Cardiac Hormones Are c-Jun-*N*-Terminal Kinase 2-inhibiting Peptides

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Abstract. *Background: Four cardiac peptide hormones, namely vessel dilator, long-acting natriuretic peptide (LANP), kaliuretic peptide, and atrial natriuretic peptide (ANP) have anticancer effects. Materials and Methods: The effects of these four cardiac hormones on human c-Jun-N-terminal kinase 2 (JNK2) were examined in human small cell lung cancer and human prostate cancer cells. Results: Vessel dilator, LANP, kaliuretic peptide and ANP maximally reduced expression of JNK2 by 89%, 56%, 45%, and 28%, respectively (each at p<0.0001) in human small cell lung cancer cells. In human prostate adenocarcinoma cells, JNK2 was maximally decreased 76%, 56%, 45%, (each at p<0.0001), and 28% (p<0.01) secondary to vessel dilator, LANP, kaliuretic peptide and ANP, respectively. Conclusion: These results indicate that four cardiac hormones are significant inhibitors (by up to 89%) of JNK2 in human small cell lung cancer cells and up to 76% in human prostate adenocarcinoma cells as part of their anticancer mechanism(s) of action.*

c-Jun-*N*-terminal kinases (JNK), also known as stress-activated protein kinase 1 alpha (SAPK1α) are activated by a variety of extracellular stimuli such as growth factors and environmental stresses (1, 2). Thus, JNK is activated by tumor necrosis factor, epidermal growth factor (EGF), platelet-derived growth factor and transforming growth factor, as well as by diverse environmental stresses (3-5). Activation of JNK by epidermal growth factor is dependent upon H-RAS activation (3, 6). JNK2 has not only been associated with cancer development but also with invasion of cancer, such as breast cancer (7). Loss of JNK activation coupled with loss of extracellular signal-related kinase (ERK) activation promotes cell death (8). Lung cancer cell growth (5) and prostate cancer proliferation and prostate cancer xenograft growth (9, 10) have been shown to be dependent upon JNK2. Of the JNK kinases it is c-Jun-*N*-terminal kinase 2 (JNK2), also known as mitogen-activated protein kinase 9 (MAPK9), that is preferentially required for mediating proliferation of lung cancer (11) and prostate cancer (9).

The present investigation was designed to determine if part of the mechanism(s) of action of four cardiac hormones, namely long-acting natriuretic peptide (LANP), vessel dilator, kaliuretic peptide, and atrial natriuretic peptide (ANP), which each have anticancer effects (12), might be inhibiting JNK2, *i.e.* the kinase preferentially required for mediating proliferation of lung cancer (11) and prostate cancer (9) cells. These four cardiac hormones eliminate up to 86% of human small cell lung tumors (13), and up to 80% of human pancreatic carcinomas growing in mice and once eliminated, the pancreatic carcinomas never return in the primary site during the lifespan of the mice (14). Up to two-thirds of human breast carcinomas growing in mice are eliminated by the cardiac hormones without any surgery, and as with the pancreatic carcinomas, once eliminated, never return during the lifespan of the mice (15). The rationale for examining the effects of these four cardiac hormones on JNK2 is that JNK2 activation by EGF is dependent on rat sarcoma mitogen-activated protein kinase (RAS) activation and the four cardiac hormones inhibit EGF activation of RAS (16). JNK2 is further required for RAS-initiated lung cancer formation (17). The cardiac hormones inhibit up to 95% of the basal activation of RAS-GTP by inhibiting 95% of the

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conversion of inactive RAS-GDP to active RAS-GTP (18, 19). The present investigation was designed to determine if these cardiac hormones inhibit c-Jun-*N*-terminal kinase 2 in different types of cancer cells, focusing on small cell lung cancer and prostate adenocarcinoma cells.

Materials and Methods

Materials. Cardiac hormones. The cardiac hormones were from Phoenix Pharmaceuticals, Inc., Belmont, CA, USA.

Human small cell lung and prostate cancer cells. Human small cell lung carcinoma cells (ATCC number CRL-2195, SHP-77) and prostate cancer cells (ATCC number HTB-881, DU-145) were obtained from the American Type Culture Collection (ATCC) Manassas, Virginia, USA. The ATCC authenticated these cell lines and performed the genotype and phenotype evaluations, including short tandem repeats (STRs) DNA profiles and cytogenic analysis.

Culture of the small cell lung and prostate carcinoma cells. Propagation of the human small cell lung cancer and prostate cancer cells was performed in Roswell Park Memorial Institute (RPMI)-1640 medium with 2 mmol/l glutamine adjusted with the addition of 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mmol/l HEPES, 1 mmol/l of 90% sodium pyruvate and 10% fetal bovine serum (FBS) (Sigma Chemical Company, St. Louis, MO, USA) at a temperature of 37˚C with 5% CO_2 as recommended by the ATCC. Cells were dispensed into new flasks with sub-culturing every 6-8 days. The growth medium was changed every three days.

Human JNK2 (ELISA). The Duo Set® IC human c-Jun-*N*-terminal kinase 2 (JNK2) ELISA was from R&D Systems, Minneapolis, MN, USA. In this assay, an immobilized captive antibody specific for JNK2 binds both phosphorylated and unphosphorylated JNK2 using a standard Streptovidin-conjugated to horseradish-peroxidase. The human JNK2 Duo Set® IC ELISA specifically recognizes JNK2 as demonstrated by western blot analysis. JNK1, JNK3, ERK2 and P38α do not cross-react or interfere in this assay. Thus, JNK2 ELISA is calibrated against a highly purified *Escherichia coli*-expressed recombinant human JNK2 produced by R&D Systems. The standard curve for this assay is calculated using a computer-generated four parameter (4-PL) curve-fit. The Duo Set® IC ELISA takes approximately 6 hours to measure JNK2 in cell lysates.

JNK Protocol. The human small cell lung and prostate adenocarcinoma cells were subcultured for 24 hours, then approximately 5,000 cells of each cancer cell line in 50 μl of lysis buffer were seeded in 96-well plates with 50 μl of media containing 1 μmol/l, 0.1 μmol/l, 0.01 μmol/l, 0.001 μmol/l, and 0.0001 μmol/l concentrations of each of the four cardiac hormones separately (n=6 for each concentration). The standards from R&D Systems were added to blank wells to serve as reference points of known JNK2 concentrations. In this assay, absorbance was recorded at a 540 nM wavelength using a 96-well BioTek Gen 5, Synergy Mx microplate reader (Winooski, VT, USA). There were 32 controls for each cell line in these experiments.

Statistical analysis. Data are expressed as the means±SEM. Statistical analysis of the data were performed by Student's *t*-test for unpaired values. A value of $p<0.05$ was considered the criterion for statistical significance.

Results

Inhibition of JNK2 in human small cell lung cancer cells. There was an 89% ($p<0.0001$) decrease of JNK2 in human small cell lung cancer cells by vessel dilator at 1 μM (Figure 1). This concentration of vessel dilator caused the maximal decrease in JNK2 in small cell lung cancer cells but there was also a significant decrease in JNK2 at lower concentrations of vessel dilator. Thus, vessel dilator caused a 56% ($p<0.0001$) decrease in JNK2 at 1 nM and a 35% ($p<0.01$) decrease at its 100 pM concentration (Figure 1).

LANP reduced JNK2 in human small cell lung cancer cells by 88% ($p<0.0001$) at 1 μM (Figure 1). This was the maximal decrease in JNK2 secondary to LANP but JNK2 was also decreased at lower concentrations of LANP with JNK2, for example, decreased by 51% ($p<0.001$) in human small cell lung cancer cells at 100 nM. LANP's ability to decrease JNK2 at 1 nM and 100 pM concentrations is illustrated in Figure 1.

Kaliuretic peptide caused a maximal decrease of 77% ($p<0.0001$) in JNK2 in human small cell lung cancer cells at 100 nM concentration (Figure 1). At 1 nM and 100 pM concentrations of kaliuretic peptide, JNK2 was reduced 10% and 12% respectively ($p<0.05$). The maximal decrease (88%; $p<0.0001$) in JNK2 in human small cell lung cancer cells secondary to ANP occurred at 1 μM (Figure 1). Thus, in human small cell lung cancer cells, the order of effect was vessel dilator=LANP=ANP>kaliuretic peptide, but the differences between the cardiac hormones were minimal, with each causing an extremely significant ($p<0.0001$) decrease in JNK2.

Decrease in JNK2 in prostate adenocarcinoma cells. Vessel dilator maximally reduced JNK2 by 76% ($p<0.0001$) in human prostate cancer cells at 1 nM concentration. There was a 63% ($p<0.0001$) decrease of JNK2 in prostate cancer cells at 1 μM of vessel dilator and a 35% ($p<0.01$) inhibition of JNK2 at 100 pM of vessel dilator (Figure 2).

LANP maximally decreased JNK2 by 56% ($p<0.0001$) in human prostate cancer cells and this occurred at 1 μM (Figure 2). LANP reduced JNK2 27% ($p<0.01$) and 20% ($p<0.01$) at 1 nM and 100 pM, respectively, in prostate adenocarcinoma cells (Figure 2).

Kaliuretic peptide maximally decreased JNK2 45% ($p<0.0001$) at 1 μM (Figure 2) in human prostate cancer cells. ANP was the least effective in reducing JNK2 in prostate cancer cells, with it causing a 26% ($p<0.01$) decrease at 1 nM. Thus, in human prostate adenocarcinoma cells, with respect to their ability to reduce JNK2, the order was vessel dilator>LANP>kaliuretic peptide>ANP.

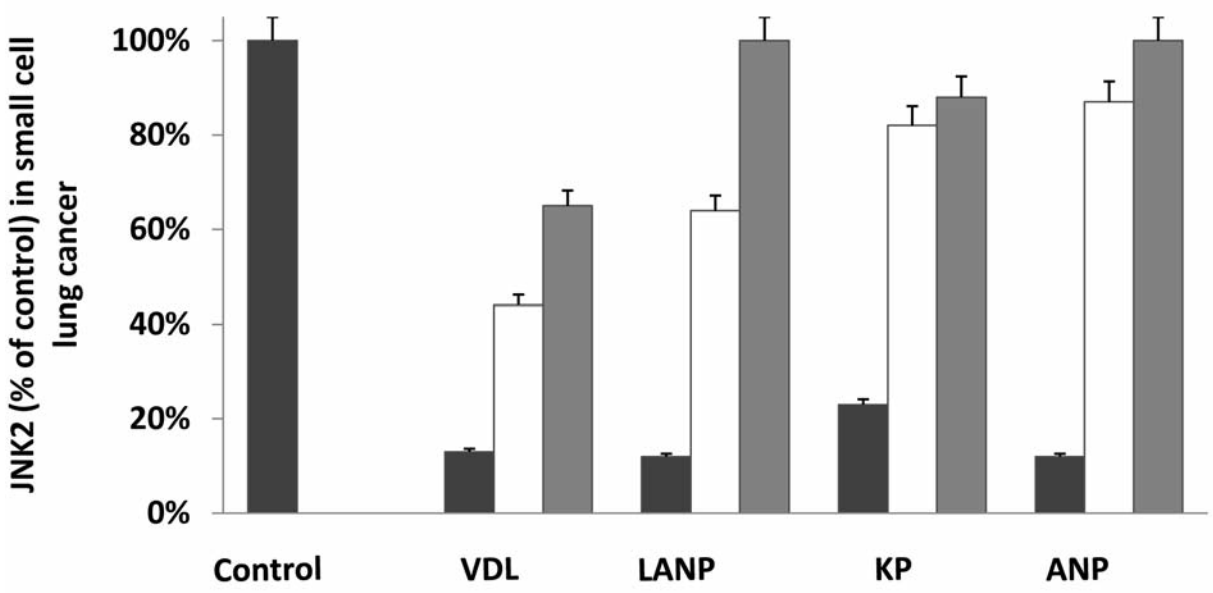

Figure 1. *In human small cell lung cancer cells, vessel dilator (VDL), long-acting natriuretic peptide (LANP), and atrial natriuretic peptide (ANP) reduced c-Jun-N-terminal kinase 2 (JNK2) the most, i.e. 88% for all three at 1 μM concentrations (■). Kaliuretic peptide (KP) maximally reduced JNK2 in human small cell lung cancer cells by 77% at 1 μM (■) concentration. The decreases in JNK2 in human small cell lung cancer cells at 1 μM concentrations were significant at p<0.0001 when evaluated by Student's t-test. In dose response studies with each of the cardiac hormones there was also a significant reduction in JNK2 at 1 nM (□) concentrations. ANP decreased JNK2 by 35% and vessel dilator reduced JNK2 56% at 1 nM (□). Vessel dilator reduced JNK2 by 35% at 100 pM (■). p<0.001 for 1 nM and for 100 pM concentrations except for ANP and LANP which had no significant effect (NS) at 100 pM concentrations. n=32 for controls, n=6 for experimental groups.*

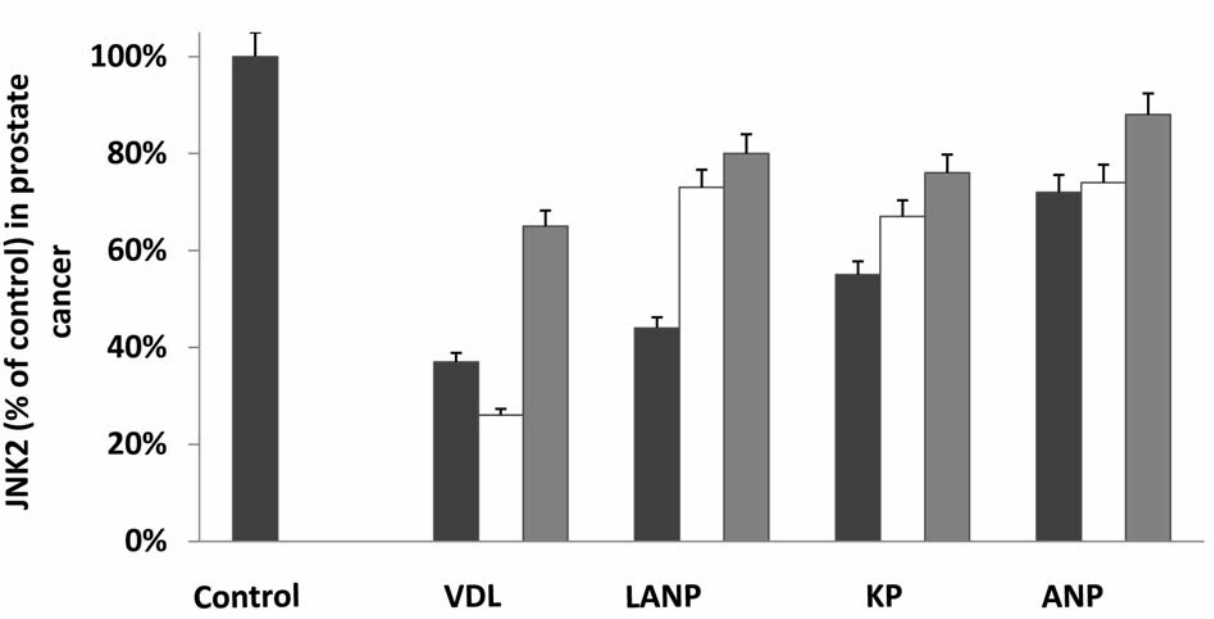

Figure 2. *JNK2 in human prostate adenocarcinoma cells was decreased the most secondary to long-acting natriuretic peptide (LANP) at 56% and vessel dilator (VDL) at 63% at 1 μM concentrations (■). The decrease in JNK2 in these adenocarcinoma cells was significant at p<0.0001 secondary to VDL and LANP when evaluated by Student's t-test. Kaliuretic peptide decreased JNK2 in human prostate cancer cells by 45% and ANP decreased JNK2 in these prostate cancer cells by 28% at 1 μM concentrations (■) which were significant at p<0.001 and p<0.01, respectively, when evaluated by Student's t-test. ANP reduced JNK2 in prostate cancer cells 28% at 1 μM concentration and 26% at its 1 nM (□) concentration (p<0.001 for both). There was no significant decrease in JNK2 in human prostate cancer cells at 100 pM of ANP (■). n=32 for controls. n=6 for the each experimental group of the cardiac hormones.*

Discussion

The present investigation demonstrates that vessel dilator, LANP, kaliuretic peptide, and ANP reduce the concentration of JNK2 by 77% to 88% in human small cell lung cancer cells. This significant ($p<0.0001$) decrease of JNK2 in human small cell lung cancer cells suggests that JNK2 is a molecular target of the cardiac hormones in cancer cells. The ability of the cardiac hormones to reduce the concentration of JNK2 in cancer cells would appear to be an additional important means of their eliminating carcinomas *in vivo* (13-15), in addition to their ability (18-23) to inhibit the RAS-MEK 1/2-ERK 1/2 kinase cascade (*i.e.* multiple kinase inhibition).

The four cardiac hormones inhibit each step in the RAS-MEK1/2-ERK 1/2 cascade (18-23). Thus, they inhibit up to 95% of Ras (18, 19), 98% of MEK 1/2 kinases (20, 21), and 96% of ERK 1/2 kinases (22, 23). The present investigation demonstrates that they also inhibit another mitogen-activated protein kinase, *i.e.* MAPK kinase 9, also known as JNK2. Each of these kinases appear to be interrelated, with RAS-MEK 1/2-ERK 1/2 kinases being activated in a stepwise fashion from the cell surface (RAS) to the nucleus, and JNK2 interacts with this cascade and is required for RAS-initiated lung cancer formation (17). Since JNK2 is the JNK kinase preferentially required for proliferation of lung cancer cells (11), it would appear to be important that these cardiac hormones inhibit JNK2 in lung cancer cells. The four cardiac hormones inhibiting JNK2 would suggest that any lung cancer cells that escape the inhibition of RAS (18, 19) will then be exposed to inhibition of JNK2 downstream in cancer signaling to help eliminate the remaining lung cancer cells that escaped the inhibition of RAS.

These cardiac hormones were also found to reduce JNK2 in another type of cancer cell, *i.e.* prostate adenocarcinoma cells in the present investigation. JNK2 is the kinase preferentially required for moderating the proliferation of prostate cancer cells (9) and with the four cardiac hormones inhibiting JNK2 in human prostate cancer cells, this would suggest they may thus modify proliferation in prostate cancer cells as they do in pancreatic cancer cells (24). There was a marked difference in the ability of ANP to maximally reduce JNK2 in prostate cancer cells (28%) compared to human small cell lung cells (88%) at their 1 μM concentrations and the reason for this difference and the difference between the ability of kaliuretic peptide to inhibit JNK2 in prostate cancer cells and in small cell lung cancer cells is unknown at present. It is to be noted, however, that the ability of vessel dilator and LANP to reduce JNK2 in human prostate cancer cells was very similar to their ability to reduce JNK2 in human small cell lung cancer cells, and correlates roughly with their ability to reduce the number of viable human prostate cancer cells in cell culture (25). This suggests that their ability to inhibit JNK2 is an important mechanism mediating their ability to reduce viability of human prostate adenocarcinoma cells.

Conclusion

These results indicate that these four cardiac hormones are significant inhibitors by up to 89% of JNK2 in human small cell lung cancer cells and up to 76% in human prostate cancer cells and these findings suggest that JNK2 is a molecular target of their anticancer effects.

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Potential Conflict of Interest Statement

Dr. Vesely has assigned the patent to treat cancer with these cardiac hormones to the University of South Florida, which has not licensed this patent to any commercial entity. There has been no pharmaceutical company funding or input into the studies described herein. None of the other authors have any potential conflict of interest.

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