

# Cyclic Guanosine Monophosphate-dependent Protein Kinase I Promotes Adhesion of Primary Vascular Smooth Muscle Cells

Pascal Weinmeister,\* Robert Lukowski,\* Stefan Linder,†  
Claudia Traidl-Hoffmann,‡ Ludger Hengst,§ Franz Hofmann,\* and Robert Feil||

\*Institut für Pharmakologie und Toxikologie, Technischen Universität München, D-80802 München, Germany; ‡ZAUM-Zentrum Allergie und Umwelt, KKG-UDA, Helmholtzzentrum München/TUM, D-80802 München, Germany; †Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten der Ludwig-Maximilians-Universität München, D-80336 München, Germany; §Sektion für Medizinische Biochemie, Medizinische Universität Innsbruck, A-6020 Innsbruck, Austria; and ||Interfakultäres Institut für Biochemie, Universität Tübingen, D-72076 Tübingen, Germany

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The cyclic guanosine monophosphate (cGMP)/cGMP-dependent protein kinase type I (cGKI) pathway regulates many cellular functions. The current study shows that 8-Br-cGMP stimulates the number of attached primary but not that of subcultured murine vascular smooth muscle cells (VSMCs). These effects of 8-Br-cGMP require the presence of cGKI. In agreement with previous studies, cGKI inhibited the number of cells in repeatedly passaged murine VSMCs. Activation of the cGMP/cGKI pathway in freshly isolated primary VSMCs slightly decreased apoptosis and strongly increased cell adhesion. The stimulation of cell adhesion by cGKI involves an inhibition of the RhoA/Rho kinase pathway and increased exposure of  $\beta_1$  and  $\beta_3$  integrins on the cell surface. Together, these results identify a novel proadhesive function of cGMP/cGKI signaling in primary VSMCs and suggest that the opposing effects of this pathway on VSMC number depend on the phenotypic context of the cells.

## INTRODUCTION

The nitric oxide (NO)/cyclic guanosine monophosphate (cGMP)/cGMP-dependent protein kinase type I (cGKI) pathway regulates important functions in many cell types, including vascular smooth muscle cells (VSMCs) (Feil *et al.*, 2003; Lohmann and Walter, 2005; Hofmann *et al.*, 2006; Lincoln *et al.*, 2006). In addition to its potent vasorelaxant properties, it has been associated with vascular proliferative diseases, such as atherosclerosis and restenosis (von der Leyen *et al.*, 1995; Janssens *et al.*, 1998; Varenne *et al.*, 1998; von der Thusen *et al.*, 2004). Several studies (an excellent summary is given in Lincoln *et al.*, 2006) suggested that activation of cGKI-dependent pathways has antimitogenic effects in smooth muscle cells (SMCs) in culture (Garg and Hassid, 1989; Boerth *et al.*, 1997; Chiche *et al.*, 1998; Brophy *et al.*, 2002) and might be vasculoprotective in the intact animal (Anderson *et al.*, 2000; Sinnaeve *et al.*, 2002). Despite a large body of evidence that active cGKI acts antiproliferative, Hassid and coworkers reported that NO/cGMP amplified the proliferative response of fibroblast growth factor-2 in primary VSMCs (Hassid *et al.*, 1994).

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Address correspondence to: Pascal Weinmeister (weinmeister@ipt.med.tu-muenchen.de).

Abbreviations used: cAK, cAMP-dependent protein kinase; cGKI, cGMP-dependent protein kinase type I; VSMC, vascular smooth muscle cell.

These results were supported by a report that NO and cGMP analogues activated rather than inhibited the mitogen-activated protein (MAP) kinase pathway in freshly isolated rat aortic VSMCs (Komalavilas *et al.*, 1999). An increased MAP kinase activity has been associated with an increase in cell proliferation. The antiproliferative effect of cGMP/cGKI signaling could not be confirmed by a study on atherosclerosis in an *in vivo* mouse model (Wolfsgruber *et al.*, 2003). This study suggested a proatherogenic role of VSMC cGKI. Furthermore, the recent analysis of several murine models for restenosis did not show significant effects of the presence or absence of cGKI in VSMCs on restenosis (Lukowski *et al.*, 2008).

Previous *in vitro* and *in vivo* studies led to the hypothesis that cGKI may affect different functions in primary and cultured VSMCs. A loss or reduction of cGKI, as it may occur during passage of VSMCs, is associated with the development of a synthetic phenotype (reviewed in Lincoln *et al.*, 2006). Because the inhibitory effects of cGKI on cell number were observed predominantly in passaged VSMCs, whereas the stimulatory effect of cGKI was found in primary VSMCs (Wolfsgruber *et al.*, 2003), the contrary findings might depend on the phenotype of the VSMCs, i.e., synthetic versus contractile smooth muscle cells.

In the present study, we analyzed the modulatory effects of cGMP on cell number in primary and passaged aortic VSMCs from control and cGKI-deficient mice (Wegener *et al.*, 2002). We conclude that cGKI acts antiproliferative on passaged VSMCs, whereas it promotes the cell number of freshly isolated VSMCs primarily via stimulation of cell adhesion.

## MATERIALS AND METHODS

### Materials

8-Bromoguanosine-3', 5'-cyclic monophosphate was purchased from Biolog (Hayward, CA), U-46619 was from Alexis Laboratories (San Diego, CA), and H1152 was from Calbiochem (San Diego). Blocking antibodies against  $\beta_1$  and  $\beta_3$  integrins (Schultz and Armant, 1995; Ridger *et al.*, 2001) were purchased from Biologend (San Diego, CA) (CD29, CD61). All other reagents and chemicals were purchased from Roth (Karlsruhe, Germany), Invitrogen (Carlsbad, CA), or Sigma-Aldrich (St. Louis, MO).

### Mouse Breeding

Wild-type, heterozygous, and homozygous cGKI knockout mice were obtained as described previously (Pfeifer *et al.*, 1998; Wegener *et al.*, 2002; Weber *et al.*, 2007). The animals used in this work were generated from heterozygous cGKI<sup>+/-</sup> mice on a 129/Sv genetic background (Wegener *et al.*, 2002). VSMCs isolated from wild-type (cGKI<sup>+/+</sup>) and heterozygous (cGKI<sup>+/-</sup>) mice showed identical results, and they were thus used as controls (ctr). Animals were handled according to German animal protection law.

### Cell Culture

Aortic VSMCs were isolated from control mice (cGKI<sup>+/+</sup> and cGKI<sup>+/-</sup>) and cGKI knockout (ko) mice (cGKI<sup>-/-</sup>) by enzymatic digestion and grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen) and 1% Pen/Strep (Invitrogen) at 37°C and 6% CO<sub>2</sub> as described previously (Kuhbandner *et al.*, 2000; Wolfsgruber *et al.*, 2003). To establish subcultures, primary VSMCs were grown to 80–90% confluence, trypsinized, counted, and replated at a density of 200,000 cells/100-mm cell culture dish for further passaging.

### Determination of Cell Number

To determine the cell number of attached cells, VSMCs were plated in 96-well plates (primary cells at 20,000 cells/well and passaged cells at 5000 cells/well) and cultured for 3 d in the absence and presence of drugs. If not stated otherwise, drugs were added before plating. The number of attached cells was determined by the CellTiter 96 AQ–NonRadioactive Cell Proliferation Assay (MTS assay) (Promega, Madison, WI), which is an indirect method of cell quantification and involves the bioreduction of a tetrazolium salt by living cells to a colored formazan product. The assay was used according to the manufacturer's protocol. Briefly, after cells were washed once with serum-free medium, 100  $\mu$ l of serum-free medium was added to each well followed by 20  $\mu$ l of MTS solution and incubation at 37°C and 6% CO<sub>2</sub>. The OD<sub>495</sub> was measured after 30 and 60 min by using a plate reader (Titertek Multiscan MCC/340; Titertek, Huntsville, AL). To validate the results obtained with the MTS assay, the toluidine blue (TB) assay was performed subsequently to the MTS assay by using the same wells. Staining of cells with TB is a more direct measure of cell number compared with the MTS assay. Cells were fixed and stained with TB solution (0.5% TB, 2% formaldehyde, and 0.2% glutaraldehyde in phosphate-buffered saline [PBS]) for 10 min at room temperature. Then, excessive TB solution was removed by five successive washing steps with PBS. Finally, the cells were destained in 100  $\mu$ l of 1% SDS, and the OD<sub>620</sub> was measured. Both assays gave comparable results, supporting the significance of our experiments. To analyze integrin-mediated adhesion, integrin blocking antibodies were added to the cells before plating. For each experiment a 24-well plate was treated accordingly to confirm the obtained values by microscopy.

For the analysis of cell attachment kinetics, freshly isolated VSMCs from control and cGKI-deficient animals were seeded at a density of 125,000 cells/ml in a  $\mu$ -Dish<sup>35 mm, low</sup> with Grid-500 (ibidi) in the absence and presence of 100  $\mu$ M 8-Br-cGMP. Pictures were taken 4 h after seeding the cells and subsequently every 24 h. Five representative fields of view were analyzed for each condition and point in time.

### Time-Lapse Microscopy

Freshly isolated VSMCs (125,000) were cultured in a 24 well plate for each condition in 1 ml of CO<sub>2</sub>-free medium (Invitrogen) supplemented with 10% FCS, 2 mM L-glutamine, and 1% Pen/Strep. Cell kinetics was recorded for 72 h in the absence and presence of 1 mM 8-Br-cGMP. Recording was started 4 h after seeding to allow sedimentation of the cells. The microscope stage and the surrounding were kept at 37°C.

### RhoA Activity Assay

RhoA activity was determined by the G-Lisa assay (Cytoskeleton, Denver, CO) according to the manufacturer's manual. To stimulate RhoA activity, cells were treated with the thromboxane mimetic U-46619 at 3  $\mu$ M for 5 min before lysis.

### Flow Cytometry

To detect apoptotic cells, a human annexin V fluorescein isothiocyanate (FITC) kit (Bender MedSystems, Vienna, Austria) was used according to the

manufacturer's protocol. Briefly, freshly isolated primary VSMCs were incubated in a Falcon tube (200,000 cells/ml) in DMEM supplemented with 10% FCS at 37°C and 6% CO<sub>2</sub> in the absence and presence of 1 mM 8-Br-cGMP. Immediately after isolation, and after 4, 6, 10, and 24 h, aliquots of 100,000 cells were taken, stained with FITC-labeled annexin V and propidium iodide (PI), and subjected to flow cytometry by using a FACSCalibur (BD Biosciences, San Jose, CA). Cells (10,000) of each sample were counted, and data were analyzed with Cell Quest Pro (4.62). Annexin V-positive and PI-negative cells were considered apoptotic and used for statistical analysis.

To analyze integrin exposure on the cell surface, freshly isolated primary VSMCs were incubated for 24 h in DMEM supplemented with 10% FCS at 37°C and 6% CO<sub>2</sub> in the absence and presence of 1 mM 8-Br-cGMP. Then, 150,000 cells were stained with antibodies against  $\beta_1$  or  $\beta_3$  integrins followed by a secondary FITC-labeled antibody and flow cytometry (in 5% FCS in PBS, pH 7.4). Dead cells were excluded by PI staining. Cells (10,000) per sample were counted, and data were analyzed with CellQuest Pro 4.62 (BD Biosciences).

### Immunocytochemistry

Cells were plated on glass coverslips in a 24-well plate at a cell density of 100,000 cells/well. After 2 to 3 d of culture, cells were washed twice with PBS and fixed in 3.7% Formalin in PBS. Subsequently, cells were permeabilized with ice-cold (–20°C) acetone for 5 min, washed with 1% bovine serum albumin in PBS and incubated with 5% normal goat serum in PBS for 10 min. VSMCs were stained for vinculin (Sigma-Aldrich) for 45 min. After three washing steps, cells were incubated with an appropriate fluorescence-labeled secondary antibody (Alexa 488) for 30 min. For F-actin staining, cells were stained with rhodamine-phalloidin (Invitrogen). After staining, the cells were embedded in Mowiol (Calbiochem) with *p*-phenylenediamine (Sigma-Aldrich). Photomicrographs were taken with a confocal microscope (TCS NT [Leica, Wetzlar, Germany] or LSM 510 [Carl Zeiss, Jena, Germany]).

### Western Blot

To analyze the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), 100,000 cells/well were seeded in a six-well culture plate. Cells were grown to 80–90% confluence, and then they were serum starved for 48 h and treated with different compounds for 30 min in serum-free medium. Cells were harvested with lysis buffer (21 mM Tris HCl, pH 8.0, 0.7% SDS, 1.7%  $\beta$ -mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride) followed by Western blot analysis using antibodies for cGKI (Pfeifer *et al.*, 1998),  $\beta$ -actin (Abcam, Cambridge, MA), and VASP (Alexis Laboratories). For detection of RhoA (Santa Cruz Biotechnology, Santa Cruz, CA) expression, primary VSMCs were cultured for 3 d in the absence and presence of 100  $\mu$ M 8-Br-cGMP.

### Statistics

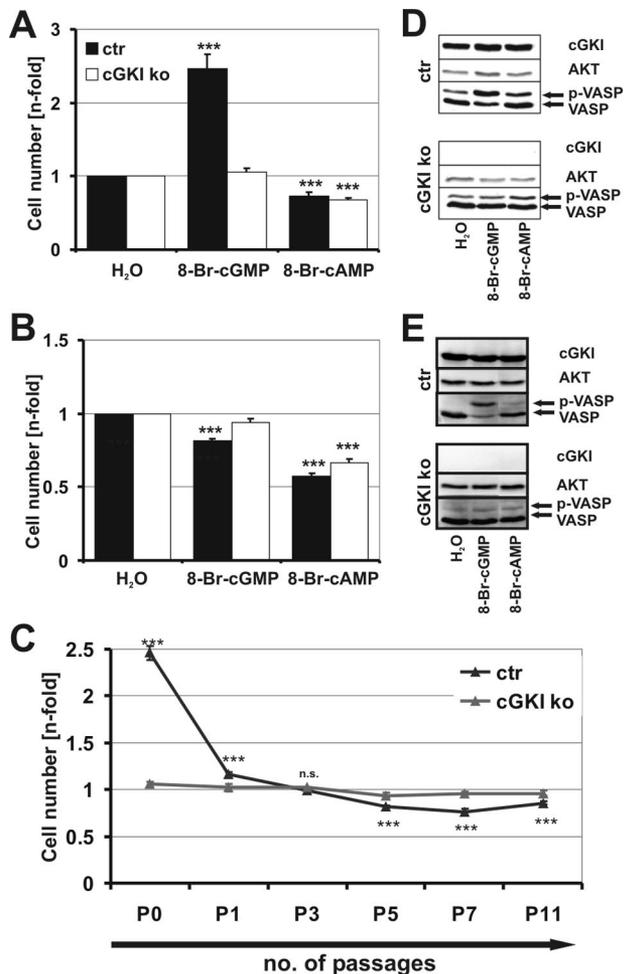
If not otherwise reported, experiments were reproduced at least three times with similar results. Data are presented as mean  $\pm$  SEM (n = 6–8) per experiment. Most data were analyzed with Origin Pro 6.1 software (Origin-Lab, Northampton, MA). To compare groups, an unpaired Student's *t* test was used. Apoptosis was analyzed by two-way analysis of variance by using the Prism 4.0 software (GraphPad Prism Software, San Diego, CA). Significance levels were ns, not significantly different; and \*, \*\*, and \*\*\* denote significant differences at p < 0.05, p < 0.01, and p < 0.001, respectively.

## RESULTS

### cGKI Promotes Recovery of Primary VSMCs in Culture

Murine primary aortic VSMCs were incubated under standard culture conditions. The number of cells was compared after 72-h incubation in the presence of different compounds. The number of viable attached cells was determined by using a colorimetric NonRadioactive Cell Proliferation Assay (CellTiter 96 AQ; Promega) and toluidine blue staining. Quantitative results were confirmed by microscopy (e.g., Figure 2B), which could exclude increase in cell size as major cause for increased cell number. Concentrations of 0.1 and 1 mM 8-Br-cGMP were chosen for cell treatment according to a dose-response curve of 8-Br-cGMP on cell attachment (Supplemental Figure 1).

The number of murine primary aortic VSMCs was not affected by the presence or absence of cGKI under standard culture conditions (Supplemental Figure 2), indicating that cGKI is dispensable for basal adhesion. Addition of the membrane-permeable cGMP analogue 8-Br-cGMP significantly increased the number of primary VSMCs in the pres-



**Figure 1.** Cell culture of control (ctr) and cGKI-deficient (ko) primary and subcultured VSMCs. Number of attached cells of primary (A) and subcultured passage 5 (B) VSMCs. Cell number was measured in the absence (H<sub>2</sub>O) and presence of 8-Br-cGMP (100  $\mu$ M) or 8-Br-cAMP (100  $\mu$ M) and normalized to control conditions (H<sub>2</sub>O). (C) cGKI-mediated growth of VSMCs in culture. Growth performance of primary (P0) up to passage 11 (P11) VSMCs in response to 8-Br-cGMP (100  $\mu$ M) (MTS assay). Cell number was normalized to control. (D and E) Phosphorylation of VASP in response to 8-Br-cGMP (100  $\mu$ M) and 8-Br-cAMP (100  $\mu$ M) in primary (D) and subcultured passage 5 (E) VSMCs. An antibody against cGKI was used to demonstrate the presence and absence of cGKI protein. Akt was used as loading control. See *Materials and Methods* for further details.

ence but not in the absence of cGKI (Figure 1A). Similar results were obtained when 8-Br-cGMP was replaced by the cGMP analogues 8-Br-PET-cGMP or 8-pCPT-cGMP (data not shown). In contrast, 8-Bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP), a stimulator of cAMP-dependent protein kinase (cAK), reduced the number of primary and subcultured VSMCs in a cGKI-independent manner (Figure 1, A and B). In line with previous reports (Garg and Hassid, 1989; Boerth *et al.*, 1997; Chiche *et al.*, 1998; Brophy *et al.*, 2002), 8-Br-cGMP acts antiproliferative on passaged murine VSMCs in a cGKI-dependent manner (Figure 1B). The inhibitory effect of cGKI in subcultured murine cells was relatively weak and became statistically significant after five passages of the VSMCs (Figure 1, B and C). Interestingly, the strong cGKI-dependent increase in cell number in primary cells is already lost with the first passage (Figure 1C). The

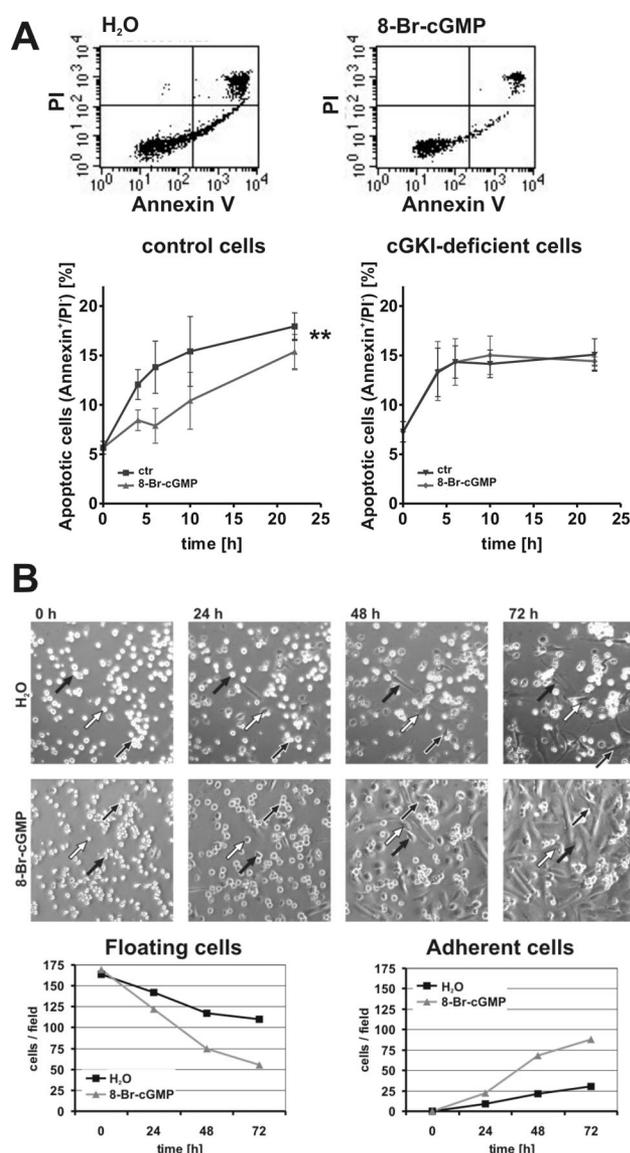
differential effects of 8-Br-cGMP on cell number of primary versus subcultured murine VSMCs was not due to a change in cGKI expression or enzyme activity during subculture as monitored by Western blot analysis of cGKI and phosphorylation of its substrate VASP (Figure 1, D and E, and Supplemental Figure 3). The phosphorylation of VASP, which is also a substrate of cAK, in response to 8-Br-cAMP was weak compared with 8-Br-cGMP-induced phosphorylation in control cells. This could be explained by the chosen stimulatory conditions that were optimized for cGKI-induced phosphorylation of VASP.

Because there is a plethora of literature describing a loss of cGKI expression during passaging (Cornwell and Lincoln, 1989; Boerth *et al.*, 1997), the obtained results for murine VSMCs were compared with VSMCs derived from rat and human to exclude an species specific effect (Supplemental Figure 4). Subcultured rat (Supplemental Figure 4A) as well as subcultured human (Supplemental Figure 4B) SMCs expressed cGKI. Furthermore, as revealed by phosphorylation of VASP, cGKI was still active.

#### *cGKI Promotes Cell Number of Primary VSMCs by Increasing Adhesion*

The above-mentioned experiments clearly demonstrated that an activation of cGKI increased the number of attached viable cells after 3 d in primary culture (Figure 1A). This effect of cGKI could be caused by several mechanisms, including 1) an antiapoptotic effect of cGKI; 2) a promitogenic effect, i.e., a proliferative response; and/or 3) an increased number of cells adhering to the culture dish. Activation of cGKI slightly but significantly decreased apoptosis during the initial time period (24 h) after isolation of the cells from the aorta (Figure 2A). This finding was unexpected, because a proapoptotic effect of cGKI has been reported in SMCs (Chiche *et al.*, 1998). However, the observed antiapoptotic effect was too small to explain the two- to threefold increase in the number of primary VSMCs attached to the culture dish after 72 h in the presence of 8-Br-cGMP (Figure 1A). To dissect the potential effects of cGMP on cell adhesion and/or proliferation, the attachment of freshly isolated VSMCs was monitored by time-lapse microscopy during the first 72 h after plating. Whereas dividing cells were not observed during this period, treatment with 8-Br-cGMP strongly increased the fraction of adherent cells (Figure 2B). Note that equal amounts of cells were plated in the absence and presence of 8-Br-cGMP (Figure 2B). To proof the cGKI-mediated increase in cell adhesion in control cells and to rule out the effect in cGKI-deficient VSMCs, the time-lapse experiment was repeated in an alternative approach. Freshly isolated VSMCs from control and cGKI-deficient animals were seeded in a  $\mu$ -Dish, and attachment of cells was documented by taking photomicrographs every 24 h (Supplemental Figure 5). The detailed analysis of this experiment confirmed a cGKI-mediated increased adhesion; hence, the absence of a proadhesive effect in response to 8-Br-cGMP in the cGKI-deficient cells (Supplemental Figure 5). The identical results of these two alternative approaches exclude the possibility that the observed effect is caused by the chosen method.

The proadhesive effect of 8-Br-cGMP was accompanied by the occurrence of stress fibers and focal adhesions as visualized by staining of fixed cells for F-actin and vinculin, respectively. These cytoskeletal changes in response to 8-Br-cGMP could not be observed in cGKI-deficient cells (Figure 3A). To exclude a proliferative effect and to strengthen the observed effect of cGKI on cell adhesion, VSMCs were allowed to attach in the absence of 8-Br-cGMP for 3 d. Subse-



**Figure 2.** Analysis of apoptosis and cell attachment kinetics. (A) Analysis of apoptosis of primary control and cGKI-deficient VSMCs. Representative original measurements of primary control VSMCs after 6 h in suspension in the absence (H<sub>2</sub>O) and presence of 8-Br-cGMP (1 mM). Cells were labeled with Annexin V and PI. The time course of the appearance of apoptotic cells (Annexin V-positive and PI-negative) in control and cGKI-deficient cell preparations is shown in the bottom diagrams. (B) Analysis of cell number and attachment by time-lapse videomicroscopy. VSMCs were plated in the absence (H<sub>2</sub>O) and presence of 8-Br-cGMP (1 mM) and monitored for 72 h. Representative pictures are shown at the indicated times. Arrows highlight individual cells during the time of observation. The number of adherent and floating cells in the absence and presence of 8-Br-cGMP was determined for each microscopic field (bottom).

quently, adherent cells were exposed to 8-Br-cGMP for further 3 d. A slight, although significant increase in cell number could be observed (Figure 3B), confirming a previous observation (Wolfsgruber *et al.*, 2003). These results indicated that the cGMP/cGKI-stimulated increase in cell number at 72 h of primary culture was mainly due to an increase in the efficiency of cell adhesion rather than to an effect on cell proliferation or apoptosis.

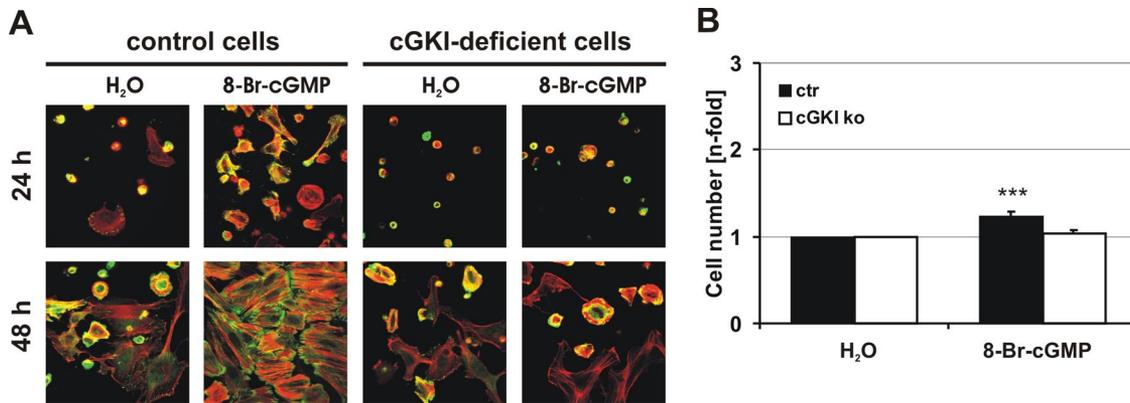
### Adhesion of Primary VSMCs Is Mediated by Inhibition of the RhoA/Rho Kinase Pathway

Cell adhesion is regulated by a number of signaling pathways, including the RhoA/Rho kinase pathway (Ridley and Hall, 1992; Rottner *et al.*, 1999; Worth *et al.*, 2004). The small GTPase RhoA is a known substrate of cGKI (Sauzeau *et al.*, 2003; Rolli-Derkinderen *et al.*, 2005). As expected from a previous report (Sauzeau *et al.*, 2003), treatment of primary VSMCs with 8-Br-cGMP increased the amount of total RhoA protein in control but not in cGKI-deficient cells (Figure 4A), indicating that activation of cGKI increased the RhoA concentration. This effect on the RhoA protein level could be mediated via phosphorylation of RhoA at Ser188, thereby possibly affecting RhoA activity by stabilization of an inactive, cytosolic RhoA-guanosine triphosphate (GTP)/GDP dissociation inhibitor (GDI) complex (Sauzeau *et al.*, 2000; Rolli-Derkinderen *et al.*, 2005). Indeed, activation of RhoA via the thromboxane receptor agonist U-46619 (Wettschreck and Offermanns, 2005; Rieken *et al.*, 2006) was efficiently blocked in the presence of 8-Br-cGMP (Figure 4B), as shown previously (Sauzeau *et al.*, 2000; Seko *et al.*, 2003). In line with a role of RhoA in VSMC adhesion, the number of adhering primary cells was suppressed by U-46619. Importantly, 8-Br-cGMP prevented the inhibitory effect of U-46619 in control but not cGKI-deficient cells (Figure 4C). The antiadhesive effect of U-46619 treatment was also reflected by a strong reduction in F-actin and vinculin staining (Figure 4D). These results support the notion that the proadhesive effect of cGMP/cGKI is mediated, at least in part, via inhibition of RhoA signaling.

A well characterized downstream target of RhoA is Rho kinase. Inhibition of this kinase has been reported to increase cell adhesion (Koga *et al.*, 2006). Addition of the Rho kinase inhibitor H1152 to the primary VSMCs mimicked the effects of 8-Br-cGMP on cell number and morphology (Figure 5). Similar results were obtained with Y27632, another well known Rho kinase inhibitor (data not shown). The observations that coapplication of 8-Br-cGMP and H1152 did not cause an additive effect (Figure 6C) and that H1152 also led to an increase in cell number of cGKI-deficient cells (Figure 5A) suggested that cGKI and Rho kinase are components of the same pathway and that Rho kinase acts downstream of cGKI.

### cGKI-stimulated Adhesion Is Mediated via Integrins

It is generally accepted that adhesion of cells is linked to integrins (Schwartz *et al.*, 1995; Berrier and Yamada, 2007). To analyze whether integrins are also involved in cGKI-mediated adhesion, primary VSMCs derived from control and cGKI-deficient mice were labeled for  $\beta_1$  and  $\beta_3$  integrins, two integrins that are known to be important for adhesion. Both integrins could be detected on the cell surface by flow cytometry. Addition of 8-Br-cGMP increased the mean fluorescence signal for  $\beta_1$  and  $\beta_3$  integrins, indicating an increase in integrin presentation at the cell membrane. This effect could only be detected in control but not in cGKI-deficient cells (Figure 6A). To determine whether  $\beta_1$  and/or  $\beta_3$  integrins were functionally involved in cGKI-dependent adhesion of primary VSMCs, the number of attached cells was analyzed in the presence of  $\beta_1$  and/or  $\beta_3$  blocking antibodies. As shown in Figure 6B, 8-Br-cGMP-stimulated adhesion was reduced by antibodies against  $\beta_1$  as well as  $\beta_3$  integrin. Moreover, coadministration of both antibodies resulted in an additive effect and abolished the proadhesive effect of 8-Br-cGMP. Importantly, the integrin-blocking antibodies decreased the proadhesive stimulation



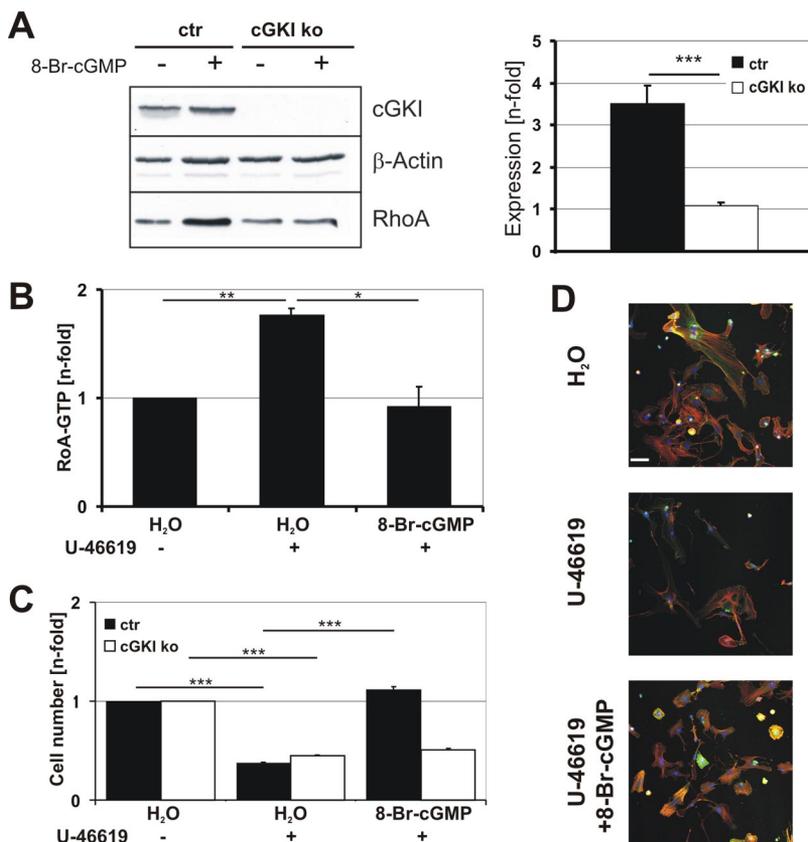
**Figure 3.** Analysis of cell adhesion and proliferation. (A) Cytoskeletal staining of primary control and cGKI-deficient VSMCs. Cells were grown on glass coverslips for 24 and 48 h in the absence (H<sub>2</sub>O) or presence of 8-Br-cGMP (1 mM). VSMCs were stained for F-Actin (red) and vinculin (green) as marker for focal adhesions. Photographs were taken with a confocal microscope (TCS NT; Leica). (B) Primary VSMCs were grown for 72 h under control conditions. Subsequently, 8-Br-cGMP (100 μM) was added for additional 72 h.

elicited by the Rho kinase inhibitor H1152 to a similar extent (Figure 6C), corroborating the view that cGMP/cGKI stimulates integrin exposure and cell adhesion via inhibition of Rho kinase.

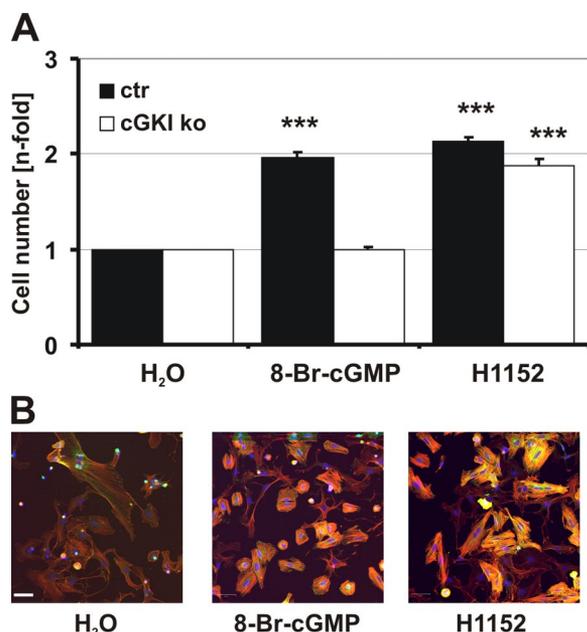
**DISCUSSION**

cGKI is an important regulator of many smooth muscle functions, including VSMC growth, adhesion, proliferation, and apoptosis (Hofmann *et al.*, 2006; Lincoln *et al.*, 2006). Many *in vitro* studies demonstrated an antiproliferative effect of cGMP and cGKI signaling in subcultured VSMCs

(Garg and Hassid, 1989; Hassid *et al.*, 1994; Li and Sun, 2005), VSMC-derived cell lines (Capey *et al.*, 2007) or VSMCs that have been transfected with cGKI (Boerth *et al.*, 1997; Browner *et al.*, 2004; Dey *et al.*, 2005). In agreement with these studies, we find that activation of the cGMP/cGKI pathway slightly inhibited proliferation of passaged murine VSMCs. In passaged murine VSMCs, the mechanism(s) leading to a reduction in cell number by cGMP is not clear. Inhibition was not associated with a loss of cGKI or its activity and in fact required the presence of cGKI. Furthermore, it is important to note that control and cGKI-deficient VSMCs showed similar basal properties and phenotypes



**Figure 4.** Adhesion of VSMCs is mediated by inhibition of RhoA. (A) Western blot analysis of total RhoA protein. Primary control (ctr) and cGKI-deficient (ko) VSMCs were grown in the absence (H<sub>2</sub>O) and presence of 8-Br-cGMP (100 μM) for 72 h. Cells were harvested, and protein lysates were subjected to Western blot (left). β-Actin was used as loading control. RhoA expression was quantified using the AIDA software by normalizing each RhoA band to its respective loading control (n = 7 ctr; n = 3 ko). The ratio of the values obtained after 8-Br-cGMP treatment to those after H<sub>2</sub>O treatment is expressed as Expression [n-fold] (right). (B) G-Lisa RhoA assay. RhoA activity (RhoA-GTP) was determined in primary control VSMCs that were grown for 72 h in the absence (H<sub>2</sub>O) or presence of 8-Br-cGMP (100 μM). Cells were treated with U-46619 (3 μM) 5 min before lysis to increase RhoA-GTP levels as indicated. All values (n = 3 for each condition) were normalized to control (H<sub>2</sub>O). (C) Primary control (ctr) and cGKI-deficient (ko) VSMCs were grown for 72 h in the absence (H<sub>2</sub>O) and presence of 8-Br-cGMP (100 μM) and/or U-46619 (3 μM) as indicated. The number of cells was determined by the MTS assay. (D) Primary control cells were grown for 72 h in the absence (H<sub>2</sub>O) or presence of 8-Br-cGMP (100 μM) or U-46619 (3 μM) and then stained for F-actin (red), vinculin (green), and DNA (Hoechst). White bar, 50 μm. Photographs were taken with a confocal microscope (LSM 510; Carl Zeiss).

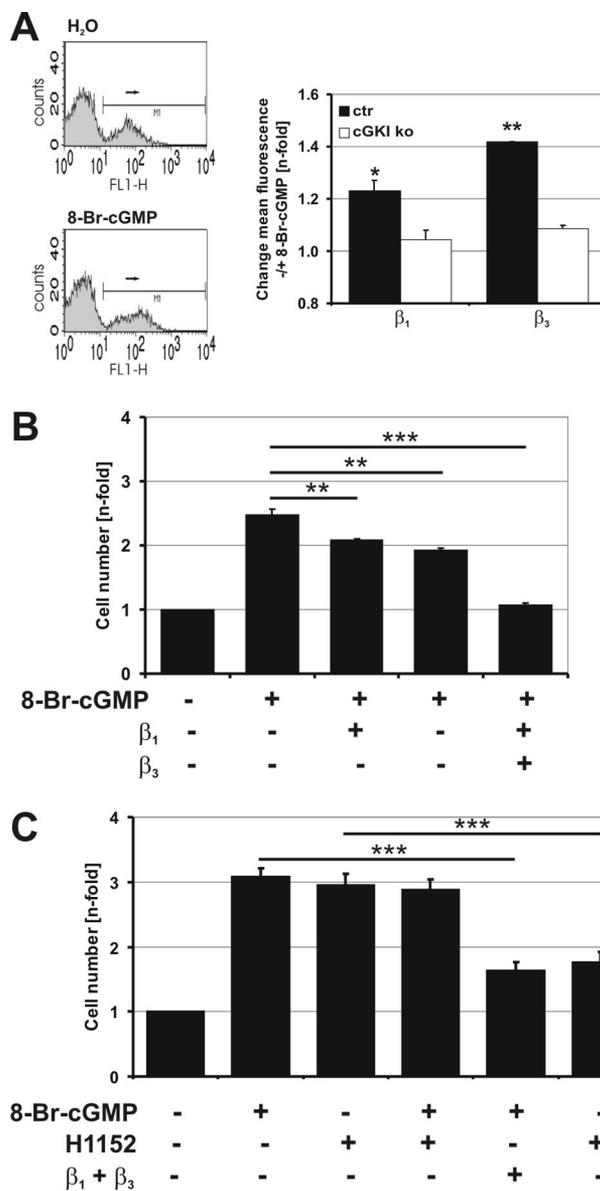


**Figure 5.** Adhesion of VSMCs is mediated by inhibition of Rho kinase. (A) Cell number of primary control (ctr) and cGKI-deficient (ko) VSMCs in the absence (H<sub>2</sub>O) and presence of 8-Br-cGMP (100  $\mu$ M) or H1152 (0.3  $\mu$ M). (B) Photographs of primary control cells were taken with a confocal microscope (LSM 510; Carl Zeiss) after 72 h in culture in the absence (H<sub>2</sub>O) and presence of 8-Br-cGMP (100  $\mu$ M) or H1152 (0.3  $\mu$ M). Cells were stained for F-actin (red), vinculin (green), and DNA (Hoechst). The merge of F-actin and vinculin staining is yellow. White bar, 50  $\mu$ m.

under standard culture conditions (Supplemental Figure 2 and Figure 3A). These results suggest that the phenotypic modulation of VSMCs during passages requires unknown factors in addition to a potential change in cGKI expression level.

In contrast to passaged VSMCs, the present work clearly demonstrates that cGKI activation increases the number of attached primary, freshly isolated VSMCs. The cGMP/cGKI-dependent increase in cell number was mainly mediated via an increase in cell adhesion as shown by 1) time-lapse microscopy, 2) enhanced exposure of integrin  $\beta_1$  and  $\beta_3$  on the cell surface; and 3) inhibition of enhanced adhesion by antibodies that block  $\beta_1$  and  $\beta_3$  integrins. The proadhesive effect is the major cause of the cGKI-dependent increase in the number of primary VSMCs after 72 h in culture. The anti-apoptotic (Figure 2A) and proliferative (Figure 3B) properties of the cGMP/cGKI pathway had a minor, although significant effect on the cell number.

The effect of cGMP/cGKI-signaling on apoptosis is discussed controversial. In SMCs, cGKI has been reported to have a proapoptotic effect (Pollman *et al.*, 1996; Chiche *et al.*, 1998). In contrast, an antiapoptotic effect of cGKI has been documented for neuronal cells (Fiscus, 2002; Ha *et al.*, 2003). The different results reported for SMCs might be related to the major differences in the chosen cell system. In contrast to our study, Chiche *et al.* (1998) used subcultured cells that were transfected with cGKI. The newly identified role for cGKI to increase adhesion of primary VSMCs might represent a protective mechanism against anoikis. It is well accepted that adhesion to the extracellular matrix is necessary for survival of differentiated adherent cells in the cardiovascular system, including endothelial cells, smooth muscle cells, fibroblasts, and cardiac myocytes (Michel, 2003).



**Figure 6.** cGKI-dependent adhesion is mediated by  $\beta_1$  and  $\beta_3$  integrins. (A) Analysis of integrin surface exposure by flow cytometry. Labeling of  $\beta_1$  and  $\beta_3$  integrins on primary control VSMCs that were grown for 24 h in suspension in the absence (H<sub>2</sub>O) or presence of 8-Br-cGMP (1 mM). The control cells show an increased mean fluorescence signal for  $\beta_3$  integrins in response to 8-Br-cGMP (left). The statistic evaluation of the  $\beta_1$  and  $\beta_3$  integrin signal in control (ctr) and cGKI-deficient (ko) VSMCs in response to 8-Br-cGMP is shown (right). Data shown were from one out of three experiments with similar results. Each experiment consisted of six independent samples. Values represent the change in fluorescence in the presence of 8-Br-cGMP compared with control (H<sub>2</sub>O). (B) Integrin-mediated adhesion of primary control VSMCs in response to 8-Br-cGMP. The MTS-assay was performed with primary VSMCs that were grown for 72 h in the absence or presence of 8-Br-cGMP (1 mM) and integrin blocking antibodies (25  $\mu$ g/ml) as indicated. Cell numbers were normalized to the value obtained in the absence of compounds. Increased adhesion in response to 8-Br-cGMP is significantly reduced upon treatment with either  $\beta$  integrin blocking antibody. (C) Comparison of integrin-mediated adhesion of primary control VSMCs in response to 8-Br-cGMP and H1152. Primary control VSMCs were grown for 72 h in the absence or presence of 8-Br-cGMP (1 mM) or H1152 (0.3  $\mu$ M) and integrin blocking antibodies (25  $\mu$ g/ml each) as indicated. Cell number was assayed by the TB method and normalized to the value obtained in the absence of compounds.

Therefore, it is likely that cGKI exerts different effects in primary and passaged VSMCs.

The cGKI is known to relax smooth muscle by several mechanisms, including inhibition of  $\text{Ca}^{2+}$  sensitization of contraction via inhibition of RhoA/Rho kinase signaling (Somlyo and Somlyo, 2003). This article adds a novel role to the interaction between the cGMP/cGKI and the RhoA/Rho kinase signaling pathways in murine VSMCs. Activation of cGKI inhibits RhoA/Rho kinase signaling, resulting in increased adhesion via  $\beta_1$  and  $\beta_3$  integrins of primary murine VSMCs. It is well accepted that RhoA/Rho kinase is involved in cell adhesion (Ridley and Hall, 1992; Burridge and Wennerberg, 2004). Here, we provide strong evidence that cGKI stimulates the attachment of primary, freshly isolated VSMCs by inhibition of the RhoA/Rho kinase pathway. The effects of 8-Br-cGMP and the Rho kinase inhibitor H1152 on cell number were not additive, and the effects of pharmacologic manipulation of RhoA and Rho kinase were independent of the genotype. In contrast, activation of cGKI efficiently suppressed RhoA activation by the thromboxane receptor agonist U-46619 as well as the U-46619-induced decrease of the number of attached cells. These results suggest that cGMP/cGKI and RhoA/Rho kinase are components of the same signaling pathway and converge in regulating cell adhesion. cGKI is downstream of the thromboxane receptor but acts upstream of RhoA and Rho kinase. Indeed, cGKI has been reported to phosphorylate and thereby stabilize the RhoA protein in an inactive RhoA-GTP/GDI complex, leading to an increase in total RhoA protein (Sauzeau *et al.*, 2000; Rolli-Derkinderen *et al.*, 2005).

Whether RhoA stimulates or inhibits adhesion seems to depend on the cell type and culture conditions (Ridley and Hall, 1992; Laudanna *et al.*, 1996; Schoenwaelder *et al.*, 2002; Worthylake and Burridge, 2003; Vielkind *et al.*, 2005; Slotta *et al.*, 2006). Increased adhesion after blockade of Rho kinase has also been described by others (Kim *et al.*, 2005; Koga *et al.*, 2006). Koga *et al.* (2006) demonstrated that inhibition of Rho kinase in human trabecular meshwork cells causes increased adhesion but that it had no influence on proliferation (Koga *et al.*, 2006). The signaling networks linking RhoA and integrins vary according to the mode of cell growth, such as adhesion of a floating cell to the substratum, spreading, or migration (Schwartz and Shattil, 2000; Danen *et al.*, 2002). For example, inhibition of RhoA/Rho kinase in leukocytes elevates integrin-mediated adhesion (Liu *et al.*, 2002; Worthylake and Burridge, 2003). The present study indicates that a similar mechanism was observed in primary VSMCs as shown by 1) a reduction in the number of attached cells upon stimulation with the thromboxane receptor agonist U-46619 that activates RhoA, 2) stimulation of the attached cell number by inhibition of Rho kinase with H1152 or Y27632, and 3) a decrease of the attached cell number in response to 8-Br-cGMP or H1152 by blocking  $\beta_1$  and  $\beta_3$  integrins.

Integrins have a pivotal role in mediating processes such as adhesion, migration, and cell-cell contacts. To allow these different processes, the expression of integrins is tightly regulated during development. For example in adult human aortic VSMCs  $\alpha_1\beta_1$  integrin is highly expressed, representing a resting, contractile phenotype, and it is strongly reduced or lost, with the beginning of cell culture (Belkin *et al.*, 1990). In line with these findings, a recent study using a conditional  $\beta_1$  integrin knockout model showed that the lack of  $\beta_1$  integrin in VSMCs causes a synthetic phenotype (Abraham *et al.*, 2008). These data support the notion that integrins have major impact on the phenotype of VSMCs that may be modulated by cGKI.

In summary, we show that cGKI promotes integrin-mediated adhesion of primary VSMCs by inhibiting the RhoA/Rho kinase pathway.

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