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Effect of Cell Passage and Density on Protein Kinase G Expression and Activation in Vascular Smooth Muscle Cells

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Abstract It has been shown that rat aortic smooth muscle cells (AoSMCs) lost PKG-I expression when propagated repetitively or grown at low densities. Conversely, AoSMCs isolated from PKG-I deficient mice are indistinguishable from those isolated from normal mice in morphology and growth characteristics. In this study, human AoSMCs were grown from passage 9 (p9) to passage 15 (p15) and rat AoSMCs were isolated and cultured from p1 through p15. Western blotting and immunofluorescence microscopy showed little difference in PKG-I expression among different passages. Next, rat AoSMCs of p4 were grown and harvested at different cell densities. Western blotting again showed little difference among cells seeded or harvested at different densities. To test the effect of cell passage on PKG-I activation, rat AoSMCs of p4 and p11 were treated with cGMP and analyzed by Western blotting for phosphorylated vasodilator-stimulated phosphoprotein (P-VASP). The results showed that p4 had higher level of PKG-I activation than p11. J. Cell. Biochem. 92: 104–112, 2004. © 2004 Wiley-Liss, Inc.

Key words: vascular smooth muscle cell; protein kinase G; cell passage; cell density; expression; activation; cGMP; smooth muscle α-actin; vasodilator-stimulated phosphoprotein; phosphorylation

Vascular smooth muscle cells (VSMCs), especially those from the aorta, are routinely isolated and cultured for vascular research. Many investigators have observed a unique phenomenon called phenotypic modulation in which the isolation and culturing of VSMCs result in an alteration of the cellular phenotype from being contractile to becoming synthetic (proliferative) [Chamley-Campbell et al., 1981; Owens, 1995]. This phenotypic modulation is accompanied by accelerated cellular growth and changes in gene expression that typically include decreased expression of contractile proteins such as calponin, myosin, and ion channels and increased expression of proliferative markers such as osteopontin. More importantly, these cell culture-incurred changes have been suggested to mimic injury-induced changes in blood vessels [Lincoln et al., 2001], as VSMCs in the neointima of injured blood vessels (by atherosclerotic insults and/or balloon angioplasty) are less contractile and more proliferative than VSMCs in the media of uninjured blood vessels [Campbell and Campbell, 1990; Newby and Zaltsman, 2000].

The contractile phenotype of VSMCs infers being able to contract and relax in response to various stimuli such as hormones and neurotransmitters. The ability to relax is critically dependent on protein kinase A (PKA) and protein kinase G (PKG-I) that are principally activated by cAMP and cGMP, respectively. In addition to regulating the contractile phenotype, the cAMP-PKA and cGMP-PKG signaling pathways appear to also regulate the proliferative phenotype, as many agents that relax blood vessels through these two pathways also inhibit VSMC growth [Garg and Hassid, 1989; Indolfi et al., 2000; Koyama et al., 2001]. While several

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studies point to the role of PKG-I *activation* in modulating cellular growth [Hofmann et al., 2000], whether PKG-I *expression* also plays a role is less clear.

Through a series of reports Lincoln and his associates have shown that loss of PKG-I expression is associated with VSMC proliferation. The first of such papers showed that repetitively propagated rat aortic smooth muscle cells (AoSMCs) lost PKG-I expression [Cornwell and Lincoln, 1989]. Later, they also showed that AoSMCs grown at low densities expressed low levels of PKG-I expression [Cornwell et al., 1994b]. Transfection of PKG-I into AoSMCs that have lost PKG-I expression (i.e., of the synthetic phenotype) resulted in increased production of marker proteins of the contractile phenotype [Boerth et al., 1997; Dey et al., 1998; Lincoln et al., 1998] and restoration of contractility [Brophy et al., 2002]. Downregulated PKG-I expression in the proliferating neointimal smooth muscle cells of coronary artery following balloon injury has also been shown [Anderson et al., 2000; Lincoln et al., 2001].

While probing PKG-I activation by western blotting, several investigators in our laboratory have independently observed stable PKG-I expression in human and rat AoSMCs that have been propagated to passages numbering in the teens. This is in disagreement with the reports by Cornwell et al. [Cornwell and Lincoln, 1989; Cornwell et al., 1994a,b] that demonstrated a dramatically reduced PKG-I expression (barely detectable) in rat AoSMCs at passage 6. Because of the extensive use of AoSMCs in vascular research and the importance of PKG-I in VSMC function, we decided to conduct a systematic investigation on the stability of PKG-I expression in cultured AoSMCs. The results indicated that PKG-I expression was stably maintained in repetitively propagated AoSMCs and was little affected by cell density.

MATERIALS AND METHODS

Isolation and Culture of Rat Aortic Smooth Muscle Cells

Care and treatment of animals were approved by the Committee on Animal Research at our institution. Two male Sprague–Dawley rats, 3 months of age, were sacrificed by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and bilateral thoracotomy. The abdominal aorta was excised, its adventitia peeled off with forceps, and the endothelium scraped off with surgical blade. The remaining aortic muscle was rinsed in sterile Hank's balanced salt solution containing 200 μg/ml penicillin, 200 μg/ml streptomycin, 5 μg/ml Fungizone, and 20 mM N-2-hydroxyethylpiperazine-iV'-2-ethanesulfonic acid (HEPES, pH 7.4). The aortic muscle was then minced to small pieces (~1 mm^3) and placed in a 100-mm dish. The dish containing the explants was left open in the cell culture hood to dry until the moisture disappeared from the edges of the explants. Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) was then gently added to the explants, which were then incubated at 37°C, under 5% CO_2, for 3 days without disturbance. The culture medium was then replaced with fresh medium and thereafter the explants were examined daily by phase contrast microscopy. When cellular outgrowth was apparent, each explant was dislodged by a stream of culture medium and then discarded. The aortic smooth muscle-derived cells were allowed to grow to approximately 80% confluence, and at that point they were considered as the primary aortic smooth muscle cells at passage 0 (p0). These cells were then subjected to successive subculturing by standard trypsinization procedure every 4–5 day until the 15th passage (p15).

Human Aortic Smooth Muscle Cells

Human aortic smooth muscle cells were purchased from Cambrex Corporation (East Rutherford, NJ) and maintained as instructed by the supplier.

Antibodies

Antibodies and their suppliers are briefly described below. Rabbit anti-PKG-I antibody from Calbiochem, Inc. (La Jolla, CA). Mouse anti-smooth muscle actin antibody from Sigma (St. Louis, MO). Fluorescein-conjugated goat anti-rabbit antibody and horseradish peroxidase-conjugated antibody from Jackson ImmunoResearch Laboratories (West Grove, PA). Texas red-conjugated sheep anti-mouse antibody from Vector Laboratories (Burlingame, CA). Rabbit anti-VASP antibody (M4) from ImmunoGlobe Antikoerpertechnik GmbH & Co. (Großostheim, Germany). Mouse anti-phosphorylated VASP antibody (VASP-16C2) from NanoTools Antikoerpertechnik GmbH & Co. (Teningen, Germany).

Immunofluorescence Microscopy

Approximately 10,000 cells were seeded on a glass coverslip inside 6-well cell culture dish and incubated overnight. They were washed with phosphate-buffered saline (PBS, without calcium and magnesium) three times, fixed with 1–2 ml of cold (−20°C) methanol for 5 min, permeabilized with 0.05% Triton X-100 in PBS for 5–8 min, and blocked with 1.5 ml of 5% horse serum in PBS for 60 min. The cells were then reacted with anti-PKG-I antibody (1:500) and anti-smooth muscle actin antibody (1:500) for 1 h in PBS with 0.5% horse serum (1 ml/well). After three washes with PBS, the cells were reacted with fluorescein-conjugated anti-rabbit antibody and Texas red-conjugated sheep anti-mouse antibody in PBS with 0.5% horse serum (1 ml/well) for 1 h. For nuclear staining, the same cells were treated with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml, Sigma, St. Louis, MO) for 5 min. The stained cells were examined with Nikon Eclipse E600 microscope and the images recorded with Retiga 1300 Q-imaging camera.

Treatment With cGMP

Cells were seeded at 4×10^5 in each well of 6-well culture plate. Twenty-four hr later, cell culture medium was removed and the cells were rinsed twice with PBS, followed by the addition of fresh medium containing 1 mM of dibutyl-cGMP. Ten minutes or 24 h later, the cells were lysed in a buffer containing 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1× PBS. After removing insoluble materials by centrifugation, the cell lysates were measured for protein concentration by the BCA method (Pierce Chemical Company, Rockford, IL).

Western Blot Analysis

Expression and activation of PKG-I in cultured cells were analyzed by Western blotting as previously described [Lin et al., 2001]. Briefly, cell lysates each containing 20 μg of protein were electrophoresed in 8% SDS–PAGE and then transferred to PVDF membrane (Millipore Corp., Bedford, MA). The membrane was stained with Ponceau S to verify the integrity of the transferred proteins and to monitor the unbiased transfer of all protein samples. The membrane was then washed with 25 ml of TBS (100 mM NaCl, 0.1% and 10 mM Tris-HCl, pH 7.5) for 5 min at room temperature and incubated in 25 ml of Blocking Buffer (TBS plus 0.1% Tween-20 and 5% nonfat milk) overnight at 4°C. To detect the protein of interest, the membrane was incubated with an appropriate primary antibody in 2 ml of Blocking Buffer for 1 h at room temperature, washed three times for 5 min each with 15 ml of TBST (TBS plus 0.1% Tween-20), incubated with horseradish peroxidase-conjugated antibody (1:15,000) in 15 ml of Blocking Buffer with gentle agitation for 1 h at room temperature, and finally washed three times for 5 min each with 15 ml of TBST. The membrane was then subject to ECL (Amersham Life Sciences, Inc., Arlington Heights, IL) for the detection of the specific antigen. Before the membrane was used for the detection of another antigen, it was stripped in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, 10 mM 2-mercaptoethanol at 55°C for 30 min and then washed four times in 1× TBS. Densitometry was performed on the resulting images by the ChemiImager-4000 program (Alpha Innotech Corporation, San Leandro, CA).

RESULTS

Effect of Cell Passages on PKG-I Expression

Ideally, primary cells (isolated from healthy tissues) that have been propagated minimally should be used in most experiments. However, certain primary cells are either difficult or costly to obtain and therefore need to be propagated to maximize their useful life span; for example, a commercially obtained human aortic smooth muscle cell strain (AoSMC) has been propagated to passage number 15 (p15) in our laboratory. Comparison among human AoSMCs of p9, p11, p13, and p15 showed step-wise decreases of smooth muscle α-actin expression but little difference in PKG-I expression (Fig. 1A).

During the course of our previous study on PKG-I activation by cyclic nucleotides [Lin et al., 2001], we also observed seemingly stable expression of PKG-I in rat AoSMCs that have been propagated to >p10. This observation appeared to contradict a study by Cornwell et al. [1994b] who reported a dramatic decrease of PKG-I in rat AoSMCs at p6. As such we decided to conduct a systematic investigation on the effect of cell passages on PKG-I expression in rat AoSMCs. To eliminate the possibility that the cell's storage in liquid nitrogen and

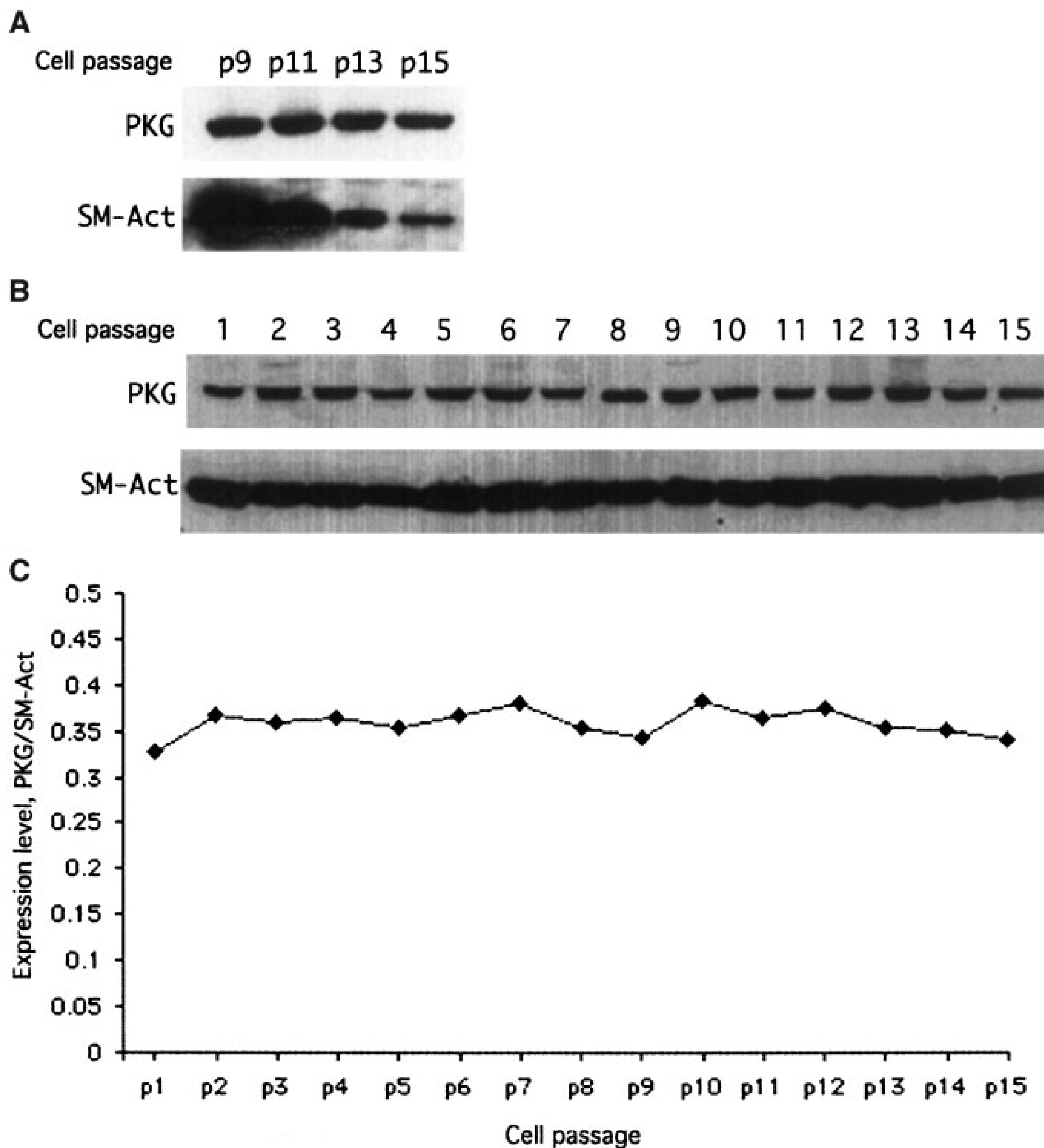

Fig. 1. Western blot analysis of PKG-I expression in different passages of human and rat AoSMCs. Equal amount (20 μg) of cellular protein from each passage was resolved in 8% SDS–PAGE and then transferred to PVDF membrane. After the detection of PKG-I, the membrane was stripped and then probed for smooth muscle α-actin (SM-Act). The levels of PKG-I expression remained essentially constant from p9 to p15 in human AoSMCs (**A**) and from p1 to p15 in rat AoSMCs (**B**). The expression levels of SM-Act decreased stepwise from p9 to p15 in human AoSMCs (A) but were essentially unchanged in rat AoSMCs of different passages (B). The reason for the difference between human and rat cells is not known. For quantitative representation, the PKG-I and SM-Act protein bands of Panel B were analyzed by densitometry and the ratio of their expression levels determined for each passage (**C**).

subsequent revival might affect PKG-I expression, we decided to derive freshly isolated rat AoSMCs and propagate them continuously to p15. The results (Fig. 1B,C) showed little differences in PKG-I expression in cells from p1 to p15. Interestingly, unlike their human counterparts, the rat cells showed little differences in smooth muscle α-actin expression.

In agreement with the well-observed phenotypic modulation, longer passaged rat AoSMCs grew faster (data not shown) and assumed a more fibroblastic morphology (example shown in Fig. 2). Still, by immunofluorescence staining, these cells were shown to express PKG-I at comparable levels as their lower passaged counterparts, as exemplified in Figure 2.

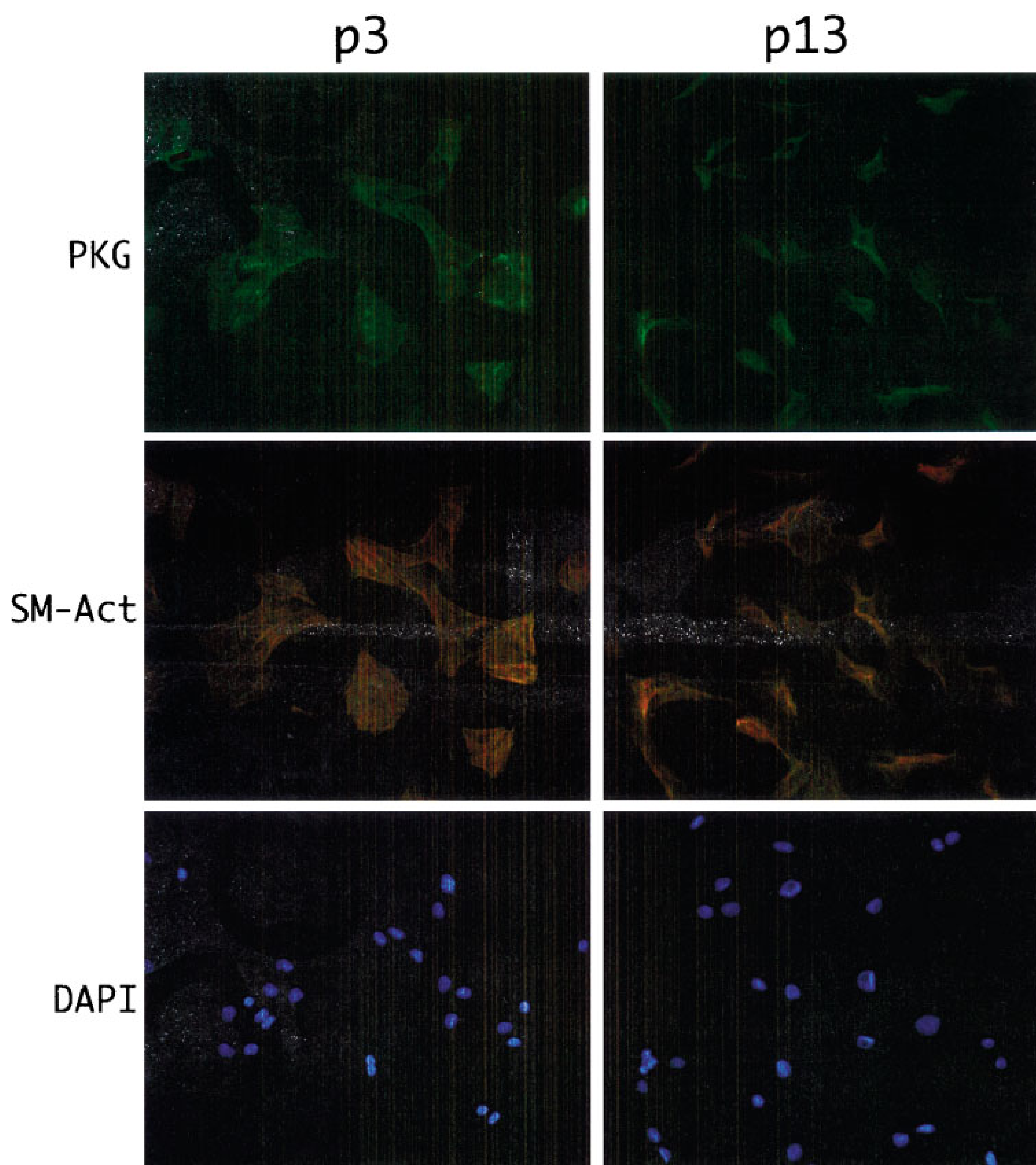

Fig. 2. Immunofluorescence microscopy of PKG-I expression in rat AoSMCs of passages 3 and 13. The cells were simultaneously stained for PKG-I and SM-Act with fluorescein-conjugated and Texas red-conjugated secondary antibodies, respectively. To facilitate the localization of each individual cell, nuclear staining with DAPI was performed. Original magnification was 200×. In agreement with the well-observed phenotypic modulation of cultured AoSMCs, p3 cells were smooth muscle-like while p13 cells were fibroblastic in morphology. However, the expression levels of PKG-I and SM-Act between these two passages were essentially identical. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

Effect of Cell Density on PKG-I Expression

Cornwell et al. [1994b] also reported that rat AoSMCs expressed much less PKG-I when cultured at low densities. To verify this observation, we seeded p4 cells at 7.5×10^4, 1.0×10^4, 0.75×10^4, and 0.2×10^4 cells/cm^2. Cells were allowed to grow for 3 or 7 days and harvested when they reached densities of 11.5×10^4, 1.73×10^4, 1.05×10^4, and 1.08×10^4 cells/cm^2,

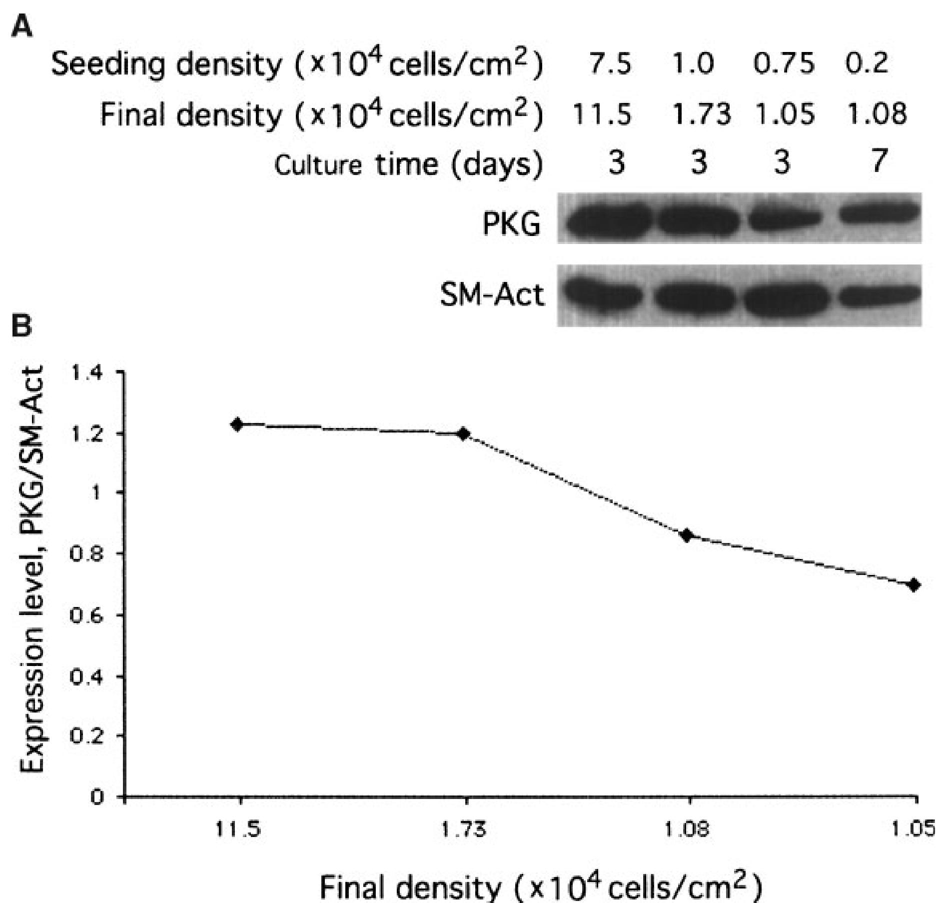

Fig. 3. Western blot analysis of PKG-I expression in rat AoSMCs seeded or harvested at different cell densities. Cells of the fourth passage were seeded at four different densities as shown at the top. Those that were seeded at higher densities (7.5, 1.0, and 0.75×10^4 cells/cm^2) were allowed to grow for 3 days, while the one seeded at 0.2×10^4 cells/cm^2 was grown for 7 days. Equal amount (20 µg) of cellular protein from each of these four different seeding/growing conditions was then resolved in 8% SDS–PAGE and subsequently transferred to PVDF membrane. After the detection of PKG-I, the membrane was stripped and then probed for SM-Act. For quantitative representation, the PKG-I and SM-Act protein bands shown in **Panel A** were analyzed by densitometry and the ratio of their expression levels determined for each cell density (**B**).

respectively. As shown in the first two lanes of Figure 3A, cells seeded at 7.5×10^4 and 1.0×10^4 cells/cm^2 (and harvested at 11.5×10^4 and 1.73×10^4 cells/cm^2, respectively) expressed identical levels of PKG (densitometry results shown in Fig. 3B). At lower seeding (0.75×10^4, and 0.2×10^4 cells/cm^2) or harvesting (1.05×10^4, and 1.08×10^4) densities, PKG expression was slightly lower (Lanes 3 and 4, Fig. 3A,B).

Effect of Cell Passages on PKG-I Activation

While we observed little differences in PKG-I expression in cells at different passages (Fig. 1), we wondered whether PKG-I activation could be affected by cell passages. We [Lin et al., 2001] have previously shown that activation of PKG-I could be detected by western blotting using antibody VASP-16C2 that detects PKG-I-phosphorylated VASP [Smolenski et al., 1998]. By using this method, we observed that, in the absence of cGMP, p4 and p11 AoSMCs expressed similar levels of P-VASP (Fig. 4). When treated with cGMP for 10 min, only p4 AoSMCs expressed an elevated level of P-VASP. When treated with cGMP for 24 h, p4 AoSMCs still expressed more P-VASP than p11 AoSMCs (Fig. 4).

DISCUSSION

The initial observation that cultured AoSMCs lost PKG-I expression after a few passages led to the hypothesis that loss of PKG-I expression might account for the phenotypic modulation in VSMCs and for the neointimal formation in injured blood vessels [Lincoln et al., 2001]. This hypothesis has been reinforced in several

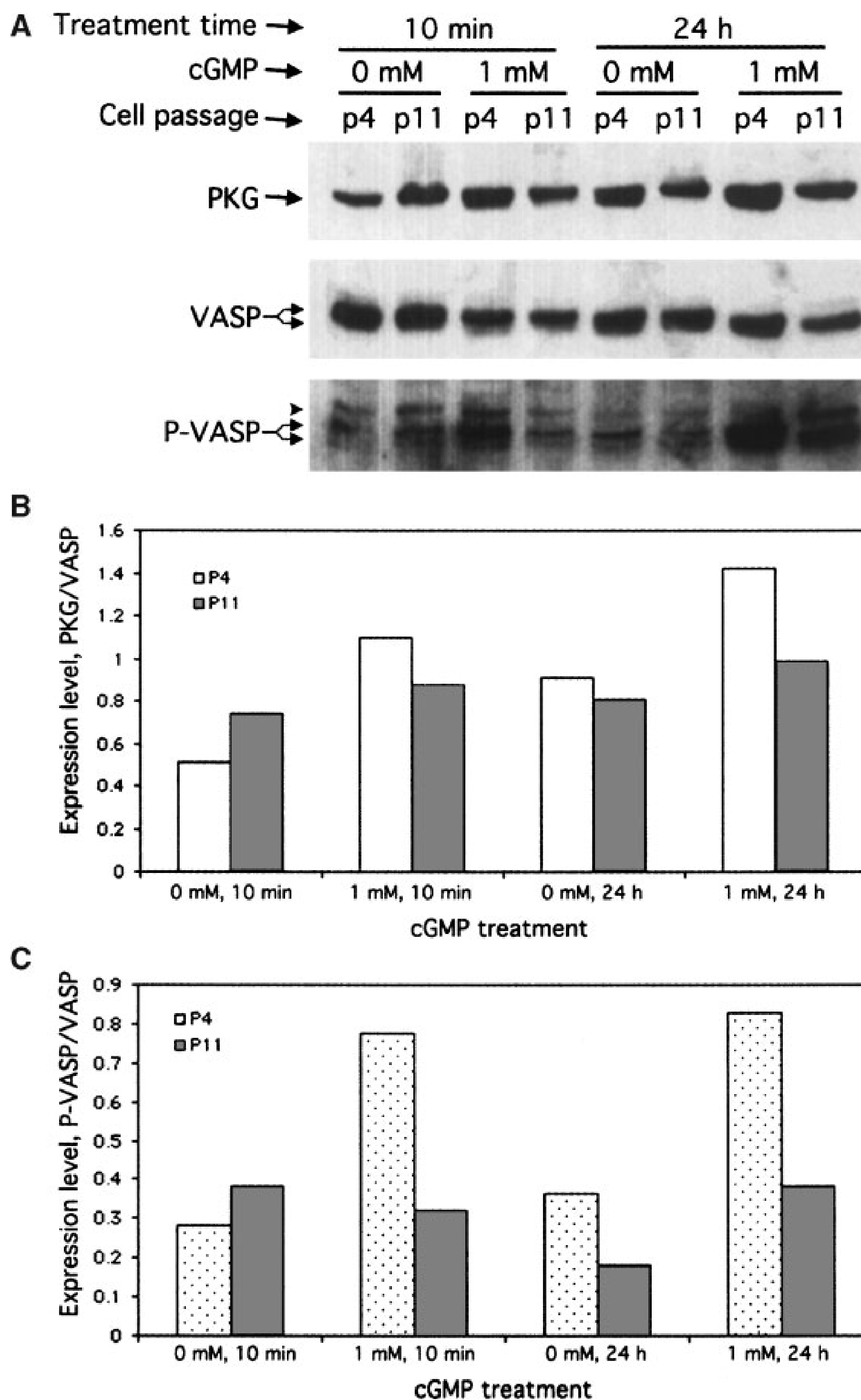

Fig. 4. Western blot analysis of PKG-I activation in rat AoSMCs of the fourth and eleventh passages. Cells were treated with 0 or 1 mM of cGMP for 10 min or 24 h. Equal amount (20 μg) of cellular protein from each of these eight treatment conditions was then resolved in 8% SDS–PAGE and subsequently transferred to PVDF membrane. Based on our experience that the detection of phosphorylated VASP (P-VASP) was technically challenging, the membrane was first incubated with antibody VASP-16C2 to detect P-VASP. It was then stripped and probed for PKG-I, stripped again and probed for VASP with antibody M4. For quantitative representation, the PKG-I, VASP, and P-VASP protein bands shown in **Panel A** were analyzed by densitometry and the ratios between PKG-I and VASP (**B**) and between P-VASP and VASP (**C**) determined for each treatment. VASP and P-VASP are sometimes resolved into two bands [Smolenski et al., 1998] as indicated by the two-headed arrows. The arrowhead indicates a nonspecific band.

follow-on studies by the same or associated authors and has been cited by other investigators to support their own work. As such, we were surprised by the seemingly constant level of PKG-I expression in our cultured AoSMCs during the course of our investigation of PKG-I activation or in efforts to extend the useful life span of these cells. To clarify the discrepancy, we conducted many repetitive experiments with many independently isolated AoSMCs, and the overall results led us to conclude that PKG-I was stably expressed in AoSMCs up to passage number 15. Furthermore, Cornwell et al. [1994b] reported that AoSMCs expressed very different levels of PKG-I when grown to different densities (PKG-I decreased from 600 ng/mg to 180 ng/mg when cell density changed from 40,000 cells/cm^2 to 10,000 cells/cm^2). However, we observed no difference in PKG-I expression between cells grown to 115,000 and 17,300 cells/cm^2. Although we did observe lowered PKG-I expression at the next lower cell density (10,500 cells/cm^2), the difference was relatively small when compared with that observed by Cornwell et al.

While our results showed stable PKG-I expression in cultured AoSMCs, two previously published studies have examined the consequences of the lack of PKG-I expression on aortic smooth muscle and on AoSMC growth characteristics. PKG-I deficient mice, despite being hypertensive and unresponsive to NO or cGMP stimulation, have histologically normal aorta [Pfeifer et al., 1998]. That is, lack of PKG-I expression did not result in vascular smooth muscle hyperplasia, as one would have predicted on the basis of the hypothesis put forth by Lincoln and associates. In addition, a more recent study showed that AoSMCs isolated from PKG-I deficient mice are indistinguishable from AoSMCs isolated from wild-type mice in the morphology and general growth characteristics [Feil et al., 2002]. As such, neither the presence nor absence of PKG-I expression seems to affect the growth characteristics of AoSMCs. This conclusion nevertheless does not dispute the importance of PKG-I in mediating vasorelaxation. Perhaps the cAMP-PKA signaling pathway, which remains intact in the PKG-I deficient mice [Pfeifer et al., 1998] and is believed to play a more significant role than the cGMP-PKG pathway does in modulating VSMC proliferation [Koyama et al., 2001], is sufficient to keep cellular growth in check. Lending support to this contention is the demonstration that the antiproliferative effects of NO and cGMP involve the activation of PKA but not PKG-I [Cornwell et al., 1994a].

In agreement with the well-observed phenomenon of phenotypic modulation, we found that AoSMCs of later passages grew faster and assumed a more fibroblastic morphology. This led us to wonder if PKG-I activation differed between cells of earlier and later passages. Based on cGMP-induced phosphorylation of VASP, we demonstrated that cells of the fourth passage indeed had higher level of PKG-I activation than cells of the eleventh passage. Whether this observation could explain the phenotypic modulation requires further studies.

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