Published in final edited form as:

Cell Signal. 2012 May ; 24(5): 1100–1108. doi:10.1016/j.cellsig.2012.01.006.

HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN A1 IS A NOVEL CELLULAR TARGET OF ATRIAL NATRIURETIC PEPTIDE SIGNALING IN RENAL EPITHELIAL CELLS

Bahar Hesabi¹, Robert S. Danziger^{1,2,3,4}, and Kumar U. Kotlo^{1,4,*}

¹Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA

² Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL, USA

³Department of Pharmacology, University of Illinois at Chicago, Chicago, IL, USA

⁴Jesse Brown Veterans Affairs Medical Center, Chicago, IL, USA

Abstract

Two classes of guanylyl cyclases (GC) form intracellular cGMP. One is a receptor for atrial natriuretic peptide (ANP) and the other for nitric oxide (NO). The ANP receptor guanylyl cyclase (GC-A) is a membrane-bound, single subunit protein. Nitric oxide activated or soluble guanylyl cyclases (NOGC) are heme-containing heterodimers. These have been shown to be important in cGMP mediated regulation of arterial vascular resistance and renal sodium transport. Recent studies have shown that cGMP produced by both GCs is compartmentalized in the heart and vascular smooth muscle cells. To date, however, how intra cellular cGMP generated by ANP and NO is compartmentalized and how it triggers specific downstream targets in kidney cells has not been investigated. Our studies show that intracellular cGMP formed by NO is targeted to cytosolic and cytoskeletal compartments whereas cGMP formed by ANP is restricted to nuclear and membrane compartments. We used two dimensional Difference in Gel Electrophoresis and MALDI-TOF/TOF to identify distinct sub-cellular targets that are specific to ANP and NO signaling in HK-2 cells. A nucleocytoplasmic shuttling protein, heterogeneous nuclear ribonucleo protein A1 (hnRNP A1) is preferentially phosphorylated by ANP/cGMP/cGK signaling. ANP stimulation of HK-2 cells leads to increased cGK activity in the nucleus and translocation of cGK and hnRNP A1 to the nucleus. Phosphodiesterase-5 (PDE-5 inhibitor) sildenafil augmented ANP-mediated effects on hnRNPA1 phosphorylation, translocation to nucleus and nuclear cGK activity. Our results suggest that cGMP generated by ANP and SNAP is differentially compartmentalized, localized but not global changes in cGMP, perhaps at different sub-cellular fractions of the cell, may more closely correlate with their effects by preferential phosphorylation of cellular targets.

© 2011 Elsevier Inc. All rights reserved.

*Address Correspondence to: Kumar U Kotlo, PhD, Department of Medicine University of Illinois at Chicago, 840 S. Wood St., Chicago, IL 60612, Tel. 312-569-7461, Fax: 312-569-8114.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures

The authors have no conflicts of interest to report.

Keywords

hnRNP A1; ANP; cGMP; cGK; phosphorylation; 2D-DIGE

1. INTRODUCTION

Cyclic guanosine monophosphate (cGMP) is an important second messenger that regulates cardio renal functions. cGMP is synthesized by two classes of Guanylyl Cyclases (GCs): membrane-bound particulate GC (pGC) activated by atrial natriuretic peptide (ANP) and soluble GCs activated by nitric oxide (NO) and carbon monoxide. GCs are a family of enzymes that metabolize GTP to cyclic GMP (cGMP). Peptide-activated or particulate GCs (pGC) are a family of seven membrane-bound proteins (GC-A to GC-G) [1–3]. Based on their ligand specificities, membrane-bound GCs have been classified into natriuretic peptide GC receptors (GC-A and GC-B), intestinal peptide-binding receptors (GC-C) and orphan receptors (GCD-GCG) [4]. GC-A and GC-B, principal receptors for atrial natriuretic peptide (ANP), are generally accepted to be homodimers consisting of subunits of approx 115 kDa [5–7]. NOGCs are a ubiquitous family of heme-containing heterodimers (α and β subunits of approx 80 and 70 kDa respectively) [8–10] and are activated by nitric oxide. Both α and β subunits contain an amino-terminal domain important in heme binding, a central dimerization domain required for subunit association and a carboxyl terminal catalytic domain.

Elevated levels of cGMP formed by ANP and NO activate cGMP-dependent protein kinase, cGK, which phosphorylates a variety of cellular substrates in the kidney including calcium-activated potassium channel, BK_{Ca} channel [11–13], Vasodilator-stimulated phosphoprotein, VASP [14], [15]. Effects of cGMP mediated by cGK include inhibition of platelet adhesion and activation, inhibition of intracellular Ca^{+} concentration leading to vasorelaxation, and reduction in renal tubular Na^{+} uptake there by controlling hypertension [16]. The renal interstitial tubular cGMP and cGK signaling pathway play a key role in pressure-natriuresis [17, 18].

There is evidence that cGMP generated by ANP and NO donors is not evenly distributed throughout the cell. Number of studies provides robust evidence that cGMP is compartmentalized to distinct sub-cellular regions in heart and vascular smooth muscle cells leading to disparate cell functions. These studies show differential responses to NO donor and ANP stimulation and sub-cellular compartmentalization of their signaling. [19, 20]. But the compartmentalization of cGMP in the kidney is not completely understood.

While these studies have demonstrated that cGMP produced by membrane-bound and soluble guanylyl cyclases is compartmentalized, a major challenge in cGMP signaling is to identify the cellular targets that are differentially phosphorylated by ANP and NO signaling and understand how it triggers these targets. What remain unclear are the mechanisms by which ANP and NO donors differentially regulate cellular processes.

hnRNP A1 is a multifunctional RNA binding protein which is involved in pre-mRNA splicing, export of mature transcripts from the nucleus, mRNA turnover, and internal ribosome entry site (IRES) –mediated translation [21–23]. hnRNP A1 is primarily located in the nucleus but a 38 amino acid domain called M9 regulates its nucleocytoplasmic shuttling [24]. It is one of the ITAFs that supports IRES-mediated translation of certain mRNAs [25, 26].

In the present study *we address* the compartmentalization of cGMP formed by ANP-stimulated guanylyl cyclase and cellular targets in Human Kidney-2 (HK-2) cells, a renal tubular epithelial cell line. We identified heterogeneous nuclear ribonucleo protein A1 (hnRNP A1) as a novel substrate which is preferentially phosphorylated by ANP/cGMP/cGK signaling.

2. Materials and Methods

Materials

S-nitroso-N-acetylpenicillamine (SNAP), Isobutyl Methyl Xanthine (IBMX), 1H-[1, 2, 4]-Oxadiazolo-[4, 3-a]-quinoxalin-1-one (ODQ), cGK inhibitor KT5823, PDE-5 inhibitor (sildenafil), and ANP are purchased from Sigma Chemical. cGKII and hnRNP A1 antibodies are from Sigma-Aldrich and Santa Cruz Biotechnology, Inc, respectively. cGMP assay kit, Proteoextract sub-cellular proteome extraction kit, and protease inhibitors are purchased from Assay design, Calbiochem and Sigma Aldrich, respectively. cGK activity assay kit is purchased from MBL Inc. Cy-3 and cy-5 dyes for 2D-DIGE analysis are purchased from GE Healthcare. Cultured Human kidney-2 (HK-2) cells (ATCC) are used at early passages and cultured in DMEM medium supplemented with 10% fetal bovine serum under 5% CO₂.

Methods

2.1 Isolation of sub-cellular fractions of HK-2 cells—is performed using a commercially available kit, Proteoextract sub-cellular proteome extraction kit (Calbiochem).

2.2 cGMP assay—is performed as described [27]. Briefly, Intracellular cGMP is measured in cultured cells pretreated with IBMX. Where, if applicable, different sub-cellular fractions of HK-2 cells are isolated. Cells are lysed with 0.1N HCl. cGMP is measured using a commercially available enzyme immuno assay (EIA) Assay Design Inc (Ann Arbor, MI). Intracellular cGMP is normalized to total protein determined by Bradford.

2.3 Two dimensional difference in gel electrophoresis (2D-DIGE)—Protein concentration is determined using the Bio-Rad protein assay method and samples are diluted with 2-D lysis buffer to the same protein concentration between 5 to 8 mg/ml. 1.0 µl of diluted Cy Dye (1:5 diluted with DMF from 1 nmol/µl stock) is added to 30 µg of protein extract. Cy3 is used for ANP-treated samples and Cy5 is used for SNAP-treated samples. The mixtures are vortexed, and then incubated under dark conditions on ice for 30 min. 1.0 µl of 10 mM Lysine is added to each of the samples, followed by vortexing and incubation under dark conditions on ice for an additional 15 min. Cy2 and Cy5 labeled samples are mixed and the appropriate volume of 2× 2D Sample Buffer (8 M urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalytes, and trace amounts of bromophenol blue) and 100 µL of destreak solution (GE Healthcare) are added to the samples. Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes, and trace amounts of bromophenol blue) is added to reach a total volume of 250 µL. The samples are mixed, spun, and equal amounts of protein are loaded onto 13 cm IPG strips (pH 3–10 linear) under 1 ml mineral oil. IEF is performed for 12 hrs at 20°C with 50 µA/strip. The focused IPG strips are incubated in fresh made Equilibration Buffer 1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, 10 mg/ml DTT, and trace amounts of bromophenol blue) for 15 min with slow shaking. The strips are rinsed with Equilibration Buffer 2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, 45 mg/ml iodoacetamide, and trace amounts of bromophenol blue) and then incubated with Equilibration Buffer 2 for 10 min with slow shaking. The IPG strips are rinsed once in SDS-gel running buffer, loaded into the 12% SDS-gels, and sealed with 0.5% w/v agarose solution (in SDS-gel running

buffer). The samples are run at 15°C until the dye front ran out of the gels. Gels are scanned immediately following SDS-PAGE using Typhoon TRIO (Amersham Biosciences) following the protocols provided. The gels are then stained with ProQ diamond phospho-protein staining kit (Invitrogen) according to the protocol provided. Image scans are carried out immediately following ProQ staining. The scanned images are then analyzed by Image QuantTL software (GE-Healthcare), and then subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE-Healthcare). The ratio change of the protein differential expression is obtained from in-gel DeCyder software analysis.

2.4 MALDI-TOF/TOF Mass Spectrometry—Protein spots chosen for analysis are excised by Ettan Spot Picker (GE Healthcare) and washed multiple times to remove staining dye and other inhibitory chemicals. Gel spots are dried and then rehydrated in digestion buffer containing sequencing grade modified trypsin. Proteins are digested in-gel at 37°C and digested peptides are extracted from the gel with TFA extraction buffer and shaking. The digested tryptic peptides are desalted using C-18 Zip-tips (Millipore) and then mixed with CHCA matrix (alpha-cyano-4-hydroxycinnamic acid) and spotted into the wells of a MALDI plate. Mass spectra (MS) of the peptides in each sample are obtained using an Applied Biosystems Proteomics Analyzer and ten to twenty of the most abundant peptides in each sample are further subjected to fragmentation and tandem mass spectrometry (MS/MS) analysis. The combined MS and MS/MS spectra are submitted for database search using GPS Explorer software equipped with the MASCOT search engine to identify proteins from the NCBI non-redundant protein database. Proteins that are identified with a confidence interval (C.I.) greater than 99.99% are reported.

2.5 cGK activity—HK-2 cells are treated with and without ANP in the presence and absence of cGK inhibitor (cGK-I), KT5823 (2uM x1 hr). The nuclear fractions from these cells are isolated and subjected to cGK activity assay using cyclex cGMP dependent protein kinase assay kit (MBL Inc) as per manufacturer's instructions.

2.6 Phosphorylation of hnRNP A1—The nuclear fractions from HK-2 cells treated with indicated reagents are immunoprecipitated with anti-hnRNPA1 antibody (Santa Cruz Biotech) and subjected to western blotting with anti-phospho serine threonine antibody (BD Biosciences).

2.7 Western Blotting—Equal amounts of protein are subjected to 10% SDS-PAGE; proteins are transferred to a nitrocellulose membrane, and then blocked in a TBS-T solution (20 mM Tris, 140 mM NaCl, and 0.05% tween-20 pH 7.6) with 5% non-fat milk. After two washes, the membrane is incubated with anti phospho serine threonine primary antibodies. The membrane is then washed three times in TBS-T and incubated for 1 h with peroxidase-linked anti-rabbit secondary antibody (Santa Cruz Biotech, Santa Cruz, CA) at a dilution of 1:2000 in TBS-T. After three additional washes in TBS-T, the membrane is incubated for 1 minute with ECL detection reagents (Amersham) and exposed to x-ray film (Kodak) for varying times to obtain desirable band intensity within linear range and optimal saturation.

2.8 Immunofluorescence—is done as described [27]. HK-2 cells are cultured in 4-well chamber slides at a density of ~ 10,000 cells/cm² in Dulbecco's modified Eagle's medium with 10% Fetal bovine serum and 1% penicillin/streptomycin solution. 36 hours after plating, cells are pre-treated with IBMX for 30 min followed by treatment with ANP and SNAP for 45 min. Then the cells are fixed in 2% Para formaldehyde for 2 hrs. The cells are washed with PBS and blocked for 1.5 hrs in PBS containing 5% normal goat serum and 0.08% saponin. The cells are incubated overnight with either 1:100 diluted un-conjugated primary antibodies against hnRNP A1 and cGKII. Cells are washed 4 times five min each

with PBS containing 0.08% saponin. Then cells are incubated with 1:80 diluted FITC-or rhodamine-conjugated anti-mouse or anti-rabbit secondary antibodies for 1 hr. Cells are washed 4 times five min each with PBS containing 0.08% saponin. The slides are then mounted with antifade mounting medium containing DAPI (In Vitrogen). Confocal images are obtained using Zeiss laser-scanning confocal microscope LSM 510 META in multi-tracking mode to prevent interference of the dyes.

2.9 Statistical analysis—Values are expressed as the mean+ SD. Differences among different treatments are determined by one way ANOVA for repeated measurements. Results are considered significant at $P < 0.05$.

3. RESULTS

3.1 cGMP pools generated by ANP signaling are confined mostly to nuclear and membrane fractions of HK-2 cells

To determine whether ANP- and NO- generated intracellular cGMP is restricted to specific sub-cellular compartments HK-2 cells are treated with 10^{-6} M ANP or 10^{-7} M SNAP in the presence of 1 mM IBMX (broad spectrum cGMP degrading PDE inhibitor). Various sub-cellular fractions of these cells are isolated and cGMP levels are monitored using cGMP assay kit. The results indicate that ANP-generated cGMP pools are more in nuclear and membrane fractions of the cell whereas NO donor-mediated cGMP pools are largely restricted to cytosolic and cytoskeletal components of the cell (Fig 1).

3.2 Identification of cellular proteins regulated by ANP signaling

The compartmentalization of cGK with substrates in the sub-cellular region(s) of the cell is an important factor involved in their phosphorylation. To analyze the proteins that are preferentially phosphorylated by ANP and NO signaling protein extracts from HK-2 cells treated with ANP and SNAP are subjected to 2D-DIGE analysis followed by phosphoprotein staining (see methods for details). Many proteins are differentially phosphorylated in ANP- vs. SNAP- treated cells (fig 2 green and red circled proteins). Five phosphoproteins are identified by MALDI-TOF/TOF analysis. Cofilin, eukaryotic translation elongation factor-2 (eEF2) and Methylene Tetrahydrofolate Dehydrogenase 1 (MTHFD1) are specifically phosphorylated by SNAP-stimulated NOGC signaling whereas Vimentin and hnRNPA1 are phosphorylated by ANP-stimulated GC-A signaling (Fig.2).

3.3 cGK activity is increased in the nuclear and membrane fraction of HK-2 cells upon treatment with ANP

Since cGMP formed by ANP/cGMP/cGK signaling is largely restricted to nuclear and membrane fraction of the cell and NO/cGMP/cGK signaling is restricted to cytoplasm (Fig 1), next we wanted to demonstrate whether ANP treatment of HK-2 cells results in the activation of cGK in these components of the cell. The results demonstrate that cGK activity is increased to a greater extent in the nuclear and membrane fraction of HK-2 cells treated with ANP compared to that of control and SNP-treated cells (Fig 3). Whereas greater cGK activity is noticed in cytoplasm of SNAP-treated cells compared to that of ANP-treated cells. These results provide evidence that nuclear and membrane compartmentalization of cGMP generated by ANP signaling leads to increased activity of cGK in these compartments.

3.4 Increased cG K activity in the nucleus correlates with phosphorylation of hnRNPA1 in the nuclear fraction of HK-2 cells

Nuclear fraction of HK-2 cells (pre-treated with IBMX) either treated or not treated with ANP, SNAP, and cGK inhibitor (KT5823) is subjected to immunoprecipitation with

antibody against hnRNPA1 followed by western blotting with anti-phospho serine threonine antibody. hnRNP A1 is specifically phosphorylated in nuclear fraction of HK-2 cells treated with ANP (Fig. 4- ANP) but not with NO donor, SNAP or control untreated cells (Fig. 4- SNAP). Treatment of HK-2 cells with cGK inhibitor in the presence of ANP abolished hnRNP A1 phosphorylation to a greater extent (Fig. 4-ANP + cGK-I). The results provide evidence that 1) hnRNP A1 is a novel cellular target of ANP signaling and 2) hnRNPA1 is preferentially phosphorylated by ANP/GC-A/cGMP/cGK signaling in the nuclear fraction of HK-2 cells.

3.5 ANP-dependent accumulation of cGK in nuclear compartment of HK-2 cells

As cGK activity is increased in the nuclear component of HK-2 cells (Fig 3) we examined the sub-cellular distribution of cGKII in untreated and ANP-treated HK-2 cells (pre-treated with IBMX). In control untreated cells cGK is predominantly in cytoplasm (Fig 5 upper panel A, B, C) whereas upon ANP treatment it is mostly in the nucleus (fig 5 upper panel D, E, F).

3.6 ANP-dependent hnRNP A1 translocation to and co-localization with cGKII in nucleus

Because hnRNP A1 is a protein that shuttles between nucleus and cytoplasm we next verified the effect of ANP on hnRNP A1 distribution in HK-2 cells. hnRNP A1 is mostly peri-nuclear and cytoplasmic (Fig 5 middle panel A, B, C) under basal conditions. However, upon ANP stimulation, its presence is noted in the nuclear compartment, demonstrating cytoplasmic to nuclear translocation (Fig 5 middle panel D, E, F). Further both hnRNP A1 and cGK are co-localized in the nucleus upon ANP stimulation (Fig 5 lower panel).

3.7. Role of cGMP-specific PDE-5 inhibitor in hnRNP A1 phosphorylation, translocation to nucleus, and cGK activity

Since cGMP-specific PDEs have been shown to play a role in the regulation of cGMP compartmentalization in cardiac myocytes [19] we looked into the effect of sildenafil, a PDE-5 inhibitor on ANP-induced phosphorylation of hnRNP A1. There is a 2 –fold increase in hnRNP A1 phosphorylation in nuclear fraction of ANP-treated cells compared to that of sildenafil- and SNAP-treated cells. PDE-5 inhibition significantly increased hnRNP A1 phosphorylation in ANP-treated but not in SNAP-treated cells (Fig 6 left and right panels) suggesting that ANP-induced hnRNP A1 phosphorylation is regulated by PDE-5.

Next we determined the effect of sildenafil on the cellular distribution of hnRNP A1. hnRNP A1 is mostly perinuclear and cytoplasmic in cells treated with sildenafil alone, and sildenafil and SNAP (Fig 7 top and middle panels). Whereas hnRNP A1 is translocated to nucleus in cells treated with ANP and sildenafil (Fig 7 bottom panel).

Lastly, we determined the effect of sildenafil on nuclear cGK activity of HK-2 cells treated with ANP and SNAP. Compared to sildenafil and SNAP, ANP increased cGK activity in nuclear fraction of HK-2 cells. But PDE-5 inhibition augmented ANP-induced but not SNAP-induced cGK activity (Fig 8, $p < 0.05$).

Together these results suggest a role for PDE-5 in the regulation of ANP-induced hnRNP A1 phosphorylation and nuclear distribution and cGK activity.

4. DISCUSSION

In the present study we identified that cGMP pools generated by ANP signaling are mainly restricted to nuclear and membrane components of HK-2 cells. Similarly, in ANP-treated HEK cells cGK is redistributed to the plasma membrane increasing cGMP pools on the

membrane, but exposure to NO donors does not [28]. Number of reports suggests ANP- and NO-specific compartmentalization of cGMP pools in a variety of cell types. In cardiac myocytes, ANP and NO had unique effects on CNG activity due to differential spatiotemporal distributions of cGMP [29]. B-type natriuretic peptide (BNP) has been shown to potentiate ATP- and thapsigargin-stimulated rise in cytosolic-free Ca^{2+} in human epithelial cells, and causes partial inhibition of cation influx. In contrast the NO donor, sodium nitroprusside, causes an increase in re-uptake of Ca^{2+} into the sarcoplasmic reticulum (SR) without affecting cation influx or Ca^{2+} efflux, thus suggesting that localized pools of cGMP play different roles in regulating Ca^{2+} homeostasis in cells [30]. NO and BNP modulated left-ventricular function in dogs with congestive chronic heart failure by preserving diastolic function through elevation of cGMP. In contrast, β -adrenergic responsiveness to dobutamine is dampened by NO but not BNP, illustrating the differential effects of NO activated guanylyl cyclase and ANP-activated GC-A-derived cGMP on cardiac inotropy [31]. ANP-mediated relaxation of airway smooth muscle is less than that produced by nitric oxide because of their differential effect on calcium sensitivity [32]. CNP reduced intracellular Ca^{2+} transients in ventricular myocytes, whereas SNAP had little or no effect [33]. NO signaling in skeletal muscles is compartmentalized to sarcolemma, contractile fibers and mitochondria [34]. Although ANP generated negligible amounts of cGMP compared to S-nitroso-N-acetyl-penicillamine (SNAP), ANP induces the activation of cyclic nucleotide-gated (CNG) channels more readily than SNAP in vascular smooth muscle cells, although SNAP generated higher intracellular cGMP levels compared to ANP suggesting that ANP and NO exhibit unique actions by compartmentalizing cGMP signals [35]. In mouse hearts nitric oxide but not ANP modulates Isoproterenol-stimulated cardiac contractility, in spite of ANP generating higher levels of cGMP, suggesting that these effects are due to compartmentalization of ANP- and NO-generated cGMP pools [36].

We report increased cGK activity by ANP stimulation in membrane and nuclear fractions of HK-2 cells (Fig 3) which correlates with the phosphorylation of hnRNP A1 in the nucleus (Fig 4) and distribution of cGMP pools in these fractions (Fig 1, top panel). SNAP-induced cGK activity is increased in cytoplasmic fraction of cells which correlates with the cGMP distribution in this fraction of the cells (Fig 1, bottom panel). Activated cGK regulates gene expression in the nucleus via class I histone deacetylase complex in *C.elegans* [37]. In Baby Hamster Kidney (BHK) cells and vascular smooth muscle cells cGMP triggers the translocation of cGK to nucleus and cGK-dependent phosphorylation of transcriptional regulators [38].

cGK phosphorylates a variety of cellular proteins in different compartments of the cells. What remain unclear are the proteins which are differentially phosphorylated by ANP and NO donors. Using phospho-specific antibodies, co-immuno precipitation studies and affinity chromatography number of cGK substrates are identified [39]. These studies are limited as whole phosphokinome cannot be analyzed in a cell or tissue. Hence we used 2D-DIGE analysis of proteins followed by pro-Q staining to identify proteins that are uniquely phosphorylated by ANP and NO donor SNAP. We identified hnRNP A1 as a novel target of ANP signaling and a substrate for cGK. Our findings suggest that ANP stimulation of HK-2 results in increased activity of cGK in nucleus followed by phosphorylation of nucleocytoplasmic shuttling protein hnRNP A1. We also identified vimentin as a target of ANP signaling which has been identified as cGK substrate earlier [15]. Whether or not vimentin has the same effect on nuclear cGMP signaling as hnRNP A1 has to be determined.

hnRNP A1 has been shown to be phosphorylated by p38 MAP Kinase and AKT ([40, 41]. AKT-mediated phosphorylation of hnRNP A1 is important for regulating internal ribosome entry site (IRES) activity of cyclin D1 and c-myc mRNAs [40]. Interleukin-6 activates IRES-mediated c-myc translation in multiple myeloma cells by phosphorylating hnRNP A1

in p38-MAP kinase dependent manner [42]. Phosphorylation of hnRNP A1 by p38 Map kinase is implicated in cellular senescence of normal human fibroblasts [43]. hnRNP A1 has been shown to regulate processing of micro RNAs with conserved loops [44, 45].

hnRNP A1 is a nucleocytoplasmic shuttling protein that plays a key role in IRES-mediated translation and alternative splicing of mRNAs. The activity of hnRNP A1 is primarily dependent on its phosphorylation which in turn regulates its cellular distribution. Our findings indicate that ANP stimulation of HK-2 cells leads to the nuclear translocation of hnRNP A1 and cGK. Similarly, stress-induced phosphorylation and sub-cellular distribution of hnRNP A1 by p38 Map Kinase regulates adenovirus E1A pre-mRNA splicing reporter [46] and cellular senescence of normal human fibroblasts [43]. Anchoring of cGK by Inositol 1,4,5-triphosphate (IP(3)) receptor-associated cGMP kinase substrate (IRAG) results in the nuclear translocation and increased activity of cGK in vascular smooth muscle cells [47]. Our results establish that hnRNP A1 may facilitate nuclear translocation of cGK which is sufficient to influence sub-cellular localization of cGK kinase and cGMP signaling in the nucleus.

cGMP-specific PDEs have been documented to regulate the compartmentalization of ANP- and NO-induced cGMP and cGK activity [36, 48]. Our results demonstrate that PDE-5 controls ANP-induced effects on hnRNP A1 activities (Figs 6, 7, and 8). Previous studies suggest that PDE-5 controls NO-induced cGMP pools but not ANP-stimulated cGMP pools in rat cardiomyocytes [29]. This discrepancy could be due to the cell types used.

Based on our findings and previous published report that disruption of ANP-mediated cGMP formation causes the renal fibrosis [49], we presume that ANP-stimulated nuclear translocation of cGKII and hnRNP A1 and phosphorylation of hnRNP A1 is probably protective in renal fibrosis of tubular epithelial cells. In view of the impact of cell cycle genes on tubular hyperplasia [50] and the role of ANP in renal fibrosis [49, 51] our studies provide novel insights into the ANP/cGMP/cGK signaling in hnRNPA1-mediated translational control of cellular proliferative genes which are implicated in the tubular damage in chronic renal failure.

HIGHLIGHTS

1. Intracellular cGMP formed by NO is targeted to cytosolic and cytoskeletal compartments.
2. Intracellular cGMP formed by ANP is restricted to nuclear and membrane compartments.
3. hnRNP A1 is preferentially phosphorylated by ANP/cGMP/cGK signaling.
4. ANP signaling leads to co-localization of cGK and hnRNP A1 to the nucleus.

Acknowledgments

This work was supported by National Institutes of Health Grant K01 DK071641-01 and 3 K01 DK071641-01-S1 (to KUK) and National Institute of Health Grant R21HL096031-01A1 and Veterans Administration Merit Review (to RSD).

REFERENCES

1. Wedel B, Garbers D. Annu Rev Physiol. 2001; 63:215–233. [PubMed: 11181955]
2. Tremblay J, Desjardins R, Hum D, Gutkowska J, Hamet P. Mol Cell Biochem. 2002; 230(1–2):31–47. [PubMed: 11952095]

3. Kuhn M. *Circ Res.* 2003; 93(8):700–709. [PubMed: 14563709]
4. Ruskoaho H. *Pharmacol Rev.* 1992; 44(4):479–602. [PubMed: 1480667]
5. Wilson EM, Chinkers M. *Biochemistry.* 1995; 34(14):4696–4701. [PubMed: 7718574]
6. Duda T, Goraczniak RM, Sharma RK. *Biochemistry.* 1994; 33(23):7430–7433. [PubMed: 7911683]
7. Thompson DK, Garbers DL. *J Biol Chem.* 1995; 270(1):425–430. [PubMed: 7814405]
8. Koglin M, Behrends S. *Biochem Pharmacol.* 2004; 67(8):1579–1585. [PubMed: 15041475]
9. Foerster J, Harteneck C, Malkewitz J, Schultz G, Koesling D. *Eur J Biochem.* 1996; 240(2):380–386. [PubMed: 8841402]
10. Krumenacker JS, Hanafy KA, Murad F. *Brain Res Bull.* 2004; 62(6):505–515. [PubMed: 15036565]
11. Sansom SC, Stockand JD. *Clin Exp Pharmacol Physiol.* 1996; 23(1):76–82. [PubMed: 8713500]
12. Stockand JD, Sansom SC. *Am J Physiol.* 1996; 271(5 Pt 1):C1669–C1677. [PubMed: 8944651]
13. Stockand JD, Sansom SC. *Physiol Rev.* 1998; 78(3):723–744. [PubMed: 9674692]
14. Burkhardt M, Glazova M, Gambaryan S, Vollkommer T, Butt E, Bader B, Heermeier K, Lincoln TM, Walter U, Palmethofer A. *J Biol Chem.* 2000; 275(43):33536–33541. [PubMed: 10922374]
15. Gambaryan S, Hauser W, Kobsar A, Glazova M, Walter U. *Histochem Cell Biol.* 2001; 116(6):535–543. [PubMed: 11810195]
16. Lincoln TM, Dey N, Sellak H. *J Appl Physiol.* 2001; 91(3):1421–1430. [PubMed: 11509544]
17. Sasaki S, Siragy HM, Gildea JJ, Felder RA, Carey RM. *Hypertension.* 2004; 43(2):286–291. [PubMed: 14718358]
18. Jin XH, McGrath HE, Gildea JJ, Siragy HM, Felder RA, Carey RM. *Hypertension.* 2004; 43(5):1133–1139. [PubMed: 15007031]
19. Fischmeister R, Castro LR, Abi-Gerges A, Rochais F, Jurevicius J, Leroy J, Vandecasteele G. *Circ Res.* 2006; 99(8):816–828. [PubMed: 17038651]
20. Beavo JA, Brunton LL. *Nat Rev Mol Cell Biol.* 2002; 3(9):710–718. [PubMed: 12209131]
21. Dreyfuss G, Kim VN, Kataoka N. *Nat Rev Mol Cell Biol.* 2002; 3(3):195–205. [PubMed: 11994740]
22. Hamilton BJ, Nagy E, Malter JS, Arrick BA, Rigby WF. *J Biol Chem.* 1993; 268(12):8881–8887. [PubMed: 8473331]
23. Henics T, Sanfridson A, Hamilton BJ, Nagy E, Rigby WF. *J Biol Chem.* 1994; 269(7):5377–5383. [PubMed: 8106521]
24. Pinol-Roma S, Dreyfuss G. *Science.* 1991; 253(5017):312–314. [PubMed: 1857966]
25. Svitkin YV, Ovchinnikov LP, Dreyfuss G, Sonenberg N. *Embo J.* 1996; 15(24):7147–7155. [PubMed: 9003790]
26. Bonnal S, Pileur F, Orsini C, Parker F, Pujol F, Prats AC, Vagner S. *J Biol Chem.* 2005; 280(6):4144–4153. [PubMed: 15525641]
27. Kotlo KU, Rasenick MM, Danziger RS. *Mol Cell Biochem.* 338(1–2):183–189. [PubMed: 20024606]
28. Airhart N, Yang YF, Roberts CT Jr, Silberbach M. *J Biol Chem.* 2003; 278(40):38693–38698. [PubMed: 12855709]
29. Castro LR, Verde I, Cooper DM, Fischmeister R. *Circulation.* 2006; 113(18):2221–2228. [PubMed: 16651469]
30. Zolle O, Lawrie AM, Simpson AW. *J Biol Chem.* 2000; 275(34):25892–25899. [PubMed: 10851228]
31. Hart CY, Hahn EL, Meyer DM, Burnett JC Jr, Redfield MM. *Am J Physiol Heart Circ Physiol.* 2001; 281(1):H146–H154. [PubMed: 11406479]
32. Rho EH, Perkins WJ, Lorenz RR, Warner DO, Jones KA. *J Appl Physiol.* 2002; 92(1):257–263. [PubMed: 11744668]
33. Su J, Scholz PM, Weiss HR. *Exp Biol Med (Maywood).* 2005; 230(4):242–250. [PubMed: 15792945]
34. Buchwalow IB, Minin EA, Samoilova VE, Boecker W, Wellner M, Schmitz W, Neumann J, Punkt K. *Biochem Biophys Res Commun.* 2005; 330(2):615–621. [PubMed: 15796928]

35. Piggott LA, Hassell KA, Berkova Z, Morris AP, Silberbach M, Rich TC. *J Gen Physiol.* 2006; 128(1):3–14. [PubMed: 16769793]
36. Takimoto E, Belardi D, Tocchetti CG, Vahebi S, Cormaci G, Ketner EA, Moens AL, Champion HC, Kass DA. *Circulation.* 2007; 115(16):2159–2167. [PubMed: 17420342]
37. Hao Y, Xu N, Box AC, Schaefer L, Kannan K, Zhang Y, Florens L, Seidel C, Washburn MP, Wiegraabe W, Mak HY. *PLoS Genet.* 7(5):e1002065. [PubMed: 21573134]
38. Sugiura T, Nakanishi H, Roberts JD Jr. *Circ Res.* 2008; 103(1):53–60. [PubMed: 18535260]
39. Schlossmann J, Desch M. *Handb Exp Pharmacol.* 2009; (191):163–193. [PubMed: 19089330]
40. Jo OD, Martin J, Bernath A, Masri J, Lichtenstein A, Gera J. *J Biol Chem.* 2008; 283(34):23274–23287. [PubMed: 18562319]
41. Guil S, Long JC, Caceres JF. *Mol Cell Biol.* 2006; 26(15):5744–5758. [PubMed: 16847328]
42. Shi Y, Frost P, Hoang B, Benavides A, Gera J, Lichtenstein A. *J Biol Chem.* 286(1):67–78. [PubMed: 20974848]
43. Shimada N, Rios I, Moran H, Sayers B, Hubbard K. *RNA Biol.* 2009; 6(3):293–304. [PubMed: 19430204]
44. Michlewski G, Guil S, Caceres JF. *Adv Exp Med Biol.* 700:28–35.
45. Michlewski G, Guil S, Semple CA, Caceres JF. *Mol Cell.* 2008; 32(3):383–393. [PubMed: 18995836]
46. van der Houven van Oordt W, Diaz-Meco MT, Lozano J, Krainer AR, Moscat J, Caceres JF. *J Cell Biol.* 2000; 149(2):307–316. [PubMed: 10769024]
47. Casteel DE, Zhang T, Zhuang S, Pilz RB. *Cell Signal.* 2008; 20(7):1392–1399. [PubMed: 18450420]
48. Kass DA, Takimoto E, Nagayama T, Champion HC. *Cardiovasc Res.* 2007; 75(2):303–314. [PubMed: 17467673]
49. Das S, Au E, Krazit ST, Pandey KN. *Endocrinology.* 151(12):5841–5850. [PubMed: 20881240]
50. Nishihara K, Masuda S, Nakagawa S, Yonezawa A, Ichimura T, Bonventre JV, Inui K. *Am J Physiol Renal Physiol.* 298(4):F923–F934. [PubMed: 20071461]
51. Nishikimi T, Inaba-Iemura C, Ishimura K, Tadokoro K, Koshikawa S, Ishikawa K, Akimoto K, Hattori Y, Kasai K, Minamino N, Maeda N, Matsuoka H. *Regul Pept.* 2009; 154(1–3):44–53. [PubMed: 19223006]

Fig 1. Nuclear and membrane compartmentalization of cGMP formed by ANP signaling
 HK-2 cells are pre-treated with non-selective broad-spectrum PDE inhibitor IBMX for 30 min followed by treatment with 10^{-6} M ANP and 10^{-7} M SNAP for 45 min. At the end of treatment periods, nuclear (top panel left), membrane (top panel right), cytosolic (bottom panel, left), and cytoskeletal (bottom panel, right) fractions of cells are harvested and subjected to cGMP assay as described in materials and methods. $n = 4$. * $p < 0.05$, ** $p < 0.01$.

Fig 2. Identification of differentially expressed proteins from HK-2 cells treated with ANP or SNAP by 2-DIGE

Left panel. Cell lysates are harvested from HK-2 cells pre-treated with non-selective broad-spectrum PDE inhibitor IBMX for 30 min followed by treatment with 10^{-6} M ANP and 10^{-7} M SNAP for 45 min. Protein is labeled with Cy3 (*green*) for ANP-treated samples and Cy5 (*red*) for SNAP-treated samples, respectively. Isoelectric focusing was carried out at pH 3–10, and the two-dimensional separation was carried out in an 8–14% gradient SDS-PAGE. On the left is gel image revealing differential expression of proteins in ANP vs. SNAP treated samples after merging. Protein spots shown in *red and green* are up regulation by SNAP and ANP treatment of HK-2 cells, respectively. Yellow represent no change in the protein levels between two treatments.

Right panel. Phosphoprotein staining of the 2D-DIGE gel showing the phosphoproteins differentially expressed in ANP vs. SNAP treated samples. The proteins circled in green and pink represent proteins differentially phosphorylated in ANP and SNAP-treated HK-2 cells, respectively. $n=3$.

Fig 3. ANP stimulation increases cGK activity in the nuclear and membrane fractions of HK-2 cells whereas SNAP treatment leads to increased cGK activity in cytoplasmic portion
 Nuclear, cytoplasmic, and membrane fractions from HK-2 cells pre-treated with non-selective broad-spectrum PDE inhibitor IBMX for 30 min followed by treatment with 10^{-6} M ANP and 10^{-7} M SNAP for 45 min are isolated and subjected to cGK activity assay as described in methods. $n=4$, ** $p<0.01$ and * $p<0.05$.

Fig 4. hnRNP A1 is selectively and specifically phosphorylated by ANP/GC-A/cGMP/cGK pathway

hnRNP A1 from nuclear fractions of control untreated, HK-2 cells pre-treated with non-selective broad-spectrum PDE inhibitor IBMX for 30 min followed by treatment with 10^{-6} M ANP and 10^{-7} M SNAP for 45 min and cGK inhibitor (KT-5823) are immunoprecipitated with hnRNP A1 antibody followed by western blotting with anti-phospho serine threonine antibody. For cGK inhibitor studies cells are pre-treated with KT-5823 (2 μ M X1 hr) followed by ANP treatment. A representative western blot (left panel) and summarized data (right panel) are shown. Phospho hnRNP A1 is indicated by an arrow. $n=4$. $p<0.05$.

Fig 5. Sub-cellular localization of cGK II and hnRNP A1 in HK-2 cells

HK-2 cells are plated in 4-well chamber slides and treated with or without ANP ($10^{-6}\text{M} \times 45 \text{ min}$) as indicated. Immunofluorescence staining using a rabbit antibody directed against cGK II (green fluorescence, top panel), a mouse monoclonal antibody against hnRNP A1 (red fluorescence, middle panel), and both antibodies (lower panel) is performed as described in Materials and Methods. Merger of cGK and hnRNP A1 signals (yellow fluorescence) is indicated. Blue represents the nuclei stained by DAPI. Experiments are repeated for four times.

Fig 6. PDE-5 inhibition increases ANP-stimulated hnRNP A1 phosphorylation
 hnRNP A1 from nuclear fractions of control untreated, HK-2 cells treated with 10^{-6} M ANP and 10^{-7} M SNAP in either alone or in the presence of sildenafil (10 nM) for 45 min are immunoprecipitated with hnRNP A1 antibody followed by western blotting with anti-phospho serine threonine antibody. A representative western blot (left panel) and quantification of data (right panel) are shown. $n=3$. $p < 0.05$.

Fig 7. nRNP A1 is translocated to nucleus in HK-2 cells treated with sildenafil and ANP
Methods are same as described in figure legend 5 except that cell are treated with either ANP and SNAP in the presence of sildenafil (10 nM) or sildenafil (10 nM) alone.

Fig 8. Nuclear cGK activity is increased in ANP-stimulated HK-2 cells in the presence of PDE-5 inhibitor sildenafil

Nuclear fractions from HK-2 cells treated with sildenafil (10 nM) alone or in the presence 10^{-6} M ANP and 10^{-7} M SNAP for 45 min are isolated and subjected to cGK activity assay as described in methods. $n=4$. * $p<0.05$.