

## Atrial Natriuretic Peptide and Long Acting Natriuretic Peptide Inhibit MEK 1/2 Activation 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 adenocarcinomas. *Materials and Methods:* Atrial natriuretic peptide, long acting natriuretic peptide and cyclic GMP's effects on MEK 1/2 kinase were examined in human prostate adenocarcinoma cells. *Results:* Atrial natriuretic peptide and long acting natriuretic peptide decreased the activation of MEK 1/2 over a concentration range of 0.01  $\mu$ M to 10  $\mu$ M. Long acting natriuretic peptide and atrial natriuretic peptide (each 10  $\mu$ M) inhibited the phosphorylation of MEK 1/2 kinase 97% ( $p < 0.00001$ ) and 88% ( $p < 0.00001$ ), respectively. The inhibition of MEK 1/2 was maximal at two hours, and ceased by four hours, secondary to both peptides. The ability of peptides to inhibit MEK 1/2 was inhibited by cyclic GMP antibody and cyclic GMP itself inhibited MEK 1/2 phosphorylation by 93%. *Conclusion:* Atrial natriuretic peptide and long acting natriuretic peptide both inhibit MEK 1/2 kinase mediated via cyclic GMP as part of their anticancer mechanism(s) of action.

The Ras/Raf/MEK/ERK mitogen-activated protein kinase (MAPK) signaling pathway is very important for the development of cancers and is constitutively activated in many types of cancer (1). This pathway is frequently constitutively activated in prostate and breast cancer and its increased expression is associated with a poor prognosis (2). A family of protein kinases located upstream of the MAP kinases (ERK 1/2) and responsible for their activation are the MAP kinase kinases (3). The prototype member of this

family, designated MAP kinase kinase (MKK-1)/or MEK-1, specifically phosphorylates the MAP kinase regulatory threonine and tyrosine residues present in the Thr-Glu-Tyr motif of ERK 1/2 (3, 4). A second MEK family member, namely MEK-2, resembles MEK-1 in its substrate specificity but is seven residues longer than MEK-1 with its amino acid sequence being 81% identical to MEK-1 (4). Recently, we found that two cardiac hormones, atrial natriuretic peptide (ANP) and long acting natriuretic peptide (LANP) inhibit the phosphorylation (activation) of ERK 1/2 kinases by 94% and 88%, respectively (5).

The present investigation was designed to determine if these two cardiac hormones have upstream affects on mitogen-activated protein kinase (MAPK)/ERK kinase (MEK 1/2), central key mediators in cancer formation. Inhibition of MEK 1/2 would effectively block the phosphorylation of the downstream kinases such as ERK 1/2 (1-4). Given the central role of MEK 1/2 in mediating the growth-promoting signals from a diverse group of upstream stimuli, any newly found inhibitors of MEK could have significant clinical benefit in the treatment of prostate and other types cancer (4). When both ANP and LANP were demonstrated to inhibit MEK 1/2 kinase 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 (6, 7).

### Materials and Methods

*Human prostate adenocarcinoma cells.* A cell line (ATCC number HTB-81; DU 145) 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.* (8) from a 69-year-old man. These homogenous cells when injected into athymic mice form moderately differentiated prostate adenocarcinomas within 21 days (8).

*Culture of the prostate adenocarcinoma cells.* Propagation of these cells was carried out in Roswell Park Memorial Institute (RPMI) 1640 medium with 2 mM/L-glutamine adjusted with the addition of 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1 mM sodium

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*Key Words:* Adenocarcinoma, prostate neoplasm, natriuretic peptides, atrial natriuretic peptide, long acting natriuretic peptide, MEK 1/2 kinase.

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<sub>2</sub> as recommended by the ATCC. Cells were subcultured every 6-8 days. The growth medium was changed every three days. The peptide hormones were from Phoenix Pharmaceuticals, Inc., Belmont, CA, USA.

**Research protocol.** The human prostate cancer cells were incubated for 5, 10, 15, 30, 45, 60 and 120 minutes, respectively, in dose-response curves with 0.01 µM to 10 µM of ANP or LANP. When it was found that ANP and LANP had maximal effects at two hours, these experiments were extended to 3, 4, 5, 6, 8 and 24 hours to determine when their effects on phosphorylation of MEK 1/2 ceased. After the respective time periods, prostate cancer cells were placed on ice, washed with cold phosphate-buffered saline (PBS) and lysed in lysis buffer (1% Triton X-100, 50 mM Tris-Cl, PH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 25 mM NaF, 0.5 mM sodium orthovanadate, 1 mM dithiothreitol, 1 µg/ml pepstatin, 2 µg/ml leupeptin, 2 µg/ml aprotinin and 0.1 mg/ml phenylmethanesulfonyl fluoride [PMSF]). Cell lysates were centrifuged at 13,400 rpm for 15 min at 4°C. Protein concentrations in cell lysate supernatants were determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). A total of 30 µg of each protein sample were utilized for SDS-polyacrylamide gel electrophoresis and immunoblotting analysis.

**Immunoblotting analysis.** After heating (100°C) in 1xSDS loading buffer for 5 min, proteins 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 5% nonfat dry milk for 1 hour with gentle rocking and incubated overnight with primary antibody p-MEK 1/2 at 4°C with gentle rocking. These membranes were washed three times (8 min each time) with Tris-buffered saline. The membranes were then immediately incubated with goat anti-rabbit, horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad) at a dilution of 1:1000 for MAP kinase kinase (MEK 1/2) for 2 hours at room temperature. The membranes were washed again and then examined by the chemiluminescent method. The primary antibody p-MEK-1/2 (Ser 218/Ser 222):Sc-7995 is a rabbit polyclonal affinity purified antibody raised against a short amino acid sequence containing phosphorylated Ser 218 and Ser 222. Using Western blotting this antibody recognizes MEK-1 phosphorylated at Ser-218 and MEK-2 phosphorylated at Ser-222 of human origin. This antibody was utilized at a 1:1000 dilution and was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Reprobing with monoclonal anti-β actin (42 kDa) in a 1:1000 dilution (Sigma) was utilized as a loading control.

**Cyclic GMP effects on MEK 1/2 kinase.** Cyclic GMP is one of the known mediators of the biological effects of these peptide hormones (6, 7). For the mechanism of action of these peptide hormones in inhibiting MEK 1/2 kinase in prostate adenocarcinoma cells, 1 µM of 8-bromoguanosine 3',5'-cyclic monophosphate (8-bromo-cyclic GMP; Sigma), the cell-permeable analog of cyclic GMP, was utilized for this investigation.

**Cyclic GMP in MEK 1/2 inhibition.** To determine if the inhibition of MEK 1/2 kinase by the cardiac hormones in prostate adenocarcinoma cells was cyclic GMP-specific, these peptide hormones (1 µM each) and the specific cyclic GMP antibody in 1:80 dilution (Sigma) were incubated together for 30 min followed by the above research protocol and immunoblotting.

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

## Results

**Dose-response and time sequence studies.** ANP inhibited the activation of MEK 1/2 by a maximum of 88% ( $p < 0.00001$ ) which occurred at 45 minutes (Figure 1A). There was a 70% decrease in the phosphorylation of MEK 1/2 within 30 minutes and this decreased activation of MEK 1/2 persisted with an 85% decrease in its phosphorylation at two hours with 10 µM of ANP (Figure 1A) ( $p < 0.00001$ ). Decreasing the concentration of ANP 10-fold in dose-response studies to 1 µM resulted in a 51% ( $p < 0.01$ ) decrease in the activation of MEK 1/2 at 45 minutes and a 74% decrease ( $p < 0.001$ ) in the phosphorylation of MEK 1/2 at two hours (Figure 1B). A 100-fold decrease in ANP in these dose-response experiments to 0.1 µM revealed that this concentration of ANP caused a maximal decrease (66%,  $p < 0.01$ ) at 120 minutes and a 46% decrease ( $p < 0.05$ ) in the phosphorylation of MEK 1/2 at 60 minutes (Figure 1C). At 45 minutes, 0.1 µM of ANP reduced the activation of MEK 1/2 by 51% ( $p < 0.01$ ) (Figure 1C). A reduction to 0.01 µM of ANP resulted in a maximal 54% ( $p < 0.01$ ) decrease in the phosphorylation of MEK 1/2 at 120 minutes (Figure 1D). There was a 32% ( $p < 0.05$ ) decrease in the phosphorylation of MEK 1/2 within five minutes and at 30 minutes there was a 46% ( $p < 0.05$ ) decrease in the phosphorylation of MEK 1/2 with 0.01 µM of ANP (Figure 1D).

LANP (10 µM) caused a maximal 97% ( $p < 0.00001$ ) decrease in the activation of MEK 1/2 in human prostate adenocarcinoma cells and this maximal decrease occurred at 120 minutes (Figure 2A). At this concentration of LANP, there was a 43% ( $p < 0.05$ ) decrease in the phosphorylation of MEK 1/2 within 30 minutes and at one hour there was an 88% decrease ( $p < 0.0001$ ) in the activation of MEK 1/2 (Figure 2A). When the concentration of LANP was reduced 10-fold to 1 µM, there was a 64% ( $p < 0.01$ ) decrease in the phosphorylation of MEK 1/2 at 30 minutes with a further decrease in the phosphorylation by 74% ( $p < 0.001$ ) at 45 minutes (Figure 2B). When the concentration of LANP was decreased to 0.1 µM, the inhibition of phosphorylation of MEK 1/2 was 63% ( $p < 0.01$ ) with 30 minutes (Figure 2C). The effect of LANP at 0.1 µM on the activation of MEK 1/2 was maximal at 30 minutes and at 120 minutes (Figure 2C).

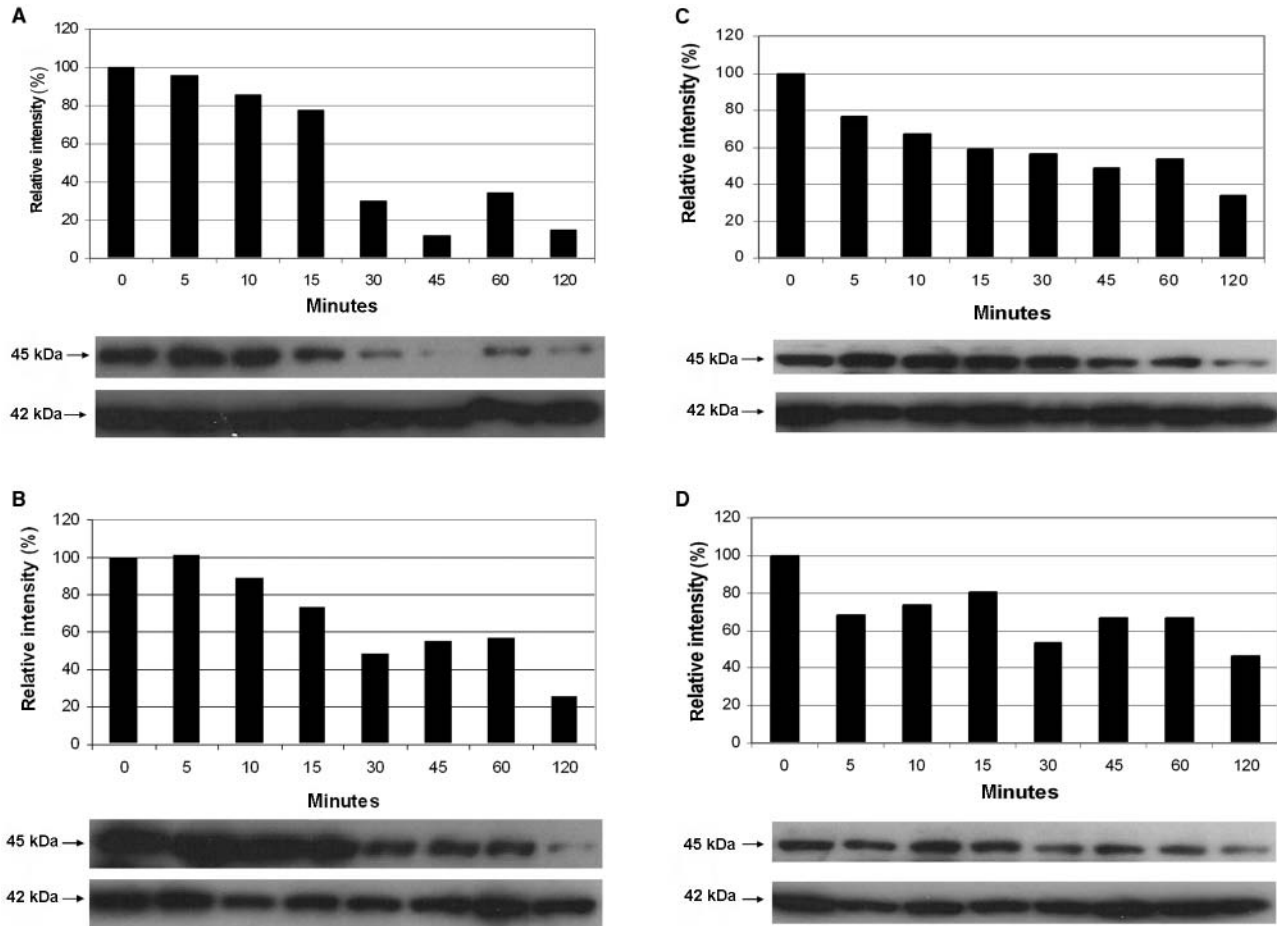


Figure 1. Effect of atrial natriuretic peptide (ANP) on phosphorylation of mitogen-activated protein kinase kinase (MEK 1/2). ANP at A) 10  $\mu$ M, B) 1 mM, C) 0.1  $\mu$ M and D) 0.01  $\mu$ M. Relative intensity was measured against untreated MEK 1/2 (100%). Lower panels indicate the results of Western blotting of proteins at each time interval. MEK 1/2 band at 45 kDa,  $\beta$ -actin (loading control) at 42 kDa.

When the concentration of LANP was reduced further to 0.01  $\mu$ M, the decrease in the activation of MEK 1/2 was significant with 15 minutes (47% decrease,  $p < 0.05$ ) and the maximal decrease of 80% ( $p < 0.0001$ ) took place at 60 minutes, followed by a 69% ( $p < 0.001$ ) decrease in the phosphorylation of MEK 1/2 at 120 minutes (Figure 2D).

*The inhibition of phosphorylation by ANP and LANP ceased by four hours.* When these experiments were extended for 24 hours to determine when the effects of ANP and LANP (each at 1  $\mu$ M) on the phosphorylation of MEK 1/2 ceased, it was found that their effects ceased by four hours (Figures 3 and 4). ANP had its most significant inhibiting effects on the phosphorylation of MEK 1/2 at two hours ( $p < 0.001$ ); by three hours its effects became non-significant (Figure 3). LANP also had its most significant inhibiting effects ( $p < 0.01$ ) on the phosphorylation of MEK 1/2 at two hours but still had significant effects ( $p < 0.05$ ) at three hours

(Figure 4). By four hours, however, LANP had no further inhibiting effects on the phosphorylation of MEK 1/2 (Figure 4). These experiments were continued for 6, 8 and 24 hours and there was no inhibitory effect of either ANP or LANP on the phosphorylation of MEK 1/2 at these time periods. Thus, the inhibitory effects of ANP and LANP are maximal at two hours and their inhibitory effects on the phosphorylation of MEK 1/2 had ceased by four hours (Figures 3 and 4).

*Cyclic GMP antibody specifically blocks ANP and LANP inhibition of MEK 1/2 kinase.* Cyclic GMP (1  $\mu$ M) inhibited the phosphorylation of MEK by 93% ( $p < 0.0001$ ) while ANP and LANP (each at 1  $\mu$ M) in the same experiment inhibited the phosphorylation of MEK 1/2 by 23% and 60%, respectively (Figure 5). When cyclic GMP antibody was added to LANP and ANP there was no inhibition of the activation of MEK 1/2 (Figure 5).

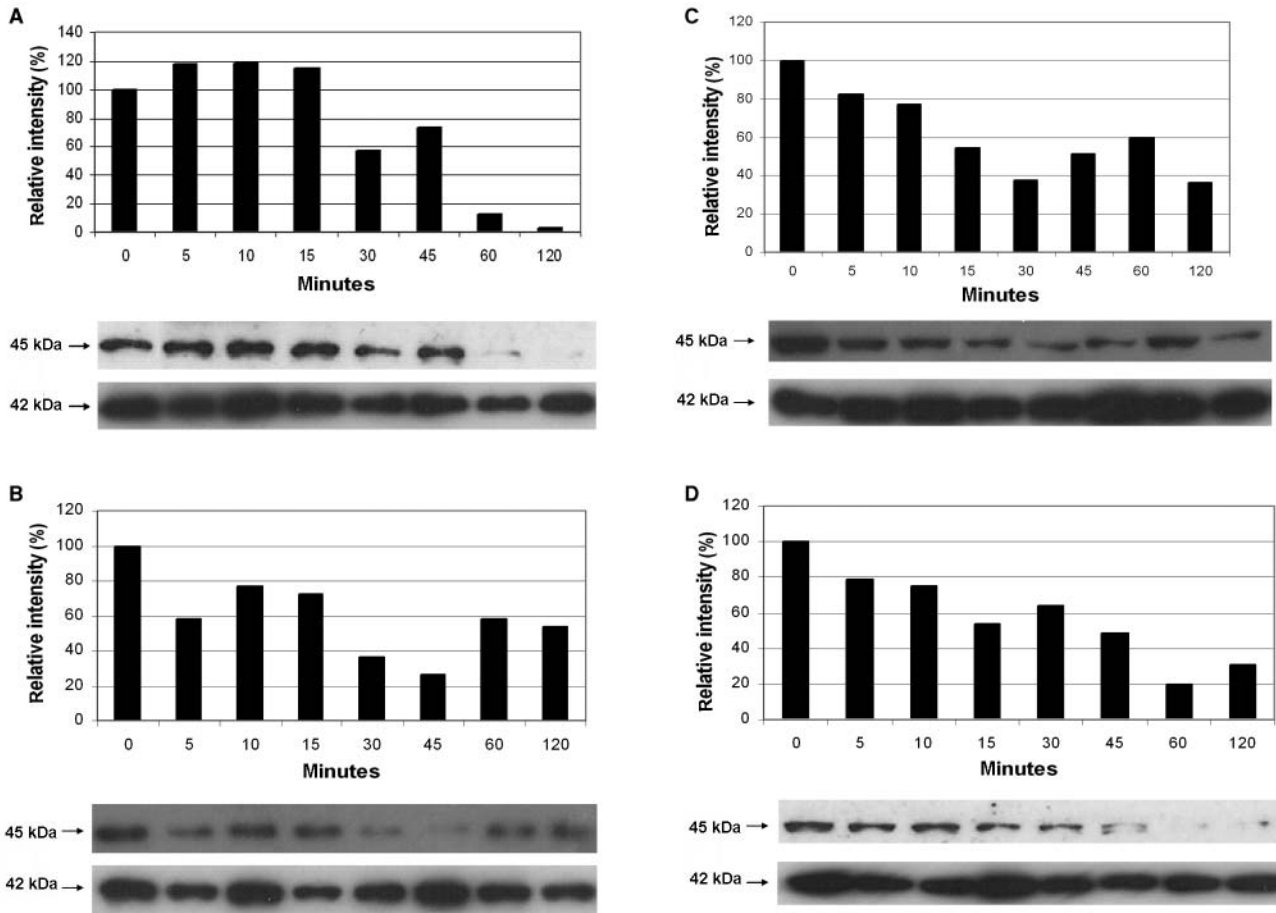


Figure 2. Long acting natriuretic peptide (LANP) decreases the phosphorylation of mitogen-activated protein kinase kinase (MEK 1/2). LANP at A) 10  $\mu$ M, B) 1  $\mu$ M, C) 0.1  $\mu$ M, and D) 0.01  $\mu$ M. Relative intensity was measured against untreated MEK 1/2 (100%). Lower panels indicate the results of Western blotting of proteins at each time interval. MEK band at 45 kDa,  $\beta$ -actin (loading control) at 42 kDa.

## Discussion

The results of this investigation define part of the mechanism(s) of action of these peptide hormones in cancer cells. This is the first investigation demonstrating that LANP can decrease the activation of MEK 1/2 whose phosphorylation it decreased by 97% (Figure 2A). It is also the first demonstration that LANP can decrease the activation of MEK 1/2 in a cancer cell. ANP was investigated for the first time for its ability to inhibit phosphorylation of MEK 1/2, which it did by 88% (Figure 1A) at 10  $\mu$ M concentration at 45 minutes. The data of the present investigation that ANP and LANP inhibit 88% and 97% of the phosphorylation of MEK 1/2, respectively, are consistent with the ability of ANP to kill 89% of human prostate cells and the ability of LANP to kill 80% of human prostate cells in culture within 24 hours (9).

The present investigation suggests that the inhibition of the phosphorylation of MEK 1/2 is one of the mechanisms involved in the reduction of the number of viable cancer cells *in vitro* (9-17) by ANP and LANP and their ability to stop the growth and decrease the volume of human cancers *in vivo* (18, 19). Thus, in addition to inhibiting DNA synthesis within cancer cells (9-17), the anticancer effects of LANP and ANP involve their ability to reduce the activation of the mitogen-activated MAP kinases (MEK 1/2), dual-specific recognition serine/threonine protein kinases that regulate extracellular-signal regulated kinases (ERK 1/2) by reversible phosphorylation of 44 kDa ERK 1 and 42 kDa ERK 2. ANP and LANP inhibit the phosphorylation of these downstream kinases from MEK 1/2 (5). These findings taken together indicate that ANP and LANP, which had significant inhibitory effects on the phosphorylation of MEK 1/2 in the present investigation, have their effects continued downstream in the RAS/RAF/MEK/ERK (MAPK) pathway

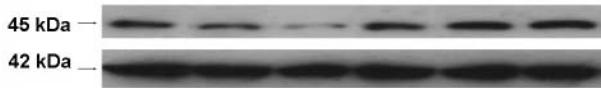
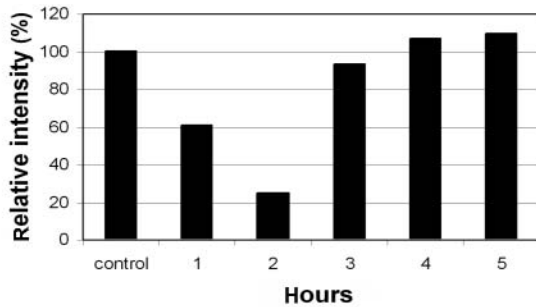


Figure 3. Atrial natriuretic peptide (ANP; 1  $\mu$ M) inhibition of the phosphorylation of MEK 1/2 ceased by 3 hours of exposure. The inhibition at 2 hours was significant at  $p < 0.001$  when evaluated by ANOVA. The effects of ANP on the phosphorylation of MEK 1/2 was no longer significant at 3, 4, 5, 6, 8 and 24 hours when evaluated by ANOVA. MEK 1/2 is at 42 kDa while  $\beta$ -actin is at 45 kDa.

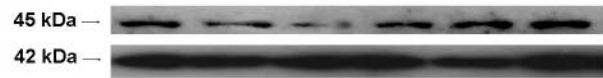
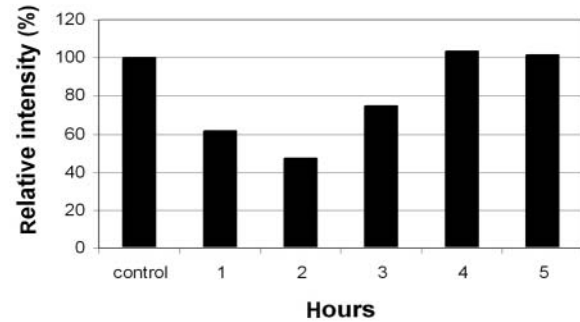


Figure 4. Long acting natriuretic peptide (LANP) had its most significant inhibiting effects on the phosphorylation MEK 1/2 at 2 hours. LANP still had significant effects at 3 hours ( $p < 0.05$ ) when evaluated by ANOVA. The effect of LANP on the phosphorylation of MEK 1/2 became insignificant at 4 hours when evaluated by ANOVA. MEK 1/2 is at 45 kDa while  $\beta$ -actin is at 42 kDa.

(5). Since MEK 1/2 is constitutively activated (1, 2) in many cancers, including human prostate cancer of the present investigation, the ability of LANP to inhibit 97% of the constitutively activated MEK 1/2 suggests that it, and possibly ANP, which inhibits 88% of the constitutively activated MEK 1/2, may be useful in the treatment of prostate and other types of cancer in the future. The present investigation further suggests that a major target for the anticancer effects of ANP and LANP (9-19) is MEK 1/2. The inhibition of phosphorylation of MEK 1/2 secondary to ANP and LANP lasted four hours. The effects of ANP and LANP on the phosphorylation of MEK 1/2 are, thus, reversible. They do not cause the phosphorylation of MEK 1/2 kinases to be permanently disabled. They are, thus, not toxic to MEK 1/2 kinases but rather cause a reversible inhibition of the activity of these kinases. This information suggests that a cancer cell would need to be exposed to fresh ANP and LANP every three hours or less to inhibit the phosphorylation of MEK 1/2 and thereby inhibit cancer growth.

With respect to the mechanism of how ANP and LANP inhibit the phosphorylation of MEK 1/2, one of the second messengers of their biological effects, namely cyclic GMP (6,7), was found using 8-bromo-cyclic GMP (1  $\mu$ M) to inhibit the phosphorylation of MEK 1/2 (by 93%) in human prostate cancer cells, an inhibition similar to that of ANP and LANP. The mimicking of the effects of ANP and LANP on the activation of MEK 1/2 in the same human prostate cancer cells by cyclic GMP suggests that it is one of the mediators of the decrease in prostate cancer cell number (9) and inhibition of the activation of MEK 1/2 in prostate adenocarcinoma cells brought about by ANP and LANP. This was further

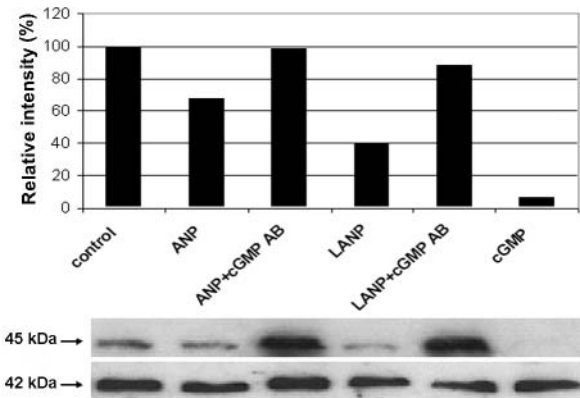


Figure 5. Cyclic GMP (cGMP) inhibits the phosphorylation of MEK 1/2. Atrial natriuretic peptide and long acting natriuretic peptide activation of MEK 1/2 was blocked completely by the cyclic GMP antibody (AB) which was significant at  $p < 0.00001$  when evaluated by ANOVA. MEK 1/2 is at 45 kDa while  $\beta$ -actin (loading control) is at 42 kDa.

defined in the present investigation where, utilizing a cyclic GMP antibody, it was demonstrated for the first time that this antibody could block all of the effects of ANP and LANP on the activation of MEK 1/2 (Figure 5). Utilizing this antibody suggests that reductions of the activation of MEK 1/2 are specifically mediated by cyclic GMP.

### Acknowledgements

We thank Charlene Pennington for excellent secretarial assistance. This work was supported in part by the Department of Veterans Affairs Merit Review Grant Award (DLV).

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Received May 21, 2007

Revised July 30, 2007

Accepted August 2, 2007