J. Biochem. 2011;149(4):433–441 doi:10.1093/jb/mvr003

JB THE JOURNAL OF BIOCHEMISTRY

Stabilization of cGMP-dependent protein kinase G (PKG) expression in vascular smooth muscle cells: contribution of 3′UTR of its mRNA

Received September 27, 2010; accepted December 15, 2010; published online February 3, 2011

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The type-I cGMP-dependent protein kinase (PKG-I) expression regulation is not yet completely understood. In this study, we examined the role of 3′-untranslated region (3′UTR)-PKG-I messenger RNA (mRNA) in the control of PKG-I expression in vascular smooth muscle cells (VSMCs). Using a 3′-rapid amplification of cDNA ends (RACE) for the amplification of complementary DNA (cDNA) ends, we generated and cloned a 1.2-kb-3′UTR mRNA PKG-I in pGL3 control vector downstream of the luciferase reporter gene. Serial deletions and functional studies revealed that among the deleted constructs, only the 1.2-kb-3′UTR PKG-I mRNA possesses the highest activity in transfected VSMC. Kinetic luciferase assays in the presence of actinomycin D showed that this construct stabilizes luciferase activity compared to the control vector. Sequence analysis of 3′UTR-PKG-I mRNA revealed the existence of four AU-rich regions (AU1 through AU4) in addition to a potential poly(A) site. Different riboprobes were generated either by 5′-end-labelling of designed ribonucleotides, containing individual AU-rich regions or by *in vitro* transcription assay using cloned 1.2-kb cDNA as a template. RNA-elecrophoretic mobility shift assay (EMSA) and ultra-violet cross-linking (UV-CL) assays showed that AU1, AU3, AU4 and 1.2-kb probes were able to retard cytosolic and nuclear proteins. Taken together, these data suggest that PKG-I expression is subjected to post-transcriptional regulation in VSMC through the 3′UTR of its mRNA.

Keywords: cyclic nucleotide/cGMP-dependent protein kinase/gene regulation/vascular smooth muscle cells/3′UTR mRNA.

Abbreviations: ActD, actinomycin D; CHX, cycloheximide; CN, cyclic nucleotide; EMSA, elecrophoretic mobility shift assay; IVT, *in vitro* transcription; LPS, lipopolysaccharide; mRNA, messenger RNA; PKG, cGMP-dependent protein kinase; UTR, untranslated region; UV-CL, ultra-violet cross-linking; VSMC, vascular smooth muscle cells.

cGMP is a potent biological mediator involved in diverse physiologic and pathophysiologic situations. It is produced following the stimulation of two guanylyl cyclases (sGC and pGC) (*1*, *2*). The consequence of the increase in cGMP is the activation of several cGMP-dependent proteins including cGMP-dependent protein kinase (PKG-I). PKG-I controls many cell functions including growth (*3*, *4*), differentiation (*5*), apoptosis (*6–8*) and gene regulation (*9*, *10*). PKG-I protein is constitutively expressed in several cell lines including vascular smooth muscle cells (VSMCs). However, PKG-I expression decreased in passaged VSMC in culture such as rat aortic smooth muscle cells. Under certain conditions, PKG-I decrease was accompanied with phenotype switch from contractile to synthetic, suggesting that PKG-I somehow is helping cells to maintain contractile phenotype (*11*, *12*). Several reports have shown that PKG-I expression is down-regulated in many diseases such as atheroma (*13*), diabetes (*14*, *15*) and cancer (*16–18*). Despite some progress in the understanding of PKG-I role and function in the cells, the molecular mechanisms underlying altered PKG-I expression in these and other conditions are not yet revealed.

PKG is expressed as two gene products in eukaryotic cells: PKG-I and PKG-II. PKG-I, in turn, is expressed as two alternatively spliced variants wherein the first or second exon is spliced into the initial coding sequences to yield PKG-Iα or PKG-Iβ, respectively (*19–21*). The two isoforms of PKG-I (76 kDa) are identical except for the N-terminal ~100 amino acids, which comprise the autoinhibitory, autophosphorylation and dimerization subdomains. Traditionally, PKG-I expression is believed to be regulated mainly at the transcriptional level. This regulation involves couple of described transcription factors including Sp1, upstream stimulatory factors (USFs), Krüppel-like transcription factor and FoxOa1 (*22–25*). Although PKG-I promoter is a TATA-less and CAAT-less promoter, it still contains potential binding sites for a number of transcription factors.

It is well documented that PKG-I expression varies greatly depending on cells (tissues) origin and growth conditions (*26*, *27*). The mechanism(s) underlying this differential expression is not known but a negative correlation between PKG-I and soluble quanylyl cyclase (sGC) expression was established in some VSMCs (*28*). In marked contrast, PKG-I protein is normally present in resting cells at basal levels and, to date, there is no stimuli reported to induce PKG-I expression in VSMC. Instead, PKG-I protein and messenger

RNA (mRNA) expressions were demonstrated to be subjected to down-regulation in different type of cells and tissues following exposure to immunologic or inflammatory stimuli (*29*, *30*), as well as chronic exposure to nitric oxide and cyclic nucleotide (CN) analogues (*31*, *22*). However, the steady-state levels of a particular mRNA depend not only on its synthesis but also on its rate of degradation. Indeed, nitric oxide (NO donors) (*22*), CN analogues (*30*) and inflammatory cytokines have been reported to reduce PKG-I expression, in part, by destabilizing its mRNA. The molecular mechanisms underlying altered PKG-I mRNA expression in these and other conditions have not yet been revealed. The objective of the present investigation is to characterize the mechanism accounting for PKG-I mRNA stabilization by analysing the post-transcriptional events involving its 3′-untranslated region (3′UTR).

Materials and Methods

Chemicals and reagents

[γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]UTP were purchased from Perkin Elmer Life Sciences. Restriction and modifying enzymes, reporter gene vector (pGL3-basic), luciferase system, β-galactosidase reporter gene, transfection reagent Tfx-20 and other biological compounds were purchased from Promega (Madison, WI, USA). *In vitro* transcription (IVT) kit was purchased from Ambion (Austin, TX, USA). Lipofectamine 2000 was from Invitrogen (Carlsbade, CA, USA); primers were ordered from MWG (High Point, NC, USA).

Cell culture

Bovine aortic SMCs (passage 4–8) were cultured as described previously (*30*). Embryonic rat aortic A7r5 cells and COS7 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Rat aortic SMCs were harvested from male adult rat as described previously (*31*, *32*). All cells were maintained in Dulbecco's minimal essential medium containing 10% fetal bovine serum and 50 μg/ml gentamicin. The routine subculturing procedure was performed to split the cells 1 : 4.

Rapid amplification of cDNA ends

The fragment containing the 3′-end of the PKG-I cDNA was generated using 3′-rapid amplification of cDNA ends (RACE) system. The target cDNA was amplified using several sense and antisense oligonucleotides determined from the published sequence of human PKG-Iβ (GenBank accession No, Z92868). The first strand cDNA was synthesized from total RNA and an adapter primer (AP) according to the protocol provided by the manufacturer. The amplification of targeted cDNA was performed using sense (5′-agagttcat gtcacacc-3′) and antisense (5′-cggggttacttatgac-3′) primers. All RACE amplifications were for 35 cycles with the following parameters: 94°C for 1 min, 55°C for 1 min and 74°C for 2 min. Products from both RACE procedures were gel purified and inserted into pGL3 control vector digested with XbaI–BamH1 to eliminate both SV40 poly(A) signal and the enhancer.

Generation of reporter plasmids, serial deletions and transient transfections

The cloned 3′UTR mRNA PKG-I was inserted in pGL3 control vector predigested with XbaI–BamHI downstream of luciferase cDNA to eliminate the poly(A) signal and SV40 enhancer. This vector was used to test the effect of 3′UTR mRNA PKG-I on the stability of luciferase under the control of SV40 promoter. Serial deletions of 3′UTR PKG-I mRNA were generated by polymerase chain reaction (PCR) using the cloned 3′UTR mRNA as template and primers indicated in Table I. Transient transfection of bovine and A7r5 cells was carried using 250 ng of each construct and Tfx-20 or lipofectamine 2000 (1 μl per well) as transfection reagents for A7r5 and bovine aortic SMC, respectively, following the protocol designed by the manufacturer. Forty-eight hours later, cells were lysed and luciferase assays were performed. As internal control for transfection normalization, cells were cotransfected with 100 ng of β-galactosidase reporter plasmid (pSV-β-galactosidase vector, Promega).

Table I. Oligonucleotides used for cloning serial deleted 3′UTR mRNA PKG.

Name	Sense (S, 5′→3′), Reverse (R, 5′→3′)
P1	S, gctctctagagagttcatgtcacaccagtg
P2	R, gcgcggatcccagttgtgcttttatc
P3	R, ccgcggatccgcattatttttcagtcttctgtg
P4	R, gcgcggatccggctgctagtcatttacttcc
P5	R, gcgcggatccgggttttaatccctcccc
P6	R, gcgcggatcccaaattggacaggagtttgg
P7	R, gcgcggatccggttcaggcctaagacaag
P8	R, gcgcggatccgttctctgacctcaaataag
P13	R, gcgcggatcccggggttacttatgac

IVT assay

IVT assays were performed using T7 promoter kit (Ambion INC.), according to the manufacturer's instructions. Briefly, the inserts of interest encompassing 3′UTR mRNA [Z1 (1-300), Z7 (1-670), Z8 (1-800)] were generated by PCR using different sets of primer containing T7 promoter sequence in the 5′ region and P13 (full-length 3′UTR as a template). Z13 was generated by subcloning full-length PKG-I 3′UTR mRNA in pGEM(7) vector (Promega). This construct was linearized before proceeding to IVT assay. One microgram of Z4, Z7, Z8 or Z13 was added to a mixture of ribonucleotides (rATP, rGTP, rTTP, 2.5 mM each; rUTP, 0.2 mM), transcription buffer (1X) and 50 μCi of [α-^{32}P]UTP. The reaction was started by adding 2 U of T7 RNA polymerase and incubated for 60 min at 37°C. At the end of the incubation, the template (DNA) was digested with DNase for 15 min at 37°C. The size of the transcript was verified on the denatured agarose gel and unincorporated nucleotides were removed by using RNA clean up kit (Qiagen, Santa Clarita, CA, USA).

RNA elecrophoretic mobility shift assay

Ribonucleotides used in RNA-elecrophoretic mobility shift assay (RNA-EMSA) are derived from the regions of 3′UTR encompassing AU-rich motifs of the 3′UTR of human PKG-I mRNA (underlined in Fig. 2A). Nuclear and cytosolic proteins were purified using NE-PER-extraction kit (Pierce, Rock Ford, IL, USA) following the protocol provided by the manufacturer. The ribonucleotides (AU1, AU2, AU3 and AU4) were end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reactions were carried out for 30 min on ice using 20 μg of protein extracts, 50,000 c.p.m. of labelled ^{32}P-transcript in a final volume of 20 μl containing 10 mM Tris–HCl (pH 8.0), 100 mM KCl, 1 mM DTT, 1 μg of yeast transfer RNA (tRNA) and 10% glycerol. Heparin (0.2 μg/μl) was included to reduce nonspecific RNA-binding activity. RNA–protein complexes were resolved by 5% non-denaturing polyacrylamide gel electrophoresis at 12 V/cm for 3 h in low ionic strength buffer (0.25X Tris-Borate-EDTA). Gels were then dried and exposed to autoradiography film.

Ultra-violet cross-linking assay

Binding reactions were performed as described in RNA-EMSA assays. Samples were then placed on ice and exposed to ultra-violet (UV) lights for 15 min in a spectro-linker [wave length 254 nm, energy = 1.7 J/cm^2, UV-cross linker (UV-CL)]. Subsequently, unbound RNA was digested with 2 U of RNase A at 37°C for 15 min. Samples were suspended in loading buffer then separated on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was dried and exposed to a film overnight at −80°C.

Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to detect PKG-I mRNA expression in VSMC and its kinetic

modulation after transcription inhibition with actinomycin D (Sigma, St. Louis, MO, USA). Total RNA was prepared using Stat-60 (Tel-Test, TX, USA) following the protocol provided by the manufacturer. RT-PCR kit one-step (Invitrogen) and specific primer (sense: atgagcgagctggaggaagactttgccaag, antisense: cagctccaagttcttc) for PKG-Iα were used to amplify a 300-bp within PKG-I mRNA. As internal control, we used specific primer to generate a fragment from GAPDH. In a separate gel, we loaded 1 μg of total RNA for each sample to make sure of equal loading for 1 μg total RNA used in RT-PCR.

Western blotting

Western blots were performed to verify the expression of PKG-I in control and SMC pre-incubated with cycloheximide (CHX, Sigma). Total protein extracts (50 μg) were separated on 10% SDS–PAGE and transferred to nitrocellulose membranes. The blots were probed with anti-PKG-I antibody (Stressgen) or anti-β-actin as a control for protein equal loading. The signal was detected by enhanced chemiluminescence (Pierce).

Statistical analysis

Data are presented as mean ± SD and analysed using paired Student's *t*-test. $^{*}P<0.05$ or $^{**}P<0.001$ were considered statistically significant.

Results

PKG-I mRNA and protein half-life estimation

We previously reported that PKG-I mRNA and protein expressions were inhibited by inflammatory mediators such as IL1-β and lipophosphosaccharide (LPS) (*31*). As a first attempt to define the possible role of mRNA destabilization in this process, the half-lives of PKG-I mRNA and protein were determined. Bovine aortic SMCs, at passage 4–8, were treated with actinomycin D (ActD, 1 μg/ml) or cycloheximide (CHX, 1 μg/ml) for different periods of time. The concentrations of ActD and CHX, which according to the literature, are suitable for blocking mRNA and protein synthesis, respectively (*33*). Total RNA was isolated and subjected to RT-PCR using specific primers designed from human PKG-Iα cDNA sequence. The detection of PKG-I protein and mRNA was performed after exposure of cells to ActD or CHX at different periods of time. As shown in Fig. 1A, PKG-I protein half-life can be estimated ~48 h following CHX treatment. The results in Fig. 1B show that the PKG-I mRNA half-life was ~10–12 h after inhibition of transcription by ActD. It is well documented that actinomycin D and CHX induce cell cytotoxicity and apoptosis. In our experiments, at the concentration used, these drugs did not show any sign of cytotoxicity. Taken together, these results suggest that PKG-I mRNA is relatively stable and reflects the stability of PKG-I protein in VSMC.

PKG-I mRNA contains a relatively long 3′ UTR with putative AU-rich sites

Although the results reported above suggest that PKG-I protein expression seems relatively stable in SMC, acute regulation of PKG-I mRNA levels could contribute to the fully expressed levels of PKG-I. It is well established that the stability of many mRNA species is regulated by sequences harboured by the 3′UTR mRNA regions. We explored the possibility that the 3′UTR mRNA was involved in controlling PKG-I expression in VSMC. We used a RACE strategy followed by RNA amplification to generate human PKG-I 3′UTR mRNA. The amplified 3′UTR mRNA full-length was cloned in pGL3 control vector downstream of luciferase cDNA. The cloned 3′UTR mRNA served as a template to generate several serial deleted constructs (Fig. 2A). An examination of the cloned PKG-I 3′UTR mRNA sequence revealed the presence of several AU-rich regions, which might play a role in stabilizing (or destabilizing) PKG-I mRNA. Thus, the 3′UTR mRNA contains two AUUUA and two AUU UUA sequences (underlined in Fig. 2A), in addition to a potential polyadenylated [poly(A)] signal (bolded in Fig. 2A). To assess the possibility that the 3′UTR

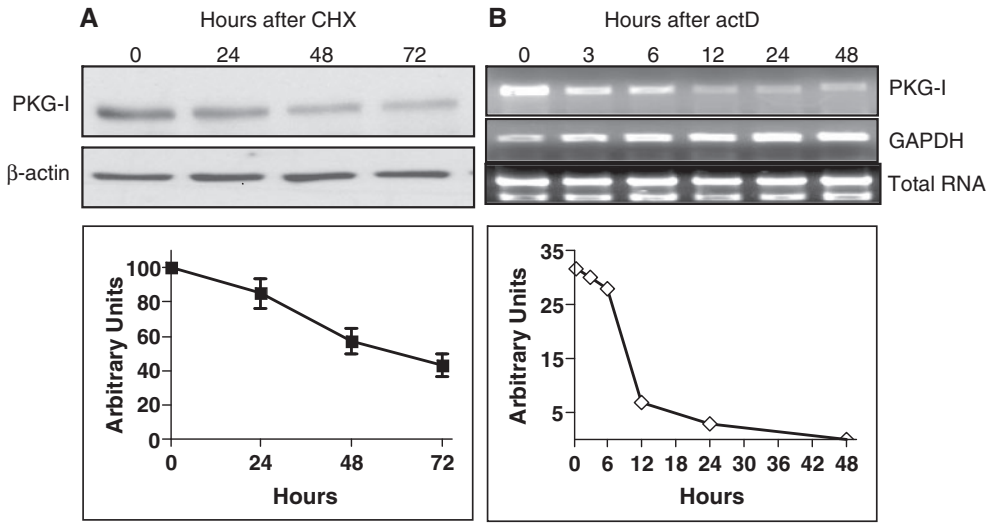

Fig. 1 Determination of PKG-I mRNA and protein half-lives. Bovine SMC were treated with actinomycin D (ActD, 1 μg/ml) or cycloheximide (CHX, 1 μg/ml) at different period of times. Western blots (A) and mRNA (B) detection were performed for each time of treatment. β-Actin was used as internal control for protein loading. RNA was extracted using stat-60 following the protocol from the company (Tel-Test Inc., TX, USA). RT–PCR was performed using a kit from Invitrogen and specific primer for PKG-Iα. GAPDH was used as an internal control and total RNA (1 μg) was shown as a representative of equal total RNA loading.

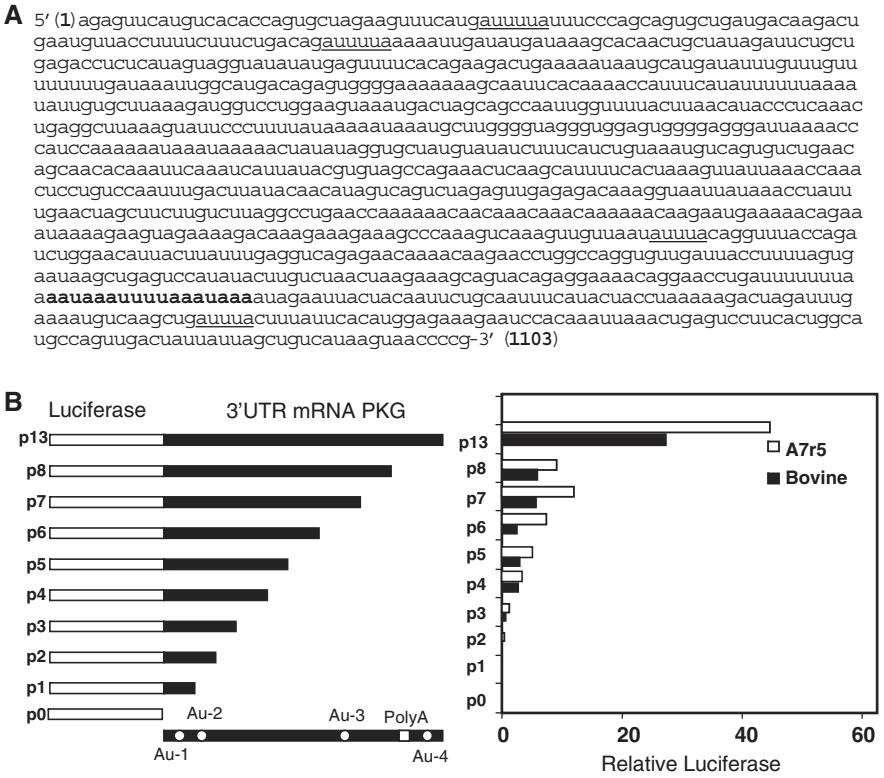

Fig. 2 Role of 3′UTR mRNA PKG-I full length in activating luciferase reporter gene. (A) Sequence of the cloned 3′UTR PKG-I mRNA showing the location of AU-rich regions (underlined) and the potential poly(A) site (bold). (B) Bovine (filled square) or A7r5 (open square) cells were transfected with 250 ng of each serial deleted construct plus 1 μl of TFx-20 (A7r5) or lipofectamine (bovine) in the absence of serum. One hour later, 2.5% of serum was added. Forty-eight hours later, cells were lysed and luciferase activities were assayed and expressed as relative luciferase (RL) after normalization to β-galactosidase activity (internal control).

mRNA contributes to luciferase expression, A7r5 or bovine aortic SMC were transfected with these constructs and luciferase activities were determined 48 h post-transfection. Data in Fig. 2B show that the full-length 3′UTR mRNA construct (p13) generated the highest luciferase activity. Luciferase activities from constructs designated p0 through p4 were low compared to the full-length construct. As the length of the 3′UTR mRNA increases, there was a corresponding increase in the activity of the luciferase reporter gene, but still less than one-third the activity of the full-length 3′UTR mRNA. In a control experiment, the same serial deleted constructs were generated in such way that they all contain SV40 poly(A) signal at their ends. The luciferase activities were high but the trend of these activities was similar to the one in Fig. 2 (data not shown).

AU-rich regions interact with cytosolic and nuclear proteins prepared from different cells

In addition to the poly(A) site revealed above, several AU-rich regions were present in the 3′UTR mRNA. Since the alteration in 3′UTR mRNA activity might be under the control of AU-specific RNA-binding proteins, we analysed the binding activities of the four AU-rich regions existing within PKG-I 3′UTR mRNA sequence. The RNA-EMSA assay shows that AU2 region generates a detectable weak band, while AU1, AU3 and AU4 retarded strong bands when cellular extracts from different VSMC were used (Fig. 3A). Figure 3B shows a representative autoradiography from UV-CL assay. It confirms the failure of probe AU2 to retard any band, and shows that AU1, AU3 and AU4 generated strong bands having molecular weights ranging from 35 to 55 kDa. The comparison between the binding of these probes (AU1 through AU4) to cytosolic or nuclear extracts prepared from bovine aortic smooth muscle cell (BASMC) shows that probes AU2 and AU4 do not bind to nuclear proteins (Fig. 3B). In contrast, probe AU3 generates a lower-molecular-weight bands with the nuclear extract (Fig. 3B). Interestingly, all AU probes, except AU2, bind to a common cytosolic protein around 50 kDa.

The 3′UTR mRNA of PKG-I stabilizes luciferase activity

In an attempt to better define the role of the 3′UTR mRNA of PKG-I in regulating PKG-I expression, the 3′UTR of the PKG-I mRNA was inserted downstream of luciferase cDNA. Bovine or A7r5 cells were transfected for 24 h and luciferase kinetics were performed at different times after transcription inhibition with (ActD, 1 μg/ml). In this experiment, time 0 corresponds to the end of the transfection and the addition of the ActD. The results in Fig. 4A show that while the control luciferase activity started to decrease immediately after transcription inhibition, the PKG-I 3′UTR mRNA maintained luciferase activity stable for at least 48 h after the addition of transcription drug inhibitor.

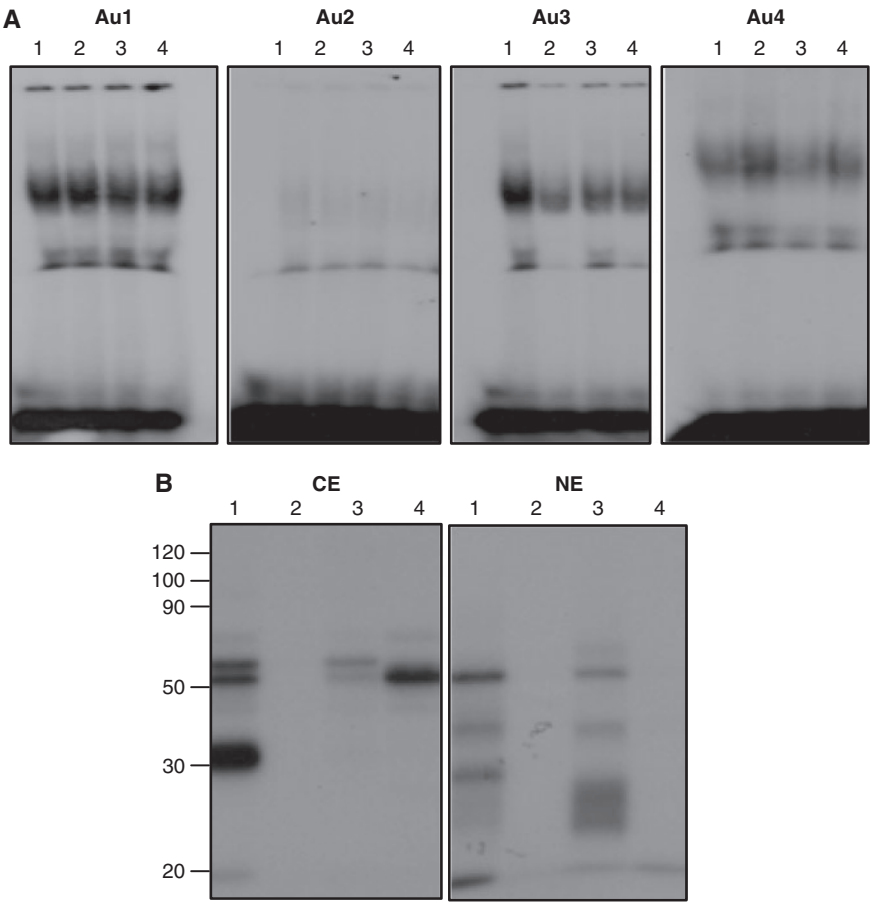

Fig. 3 Individual AU-rich regions within the 3′UTR-mRNA of PKG-I bind to several cytosolic and nuclear proteins. (A) Representative autoradiogram of RNA-EMSA assays using ribonucleotides corresponding to different AU-rich regions (AU1: GuuuCAuGAuuuuAuuuCCCAG; AU2: uCuGACAGAuuuuAAAAAuuGAu; AU3: GuuGuuAAuAuuuACAGGuuuAC; and AU4: GuCAAGCuGAuuuACuuuAuuCAC). Cytosolic extracts (CE) were prepared from A7r5 (1), rat aortic VSMCs (2), bovine aortic VSMCs (3) or COS7 cells (4). EMSA-RNA assays were performed as described in 'Materials and Methods' section. (B) Representative autoradiogram of UV-CL assays using different AU-probes (1: Au1, 2: Au2, 3: Au3, and 4: Au4) comparing the binding of cytosolic extracts (CE) versus nuclear extracts (NE) harvested from bovine aortic VSMCs. The numbers on the left of the gels indicate the position of molecular weight markers (in kDa).

Since the constructs used share the same promoters for driving luciferase expression, these results suggest that the 3′UTR-PKG-I mRNA contained in the p13 plasmid-induced stabilization of the transgene expression.

CN analogues and LPS decreased luciferase activity

We previously reported that inflammatory cytokines (e.g. IL-1β), CN analogues or LPS decreased steady-state PKG-I mRNA expression in bovine aortic SMCs (*31*). In an attempt to explore if the 3′UTR mRNA contributed to this inhibition, we compared the effect of the 8-chlorophenylthio-CN analogues (8-pCPT-cGMP/cAMP) or LPS on luciferase activity generated by the p13 full-length harbouring 3′UTR-mRNA PKG-I. The rationale for using the CN analogues, as discussed in our previous study (*31*), is that cytokines and LPS increase both type II NO synthase (iNOS) expression and cylooxygenase-2 (COX-2) expression in SMCs. Hence, the cytosolic level of both cGMP and cyclic adenosine monophosphate (cAMP) is increased in the cell. In this experiment, bovine aortic SMCs or A7r5 cells were transfected with the p13 construct then treated with CN analogues or LPS. Luciferase assays were performed 48 h later as mentioned in 'Materials and Methods' section. As shown in Fig. 4B and C, the CN analogues (100 μM each) and LPS (10 μg/ml) decreased luciferase activity, albeit to different degrees in bovine and A7r5 SMCs. Bovine aortic SMCs in particular were less sensitive to the effects of CN treatment. The ActD (1 μg/ml) or CHX (1 μg/ml) added immediately after cell transfection abolished totally the luciferase activity (data not shown). In a control experiment, neither the CN analogues nor LPS induced a significant decrease in pGL3 control activity (data not shown). These results suggest that under pathophysiological conditions, destabilization of PKG-I mRNA may be occurring at the posttranscriptional level.

Binding profile of 3′UTR mRNA PKG-I-generated probe to different cytosolic extracts

We next performed IVT assays to generate different lengths of 3′UTR PKG-I mRNA to be used in binding assays. The different 3′UTR mRNA probes thus

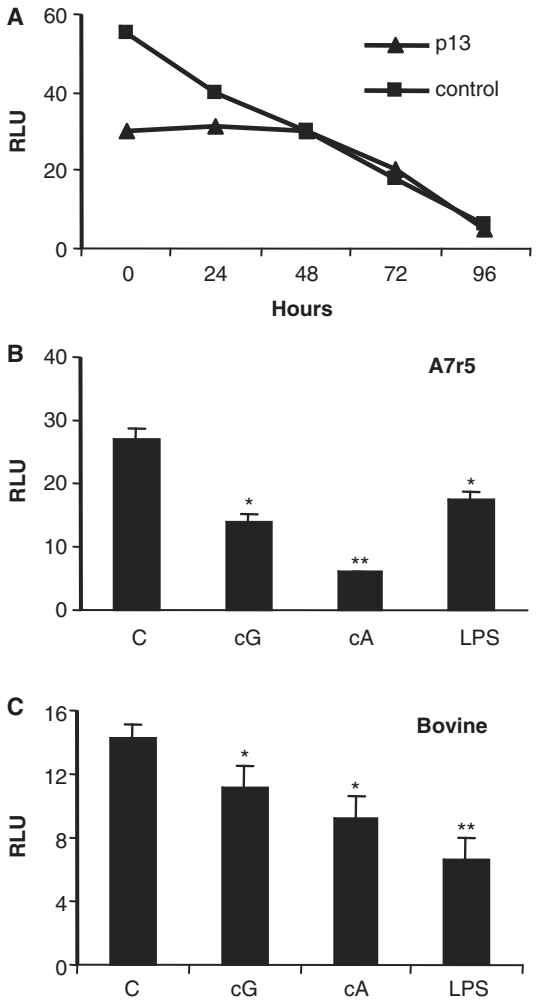

Fig. 4 3′UTR-mRNA of PKG-I stabilizes luciferase expression: effects of cyclic nucleotides and LPS. (A) A7r5 cells were transfected for 24 h with p13 or control construct as indicated in methods. After 24 h, cells were treated with ActD (1 μg/ml) and luciferase activities were performed at different times. A7r5 (B) or bovine cells (C) were transfected with 3′UTR-mRNA or control luciferase constructs then treated with cyclic nucleotide analogues (100 μM) or LPS (10 μg/ml). Luciferase activities were determined 48 h later. Data are representative of three independent experiments performed in quadruplicates.

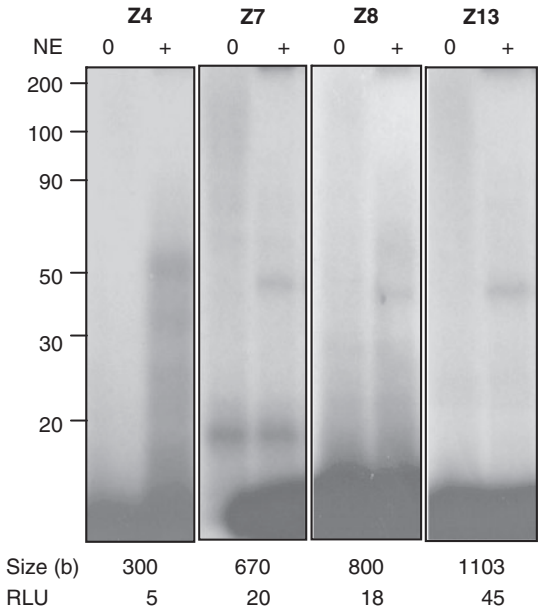

Fig. 5 Binding profile of different 3′UTR-mRNA PKG-I probes. Profile for the binding of different probes (Z4, Z7, Z8 and Z13), generated by *in vitro* transcription, as described in 'Materials and Methods' section. Z4 (1-300), Z7 (1-670) and Z8 (1-800) are inserts encompassing the 3′UTR. They were amplified by PCR using a primer containing the T7 promoter sequence in the 5′-region and P13 (full-length 3′UTR) as a template. Z13 correspond to the full-length 3′UTR cloned in pGEM7 vector. These probes were added to different concentrations of CE prepared from bovine aortic VSMCs. The sizes of different probes as well as their respective luciferase activities are represented at the bottom of the figure. The numbers on the left of the gels indicate the position of molecular weight markers (in kDa). Data are representative of three independent experiments.

generated are referred to 'Z' probes, to distinguish these from the endogenous mRNA constructs used in Fig. 2. As shown in Fig. 5, probes of increasing length (Z4 through Z13) demonstrate some similarities and differences in binding protein activity to bovine aortic SMC extract protein. The shortest probe, Z4, binds a protein of ~50 kDa, which is not found in longer probes, suggesting that this protein likely is sterically hindered from binding the 3′UTR mRNA under normal conditions. On the other hand, all probes bind a protein with an apparent mass of 40 kDa. These results suggest that 40-kDa protein represents an important mRNA-binding protein present most likely in the AU3 region, the 3′UTR mRNA. Also, the luciferase activity relative to each construct is shown in Fig. 5. Only the Z13 probe was capable of generating robust activity, most likely because only the Z13 probe contained the poly(A) site.

Figure 6A compares the binding activity of probe Z13 to cytosolic extracts (CEs) from three SMC cultures (A7r5, rat SMC and bovine SMC) and COS7 cells. When incubated with Z13, extracts (CEs) from bovine aortic SMC, A7r5 cells, rat aortic SMC and COS-7 cells generate bands between 30 and 50 kDa in addition to a lower band.

CN analogues treatment affect the profile of protein binding to the 3′UTR mRNA of PKG-I

To establish a correlation between the functional studies obtained in Fig. 3B, protein binding. UV-CL assays were performed using the 3′UTR mRNA probe, generated by IVT, and CEs harvested from bovine aortic SMC treated for 48 h with CN analogues or LPS. The results in Fig. 6B show that the retarded band 3 relative to 50 kDa was eliminated following CN treatment; instead, a new band was generated (band 2) after cell treatment with CNs. Additionally, a band 1 appears to be generated following cyclic analogues but at less intensity with LPS treatment.

Discussion

The regulation of PKG-I expression in VSMCs is likely of crucial physiological importance in the maintenance of vascular homeostasis. PKG-I has been implicated in

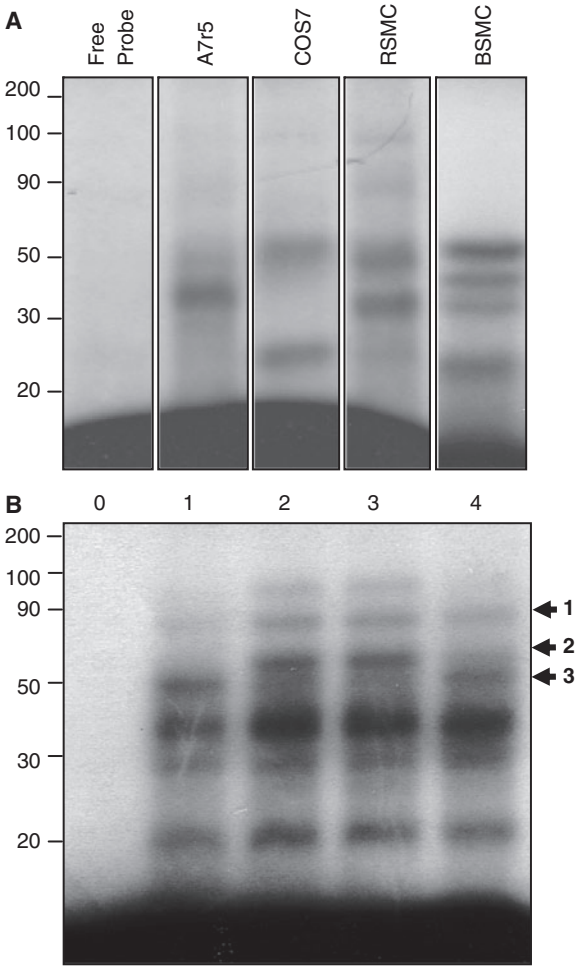

Fig. 6 Binding profile of Z13, competition, and modulation by CN and LPS. (A) Binding of Z13 probe, generated by *in vitro* transcription, to CE prepared from different cells (A7r5, COS7 cells, rat aortic VSMC and bovine aortic VSMC) as represented in the figure. (B) Effect of cyclic nucleotide analogs or LPS on the binding of 3′UTR mRNA. Bovine aortic VSMC were treated for 24 h with cyclic nucleotide analogues (8pCPTcGMP/cAMP, 100 μM each) or LPS (10 μg/ml). UV-CL assays were carried by incubation of CE with *in vitro* transcribed 3′UTR-mRNA (Z13). The numbers on the top of the Fig. 6B indicate the analysed samples (0: free probe, 1: control, 2: 8pCPTcGMP, 3: 8pCPTcAMP, and 4: LPS). The numbers on the left of the gels indicate the position of molecular weight markers (in kDa). Data are representative of three independent experiments.

the pathogenesis of vascular diseases such as atherosclerosis, diabetes, cancer, . . . etc. Our preliminary observations suggest that PKG-I expression was regulated at transcriptional and posttranscriptional levels. In this study, we report for the first time, by combination of functional and binding assays, that 3′UTR mRNA contributes to the regulation of PKG-I expression in VSMCs. The estimation of PKG-I mRNA and protein half-lives shows that mRNA, as well as protein expression, were relatively stable in VSMCs. These results suggest that PKG-I is highly stable in VSMCs under normal physiologic conditions, and that many mechanisms might contribute to its stability. On the other hand, the expression of PKG-I was reported to be altered in certain pathophysiological situations. For instance, in coronary arterial SMCs in response to injury, there was a transient decrease in the expression of PKG-I in neointimal smooth muscle cells when compared to medial SMCs (*34*). Moreover, chronic intrauterine pulmonary hypertension was reported to be accompanied with decreases in PKG-I expression at mRNA and protein levels (*35*). Recently, it was reported that diabetic situation was accompanied with a decrease in PKG-I expression in SMC from corpus cavernosum of diabetic rabbits (*14*). Finally, the same results, related to PKG-I down-regulation, were recently reported in VSMC subjected to high glucose concentration that simulates diabetic situations (*36*). Although the functional roles of PKG-I are well studied, the molecular mechanisms underlying the regulation of its expression have not been yet elucidated. In addition, the notion that PKG-I might be regulated only transcriptionally was difficult to reconcile with its long 3′UTR mRNA and its status as cell function regulator. In this study, we consider that posttranscriptional mechanism involving 3′UTR-mRNA may account for the regulation of PKG-I expression in VSMC.

In addition to transcription, post-transcriptional mechanisms play an important role in the regulation of the expression of an increasing number of mRNA(s), with specific elements involved in the regulation of RNA stability found in the 3′UTR of many messages (*37–39*). An important subset of regulatory ribonucleoprotein association relies on the presence of *cis*-RNA sequences which are U- or AU-rich regions, present in 3′- or 5′-UTRs of mRNAs. The analysis of the nucleotide sequence of 3′UTR mRNA of PKG-I shows the presence of couple of AU-rich regions that are binding to CEs from different VSMCs except AU2, which did not show any strong binding activity. These regions were highly conserved across human, bovine, chimpanzee and murine species when analysing sequence homology between species (data not shown). The conservation of these elements between species is itself of interest, since it may lead to a common mechanism governing posttranscriptional regulation of PKG-I. In addition to these potential AU-binding regions, 3′UTR PKG-I mRNA contains couple of poly(A) sites as revealed by luciferase assay analysis following serial deletion transfection assays. The major poly(A) site is situated at the end of 3′UTR mRNA and consists of 2 repeats with AUUUUA logged in between. The individual AU-rich region as well as the 3′UTR mRNA PKG-I generated by IVT were able to bind to cytosolic and nuclear proteins. In this study, we found that 3′UTR mRNA PKG-I possesses potent stabilizing activity as tested by luciferase reporter. This activity increased with the size of 3′UTR mRNA showing high activity within the full length, although our study does not exclude other possibilities contributing to this stability such as translational efficiency, metabolic stability or intracellular localization residing predominantly in the 5′- or 3′-UTRs of mRNA.

CNs were reported to play a pivotal biological role as second-messenger molecules in intra-signalling pathways. It was suggested that CNs regulate gene expression by posttranscriptional mechanisms that

affects mRNA stability (*40–42*). Supplementation of transfected A7r5 cells or bovine cells with cGMP/AMP analogues reduced luciferase activity of a construct harbouring 3′UTR mRNA PKG-I. These observations evidence a negative feedback regulation of PKG-I expression by CNs, in which posttranscriptional mechanisms may play an important role. In addition, the inhibitory effects mediated by CN analogues were mimicked by inflammatory mediators such as LPS. The suppression of luciferase activity was accompanied with a decrease of the intensity of band 3 (50 kDa) and an increase of band 2 when cells were treated with CN but not with LPS (Fig. 6B). The significance of these changes is not known but we are in the process of identifying these proteins. Since the measurement of luciferase activity was used to quantify the amount of the reporter enzyme synthesized by transfected cells, we may attribute the changes in luciferase activity to either alteration in message stability or rate of mRNA translation. The observation reported here consisting of the effect of cGMP and cAMP on gene regulation is not restricted to PKG-I. Effectively, this effect can be extended to NO/cGMP signalling pathway including NOS and sGC (*41*, *42*). Thus, decreased PKG-I expression may contribute to the development of vascular disorders such as atherosclerosis, since PKG-I is involved in cell growth, proliferation and apoptosis.

In this study, we have demonstrated the contribution of 3′UTR mRNA PKG-I in stabilizing the expression of PKG-I in VSMCs. We are reporting the involvement of cytoplasmic factors having strong binding affinities for the highly conserved AU-rich regions within the human PKG-I 3′UTR mRNA. The functional studies as well as the binding assays and their modulation by CN analogues and LPS strongly suggest a role in the posttranscriptional regulation of mRNA stability. In conclusion, our results indicate that different repertoires of RNA-binding proteins target the PKG-I 3′UTR to regulate PKG-I mRNA stability and, thus, alter PKG-I expression. These RNA-binding proteins interacting with 3′UTR PKG-I mRNA are yet to be identified and are the subject of ongoing investigations.

Funding
National Institutes of Health grant (HL066164).

Conflict of interest
None declared.

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