rap1-independent mechanism. Studies from Newby's lab indicate that altered S-phase kinase-associated protein-2 (Skp2) is a major mediator of cAMP-induced inhibition of VSMC proliferation via its regulation of the ubiquitination and degradation of several cell-cycle regulatory proteins, including the cyclin-dependent kinase inhibitor, p27Kip1 (18). The roles of Epac and Skp2 in regulation of proliferation in 3A-KO VSMCs are not certain at this time.

Mechanism of defective cell cycle cegulation in 3A KO VSMCs

Our studies also revealed that PDE3A-deficiency causes blockade of multiple proximal and distal steps in cell cycle progression by modulating several key cell cycle regulatory genes and proteins (11, 19). Most notably, there was substantial up-regulation of genes that control G1-S transition (*Rhou*), S phase and DNA replication (*Rad 17*), G2 phase and G2/M transition (*PPM1D*), M phase (*Rad21*), cell cycle check point/cell cycle arrest (*Brca2, Gadd45a, Tsg101, Macf1, and PPM1D*), and cell cycle regulation (*Ccnc, Cdc37, Gadd45a, and Rad 9*). Expression of some of the negative regulators of the cell cycle, including *Cdkn1a* ($p21^{cip}$), *Cdkn2d* (p19), and *Trp53* (p53) were also upregulated, while expression of cyclin D1, SHC1, CDC28 protein kinase 1 (*csk1b*), *Pes1, Gas2, Itgb1* and *Rbl2* (retinoblastoma (Rb) genes were decreased. Decreased expression of Cyclin-D1 was due to impaired activation of MAPK with resultant decrease in mitogen-induced Rb phosphorylation, most probably due to reductions in Cdk4 activity. As a consequence, Rb remained active and presumably associated with E2F (20), thereby causing suppression of $G_1 \rightarrow S$ phase of cell cycle progression. We also observed that PDE3A-deficiency, via increased cAMP-signaling and PKA-induced phosphorylation/activation of CREB (21), was associated with increased expression of the Cdk inhibitor protein, $p21^{cip}$, which is known to inactivate both Cdk2 and Cdk4, thereby promoting G_0/G_1 cell cycle arrest (22). Adenoviral

expression of inactive CREB in 3A-KO VSMCs partially restored the stimulatory effect of PDGF on DNA synthesis. Most notably, p53 protein expression and phosphorylation were increased in 3A-KO VSMCs, and depletion of cellular p53 with antisense siRNA decreased $p21^{cip1}$ and MKP-1 protein expression and completely restored PDGF-induced DNA synthesis. In contrast to cardiomyocytes, in which chronic downregulation of PDE3A expression and inhibition of PDE3A function is associated with apoptosis due to induction of ICER (inducible cAMP early repressor) (23), p53 upregulation in 3A-KO VSMCs did not promote apoptosis as evidenced by lack of alterations in cell viability, caspase-3 activity, and Akt-mediated pBad phosphorylation in 3A-KO VSMCs. Thus, the loss of PDE3A causes cell cycle arrest and inhibition of VSMC proliferation, primarily by utilizing two complementary mechanisms. Mitogen-induced MAPK signaling is blunted due to inactivation of the upstream Raf kinase via PKA-mediated site-specific inhibitory phosphorylation of Raf-1, as well as due to increased MKP-1 expression. Second, PDE3A deficiency results in altered expression of several cell cycle regulators and their phosphorylation, including p21, p53, and Rb. PKA-induced phosphorylation/activation of CREB may lead to increased expression of $p21^{cip1}$, an inhibitor of cell cycle progression, and upregulation of p53 protein and its phosphorylation via an unknown kinase. PKA has been reported to phosphorylate p53, in a conformation-dependent manner (24). Elevations in p53 expression and $p53^{Ser15}$ phosphorylation enhance p53 transcriptional activity, resulting in increased MKP-1 and p21 levels, and increased gene expression of Wip1, p21, and Cdkn2d (Cdk4 inhibitor). These three latter genes inhibit cell cycle progression.

Earlier studies using cAMP agonists and pharmacologic inhibitors of PDE3 and PDE4 isoforms have reported inhibition of VSMC growth (25–27). However, the exact role of PDE3A and PDE3B isoforms and PKA was difficult to discern in these studies because of the lack of availability of specific inhibitors of PDE3A and PDE3B isoforms. Our current findings define, for the first time, a specific role for PDE3A in the control of proximal as well as distal regulatory pathways of VSMC proliferation in response to growth factors and, perhaps, arterial injury. Growth inhibition in 3A-KO VSMCs is in part due to PKA-mediated ERK inactivation and as well as via up regulation of p53 and p21, two major cell cycle inhibitory proteins (17,28–32). This definitive role for PDE3A was substantiated by the absence of inhibition of VSMC growth in 3B-KO VSMCs, and by inhibition of PDGF-stimulated DNA synthesis in 3B-KO VSMCs depleted of PDE3A by transfection with PDE3A siRNA.

We also observed that in addition to PKA/CREB, other signals, especially p53, are required for growth inhibition, since inactive CREB only partially restored FBS-induced DNA synthesis in 3A-KO VSMCs. Depletion of p53 levels with TRP53 siRNA, however, completely restored the growth promoting effects of mitogens in PDE3A-KO VSMCs. Downstream targets of p53, i.e., upregulation of expression of p21 as well as Wip1 and Cdkn2d, might also be critical elements in cell cycle arrest seen in 3A-KO VSMCs. Moreover, cAMP, via CREB, is known to promote cell survival and vascular re-endothelialization by upregulating HGF (33) and, because CREB is activated in these VSMCs, it is plausible that CREB might also limit apoptosis in 3A-KO VSMCs by increasing vascular HGF, which was reported in earlier studies of human VSMCs exposed to cilostazol, a PDE3 inhibitor (33, 34). A schematic summary of the signaling pathways that mediate growth inhibition in 3A-KO VSMCs is shown in Fig. 1

Effect of PDE3A deletion on the cardiovascular system

How do the results shown above in VSMCs translate to in vivo situations? Recent studies by Sun et al. (35) showed that 3A-KO mice were protected from collagen/epinephrine-induced pulmonary thromboembolism and subsequent death, most likely due to elevated intraplatelet cAMP (due to the absence of platelet PDE3A) and reduced platelet activation. Moreover, in

3A-KO mice, heart rate was increased, and arterial blood pressure and left ventricular pressure were reduced, presumably due to peripheral vasodilation. On-going collaborative studies using WT and 3A-KO mice have further indicated that PDE3A is an important PDE isozyme that regulates basal heart function by regulating cAMP levels in microdomains containing sarcoplasmic reticulum Ca^2-stimulated ATPase (SERCA2)/Phospholamban/PDE3A macromolecular complexes (Shen, W., Becca, S., et. al., unpublished). Other studies, using human myocardial samples, have also supported the notion that PDE3A is a component of a SERCA2-containing macromolecular complex that may integrate cAMP- and SERCA-signaling in human heart (Ahmad, F. et. al., unpublished). Recent studies in rats by Zhao et al. (36) indicated that balloon angioplasty increased PDE3/PDE4 proteins and activities, with a concomitant decrease in VASP phosphorylation, which is an index of PKA activity and VSM relaxation. Treatment with PDE3 inhibitors restored VASP phosphorylation.

These observations suggest that PDE3A isoforms may play a major role in cardiovascular function by regulating cardiac contractility and peripheral vasodilation as well as VSMC growth. Given that cilostazol has been reported to inhibit neointimal injury in rats following balloon injury (34), future *in vivo* studies with balloon injury models as well as with ApoE KO and/or LDLR KO mice crossed with 3A KO mice will be needed to test the protective effect of PDE3A deletion on vessel wall thickening, smooth muscle cell migration, and neointimal growth.

PDE3A and regulation of oocyte maturation

In addition to VSMCs, PDE3A is an important regulator of cell cycle progression in murine oocytes. Female 3A-KO mice, not female 3B-KO mice, are completely sterile, due to increased cAMP/PKA signaling in oocytes, which results in meiotic arrest at prophase I of the first meiotic division, and which thereby inhibits oocyte maturation and capacity for fertilization (37). Meiotic maturation in 3A-KO oocytes was restored by inhibiting PKA with adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS) or by injection of protein kinase inhibitor peptide (PKI) or mRNA coding for phosphatase CDC25 into cultured oocytes Treated 3A-KO oocytes that underwent germinal vesicle breakdown and meiotic maturation showed activation of Maturation promoting factor (MPF) and MAPK, completed the first meiotic division and extruded polar bodies, and became competent for fertilization by spermatozoa. These studies confirmed that increased cAMP/PKA signaling was responsible for the meiotic blockade, and provided the first genetic evidence indicating that resumption of meiosis in vivo and in vitro requires PDE3A activity. 3A-KO mice represent an in vivo model where meiotic maturation and ovulation are dissociated, and which underscores inhibition of PDE3A as a potential strategy for contraception. Recent studies by Shen et al (38) suggest that elevated cAMP/PKA signaling in 3A-KO oocytes prevents dephosphorylation and activation of MPF, a key regulator of G2/M transition and resumption of meosis, and maintains meotic arrest via PKA-catalyzed phosphorylation and inactivation of Cdc25B (39) and Polo-like kinase 1 (Plk1) (38). In WT oocytes, PKA catalytic subunit (PKAc) was rapidly translocated into the nucleus, and then to the spindle apparatus, but in 3A-KO oocytes, PKAc remained in the cytosol (38). Incubation of 3A-KO oocytes with PKA inhibitors reactivated MPF (37) and Plk1 (38), and resumed meiosis. PDE3A was found to be co-localized with Plk1 in WT oocytes, and co-immunoprecipitated with Plk1 in WT ovary and Hela cells. PKAc phosphorylated recombinant Plk1 and Hela cell Plk1 and inhibited Plk1 activity in vitro. Taken together, our results suggest that Plk1 is a potential cAMP/PKA substrate target and a component of a macromolecular complex that contains PDE3A and regulates cAMP/PKA signaling pathways that may be important in progression of meiosis and regulation of female fertility.

Conclusions

These studies using 3A-KO VSMCs and oocytes clearly document the important role of the PDE3A, not PDE3B, isoform in the control of cell cycle progression in two cell types, oocytes and VSMCs, via regulation of cAMP levels. These findings suggest that therapies specifically aimed at inhibiting the PDE3A isoform might lead to amelioration of excessive VSMC growth and decrease the atherosclerosis process, which is a prominent feature of cardiovascular diseases, including metabolic syndrome and post angioplasty restenosis. In addition, PDE3A appears to be a good target for the development of new contraceptive drugs.

Acknowledgments

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Highlights

1. PDE3A, not PDE3B, regulates cAMP pools which control cell cycle progression in murine VSMCs and oocytes.
2. In VSMCs and oocytes, PDE3A-deficiency is associated with increased cAMP/PKA signaling and inhibition of MAPK signaling (but not inhibition of PI3K/Akt signaling in VSMCs).
3. in VSMCs, PDE3A-deficiency is associated with changes in key cell cycle regulatory proteins, including upregulation of p53 and p21.
4. In VSMCs, PDE3A-defiency is associated with inhibition of mitogen- and serum-induced DNA synthesis.
5. siRNA-induced depletion of p53 completely restores DNA synthesis in 3A-KO VSMCs

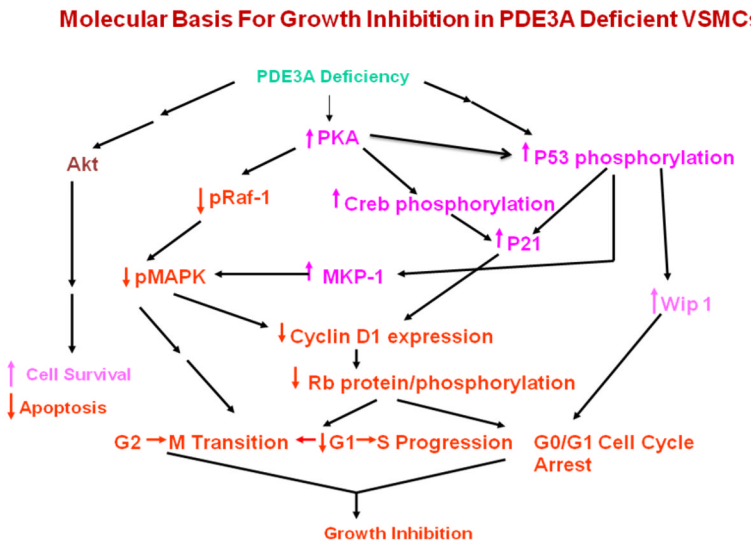

Fig. 1. Schematic diagram of signaling pathways that mediate growth inhibition in 3A-KO VSMCs

Increased PKA and p53 signaling independently and cooperatively inhibit upstream MAPK signaling via raf-1 inhibition and MAPK dephosphorylation by elevated MKP-1, which results in decreased cyclin D1and Rb protein expression. Elevations in PKA and p53 signaling also lead to increased induction of p21, MKP-1 and Wip1 expression leading to cell cycle arrest at Go/G1 and inhibition of G1->S progression.