Atrial Natriuretic Peptide and Long Acting Natriuretic Peptide Inhibit ERK 1/2 in Prostate Cancer Cells

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Abstract. *Background: Atrial natriuretic peptide (ANP) and long acting natriuretic peptide (LANP) have anticancer effects in human prostate adenocarcinomas. Materials and Methods: ANP, LANP and cyclic GMP's effects on extracellular signal-regulated kinase (ERK) 1/2 kinase were examined in human prostate adenocarcinoma cells. Results: ANP and LANP decreased the activation of ERK 1/2 over a concentration range of 0.01 μM to 10 μM. ANP and LANP's maximal inhibition of the phosphorylation of ERK 1/2 kinase were 94% and 88% ($p<0.0001$), respectively. ANP had significant effects within five min at its 10 μM concentration. The inhibition of ERK 1/2 lasted for at least two h, where it was maximal, secondary to both peptides. Their ability to inhibit ERK 1/2 was inhibited by cyclic GMP antibody and cyclic GMP itself inhibited ERK 1/2 phosphorylation. Conclusion: ANP and LANP both inhibit ERK 1/2 kinase mediated via cyclic GMP as part of their anticancer mechanism(s) of action.*

Extracellular-signal regulated kinase (ERK) 1/2 is a mitogen-activated protein kinase (MAP kinase) important for the growth of cancer(s) (1, 2). Growth factors, such as epidermal growth factor (EGF), platelet derived growth factor, fibroblast growth factor and vascular endothelial growth factor (VEGF), after binding to their specific receptor tyrosine kinases work *via* ERK 1/2 kinase to cause proliferation (1). EGF, for example, when it binds to its EGF receptor, causes this receptor to autophosphorylate on tyrosine residues and recruits the Grb2-Sos complex to turn on membrane-associated Ras, which then activates the Raf-Mek 1/2 – ERK 1/2 kinase cascade (1).

Cardiac natriuretic are a family of peptide hormones that have significant anticancer effects on human prostate, breast, colon and pancreatic adenocarcinoma cells (3-6), as well as small cell and squamous lung carcinoma cells *in vitro* (7, 8). Of this family of peptide hormones, one gene in the heart synthesizes a 126 amino acid prohormone, which, with proteolytic processing, results in four peptide hormones consisting of: i) long acting natriuretic peptide, LANP (*i.e.,* the first 30 amino acids (a.a.) of this prohormone), ii) vessel dilator (VDL; a.a. 31-67), iii) kaliuretic peptide (KP, a.a. 79-98), and iv) atrial natriuretic peptide (ANP), a.a. 99-126 of the 126 a.a. prohormone (Figure 1). Two out of these peptide hormones, *i.e.,* VDL and KP inhibit ERK 1/2 kinase as part of their mechanism of anticancer effects (9). Recently we have found that ANP has the strongest anticancer effects *in vivo* of these peptide hormones, of eliminating 80% of human pancreatic adenocarcinomas growing in athymic mice (Vesely DL *et al.*, unpublished data).

The present investigation's aim was to determine if the two peptide hormones originating from the N-terminal and C-terminal ends of the ANP prohormone, *i.e.,* ANP (the peptide hormone which eliminates the largest percentage of cancers growing *in vivo*) and LANP can inhibit ERK 1/2 kinase in human prostate cancer cells. When both ANP and LANP were demonstrated to inhibit ERK 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 these peptide hormones' effects (10, 11).

Materials and Methods

Human prostate adenocarcinoma cells. A cell line of human prostate adenocarcinoma cells was purchased from American Type Culture

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

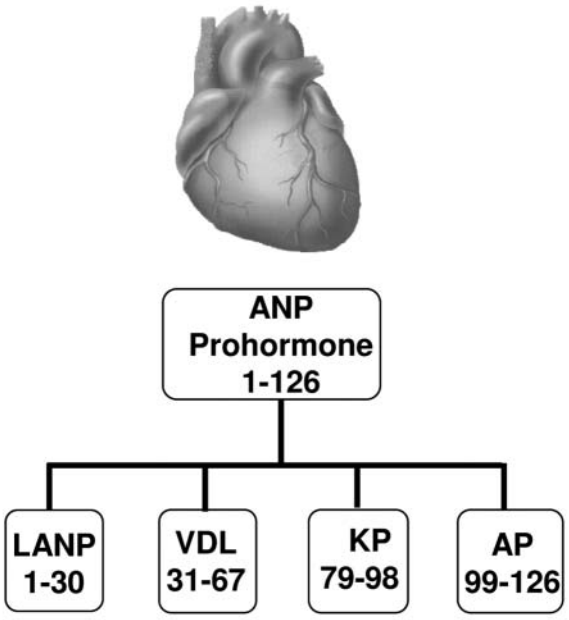

Figure 1. *The atrial natriuretic peptide gene in the heart synthesizes a 126 amino acid (a.a.) prohormone with which proteolytic processing results in the formation of four cardiac hormones. These four cardiac hormones, i.e., i) long acting natriuretic peptide (LANP) consists of the first 30 amino acids of the 126 a.a. prohormone, ii) vessel dilator (VDL), a.a. 31-67 of the prohormone, iii) kaliuretic peptide (KP), a.a. 79-98 of this prohormone, and iv) atrial natriuretic peptide (ANP), consisting of a.a. 99-126 of the 126 a.a. prohormone.*

Association (ATCC, number HTB-81; DU 145), Manassas, VA, USA. This prostate cancer cell line was derived in 1978 by KR Stone *et al.* (12) from a 69-year-old man. These homogenous cells when injected into athymic mice form moderately-differentiated prostate adenocarcinomas within 21 days (12).

Culture of the prostate adenocarcinoma cells. Propagation of these cells was in Roswell Park Memorial Institute (RPMI) 1640 medium with 2 mM/L-glutamine adjusted with addition of 1.5 g/L sodium bicarbonate, 90% 10 mM HEPES, 1 mM 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, 5% CO_2, 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 cancers were incubated for 5, 10, 15, 30 and 45 min, or 1 and 2 h, respectively, in dose-response curves with 0.01 μM to 10 μM of ANP or LANP. After the respective time periods prostate cancer cells were placed on ice, washed with cold 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 pepstanin, 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). Thirty μg of each protein sample was 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 volts for 90 min) and transferred to nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences Corporation, Piscataway, NJ, USA) for 90 min at 110 volts in transfer buffer. The membranes were washed four times with 1xTris-buffered saline (TBS), blocked with 5% non-fat dry milk for 1 h with gentle rocking, washed three more times with 1xTBS, and incubated overnight at 4˚C with gentle rocking. These membranes were washed three times (8 min each time) with TBS. The membranes were then immediately incubated with goat anti-rabbit, horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad) at a dilution of 1:1,000 for extracellular-signal-related kinase 1 and 2 (ERK 1/2) for 1 h at room temperature. The membranes were washed again and then examined using the chemiluminescent method. The primary antibody (P-ERK[E-4]) for measuring the detection of ERK 1 and 2 phosphorylation at Try-204 and the antibody for measurement of total ERK 1/2 (K-23) were both utilized at a 1:1,000 dilution and were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cyclic GMP effects on ERK 1/2 kinase. Cyclic GMP is one of the known mediators of the biologic effects of these peptide hormones (10, 11). For the mechanism of action of these peptide hormones' ability to inhibit ERK 1/2 kinase in prostate adenocarcinoma cells, 1 μM of 8-bromoguanosine 3',5'-cyclic monophosphate (*i.e.*, 8-bromo-cyclic GMP, Sigma Chemical Company), the cell-permeable analog of cyclic GMP, was utilized.

Do these peptide hormones' ability to inhibit ERK 1/2 in prostate adenocarcinoma cells specifically involve cyclic GMP? To determine if the inhibition of ERK 1/2 kinase 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 Chemical Co.) were incubated together for 30 min followed by the research protocol and immunoblotting analysis described previously.

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

Results

Dose-response and time sequence studies. ANP inhibited the activation of ERK 1/2 kinase by 94% ($p<0.00001$) at its 10 μM concentration with a 32% decrease ($p<0.05$) in the activation of ERK 1/2 within 5 min (Figure 2). At 45 min ANP (10 μM) inhibited 75% of the activation of ERK 1/2 with the phosphorylation of ERK 1/2 being decreased 86% at 60 min and the maximal 94% inhibition of ERK 1/2 phosphorylation occurring at 120 min (Figure 2). Even with

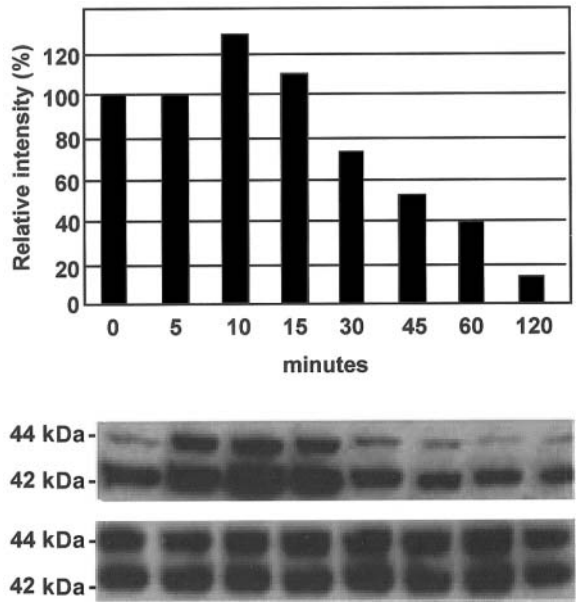

Figure 2. *ANP at 10 μM inhibits 94% of the phosphorylation of extracellular-signal regulated kinase (ERK) 1/2. This inhibition was maximal at 120 minutes which was significant at $p<0.0001$ when evaluated by analysis of variance (ANOVA). The decrease in activation of ERK 1/2 began as early as five minutes (32% decrease) which was significant at $p<0.05$ when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kDa) while ERK 2 is at 42 kDa. Upper row of immunoblots is the phosphorylation of ERK 1 and 2 at Try-204. Lower panel of immunoblots is total ERK 1 and 2. The relative intensity in the bar graphs is a comparison against untreated ERK 1/2 (100% intensity).*

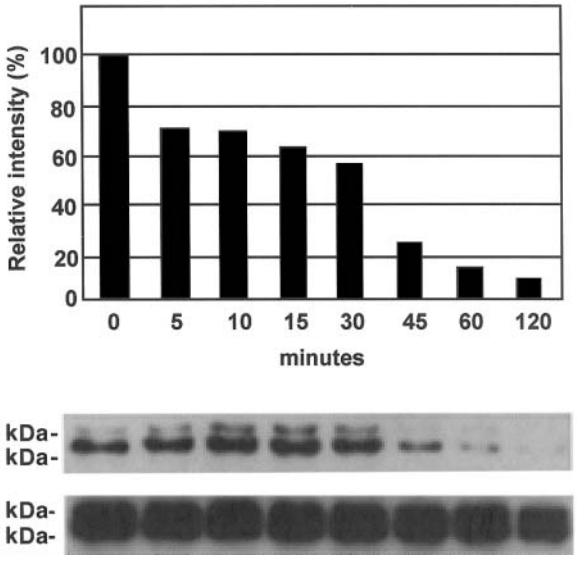

Figure 3. *Ten-fold decrease in ANP to 1 μM resulted in a 87% decreased phosphorylation of ERK 1/2 at 120 minutes which was significant at $p<0.0001$ when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kDa) while ERK 2 is at 42 kDa. Upper immunoblot panel is phosphorylation of ERK 1 and 2 at Try-204. Lower panel of immunoblots = total ERK 1/2.*

decreasing the concentration of ANP 10-fold to 1 μM there was a very significant 50% ($p<0.001$) decrease at 45 min and 87% decrease ($p<0.0001$) in the activation of ERK 1/2 at 120 min (Figure 3). A 100-fold decrease in ANP (*i.e.,* 0.1 μM) in these dose-response experiments revealed that there was a 36-39% decrease in the phosphorylation of ERK 1/2 (at 45, 60 and 120 min) ($p<0.05$) (Figure 4). With decreasing the concentration further to 0.01 μM of ANP, there was not a significant decrease in the activation of ERK 1/2 until 60 min (30% decrease; $p<0.05$) which then continued at 120 min (32% decrease; $p<0.05$, Figure 5).

LANP decreased the activation of ERK 1/2 by 31% ($p<0.05$) at 45 min with its maximal (72%, $p<0.001$) decrease in phosphorylation of ERK 1/2 occurring at 120 min (Figure 6). With decreasing the concentration of LANP to 1 μM there was a 53% ($p<0.01$) decrease in the phosphorylation of ERK 1/2 at 30 min and an 88% ($p<0.001$) decrease in the activation of ERK 1/2 secondary to LANP by 45 min which continued through 120 min where there was a 70% ($p<0.001$) in the phosphorylation of ERK 1/2 (Figure 7). When the concentration of LANP was decreased to 0.1 μM and 0.01 μM, the decrease in phosphorylation of ERK 1/2 did not

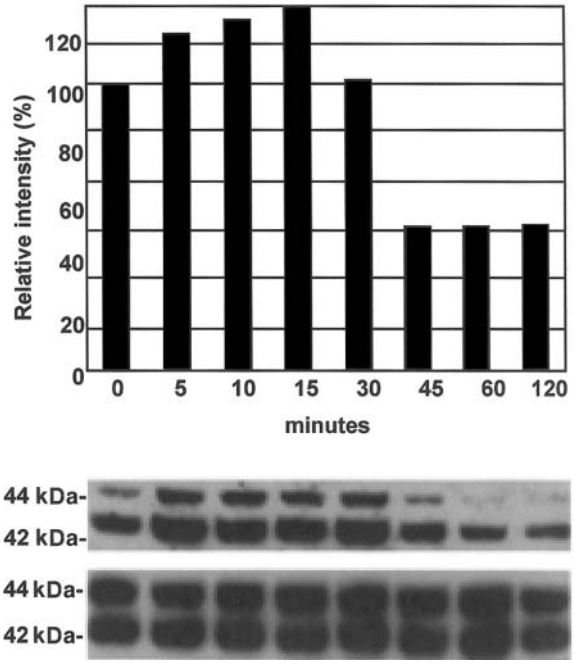

Figure 4. *0.1 μM of ANP decreased (35%) the activation of ERK 1/2 at 45 minutes with this percent of inhibition continuing at 60 and 120 minutes ($p<0.05$) when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kDa) while ERK 2 is at 44 kDa. Upper immunoblot panel is the phosphorylation of ERK 1 and 2 at Try-204. Lower panel of immunoblots = total ERK 1 and 2.*

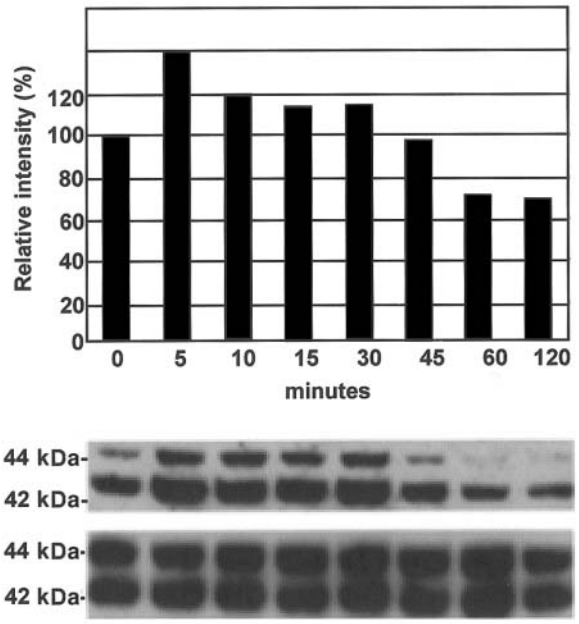

Figure 5. *ANP at 0.01 μM did not begin to significantly decrease the phosphorylation of ERK 1/2 until 60 min of exposure to ANP where there was a 30% decrease and similar decrease 32% at 120 min which were both significant at p<0.05 when evaluated by ANOVA. ERK 1 is at 44 kilo Dalton (kDa) while ERK 2 is at 42 kDa. Upper panel of immunoblots is the phosphorylation of ERK 1 and 2 at Try-204. The lower immunoblot panel = total ERK 1/2.*

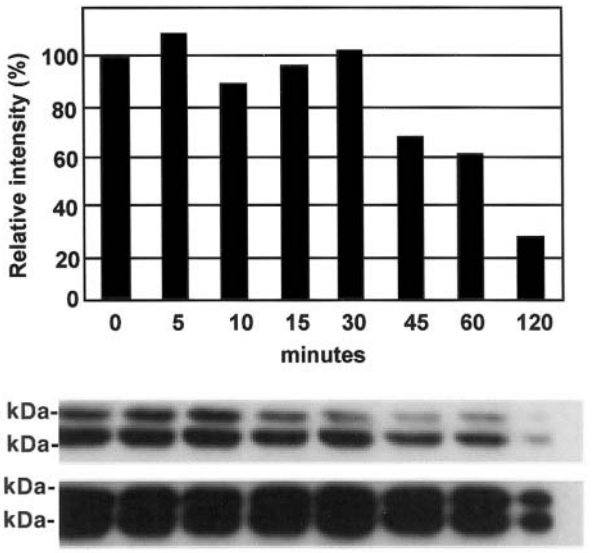

Figure 7. *There was a 53% decrease in the activation of ERK 1/2 in 30 minutes (p<0.01) with 1 μM of LANP. Maximal decrease in the phosphorylation of ERK 1/2 was at 45 min (88% decrease, p<0.0001). At 2 h (70% decrease) was significant at p<0.001 when evaluated by ANOVA. ERK 1 is 42 kilo Daltons (kDa) while ERK 2 is 44 kDa. Upper immunoblot panel is the phosphorylation of ERK 1 and 2 at Try-204. Lower immunoblot panel = total ERK 1 and 2.*

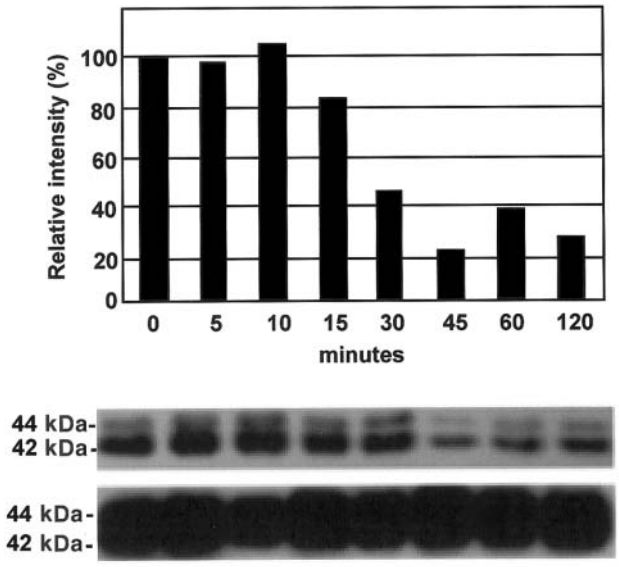

Figure 6. *Long acting natriuretic peptide (LANP, 10 μM) caused a 31% decrease at 45 min (p<0.05) and a 72% decrease (p<0.001) at 120 min in the phosphorylation of ERK 1/2 (ANOVA). ERK 1 is at 44 kilo Daltons (kDa) while ERK 2 is at 42 kDa. Upper immunoblot panel is the phosphorylation of ERK 1 and 2 at Try-204. Lower immunoblot panel = total ERK 1 and 2.*

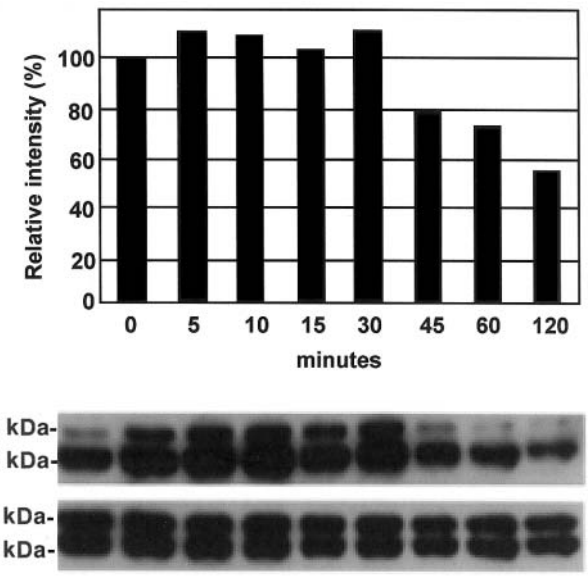

Figure 8. *Delayed onset of decreasing the activation of ERK 1/2 with a 10-fold decrease to 0.1 μM of LANP. The first significant decrease (p<0.05) occurred at 45 min while the maximal decrease of 45% occurred at 120 min and was significant at p<0.01 when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kDa) while ERK 2 is at 42 kDa. Upper immunoblot panel is the phosphorylation of ERK 1 and 2 at Try-204. Lower immunoblot panel is total ERK 1 and 2.*

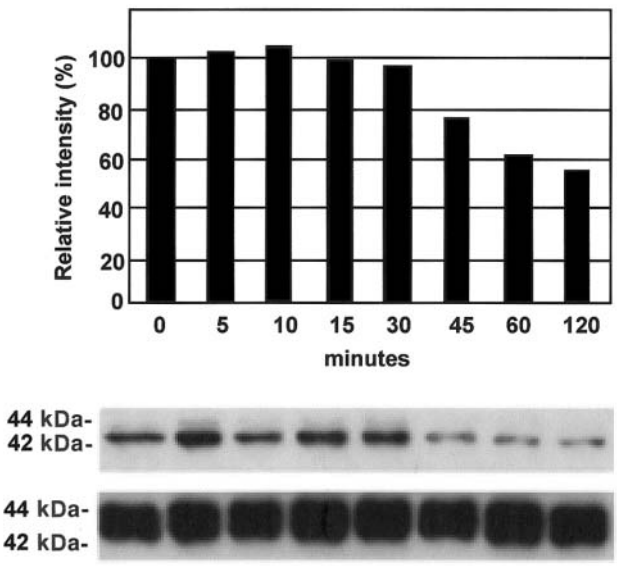

Figure 9. *The maximal decrease of the phosphorylation of ERK 1/2 by 0.01 μM of LANP of 46% occurred at 120 min similar to that observed with 0.1 μM of LANP which was significant at $p<0.01$ when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kDa) while ERK 2 is at 42 kDa. Upper panel of immunoblots is the phosphorylation of ERK 1 and 2 at Try-204. Lower immunoblot panel = total ERK 1 and 2.*

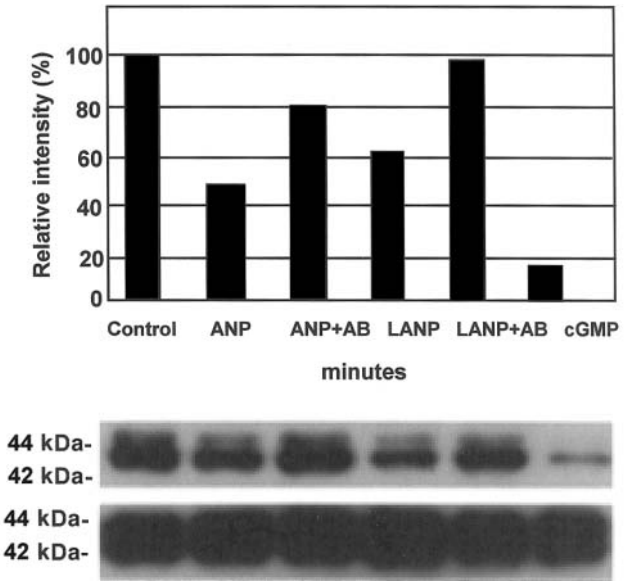

Figure 10. *Cyclic GMP inhibits 83% of the phosphorylation of ERK 1/2. LANP's activation of ERK 1/2 was blocked completely by the cyclic GMP antibody (Ab) while ANP's inhibition of ERK was decreased by 2/3rds with the cyclic GMP antibody which was significant at $p<0.0001$ and $p<0.001$ when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kDa) while ERK 2 is at 42 kDa. Upper immunoblot panel is the phosphorylation of ERK 1 and 2 at Try-204. Lower immunoblot panel is total ERK 1 and 2.*

begin until 45 min and was a maximal 45% decrease ($p<0.01$) at 120 min (Figures 8 and 9) which was considerably less than the 72% decrease in phosphorylation at 120 min with 10 μM of LANP (Figure 6).

Cyclic GMP antibody specifically blocks ANP and LANP inhibition of ERK 1/2 kinase. Cyclic GMP (1 μM) inhibited the phosphorylation of ERK 1/2 by 83% while ANP and LANP (each at 1 μM) in the same experiment inhibited the phosphorylation of ERK 1/2 by 47% and 37%, respectively, (Figure 10). When cyclic GMP antibody was added to LANP there was no inhibition of the activation of ERK 1/2 (Figure 10). The addition of the cyclic GMP antibody decreased 2/3rds of the inhibition of phosphorylation of ERK 1/2 by ANP, *i.e.*, from a 47% inhibition to a 16% inhibition (Figure 10).

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 ANP can decrease the activation of ERK 1/2 in cancer cells in which it decreased up to 94% of the phosphorylation of ERK 1/2 (Figure 2). LANP was investigated for the first time for its' ability to inhibit phosphorylation ERK 1/2 and it maximally inhibited the phosphorylation ERK 1/2 by 88% (Figure 7). In the dose-response curves (Figure 2-9) of the present investigation ANP was a stronger inhibitor of ERK 1/2 than LANP at every concentration. This information is consistent with ANP's ability to decrease (*i.e.,* eliminate) more of human prostate adenocarcinoma cells in cell culture within 24 h than LANP (5). ANP eliminates up to 89% of human prostate cancer cells in 24 h which is consistent with the present demonstrated ability to inhibit 94% of the activation of ERK 1/2. LANP kills 87% of human prostate cancer cells in 24 h (5) which is also consistent with its ability to maximally decrease the activation of ERK 1/2 by 88% in the present investigation. This suggests that the inhibition of the phosphorylation of ERK 1/2 is one of the mechanisms involved in ANP and LANP's ability to decrease the number of viable cancer cells *in vitro* (3-8) and their ability to stop the growth and decrease the volume of human cancers *in vivo* (13). Thus, in addition to inhibiting DNA synthesis within cancer cells (3-8) ANP and LANP's anticancer effects involve their ability to decrease the activation of extracellular-signal regulated kinase (ERK) 1/2, a cancer growth promoting peptide that translates from the extracellular membrane to the nucleus of the cell to promote growth (1, 2).

With respect to the mechanism of how ANP and LANP inhibit the phosphorylation of ERK 1/2, one of the second messengers of their biologic effects, *i.e.,* cyclic GMP (10, 11), was found using 8-bromo-cyclic GMP (1 μM) to inhibit the phosphorphylation of ERK 1/2 by 83% in human prostate cancer cells. Cyclic GMP's mimicking the effects of ANP and LANP on the activation of ERK 1/2 in the same human prostate cancer cells suggests that cyclic GMP is one of the mediators of these peptide hormones' ability to decrease cancer cell number (5) and inhibit the activation of ERK 1/2 in prostate adenocarcinoma cells. This was further 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 LANP's effects on the activation of ERK 1/2 and decrease by 2/3rds ANP's ability to inhibit the phosphorylation of ERK 1/2 (Figure 10). Utilizing this antibody suggests that cyclic GMP's effects of decreasing the activation of ERK 1/2 are specific for LANP, *i.e.,* not due to some other mediator and important for ANP's inhibition of the activation (*i.e.,* phosphorylation) of ERK 1/2.

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 October 9, 2006
Accepted October 18, 2006