Central Role of β-Catenin in Anticancer Effects of Cardiac Hormones

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Abstract. *Background: β-Catenin causes malignant growth of colonic, pancreatic and renal cancer. Four cardiac hormones, namely atrial natriuretic peptide (ANP), vessel dilator, long-acting natriuretic peptide (LANP) and kaliuretic peptide eliminate up to 80% of human pancreatic carcinomas growing in mice. Materials and Methods: Four cardiac hormones were evaluated for their ability to reduce the expression of human β-catenin, measured by enzyme-linked immunosorbent assay (ELISA) in human colorectal, pancreatic and renal cancer cells. Results: Vessel dilator, LANP, kaliuretic peptide, and ANP, over a concentration range of 100 pM to 10 μM, maximally reduced expression of β-catenin in human colorectal cancer cells by 78%, 71%, 69%, and 83%, respectively. Vessel dilator, LANP, kaliuretic peptide, and ANP reduced β-catenin expression in human pancreatic cancer cells by 76%, 66%, 72%, and 88%, and by 64%, 54%, 58% and 73%, in human renal cancer cells, respectively. Conclusion: Part of the anticancer action of these four cardiac hormones is a potent inhibition of β-catenin.*

β-Catenin is a multifunctional protein, located on the intracellular side of the cytoplasmic membrane, that causes malignant growth of colonic (1, 2), renal (3, 4) and pancreatic (5, 6) cancers. β-Catenin activation also leads to breast (7, 8), anaplastic thyroid (9), gastric (10), liver (11), ovarian (12), endometrial (12), and prostate (13, 14) cancer.

The present investigation was designed to determine whether four cardiac hormones, namely vessel dilator, atrial natriuretic peptide (ANP), long-acting natriuretic peptide (LANP), and kaliuretic peptide, which eliminate up to 97% of colon, pancreatic, renal, breast, ovarian, prostate, small-cell and squamous cell lung cancers *in vitro* (15-22), have part of their mechanism(s) of action by inhibiting β-catenin. *In vivo*, these four endogenous anticancer hormones eliminate up to 80% of human pancreatic adenocarcinomas (23), two-thirds of human breast carcinomas (24), and up to 86% of human small-cell lung carcinomas growing in athymic mice (25). The rationale for studying the effects of these cardiac hormones on β-catenin, is that β-catenin has a central position in the cross-talk between rat sarcoma-bound guanosine triphosphate kinase (RAS), AK mouse strain with "t" for thymoma (AKT), and vascular endothelial growth factor (VEGF), which feeds back to stimulate RAS, causing a vicious cycle of growth of cancer cells, as illustrated in Figure 1. The cardiac hormones inhibit upstream regulators of β-catenin (26, 27), *i.e.* RAS by up to 95% (28-30) and AKT (31). They also inhibit downstream targets of β-catenin (32, 33), *i.e.* c-JUN-*N*-terminal kinase-2 by 89% (34) and VEGF receptor 2 by 89% (35). The present investigation examined whether these cardiac hormones in dose–response evaluations inhibit β-catenin in three different human cancer cell lines (colorectal, pancreatic and renal) as this would break the feedback loop between RAS and VEGF, thus interrupting the vicious cycle which stimulates cancer cell growth.

Materials and Methods

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

Human colorectal, pancreatic, and renal cancer cells. Human colorectal cancer cells (ATCC number CCL-225), pancreatic carcinoma cells (ATCC number CRL-1469, panc-1) and renal

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Key Words: β-Catenin, cardiac hormones, human pancreatic cancer, human colorectal adenocarcinoma, human renal adenocarcinoma.

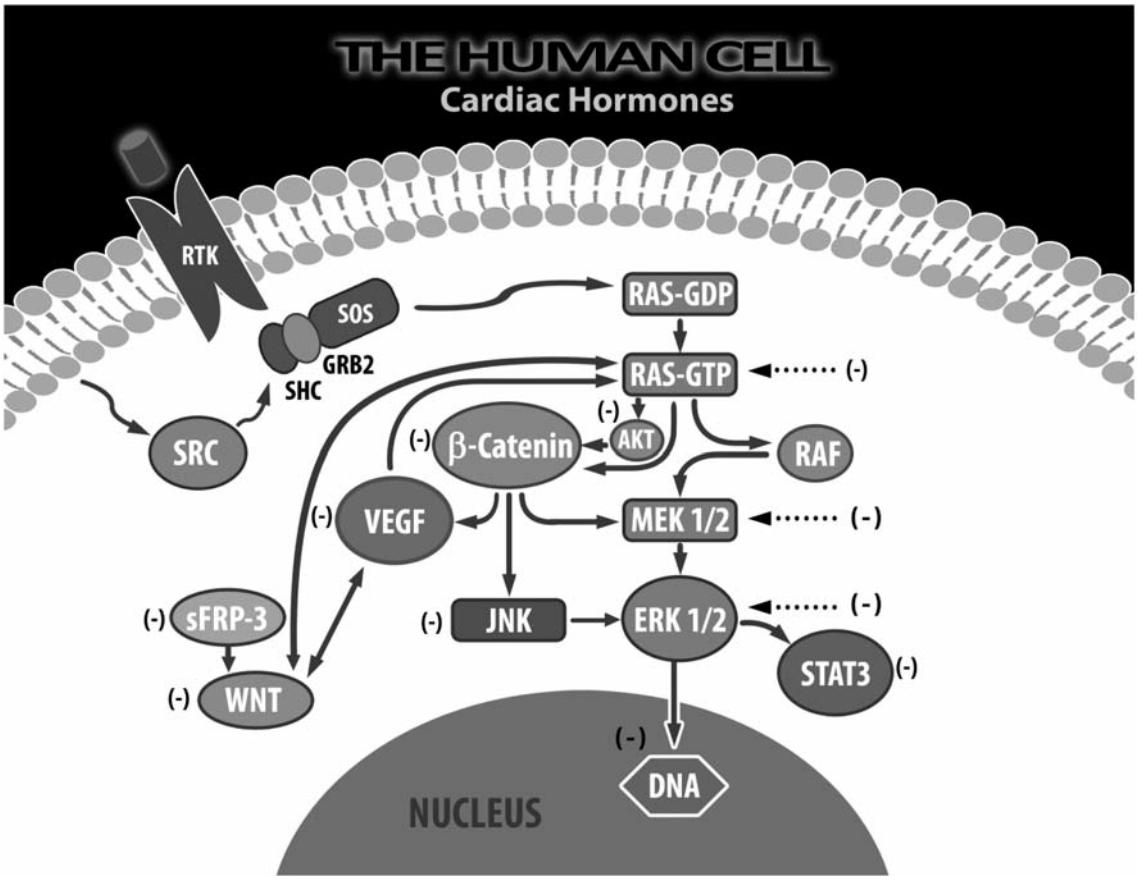

Figure 1. *Cardiac hormones inhibit β-catenin (up to 88%) and rat sarcoma-bound guanosine triphosphate (RAS-GTP), mitogen-activated protein kinase kinase (MEK) 1/2, and extracellular signal-related kinases (ERK) 1/2 of the RAS–MEK 1/2–ERK1/2 kinase cascade by 95-98%. These multiple kinase inhibitors are also strong inhibitors (i.e. 91%) of deoxyribonucleic acid (DNA) synthesis within cancer cells. Other targets which these cardiac hormones inhibit within cancer cells are vascular endothelial growth factor (VEGF), the VEGFR-2 receptor, secreted Frizzled-related protein 3 (sFRP3), c-JUN-N-terminal kinases (JNK-2), signal transducer and activator of transcription-3 (STAT3), and the WNT pathway. As illustrated here, the cardiac hormones inhibit [shown by (–)] several steps in the feedback loop between RAS kinase and VEGF that lead to a vicious cycle of stimulating cancer cell growth. RTK: Tyrosine kinase receptor; SRC: rous sarcoma viral proto-oncogene tyrosine kinase; SHC: rous sarcoma SH2 C-terminal binding domain adapter protein; GRB2: growth factor receptor-bound protein 2; SOS: son of sevenless gene; RAS-GDP: rat sarcoma-bound guanosine diphosphate; RAF: rapidly-accelerated fibrosarcoma serine/threonine protein kinase; AKT: AK mouse strain with "t" for thymoma. Modified with permission from reference 30.*

adenocarcinoma cells (ATCC CRL-1611) were obtained from the American Type Culture Collection (ATCC) Manassas, VA, USA. The ATCC authenticated these cell lines and performed the genotype and phenotype evaluations, including DNA profiles (short tandem repeat, STR) and cytogenetic analyses. The ATCC has further characterized some of the receptors and antigen expression within these well-characterized cancer cells.

Culture of human colorectal adenocarcinoma cells. Propagation of the human colorectal adenocarcinoma cells was performed in Roswell Park Memorial Institute (RPMI)-1640 medium with 2 mM glutamine adjusted with the addition of 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1 mM of 90% sodium pyruvate and 10% fetal bovine serum (FBS) (Sigma Chemical Co., 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.

Culture of human pancreatic carcinoma cells. Propagation of the human pancreatic carcinoma cells was carried out in Dulbecco's modified Eagle's plus Ham's F12A 1:1 mixture containing 1.2 g/l of sodium bicarbonate (Sigma Chemical Co.) supplemented with 15 mM of HEPES and 10% FBS with 5% CO_2 at a temperature of 37˚C, as recommended by the ATCC. Cells were dispensed into new flasks with subculturing every 6-8 days. The growth medium was changed every three days.

Culture of human renal adenocarcinoma cells. Propagation of human renal cell adenocarcinomas was performed in Eagle's minimum essential medium supplemented with 2 mM glutamine adjusted with the addition of 1.5 g/l sodium bicarbonate, 1 mM of

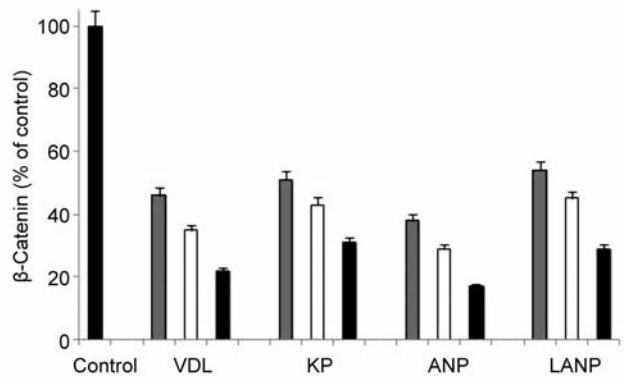

Figure 2. *Vessel dilator (VDL), kaliuretic peptide (KP), atrial natriuretic peptide (ANP) and long acting natriuretic peptide (LANP) reduce β-catenin levels in human colorectal cancer cells. VDL, kaliuretic peptide, ANP, and LANP maximally reduced β-catenin by 78%, 69%, 83%, and 71% compared to control β-catenin levels, respectively, each at 10 μM. For each of the cardiac hormones, reduction of β-catenin at100 pM (■), 100 nM (□), and 10 μM (■), was significant at p<0.0001, when evaluated by the Student's t-test for unpaired values. Each bar represents the mean±SEM of 32 determinations for the control and six determinations for the experimental groups.*

90% sodium pyruvate and 10% FBS (Sigma Chemical Co.) with 5% CO_2 at a temperature of 37˚C, as recommended by the ATCC. Cells were dispensed into new flasks with subculturing every 6-8 days. The growth medium was changed every three days.

Human β-catenin enzyme-linked immunosorbent assay (ELISA). The DuoSet Human Total β-catenin immunoassay (R&D Systems, Inc., Minneapolis, MN, USA) is a sandwich ELISA designed to measure β-catenin in cell lysates. In this assay, an immobilized capture antibody specific for β-catenin binds both phosphorylated and unphosphorylated β-catenin using a standard Streptavidin-conjugated to horseradish-peroxidase format to detect captured protein. β-Catenin ELISA is calibrated against a highly purified recombinant human β-catenin produced by R&D Systems. The standard curve for this assay was calculated using a 4 parameter logistic (4-PL) curve-fit. The lowest quantification of this assay is 312 pg/ml. The range measured by the standard curve is 312–20,000 pg/ml.

β-Catenin protocol. The human colorectal cancer, pancreatic carcinoma, and renal adenocarcinoma cells were subcultured for 24 hours, then approximately 5,000 cells of each line in 50 μl of their respective media were seeded in 96-well plates with 50 μl of media containing 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, and 100 pM concentrations of each of the four cardiac hormones separately (*i.e.* six concentrations of four cardiac hormones measured six times at each concentration; n=6 for each concentration). The cardiac hormones were incubated with the cancer cells for two hours at room temperature. The standards from R&D Systems were diluted using Reagent Diluent and added to blank wells to serve as reference points of known β-catenin concentration. In this assay, absorbance was examined at a 540 nM wavelength using a 96-well BioTek Gen5, Synergy Mx microplate reader (Winooski, VT, USA) set according to the parameters recommended by the kit manufacturer. There were 32 controls for each cell line in these experiments (n=32) and six experimental determinations for each of the six concentrations of the four cardiac hormones in the three cancer cell lines (n=6).

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

Human colorectal cancer cells. In human colorectal cancer cells, there was a maximal 78% ($p<0.0001$) decrease of β-catenin by vessel dilator at 10 μM (Figure 2). Kaliuretic peptide caused a maximal decrease of 69% ($p<0.0001$) of β-catenin in human colorectal cancer cells (Figure 2) at 10 μM. The maximal decrease of β-catenin in colorectal cancer cells treated with ANP was 83% ($p<0.0001$), which occurred at 10 μM (Figure 2). LANP caused a maximal decrease of 71% ($p<0.0001$) of β-catenin in human colorectal cancer cells at 10 μM. In reducing the concentration of the four cardiac hormones 100,000-fold in dose–response evaluations to 100 pM, there was still a significant ($p<0.0001$) inhibition of β-catenin by the four cardiac hormones (Figure 2). In human colorectal cancer cells, the ability of the respective cardiac hormones to inhibit β-catenin was greatest with ANP and vessel dilator, followed by LANP and lastly kaliuretic peptide.

Human pancreatic cancer. Next, in order to determine if the effects of the four cardiac hormones on β-catenin only occur in colorectal cancer cells or are more generalized, β-catenin expression in pancreatic cancer cells was evaluated in response to the cardiac hormones. Vessel dilator maximally reduced β-catenin by 76% ($p<0.0001$) at 10 μM (Figure 3). Kaliuretic peptide maximally reduced β-catenin in human pancreatic cancer cells by 72% ($p<0.0001$), which occurred at 10 μM (Figure 3). ANP caused a maximal decrease of 88% of β-catenin expression in human pancreatic cancer cells ($p<0.0001$) at 10 μM (Figure 3). LANP reduced β-catenin expression in human pancreatic cancer cells by 66% at 10 μM ($p<0.0001$) (Figure 3). In reducing the concentration(s) of these cardiac hormones 100,000-fold to 100 pM, there was still a significant inhibition of β-catenin ($p<0.0001$) as can be seen in Figure 3. ANP inhibited β-catenin in human pancreatic cancer cells most strongly, followed by vessel dilator, kaliuretic peptide, and LANP.

Human renal adenocarcinoma cells. We then tested the hypothesis that ANP and vessel dilator, which inhibited β-catenin the strongest in human pancreatic cancer and colorectal cancer calls, are the strongest inhibitors of β-catenin of cancer cells in general by examining the effects of these four hormones on β-catenin expression in renal cancer cells. Vessel

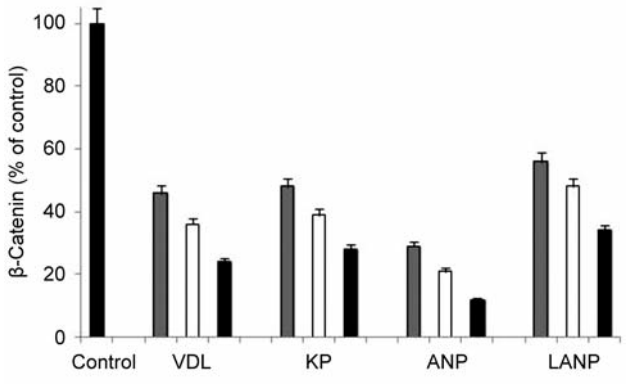

Figure 3. *β-Catenin expression in human pancreatic cancer cells is markedly reduced by four cardiac hormones. Vessel dilator (VDL), kaliuretic peptide (KP), atrial natriuretic peptide (ANP), and long acting natriuretic peptide (LANP) maximally reduced β-catenin by 76%, 72%, 88%, and 66%, respectively, which at 10 μM. For each of the cardiac hormones, reduction of β-catenin at100 pM (■), 100 nM (□), and 10 μM (■), was significant at p<0.0001, when evaluated by the Student's t-test for unpaired values. Each bar represents the mean±SEM of 32 determinations for the control and six determinations for the experimental groups.*

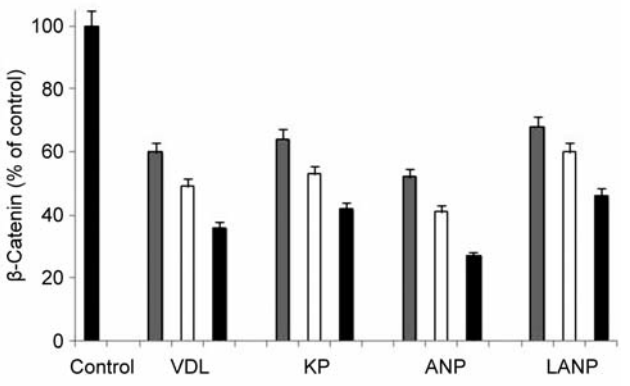

Figure 4. *β-Catenin expression is reduced in human renal adenocarcinoma cells by vessel dilator (VDL), kaliuretic peptide (KP), atrial natriuretic peptide (ANP), and long acting natriuretic peptide (LANP). VDL, kaliuretic peptide, ANP, and LANP maximally reduced β-catenin by 64%, 58%, 73%, and 54%, respectively, at 10 μM. For each of the cardiac hormones, reduction of β-catenin at100 pM (■), 100 nM (□), and 10 μM (■), was significant at p<0.0001, when evaluated by the Student's t-test for unpaired values. Each bar represents the mean±SEM of 32 determinations for the control and six determinations for the experimental groups.*

dilator reduced β-catenin in human renal cancer cells by 64% at 10 μM ($p<0.0001$) (Figure 4). Kaliuretic peptide maximally reduced β-catenin by 58% ($p<0.0001$) in human renal cancer cells at 10 μM (Figure 4). ANP maximally reduced β-catenin by 73% ($p<0.0001$) in human renal cancer cells at 10 μM (Figure 4). There was a 54% decrease of β-catenin in human renal cancer cells induced by LANP at 10 μM (Figure 4) ($p<0.0001$). As in the other cancer cell lines, reducing the concentration of the four cardiac hormones 100,000-fold to 100 pM, there was still a significant ($p<0.0001$) inhibition of β-catenin in human renal adenocarcinoma cells (Figure 4).

Comparison of the ability of cardiac hormones to reduce β-catenin expression in different human cancer cells. In human renal cancer cells, the differences in inhibition of human β-catenin in the different cancer cell types was most pronounced with ANP, followed by vessel dilator, and then by kaliuretic peptide and LANP, which had similar effects (comparing Figures 2-4). It is also evident by examining the data in these figures that the four cardiac hormones reduced β-catenin expression the most in human pancreatic carcinoma cells and human colorectal cancer cells, with that in renal adenocarcinoma cells being less (Figures 2-4).

Discussion

The present investigation demonstrates that vessel dilator, kaliuretic peptide, ANP and LANP inhibit β-catenin by up to 83% in human colorectal cancer cells, up to 88% in human pancreatic carcinoma cells, and up to 73% in human renal cancer cells (Figures 2-4). With each of the human cancer cell lines, there was a definite dose–response relationship. The significant reduction of β-catenin expression in these cells suggests that β-catenin is a key target of anticancer effects of these cardiac hormones in a variety of human cancer cell lines. Epidermal growth factor (EGF) phosphorylates β-catenin on its tyrosine residues, resulting in tumor progression (26). One upstream regulator of β-catenin resulting in tumor growth is RAS kinase (27) (Figure 1). The four cardiac hormones interact with these upstream regulators of β-catenin to reduce the basal activity of RAS by up to 95% (28, 29) and also reduce EGF stimulation of RAS by up to 79% (30). RAS stimulates AKT which, in turn, activates β-catenin (Figure 1) and the activation of AKT is also inhibited by the cardiac hormones (31). There is, thus, a complex interaction of intracellular enzymes upstream of β-catenin in cancer cells that contribute to cancer cell growth and these four cardiac hormones inhibit these enzymes at multiple steps, as outlined in Figure 1.

The targets downstream of β-catenin causing cancer, include c-JUN-N-terminal kinase 2 (32) and VEGF (33); (see Figure 1). The four cardiac hormones reduce expression of β-catenin's downstream targets, *i.e.* JNK (34) (89% decrease), VEGF and its receptor VEGFR-2 (89% decrease), which is its receptor associated with cancer growth (35). These results suggest a unique interrelationship of the cardiac hormones with β-catenin, its signaling and its downstream, as well as upstream regulators. As can be seen in Figure 1, the ability to inhibit β-

catenin is crucial in stopping the growth of cancer cells as β-catenin stimulates VEGF which, in turn, enhances the whole RAS–MEK 1/2–ERK 1/2 kinase cascade. RAS, in turn, also stimulates β-catenin (26, 27), resulting in a vicious cycle of cancer growth. β-Catenin is also a major effector of the canonical WNT signaling pathway (36, 37) (Figure 1). WNT feeds back to stimulate RAS-GTP (38) and VEGF (33), creating further feedback loops enhancing cancer cell growth. WNT also feeds back to stabilize β-catenin (36, 37) (Figure 1). WNT family members utilize the β-catenin-dependent signaling pathway to enhance cell proliferation (39). These four cardiac hormones inhibit the cross-talk between these pathways by reducing WNT-3a by up to 68% in human pancreatic cells and by up to 53% in the same human colorectal cancer cells utilized in the present investigation (40). These four cardiac hormones break the vicious cycle by inhibiting β-catenin as well as WNT, VEGF and multikinases (28, 29, 34, 35, 40). That the cardiac hormones reduce β-catenin levels roughly correlating with their ability to eliminate human pancreatic carcinomas *in vivo* (23) would suggest that such decrease in β-catenin may be an important contributor to their ability to eliminate human carcinomas *in vivo*.

Conclusion

Four cardiac hormones, namely ANP, vessel dilator, LANP and kaliuretic peptide, are potent inhibitors of β-catenin, as part of their anticancer mechanism(s) of action. By inhibiting β-catenin, one is able to break the feedback loop between RAS kinase and VEGF that stimulates cancer cell growth (Figure 1).

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