Atrial Natriuretic Peptide and Long-acting Natriuretic Peptide Inhibit Ras in Human Prostate Cancer Cells

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Abstract. *Background: Atrial natriuretic peptide and long-acting natriuretic peptide have anticancer effects in human prostate adenocarcinoma. Materials and Methods: The effects of atrial natriuretic peptide and long-acting natriuretic peptide and cyclic GMP on Ras were examined in human prostate adenocarcinoma cells. Results: Atrial natriuretic peptide and long-acting natriuretic peptide reduced the activation of Ras-GTP over a concentration range of 0.01 μM to 1 μM. Atrial natriuretic peptide and long-acting natriuretic peptide (each 0.1 μM) inhibited the phosphorylation of Ras 90% ($p<0.0001$) and 83% ($p<0.0001$), respectively. At 0.01 μM of long-acting natriuretic peptide, the maximal inhibition was 89%, which occurred within 5 minutes. Both peptide hormones inhibited Ras for 24 hours. Their ability to inhibit Ras was inhibited by cyclic GMP antibody and cyclic GMP itself inhibited Ras phosphorylation (72%; $p=0.009$). Conclusion: Atrial natriuretic peptide and long-acting natriuretic peptide both inhibit Ras partially mediated via cyclic GMP as part of their anticancer mechanism(s) of action.*

The Ras mitogen-activated protein kinase (MAPK)/ extracellular signal-related kinase (ERK) kinase (MEK) ERK cascade, hereafter referred to as the Ras-MAPK pathway, is the prototypical signal transduction pathway in cancer (1, 2). This pathway is aberrantly activated in many types of neoplasms, including prostate and breast cancer, with this activation being associated with a poor prognosis (1, 2). The integral role of the Ras–MEK-ERK pathway in mediating multiple hallmarks of cancer has suggested that the different kinases in this pathway may be targets for the treatment of cancer (3-6). Structural alteration in the GTPase Ras occur in 25% to 30% of human cancers which allows them to relay mitogen signals in a ligand-independent manner, thereby obviating the need for ligand activation of growth factor receptors that occurs in normal cells (3, 4). Attempts to target Ras by perturbing its interaction with either Son of Sevenless gene (SOS) or growth factor receptor-bound 2 (GRB2) have not yielded viable drug development candidates largely because of the inherent difficulties of disrupting protein protein interactions with drug-like molecules (3). Several drug discovery programs have also been devoted to finding inhibitors of farnesyltransferase as a means to prevent the membrane localization of Ras (3). Despite the successful identification of several chemical leads that effectively inhibited this prenylation enzyme, tumor cells, however, have proved generally to be impervious to the action of this class of inhibitors (3).

The present investigation was designed to determine if two endogenous cardiac peptide hormones, namely atrial natriuretic peptide (ANP) and long-acting natriuretic peptide (LANP), which eliminate up to 80% of human pancreatic tumors in athymic mice (7) and two-thirds of human breast tumors in athymic mice (8), affect Ras. These two cardiac hormones are known to inhibit the phosphorylation (*i.e.* activation) of downstream kinases MEK 1/2 (9) by 97% ($p<0.0001$) and ERK 1/2 by 94% ($p<0.0001$) (10). These cardiac hormones have never been investigated for their effects on Ras. When both ANP and LANP were demonstrated to inhibit Ras in dose-response and time-sequenced experiments, it was then investigated whether their effects were specifically mediated by cyclic GMP, the intracellular mediator of many of the effects of these peptide hormones (11, 12).

Materials and Methods

Human prostate adenocarcinoma cells. The DU 145 cell line (ATCC number HTB-81) of human prostate adenocarcinoma cells was purchased from the American Type Culture Association (ATCC, Manassas, VA, USA). This prostate cancer cell line was derived in 1978 by KR Stone *et al*., (13) from a 69-year-old man. When injected into athymic mice, these homogenous cells form moderately differentiated prostate adenocarcinomas within 21 days (13).

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Culture of the prostate adenocarcinoma cells. Propagation of these cells was carried out in Roswell Park Memorial Institute (RPMI)-1640 medium with 2 mmol/l-glutamine adjusted by the addition of 1.5 g/l sodium bicarbonate, 10 mM HEPES, 1 mmol/l sodium pyruvate and heat-inactivated 10% fetal bovine serum (Sigma Chemical Company, St. Louis, MO, USA) with penicillin, streptomycin and fungizone, at a temperature of 37˚C, with 5% CO_2 as recommended by the ATCC.

Ras activity assay protocol. The activation of Ras was evaluated using a Ras activation assay kit (Upstate Cell Signaling Solutions, Temecula, CA, USA) according to the manufacturer's protocol. The DU 145 cells were incubated at a density of 1×10^7 cells at 90% confluence for 5,15, 30, and 45 minutes as well as 1, 2, 3, and 4 hours respectively, in dose-response curves with 0.01 μmol/l to 1 μmol/l of ANP or LANP. After the respective time periods, DU 145 cells were placed on ice, washed with cold phosphate-buffered saline (PBS), and lysed in magnesium lysis wash buffer (MLB) (25 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 1% Igepal CA-630, 10% glycerol, 25 mmol/l NaF, 10 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell lysates were centrifuged at 14,000 rpm for 5 min at 4˚C. Protein concentrations in cell lysate supernatants were determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Fresh lysates were utilized in these experiments because GTP-Ras (*i.e.* the activated form of Ras) is quickly hydrolyzed to GDP-Ras.

GTPγS/GDP loading for positive and negative controls. A volume of 0.5 ml of the DU 145 cell extract was aliquoted to two microfuge tubes to which 10 mM of EDTA was added. For the positive control 100 μmo/l of GTPγS was added while the negative control had 1 mmol/l of GDP added (both were from Upstate Cell Signaling Solutions). These tubes were incubated for 30 minutes at 30˚C with agitation. Loading was stopped by placing the tubes on ice and adding 60 mmol/l of $MgCl_2$.

Ras pull-down assay. To each 0.5 ml of DU 145 cell extract, 10 μg of Ras Assay Buffer Reagent (Upstate Cell Signaling Solutions) consisting of glutathione. S-transferase fusion protein, corresponding to the human Ras binding domain residues 1-149 of Raf-1 expressed in *Escherichia coli*, and provided bound to glutathione-agarose beads were added. This reagent specifically binds to and precipitates Ras-GTP from cell lysates. These reaction mixtures plus ANP, or LANP, or controls without either cardiac hormone were incubated for 45 minutes at 4˚C with gentle rocking followed by centrifugation at 14,000 × *g* at 4˚C for 10 sec to pellet the agarose beads. The beads were washed three times with MLB and then resuspended in 40 μl of 2× Laemmli reducing sample buffer with 2 μl of 1 M dithothreital and boiled for 5 minutes. These beads were then pelleted by centrifugation (14,000 × *g* at 4˚C) and collected by microcentrifuge pulse.

Immunoblotting analysis: Western blots. Protein samples (20 μl) were separated by 10% SDS-polyacrylamide gels (120 V for 90 min) and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences Corporation, Piscataway, NJ, USA) for 90 min at 110 V in transfer buffer. The membranes were blocked with 3% nonfat dry milk for 2 hours with gentle rocking and incubated overnight with anti-Ras, clone Ras 10 containing 0.05 ng/l of purified Ig G2aκ (Upstate Cell Signaling Solutions) at 4˚C with gentle rocking. These membranes were then washed three times (6 min each time) with PBS. The membranes were then immediately incubated with goat anti-mouse, horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad) at a dilution of 1:1000 for Ras for 1 hour at room temperature. The membranes were washed again two times and then examined by the chemiluminescent method.

Cyclic GMP effects on Ras. Cyclic GMP is one of the known mediators of the biological effects of ANP and LANP (11, 12). To investigate the mechanism of action of these peptide hormones in inhibiting Ras in DU 145 cells, 1 μM of 8-bromoguanosine 3',5'-cyclic monophosphate (8-bromo-cyclic GMP, Sigma), a cell-permeable analog of cyclic GMP, was added to the DU 145 cells in culture to determine if it could mimic the effects of ANP and LANP on Ras.

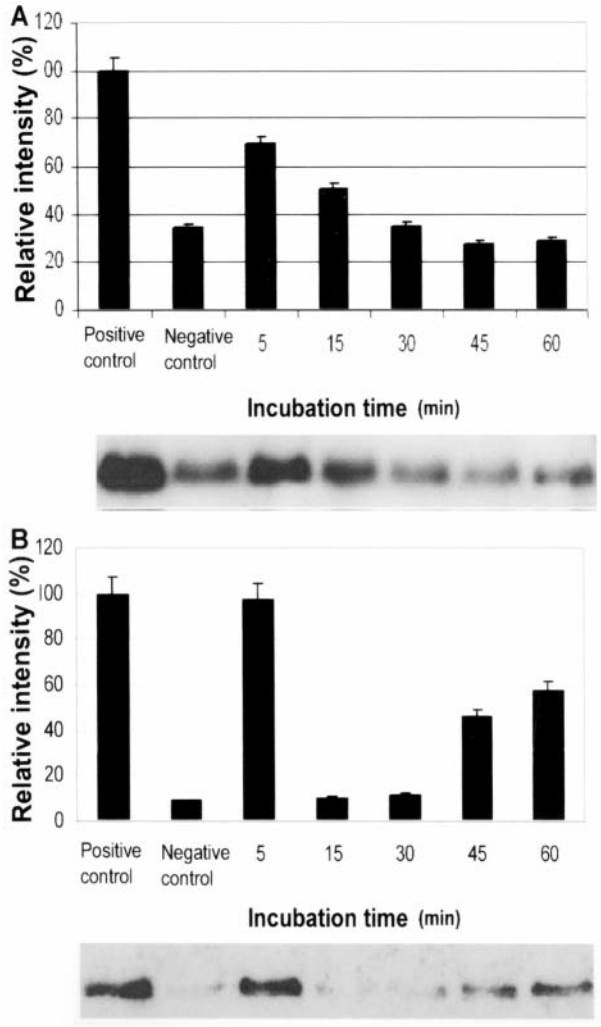
To determine if the inhibition of Ras in DU 145 cells was cyclic GMP specific, the peptide hormones (1 μmol/l each) and the specific cyclic GMP antibody in 1:80 dilution (Sigma) were incubated together with cells for 30 min followed by the above research protocol with immunoblotting.

Statistical analysis. Data are expressed as means±SEM and evaluated using analysis of variance (ANOVA) with repeated measures design for within-group comparisons. A value of $p<0.05$ was considered the criterion for statistical significance.

Results

Dose-response and time sequence studies. ANP at a concentration of 0.1 μmol/l inhibited the activation of Ras by a maximum of 90% ($p<0.00001$) at 15 minutes and inhibited Ras 88% ($p<0.00001$) at 30 minutes (Figure 1B). With 1 μmol/l of ANP, at 30 minutes there was a 65% decrease in the phosphorylation of Ras with the activation of Ras being maximally inhibited by 73% at 45 minutes (Figure 1A; $p<0.0088$). At one hour, with 1 μmol/l of ANP there was still a 71% decrease in the activation of Ras compared to control ($p<0.009$) (Figure 1A). When the effects of 1 μmol/l of ANP were investigated to determine when ANP no longer inhibited the phosphorylation of Ras, it was found that ANP effects began to wane at 24 hours (39% decrease, $p<0.05$) and at 48 hours became non-significant (0% decrease, Figure 2). When ANP in dose-response experiments was reduced to 0.01 μmol/l, the maximal decrease (83%, $p<0.0001$) was at 45 minutes (Figure 1C). After 60 minutes of exposure to 0.01 μmol/l of ANP the phosphorylation of Ras was still significantly lower (by 75%, $p<0.0088$) (Figure 1C).

LANP at 1 μmol/l caused a maximal (71%; $p=0.009$) decrease in Ras at 30 minutes with a still significant ($p<0.03$) 63% decrease in the activation of Ras in DU 145 cells at 45 minutes (Figure 3A). When the concentration of LANP was reduced 10-fold to 0.1 μmol/l there was an 83% ($p<0.0001$) decrease in the phosphorylation of Ras within five minutes, while phosphorylation remained significantly lower by 51% ($p<0.05$) at 60 minutes (Figure 3B). When the concentration of LANP was further reduced to 0.01 μmol/l, the inhibition of phosphorylation of Ras began as early as five minutes (89%



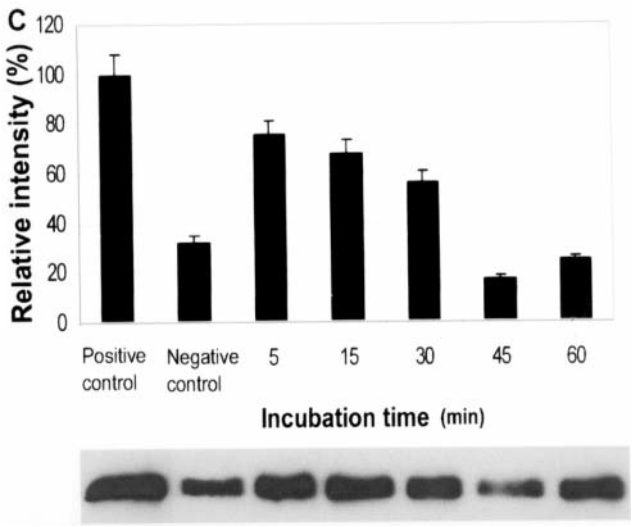


Figure 1. *Dose response of atrial natriuretic peptide on the activation of Ras in human prostate cancer cells at A, 1 μmol/l, B, 0.1 μmol/l and C, 0.01 μmol/l in time-sequenced experiments at 5, 15, 30, 45, and 60 minutes. There was a significant ($p<0.0001$) inhibition of the activation (i.e. phosphorylation) of Ras at each time point when evaluated by analysis of variance (ANOVA). Ras-GTP (i.e. active Ras) is at 21 kDa. The relative intensity in these bar graphs is a comparison of three Western blots against the positive control (untreated Ras-GTP) with one typical Western blot illustrated. The illustrated negative controls in these graphs is Ras-GDP at 21 kDa.*

decrease, $p<0.0001$), at which time it was maximal (Figure 3C). At this concentration, the effects of LANP on Ras did not last as long, with only 57% lower phosphorylation of Ras after 15 minutes ($p<0.04$) and 26% lower ($p<0.05$) at 1 hour (Figure 3C). The effects of LANP on Ras began to wane at 24 hours (27% decrease, $p<0.05$) and became non-significant at 48 hours (6% decrease; Figure 2).

ANP and LANP inhibition of Ras kinase is specifically blocked by cyclic GMP antibody. Cyclic GMP (1 μmol/l) inhibited the phosphorylation of Ras by 72% ($p<0.009$) (Figure 4). When DU 145 cells were incubated with cyclic GMP antibody and ANP, the inhibition of the activation of Ras decreased from 82% with ANP alone to 21% ($p<0.036$ for ANP alone *versus* ANP with cyclic GMP antibody). The addition of the cyclic GMP antibody also reduced the inhibition of phosphorylation of Ras by LANP down to 21% ($p<0.033$ for LANP alone *versus* LANP with cyclic GMP antibody) (Figure 4).

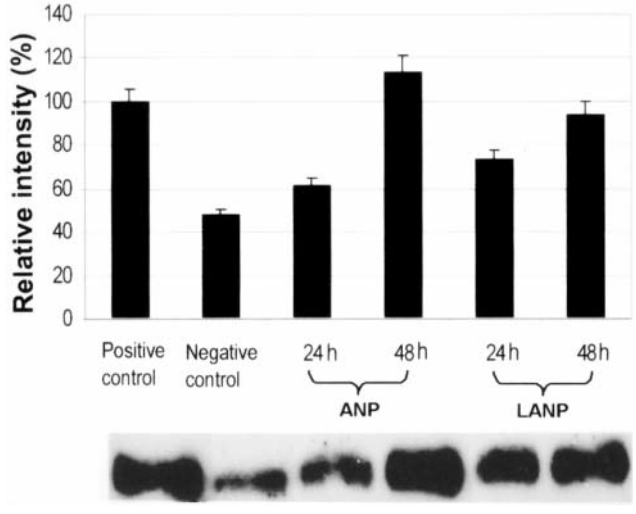
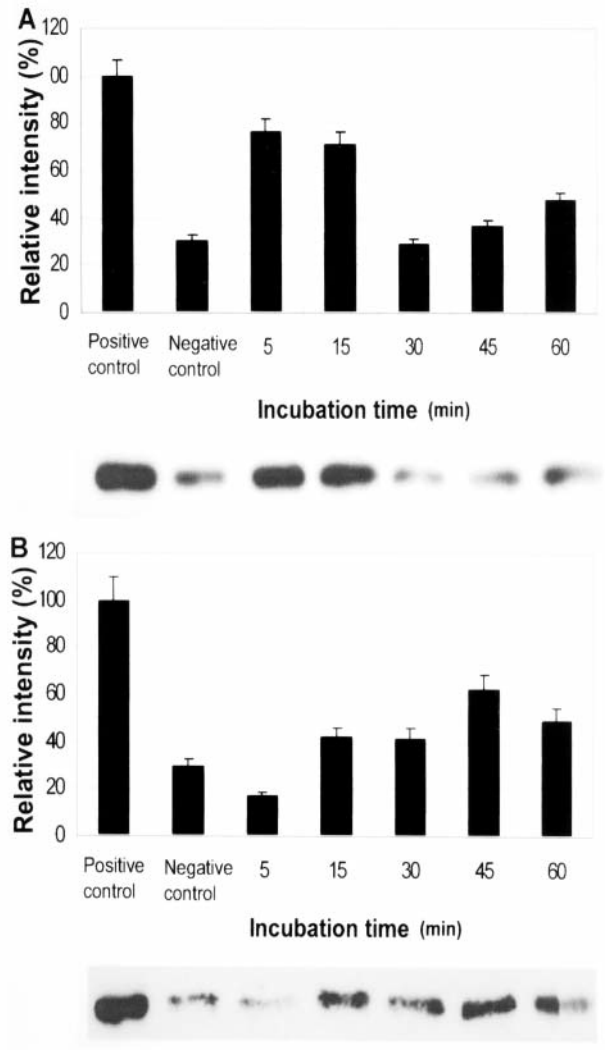


Figure 2. *Time-sequenced experiments to determine when the inhibitory effects of atrial natriuretic peptide and long-acting natriuretic peptide on Ras were no longer significant. Effects on Ras were no longer significant after 48 hours when evaluated by ANOVA. Ras-GTP is at 21 kDa. The relative intensity in this bar graph is a comparison of the Western blots, with the positive control (untreated) Ras-GTP intensity designated as 100%. The negative control illustrated in these graphs is Ras-GDP at 21 kDa.*



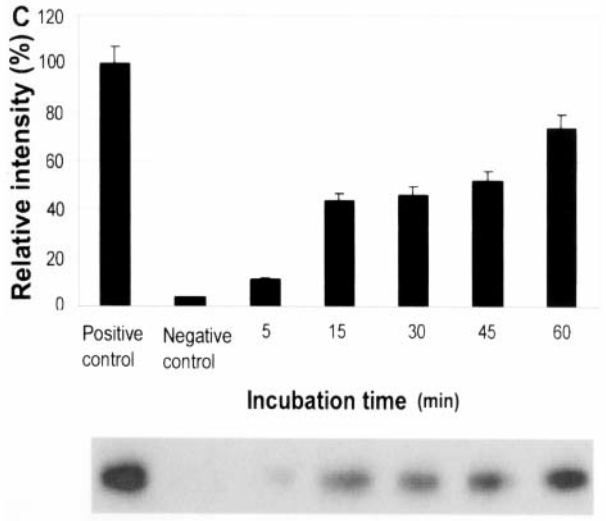


Figure 3. *Dose response of long-acting natriuretic peptide on the activation of Ras in human prostate adenocarcinoma cells at A, 1 μmol/l, B, 0.1 μmol/l, and C, 0.01 μmol/l in time-sequenced experiments at 5, 15, 30, 45 and 60 minutes. There was a significant inhibition of the phosphorylation (i.e. activation) of Ras at each time point when evaluated by analysis of variance (ANOVA). Ras-GTP (positive control) is at 21 kDa. The illustrated negative controls in these graphs is Ras-GDP. The relative intensity in this bar graph is a comparison of three Western blots, with the control blot intensity designated as 100% .*

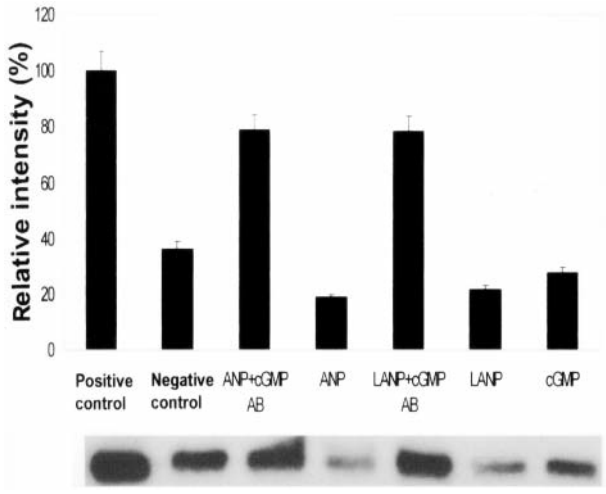


Figure 4. *Cyclic GMP inhibits the phosphorylation of Ras by 72% and a cyclic GMP antibody (AB) blocked inhibition of Ras by atrial natriuretic peptide and long-acting natriuretic peptide which were both significant at p<0.001 when evaluated by ANOVA. Ras-GTP is at 21 kDa. The positive control of Ras-GTP is illustrated as 100% intensity.*

Discussion

Ras is a small GTPase that cycles between an inactive GDP-bound and an active GTP-bound form (14). Ras has become important in cancer research with the finding that Ras protein is constitutively active in the GTP-bound form (3, 4) in 25-30% of human cancers. This is usually due to point mutation in one of three Ras genes namely *H-Ras, K-Ras* or *N-Ras*, which encode for highly similar proteins with a molecular weight of 21,000 (15). This point mutation abolishes the intrinsic GTPase activity of Ras protein (15).

The present investigation demonstrates that endogenous cardiac hormones can significantly ($p<0.0001$) inhibit the activity of Ras. Previous studies to prevent the membrane localization of Ras proved unfruitful as tumor cells proved generally impervious to their action (3). Likewise, attempts to target Ras by perturbing interaction with SOS or GRB2 have not yielded any viable drugs because of the inherent difficulties in disrupting protein protein interactions (3). Thus, the present endogenous inhibitors of Ras are unique in that they markedly decrease the phosphorylation (*i.e.* activation) of Ras and were shown to eliminate up to 80% of human pancreatic carcinomas (7) and two-thirds of human breast carcinomas (8) in athymic mice.

The present investigation suggests that one of the metabolic targets of ANP and LANP for the elimination of cancers is Ras. These cardiac hormones thus have three metabolic targets within cancer cells to mediate their effects. The other two metabolic targets in addition to Ras are part of the Ras-MEK 1/2-ERK 1/2 (Ras-MAPK kinase) cascade. The second metabolic target of ANP and LANP on cancer cells is MEK 1/2 (9). These two hormones inhibit up to 97% of MEK 1/2 kinases (9). The third metabolic target in cancer cells is the downstream ERK 1/2 kinases, whose activation is inhibited up to 94% by ANP and LANP (10). In addition to eliminating up to 94% of the basal phosphorylation of ERK 1/2, these cardiac hormones completely block the ability of mitogens such as epidermal growth factor (EGF) to stimulate ERK 1/2 (16). It is important to note that ANP and LANP do not inhibit the basal phosporylation of ERK1/2 kinase in normal human cells (17). Thus, with the demonstration in the present investigation that these two peptides are very potent ($p<0.0001$) inhibitors of Ras, there are now three known metabolic targets within cancer cells for these hormones to mediate their anticancer effects.

With respect to the mechanism of how ANP and LANP inhibit the phosphorylation of Ras, one of the second messengers of their biological effects, cyclic GMP (11, 12) was here found to inhibit the phosphorylation of Ras by 72% in human prostate cancer cells (Figure 4). That cyclic GMP mimics the effects of ANP and LANP on the activation of Ras in the same human prostate cancer cells suggests that cyclic GMP is one of the mediators of significant decrease (97%) in prostate cancer cell numbers brought about by ANP and LANP (18). This was further defined in the present investigation where a cyclic GMP antibody was demonstrated to significantly ($p<0.001$) block the effects of ANP and LANP on the activation of Ras (Figure 4). Utilizing this antibody suggests that decrease in the activation of Ras caused by ANP and LANP is specifically mediated in part by cyclic GMP.

Conclusion

ANP and LANP both inhibit Ras in human prostate cancer cells partially mediated *via* cyclic GMP as part of their anticancer mechanism(s) of action.

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