Discovery of an alternate mechanism for the inhibition of particulate guanylyl cyclases by the natriuretic peptide 'clearance' receptor

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Abstract

The cardiac natriuretic peptides (NPs) control pivotal physiological actions such as fluid and electrolyte balance, cardiovascular homeostasis, and adipose tissue metabolism by activating their receptor enzymes (NPRA and NPRB). These receptors are homodimers that generate intracellular cyclic guanosine monophosphate (cGMP). The so-called 'clearance' receptor NPRC lacks a guanylyl cyclase domain; instead it can bind the NPs to internalize and degrade them. The conventional paradigm is that by competing for and internalizing NPs, NPRC blunts the ability of NPs to signal through NPRA and NPRB. Here we show a previously unknown mechanism by which NPRC interferes with the cGMP signaling function of the NPs: by forming a heterodimer with monomeric NPRA or NPRB, NPRC prevents the formation of a functional guanylyl cyclase domain and thereby suppressing cGMP production.

Significance Statement

Natriuretic peptides (NP) are hormones that are important regulators of vascular and cardiac function, in part via their regulation of fluid and electrolyte balance. NPs signal through particulate guanylyl cyclases (NPRA and NPRB), which are homodimeric membrane-bound receptor enzymes that generate cGMP upon NP binding. Additionally, a 'silent' NP receptor (NPRC) lacks the guanylyl cyclase domain and is a negative regulator of NP signaling. Until now, it has been thought that inhibition of NP signaling by NPRC was achieved via competition for and degradation of NP ligands. Here, we demonstrate a new mechanism by which NPRC inhibits NP signaling: NPRC forms non-functional heterodimers with NPRA and NPRB thereby abrogating NP signaling. This finding establishes a novel mechanistic role for NPRC. (120 words)

Main Text

Introduction

In 1981 de Bold and colleagues (1) made the observation that extracts from atria when injected into rats caused a striking drop in blood pressure. These natriuretic factors were then shown by de Bold to be a peptide (2). We now know these factors as atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP). These discoveries unexpectedly placed the heart in the category of an endocrine organ. It was subsequently determined that the action of these natriuretic factors was mediated by a receptor, natriuretic peptide receptor-A (NPRA, also referred to as GC-A), in the kidney to cause natriuresis and diuresis. NPRA is an integral membrane protein that in dimeric form contains guanylyl cyclase activity (3); consequently, cyclic GMP mediates NP action. Another related peptide, CNP, was later discovered that has its own guanylyl cyclase-containing receptor NPRB (or GC-B) (4). A third NP receptor, NPRC, was discovered (5) and referred to as the 'silent' receptor (6), as it has a short cytoplasmic domain and lacks guanylyl cyclase activity. Administration of variants of ANP_{1-28}, including ANP_{4-23} and a modified ring-deleted form are selective for binding NPRC (6, 7), were found to increase endogenous levels of ANP and BNP in circulation (6-9), suggesting it played some role in antagonizing the actions of the GC-containing receptors. NPRC-mediated endocytosis of ANP was observed in several subsequent studies (10-12), further cementing NPRC's nickname as the 'NP clearance receptor'. As a result, NPRC can be considered to function as an inhibitor of NPRA/NPRB-cGMP signaling by competing for the peptide ligands.

Since these initial discoveries, NPs have been found to exert a broader range of physiological functions beyond natriuresis and vasodilation, ranging from protection from cardiac fibrosis to stimulating adipose, bone and skeletal muscle metabolism (13, 14). The NP signaling pathway has recently been harnessed for the therapeutic treatment of heart failure with a new class of drugs, Angiotensin Receptor-Neprilysin inhibitor (ARNi), which includes inhibition of neprilysin (also known as neutral endopeptidase) (15). Neprilysin is a metalloprotease that degrades NPs along with several other vasoactive peptides. Clinically, the level of NPRC in

tissues has been correlated with certain disease states. For example, in large population studies, subjects with obesity and hypertension have been shown to have lower circulating ANP and BNP levels (16-22), coupled with a dramatic increase in the level of NPRC in their adipose tissue (23-27). This has led to the concept of a 'natriuretic handicap', and it has been proposed that possibly the increased adipose NPRC levels may serve as a 'sink' to remove NPs from the circulation and thus contribute to disease pathology in terms of blunted adipose tissue lipolysis as well as increased blood pressure and risk of heart failure. This increase in NPRC levels with obesity has also been observed in preclinical models, including rats and mice (28-30). Conversely, in both rodents and humans, dieting/fasting and cold exposure raises the NPRA/NPRC ratio in adipose tissue, largely by decreasing the levels of NPRC, resulting in improved NP signaling (31-33). In support of this we recently reported that adipose tissue-specific disruption of the *Nprc* gene in mice resulted in improvements in several aspects of metabolic disease in an obesity paradigm (30). As we and others showed in earlier work, raising the level of NPRC shifts the dose-response curve for cGMP production through NPRA to the right in the same manner as a receptor antagonist (30, 34). Thus, the biological significance of the relative ratio of NPRA to NPRC *in vivo* raises a number of questions, such as how NPRC expression is regulated and how it interferes with NP signaling through their signaling receptors. In this study, we asked whether the ability of elevated NPRC to impair NPRA activity is solely due to the clearance function of NPRC, or whether NPRC, lacking a guanylyl cyclase domain, might form heterodimers with NPRA or NPRB. In this report we present evidence for the existence of such heterodimers using several independent approaches.

Results

HEK-293 cells were transfected to express either NPRA alone or NPRA and NPRC together, followed by preparation of plasma membranes and ANP-evoked cGMP assays. In this approach, unlike using intact cells as in our previous work (30), NPRC internalization to remove ANP cannot occur in a plasma membrane preparation. As shown in **Figure 1**, when NPRA and

NPRC were expressed together in the same cell, the cGMP generated over time was significantly reduced, compared to plasma membranes from cells expressing NPRA alone. Therefore, if the effect of NPRC on NPRA function is solely due to endocytosis and clearance, we would expect that there should be no difference in the rate of cGMP production. This apparent 'discrepancy' from the 'clearance' model raised the possibility that heterodimers between NPRA-NPRC and NPRB-NPRC might form when expressed in the same cell. Such heterodimers would block cGMP generation because the heterodimer only contains one-half of the guanylyl cyclase enzyme, which to function is an obligate homodimer (35, 36).

Figure 2A shows that following transfection of Flag-tagged NPRA into HEK-293 cells, immunoprecipitation (IP) of the cell lysate with anti-Flag antibody retrieves the endogenously expressed NPRC protein. **Figure 2B** shows that this interaction is independent of ligand, whether using ANP_{1-28} or the NPRC-selective ANP_{4-23}. In a similar fashion, Flag-tagged NPRB was introduced into HEK-293 cells, and IP with anti-Flag antibody also recovers the endogenous NPRC protein (**Fig 2C**). In order to understand which cellular domain is responsible for this protein-protein interaction, a truncated form of NPRA was generated that retains the transmembrane domain but lacks all but 37 amino acids of the intracellular domain: thus, making its intracellular domain the same length as NPRC (**Suppl. Fig. 1A**). As shown in **Figure 2D**, similar to the full length NPRA, the truncated form could also immunoprecipitate the endogenous NPRC. Since the majority of the kinase homology domain and the entire hinge region and guanylyl cyclase catalytic domain were removed, it can be concluded that these are not necessary for heterodimer formation. In support of this a crystal structure of the dimeric extracellular hormone binding domain of NPRA (37) suggests that this region is sufficient for dimer formation.

In another approach, we used the NanoLuc® Binary Technology (NanoBiT) (38) to demonstrate this interaction in cells. We made NPRA, NPRB, and NPRC constructs that contain a 'two-hybrid' type luciferase reporter at their C-termini (see **Suppl. Fig. 1B**). In this assay, the luciferase signal is only produced when proteins containing the complementary halves of the reporter subunits (Large BiT, *lg*, and Small BiT, *sm*) form a functional luciferase enzyme. For this

assay HAP-1 cells were chosen due to low endogenous expression level of the three natriuretic peptide receptors (**Suppl. Fig. 2**). **Figure 3A** shows that when NPRA and NPRC are tagged with *lg* and *sm* complementary halves of the reporter subunits, significant luminescence is observed. The relative expression of these proteins is shown in the Western Blots in **Figure 3B, D**. While the strength of the luciferase signals varied across constructs, this is likely due to the relative levels of expression of the proteins and the size of the intracellular domains that could increase the distance between the two parts of the luciferase enzyme. This is especially true for the NPRC-*lg*/NPRA-*sm* in **Figure 3A**, which was expected due to the presence in full-length NPRA of the intracellular kinase homology domain, hinge region, and guanylyl cyclase catalytic domain, for which there is a difference in 528 intracellular amino acid length between them. Because results from others (35, 37) and our data here (**Fig. 2D**) demonstrated that the intracellular guanylyl cyclase domain is not required for dimer formation, we generated luciferase reporters for NPRA and NPRB that lack all but 37 amino acids of the intracellular domain termed: $NPRA_{1-532}$ and $NPRB_{1-515}$, thus making their intracellular regions the same length as NPRC. As shown in **Fig. 3A** and **3C,** NPRC formed heterodimers with both $NPRA_{1-532}$ and $NPRB_{1-515}$ producing a strong luciferase response. To determine if expressing NPRC is capable of disrupting NPRA or NPRB homodimers in this assay, cells expressing $NPRA_{1-532}$ or $NPRB_{1-515}$ were co-transfected with Flag-tagged-NPRC. As shown in **Figure 3E,F** the presence of NPRC significantly reduced the luciferase signal indicating the blunted ability of NPRA and NPRB to form homodimers.

Finally, we employed the DuoLink® proximity ligation assay using HeLa and A-10 cells as models to test the NPRA and NPRC interaction because these cells endogenously express relatively high levels of both receptors (39). **Figure 4** shows that for both cell types fluorescent puncta are detected only when antibodies for both receptors are present.

Discussion

Natriuretic peptides are important clinically as both a biomarker for heart failure as well as playing a key role mediating the therapeutic action of ARNi's, a new and promising class of heart

failure medication (40-42). Our novel findings reported here significantly alter the understanding of how NP signaling operates and has broad implications for many fields due to the expression of NPRC in diverse organs such as kidney, heart, bone, and adipose tissue. It has long been known that increasing levels of NPRC can progressively interfere with the signaling and biological actions of ANP through NPRA; this being generally attributed to the peptide clearance function of NPRC (43). Our identification of this new mechanism by which NPRC inhibits NPRA and NPRB indicates that the level of NPRC expression in the individual cell, instead of just at the organ or tissue level, is likely to be critically important for the regulation of NP signaling. Furthermore, as we begin to realize the importance of NPRC to the individual cell's regulation of NP signaling, new questions are raised such as how cells control the amount of NPRC at the plasma membrane and what governs the formation of NPR homodimers versus heterodimers. All together our findings demonstrate that NPRC interacts with both NPRA and NPRB and competes with NPRA and NPRB for homodimer formation, with the net effect to reduce NP-evoked cGMP production. Our data indicate that in addition to its role as a clearance receptor, a previously unrecognized function of NPRC is to act as a dominant-negative NP receptor by forming catalytically inactive heterodimers with NPRA and NPRB. Our results in aggregate provide evidence that monomers of NPRA (and NPRB) can form non-functional heterodimers with NPRC, with the result being an inability to generate cGMP. We suggest that this heterodimer formation may be another physiologic mechanism by which NPRC inhibits or dampens NP-cGMP signaling.

Materials and Methods

Plasmids

A summary of the plasmids used in this study can be found in Supplemental Table 1. NanoLuc plasmids were made by cloning full-length and truncated human *NPR1*, *NPR2*, and *NPR3* genes from the FLAG-tagged plasmids purchased from GenScript into the appropriate vectors from the NanoBiT® PPI MCS Starter System (Promega, #N2014). Intracellular domain deletion of Flag-rNPRA$_{(1-527)}$ was performed by site-directed mutagenesis to introduce an early stop codon and all

plasmid constructs were sequenced and confirmed by Genewiz (South Plainfield, NJ). Primers used for cloning and sequencing can be found in Supplemental Table 2.

Reagents and Antibodies

The following reagents were purchased from Sigma: bovine serum albumin (BSA) (#A7906), creatine phosphokinase (#C3755), guanosine 5′-triphosphate sodium salt (GTP) (#G8877), phenylmethanesulfonyl fluoride (PMSF) (#P7626), phosphocreatine disodium salt (#P7936). ANP_{1-28} was from AnaSpec (AS-20648). ANP_{4-23} was from Phoenix Pharmaceuticals (#005-26). 3-Isobutyl-1-methylxanthine (IBMX) was from Acros Organics (#228420050). PF-04447943 was purchased from MedChem Express (#HY-15441/CS-0942). Polyethylenimine (PEI) was from Polysciences, Inc. (#23966). Sucrose was from EMD Chemicals (#SX1075-1). From Roche, PhosSTOP™ (#04906837001) and cOmplete™, Mini Protease Inhibitor Cocktail (#04693124001) were purchased. Protein concentration was measured using Micro BCA™ Protein Assay Kit from Thermo Scientific (#23235). cGMP was measured using Cyclic GMP XP® Assay Kit from Cell Signaling (#4360).

For Western Blotting the following primary antibodies were used: Flag (Sigma #F1804), NPRA (Novus Biologicals #NBP1-31333), NPRB (Proteintech #55113-1-AP), NPRC (Novus Biologicals #NBP1-31365), NanoLuc® Luciferase (R&D Systems #MAB10026), β-actin (Cell Signaling #4967). Secondary antibodies were goat anti-Mouse IgG−Alkaline Phosphatase (Sigma #A3562) and goat anti-Rabbit IgG–Alkaline Phosphatase (Sigma #3687). For immunoprecipitation Anti-FLAG® M2 Affinity gel (Sigma #A2220) was used.

Cell culture and transfection

HEK-293T, HEK-293FT, HeLa, and A-10 cells were cultured with DMEM high glucose containing 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin. HAP-1 cells were maintained in Iscove's modified Dulbecco's medium with 10% FBS and 50 units/ml penicillin and 50 µg/ml streptomycin. For plasma membranes, 8 µg total plasmid was transfected using 21 µg of PEI per 10 cm plate. For co-immunoprecipitation experiments, 2 µg total plasmid was transfected using 6 µg of PEI per 3.5 cm diameter well. For luciferase assay, 0.4 µg total plasmid was

transfected using 1.2 µg of PEI per well of a 96-well plate. Cells were collected twenty-four hours post transfection.

cGMP Production Assay

HEK-293T cells were transfected with equimolar concentrations of either hNPRA-Flag and hNPRC-Flag or hNPRA-Flag and pcDNA3-Clover plasmids. At the time of harvesting, cells were rinsed with ice-cold Membrane Lysis Buffer (15 mM HEPES, 5.0 mM EDTA, 5.0 mM EGTA, 1.5 mM $MgCl_2$, 2 mM PMSF, pH 7.4-7.6). 5 ml ice-cold Membrane Lysis Buffer was added to each 10 cm plate, cells were scrapped off with a rubber policeman, and were triturated through a 21-gauge needle 5 times. This lysate was gently layered onto a cushion of 60% sucrose solution in Membrane Lysis Buffer in 10 cm x 1.5 cm diameter Sorvall centrifuge tube. Lysates were centrifuged at 35,000 ×g for one hour at 4 °C in a Sorvall SS34 rotor (Du Pont). Membranes were isolated from the sucrose-buffer interface, triturated through a 25-gauge needle 5 times, aliquoted, and stored at -80 °C. For guanylyl cyclase reaction, membranes were diluted to a concentration of 25 µg per 10 µl in Membrane Lysis Buffer. 10 µl (25 µg) membranes were added to a tube on ice containing 90 µl of Guanylyl Cyclase Reaction Buffer (50 mM Tris-HCl pH 7.6, 4 mM $MgCl_2$, 1 mM GTP, 2 mM IBMX, 300 nM PF-04447943, 1 mg/ml BSA, 7.5 mM phosphocreatine, 55 units/ml creatine phosphokinase) containing 100 nM ANP. The reaction was started when tubes were placed in a 37 °C water bath. At the indicated timepoints, 20 µl was removed and placed in 180 µl Stop Reaction Buffer (50 mM Sodium acetate, pH 6.2), boiled for 3 minutes, and subsequently incubated on ice for 15 minutes. Samples were centrifuged at 12,000 ×g for 5 minutes 4 °C and supernatant was saved and stored at -80 °C. cGMP was measured in duplicate according to manufacturer's directions using the Cyclic GMP XP® Assay Kit (Cell Signaling #4360) after the supernatant was diluted 1:10 in 1× Cell Lysis Buffer (Cell Signaling #9803). Plates were read on an Epoch Microplate Spectrophotometer with Gen5 Software (Biotek Instruments). Results were from four independent experiments using two independent preparations of membranes. The average from each sample duplicate was plotted as percent cGMP at time 0 and the linear regression function in Prism was

used to calculate and determine if the slopes of cGMP concentration over time were different between membranes from NPRA+NPRC and NPRA transfected cells.

Immunoprecipitation and Western Blotting

Cells were lysed and sonicated in a buffer containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM glycerophosphate, 0.9% Triton X-100, 0.1% IGEPAL, 5 mM sodium pyrophosphate, 10% glycerol, plus 1 tablet each of PhosSTOP™ and cOmplete™, Mini Protease Inhibitor Cocktail per 10 ml of lysis buffer. For immunoprecipitations, 1 mg total protein from the cell lysate was incubated with Anti-FLAG® M2 Affinity gel at 4 °C for 2 hours, followed by three 5 min washes with the lysis buffer. For Western Blot analysis, 50 µg total protein ("Lysate"), the immunoprecipitate, or 25 µg plasma membrane was resolved in 10% Tris-glycine gels, transferred to nitrocellulose membranes, which were blocked in 5% milk for 1-hour at room temperature, then incubated overnight at 4 °C with specific primary antibodies, followed by secondary antibody incubations for 1-hour at room temperature. Image acquisition was performed on The Digital ChemiDoc MP (Bio-Rad) at the VUMC Molecular Cell Biology Resource Core.

NanoLuc Luminescence Assay

NanoLuc® Binary Technology (NanoBiT) is a two-subunit luciferase system used for detection of protein:protein interactions. Following the NanoBiT® PPI MCS Starter System protocols, plasmids containing Large BiT (LgBiT; 17.6kDa, 156 amino acids) and Small BiT (SmBiT; 11 amino acids) subunits fused to the C-terminal of full length and truncated NPRA, NPRB, NPRC were generated (Supplemental Figure 1, Supplemental Table 1). In addition, positive control vectors containing the PKA regulatory (LgBiT-PRKAR2A) and catalytic (SmBiT-PRKACA) subunits were utilized for protocol validation (data not shown). Equimolar ratios of LgBiT and SmBiT plasmids were transiently co-transfected into HAP-1 cells growing in flat, clear-bottom, black-wall 96-well plates (Greiner Bio-One #655090). 36-hours post transfection, protein:protein interactions were detected in real-time using live cells with the Nano-Glo® Live Cell Assay System, a nonlytic detection reagent that contains the cell-permeable substrate furimazine. Experiments were performed on a Synergy™ NEO HTS Multi-Mode Microplate Reader (Biotek Instruments) in the

Vanderbilt High-Throughput Screening (HTS) Core Facility. The HTS Core receives support from the Vanderbilt Institute of Chemical Biology and the Vanderbilt Ingram Cancer Center (P30 CA68485). For each experiment, conditions were run in triplicate. Replicates were excluded if contaminated by light from neighboring wells. Data are from four independent experiments. Data were log transformed and analyzed by paired t-test comparing each condition to the untransfected control using Prism 8.0.

Duolink Proximity Ligation Assay

Duolink proximity ligation assays were performed using a Duolink™ In Situ PLA Kit Goat/Rabbit (Olink Biosciences) purchased from Sigma. Briefly, HeLa and A-10 cells were grown on 8-well glass chamber slides overnight, washed three times with cold PBS, and fixed for 10 min with 4% PFA. The chamber was removed, washed once more with cold PBS, then incubated in 70 ml 300mM glycine PBS-T for 20 min, and one final PBS wash for 10 min. The antibodies used in the Duolink assays were rabbit Anti-NPR-A antibody from Abcam (#ab14356), at 1:200 dilution; and the goat Anti-NPR-C Antibody (N-20) from Santa Cruz Biotechnology (#sc-16871) at 1:100 dilution. Forty µl primary antibody solution was used for each well and was incubated in a humidified chamber at 4°C overnight. Secondary antibodies conjugated with the PLA probes provided by Duolink were added, and the remainder of the protocol was as described by the manufacturer. Fluorescence was detected by Nikon Eclipse-Ti TIRF microscope with Element software.

Data and materials availability: Plasmid constructs used in heterodimer studies are available upon completion of a Material Transfer Agreement. All data is available in the main text or the supplementary materials.

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Figures and Tables

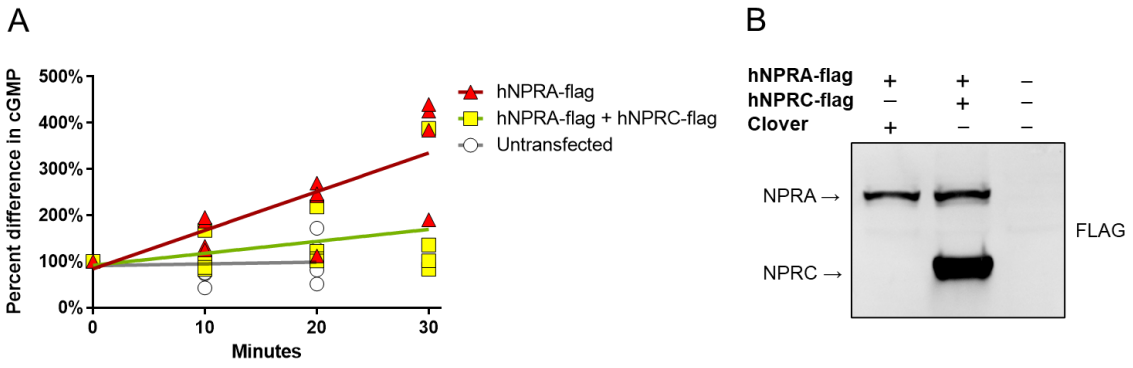

Figure 1. Co-expression of NPRC reduces ANP-evoked cGMP production in plasma membranes. Plasma membranes were made from HEK-293T cells transfected with either NPRA-Flag plus pcDNA3-Clover control or NPRA-Flag plus NPRC-Flag. (**A**) Co-expression of hNPRC along with hNPRA reduced the cGMP generated in the presence of 100 nM ANP (P=0.0163, test for difference in slopes between NPRA and NPRA+NPRC). Untransfected cells did not appear to generate cGMP. (**B**) Western Blot detecting the Flag epitope from the plasma membranes used in the ANP-stimulated cGMP assay.

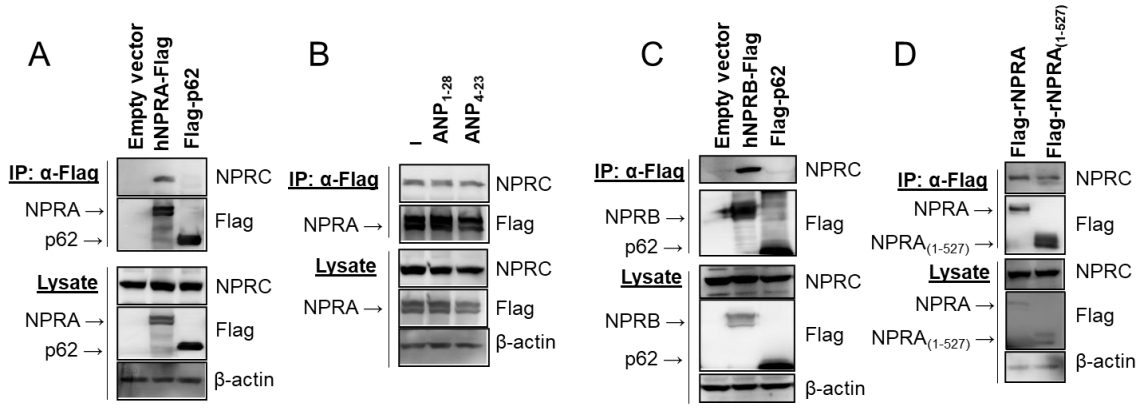

Figure 2. NPRC co-immunoprecipitates with NPRA and NPRB. HEK-293FT cells were transfected with plasmids expressing Flag-tagged natriuretic peptide receptors. Twenty-four hours after transfection, cell lysates were immunoprecipitated (IP) with anti-Flag Affinity agarose. (**A**) The endogenous NPRC coimmunoprecipitated with hNPRA-Flag. Flag-p62 served as a negative control for the Flag-IP. (**B**) hNPRA-Flag expressing HEK-293FT cells were treated with ANP (ANP_{1-28}, 200 nM) or a truncated form of ANP that preferentially binds NPRC (ANP_{4-23}, 200 nM) for 30 minutes. Neither ANP_{1-28} or ANP_{4-23} affected the ability of endogenous NPRC to coimmunoprecipitate with hNPRA-Flag. (**C**) Endogenous NPRC coimmunoprecipitated with hNPRB-Flag. Flag-p62 served as a negative control. (**D**) Endogenous NPRC coimmunoprecipitated with Flag-rNPRA and a truncated version of Flag-rNPRA lacking the C-terminal amino acids 528-1057 (Flag-$rNPRA_{(1-527)}$).

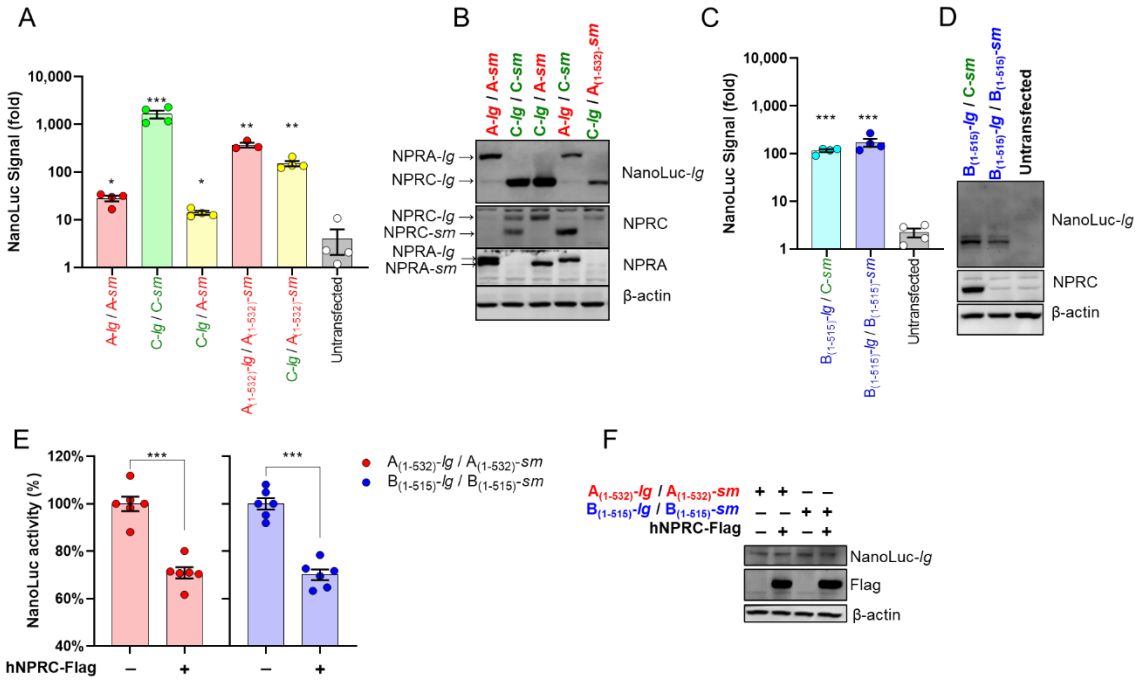

Figure 3. NPRC can form a heterodimer with either NPRA or NPRB and interferes with NPRA and NPRB homodimer formation. HAP-1 cells were transfected with natriuretic peptide receptors tagged with opposite halves of the NanoBiT complementation reporter. (**A**) A strong luciferase signal was observed for NPRA and NPRC homodimer as well as NPRA:NPRC heterodimer formation. A truncated version of NPRA containing only 37 intracellular amino acids ($NPRA_{(1-532)}$) also formed homodimers and heterodimers with NPRC. (**B**) Transfected proteins were visualized by Western Blot. (**C**) The truncated version of NPRB containing only 37 intracellular amino acids ($NPRA_{(1-515)}$) formed homodimers and heterodimers with NPRC. Full length NPRB exhibited poor expression, consequently the luciferase signal was not significantly greater than background (data not shown). (**D**) Western Blot was used to visualize expression of the transfected proteins. (**E**) HAP-1 cells were transfected with complementary halves of truncated NPRA or NPRB plus either unlabeled NPRC or empty vector. Both NPRA and NPRB expressing cells demonstrated reduced luciferase activity when expressing NPRC. (**F**) The transfected proteins were visualized by Western Blot. For all experiments, each point represents the average of 3 replicates from independent

experiments. Paired t-tests were performed on the log transformed data comparing each treatment group to the untransfected control. * $P<0.05$, ** $P<0.01$, *** $p<0.001$.

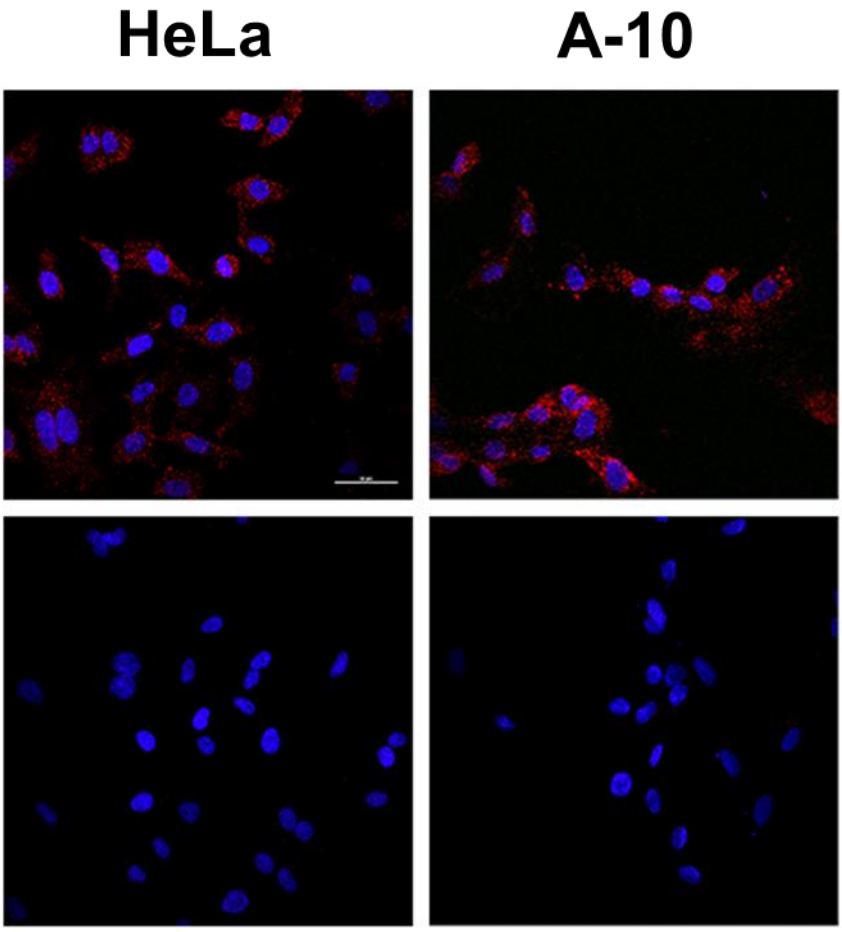

Figure 4. Endogenous NPRA:NPRC heterodimer formation in HeLa and A-10 cells.

HeLa cells (left panels) and A-10 cells (right panels) were used in the Duolink Proximity Ligation Assay as described in the Methods with either NPRA and NPRC antibodies together (top) or NPRC antibody alone (bottom).