Four Cardiac Hormones Cause Death of Human Cancer Cells but Not of Healthy Cells

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Abstract. *Background: Four cardiac hormones, namely, vessel dilator, long-acting natriuretic peptide, atrial natriuretic peptide and kaliuretic peptide, have anticancer effects but whether they cause cell death of human cancer cells or normal cells is unknown. Materials and Methods: These cardiac hormones were examined for their ability to cause cell death quantified by measurement of nuclear matrix proteins 41/7 which is a function of the number of dead or dying cells. Results: Each of these cardiac hormones caused cell death in up to 36% ($p<0.0001$) of the pancreatic adenocarcinoma cells and up to 28% ($p<0.0001$) of the prostate cancer cells over a concentration range of 100 pmol/l to 10 μmol/l. There was no cell death of normal human prostate, kidney, or lung cells at the above concentrations. Conclusion: Four cardiac hormones cause death of pancreatic and prostate cancer cells but not of normal prostate, lung, or kidney cells.*

Nuclear matrix proteins (NMPs) make up the internal structure (framework) of the nucleus and are associated with such functions as DNA replication, RNA synthesis, and hormone receptor binding (1, 2). NMPs are involved in regulation and coordination of gene expression (3-5). The identification of cell-type-specific NMPs supports their potential contribution to cellular differentiation and tissue development (2). Although the nuclear matrix has been shown to be highly insoluble *in vitro*, it is now known that cell death releases soluble NMPs that can be detected in culture supernatant and other fluids containing dead and dying cells (3). Because the level of NMP 41/7 detected in culture supernatant is a function of the number of dead or dying cells, measurement of NMP 41/7 is useful to quantify cell death.

Four cardiac hormones synthesized by the same gene, namely, atrial natriuretic peptide (ANP), long-acting natriuretic peptide (LANP), vessel dilator and kaliuretic peptide have anticancer effects (6) and eliminate up to 80% of human pancreatic adenocarcinomas growing in athymic mice (7). These cardiac hormones have never been examined to determine if they cause cancer cell death at the concentrations at which they eliminate human cancer growing in mice (7). A second part of this investigation was to test whether these four cardiac hormones cause death of normal cells at the same concentrations that they have anticancer effects on human pancreatic and prostate cancer cells (8, 9). Thus, normal human prostate, lung, and kidney cells were examined simultaneously with examination of cell death of human pancreatic and prostate cancer cells using these four hormones to determine if they may have deleterious effects on normal cells at the same concentrations (7-9) that they have anticancer effects.

Materials and Methods

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

Cell lines. All cell lines used were purchased from the American Type Culture Association (ATCC), Manassas, VA, USA. Cells were investigated within 6 months of obtaining from them the ATCC, which authenticated these cell lines and performed the genotype and phenotype evaluations, including DNA profiles (STR) and cytogenic analyses.

Human pancreatic adenocarcinoma cells. A cell line (ATCC number CRL-2119) of human pancreatic adenocarcinoma cells was derived in 1994 from a nude mouse xenograft of a primary tumor

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removed from the head of a human pancreas (10). These adenocarcinoma cells are tumorigenic in that they form tumors in athymic nude mice at the site of inoculation that are histologically similar to the tumor of origin (10).

Human prostate adenocarcinoma cells. A cell line (ATCC number HTB-81; DU 145) of human prostate adenocarcinoma cells was derived in 1978 by KR Stone *et al*. (11) from a 69-year-old man. These homogenous cells, when injected into athymic mice, form moderately differentiated prostate adenocarcinomas within 21 days (11).

Normal human prostate, kidney and lung cells. Cell lines of normal human prostate (ATCC number CRL-11609), kidney (ATCC number CRL-1573), and lung (ATCC number CLL-215) were used. The ATCC authenticated these cell lines and characterized some of their receptors and antigen expression.

Culture of human pancreatic adenocarcinoma cells. Propagation of the human pancreatic adenocarcinoma cells was in Dulbecco's modified Eagle's plus Ham's F12A 1:1 mixture containing 1-2 g/l of sodium bicarbonate (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 15 mmol/l of HEPES and 10% fetal bovine serum (Sigma Chemical Co.), 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 medium was changed every 3 days.

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

Culture of human prostate, kidney and lung cells. The normal human kidney and lung cells were cultured in Eagle's minimum essential medium with 10% fetal bovine serum as recommended by the ATCC and subcultured as above. The normal human prostate cells were propagated in keratinocyte serum-free medium (Gibco, Invitrogen, Carlsbad, CA, USA) with 0.05 mg/ml of bovine pituitary extract and 5 ng/ml of epidermal growth factor recommended by ATCC. Subculturing was the same as above. Cells were subcultured every 6-8 days.

Cell death assay. Cell death of the cancer and normal human cells was examined with a Calbiochem Cell Death Detector (Nuclear Matrix Protein) ELISA kit (Calbiochem, San Diego, CA, USA) which quantitatively determines nuclear matrix protein 41/7 released from dead or dying cells. (NMP 41/7 appears to be the same protein as that designated as NuMA [nuclear mitotic apparatus] protein.) The antibodies utilized in this assay have been shown to react with lambda gt11 cDNA clones carrying the published sequence from nuclear mitotic apparatus protein. More specifically, the Capture Antibody for NMP 41/7 reacts with a lambda gt11 cDNA clone encoding a peptide of approximately 60 kDa, located in the amino terminus of the published sequence for NuMA (12), and the FITC-anti-NMP 41/7 detector antibody reacts with a lambda gt11 cDNA clone encoding a peptide of ~96 kDa, located in the carboxy-terminus of the published sequence for NuMA. The antibodies in this ELISA are specific for human NMP 41/7 and are not known to cross-react with non-human proteins.

Research protocol. The pancreatic and prostate adenocarcinoma and normal cells were subcultured for 24 hours and then approximately 5,000 cells of each cell line in 50 μl of their respective media were then seeded in 96-well plates (Sarstedt, AG & Co., Nuembrecht, Germany) with 50 μl of media containing 10 μmol/l, 1 μmol/l, 100 nmol/l, 10 nmol/l, 1 nmol/l, and 100 pmol/l of each of the four cardiac hormones separately (*i.e*. six concentrations of four cardiac hormones in triplicate = 72 wells). The experimental groups with the respective peptide hormones at the above concentrations for two hours were examined as recommended by Calbiochem. NMP standard (200 nl) was added to each of the 8 blank wells to serve as a reference point for the absorbance at a dual wavelength reading of 450 nm and 595 nm, using a Benchmark 96-well plate Microplate Reader (Bio-Rad, Hercules, CA, USA). These experiments were repeated twice in order that there were six observations at each of the concentrations of vessel dilator, kaliuretic peptide, ANP, and LANP, respectively. There were 32 controls for each cell line in these experiments, *i.e*. 16 wells per plate × 2=32.

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

Results

Dose–response pancreatic cancer cell death secondary to cardiac hormones. LANP caused cell death in two hours of 28% of human pancreatic cancer cells at 100 pmol/l concentration (Figure 1 F). Cell death of pancreatic cancer cells ranged from 18% to 31% (Figure 1 A-E) in dose–response curves with 1 nmol/l to 10 μmol/l of LANP. The maximal 31% decrease occurred at 10 nmol/l of LANP (Figure 1 D). Nineteen percent of the human pancreatic adenocarcinoma cells underwent cell death with 100 pmol/l of vessel dilator for two hours (Figure 2 F). Dose–response curves revealed that 10% to 20% of human pancreatic adenocarcinoma cells died within two hours when exposed to 1 nmol/l to 10 μmol/l of vessel dilator (Figure 2 A-E). Maximal number of pancreatic cancer cells undergoing cell death (20%) secondary to vessel dilator occurred at 100 nmol/l (Figure 2 C). ANP caused cell death in 20% of human pancreatic adenocarcinoma cells at 100 pmol/l in two hours (Figure 3 F). Maximal cell death, of 35% of the pancreatic cancer cells, occurred at 100 nmol/l of ANP (Figure 3 C). Kaliuretic peptide caused 26% of human pancreatic adenocarcinoma cells to undergo cell death in two hours at 100 pmol/l concentration (Figure 4 F). This was the maximal cell death in dose–response curves with kaliuretic peptide where the amount of cell death ranged from 10% to 26% with 100 pmol/l to 10 μmol/l of kaliuretic peptide (Figure 4 A-F). Thus, each of the cardiac hormones causes cell death of human pancreatic adenocarcinoma cells.

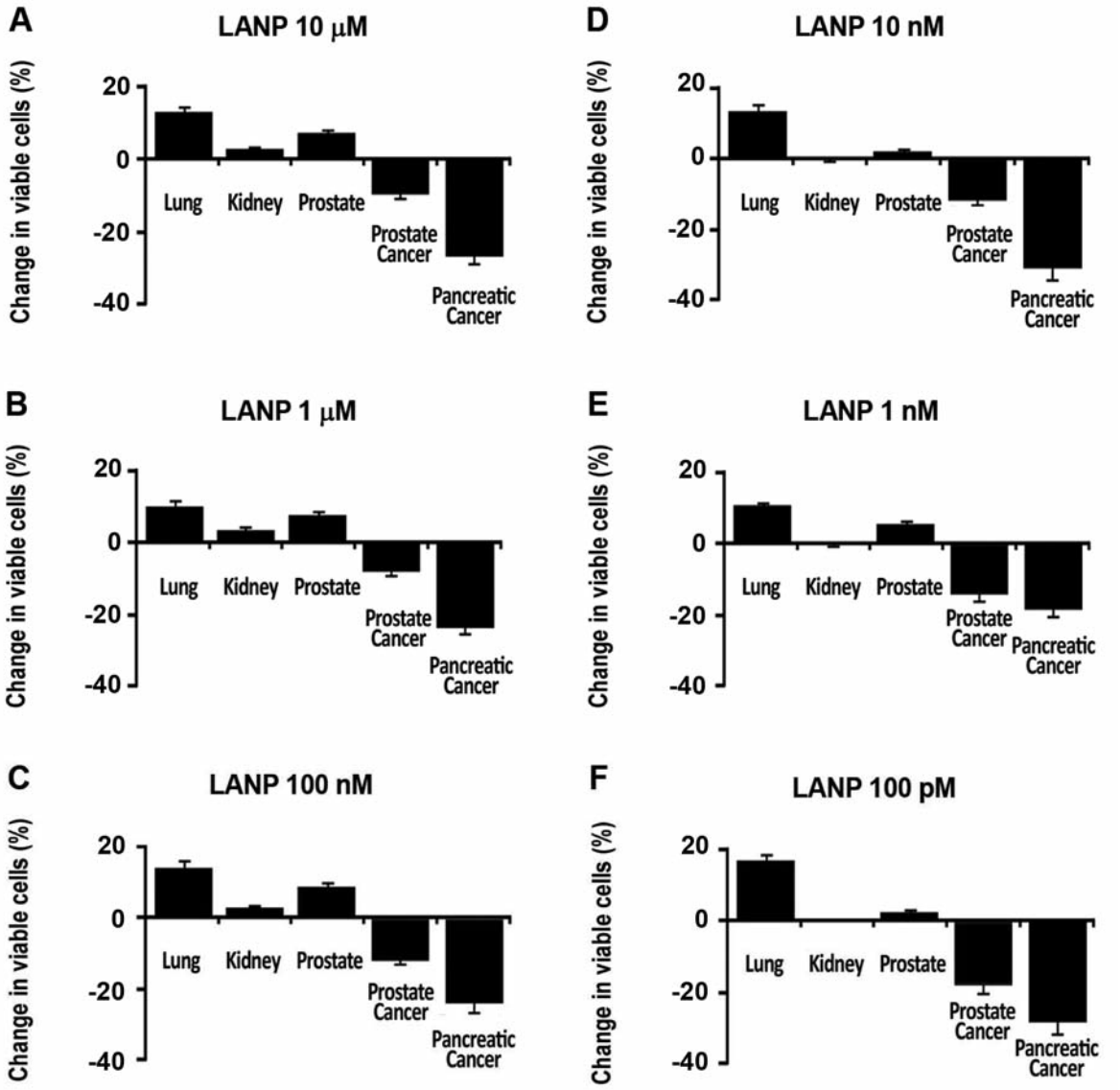

Figure 1. *Dose-response of long-acting natriuretic peptide (LANP) on cell death of human pancreatic and prostate adenocarcinoma cells and normal prostate, kidney, and lung cells after a 2-hour exposure at concentrations shown. p>0.05 for normal cells and p<0.001 for pancreatic cancer and p<0.002 for prostate cancer at all concentrations for change in % of viable cells when evaluated by analysis of variance (ANOVA).*

Cell death of human prostate cancer cells secondary to cardiac hormones. LANP for two hours caused cell death in 18% of human prostate adenocarcinoma cells at 100 pmol/l (Figure 1F). In addition, there was cell death of the human prostate cancer cells at each concentration of LANP from 10 μmol/l to 1 nmol/l (Figure 1 A-E). Vessel dilator for two hours induced a 9% cell death of human prostate cancer cells at 100 pmol/l (Figure 2 F). Cell death of the prostate cancer cells ranged from 2 to 10% with vessel dilator for two hours with 1 nmol/l to 10 μmol/l in dose–response studies. ANP caused cell death of 11% of human prostate cancer cells in 2 hours at100 pmol/l and 1 nmol/l (Figure 3 E and F). ANP also significantly enhanced prostate cancer cell death at 10 nmol/l and 100 nmol/l (Figure 3 C and D). The effects of ANP on prostate cancer cell death of 6% at 10 μmol/l and 2% at 1 μmol/l were not significant (Figure 3 A and B). Kaliuretic peptide caused cell death of 6% of the human prostate cancer cells at 100 pmol/l (Figure 4 F). Prostate cancer cell death secondary to kaliuretic peptide was significant using all concentrations of kaliuretic peptide except 10 nmol/l (Figure 4 A-C, E and F). Thus, each of the cardiac hormones was able to cause death of human prostate adenocarcinoma cells at some of their concentrations.

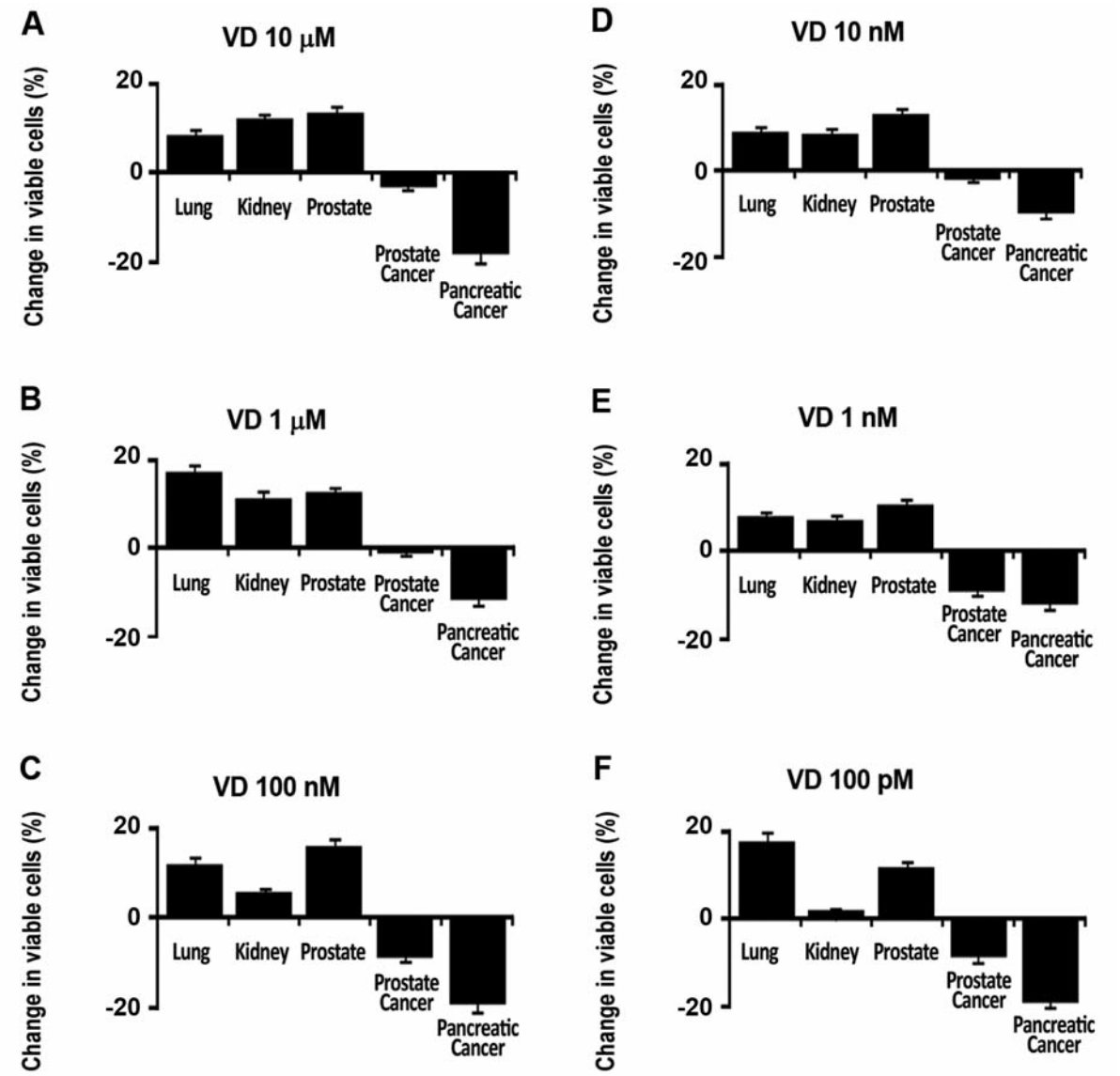

Figure 2. *Vessel dilator causes death of human pancreatic and prostate cancer cells but not of normal prostate, kidney, and lung cells when evaluated in dose–response curves at concentrations shown. p>0.05 for normal cells and p<0.03 for pancreatic cancer and p<0.05 for prostate cancer at all concentrations (except for 1 μmol/l and 10 nmol/l in prostate cancer cells) for change in % of viable cells when evaluated by ANOVA.*

Normal human prostate cells do not undergo cell death secondary to cardiac hormones. There was no cell death of normal human prostate cells with 100 pmol/l to 10 μmol/l of LANP when exposed to this cardiac hormone for 2 hours (Figure 1 A-F). There was no cell death but rather a 2% to 9% increase in normal prostate cells when incubated with LANP for 2 hours (Figure 1 A-F). Vessel dilator did not cause death of normal human prostate cells at any of the concentrations (Figure 2 A-F). Rather, with vessel dilator there was a 10% to 16% increase in prostate cell number during incubation (Figure 2 A-F). ANP did not cause cell death when incubated with human prostate cells for 2 hours at its various concentrations in dose–response curves (Figure 3 A-F). When incubated for 2 hours with ANP there was a 7% (10 μmol/l; Figure 3A) to 13% increase (100 nmol/l; Figure 3 C) in cell numbers. Kaliuretic peptide did not cause death of normal prostate cells at any of the concentrations used in dose–response studies (Figure 4 A-F). The number of normal prostate cancer cells increased by 7% (1 μmol/l) to 16% (at 100 pmol/l) when incubated with kaliuretic peptide for 2 hours.

Cardiac hormones do not cause cell death of normal human kidney cells. There was no significant death of human kidney

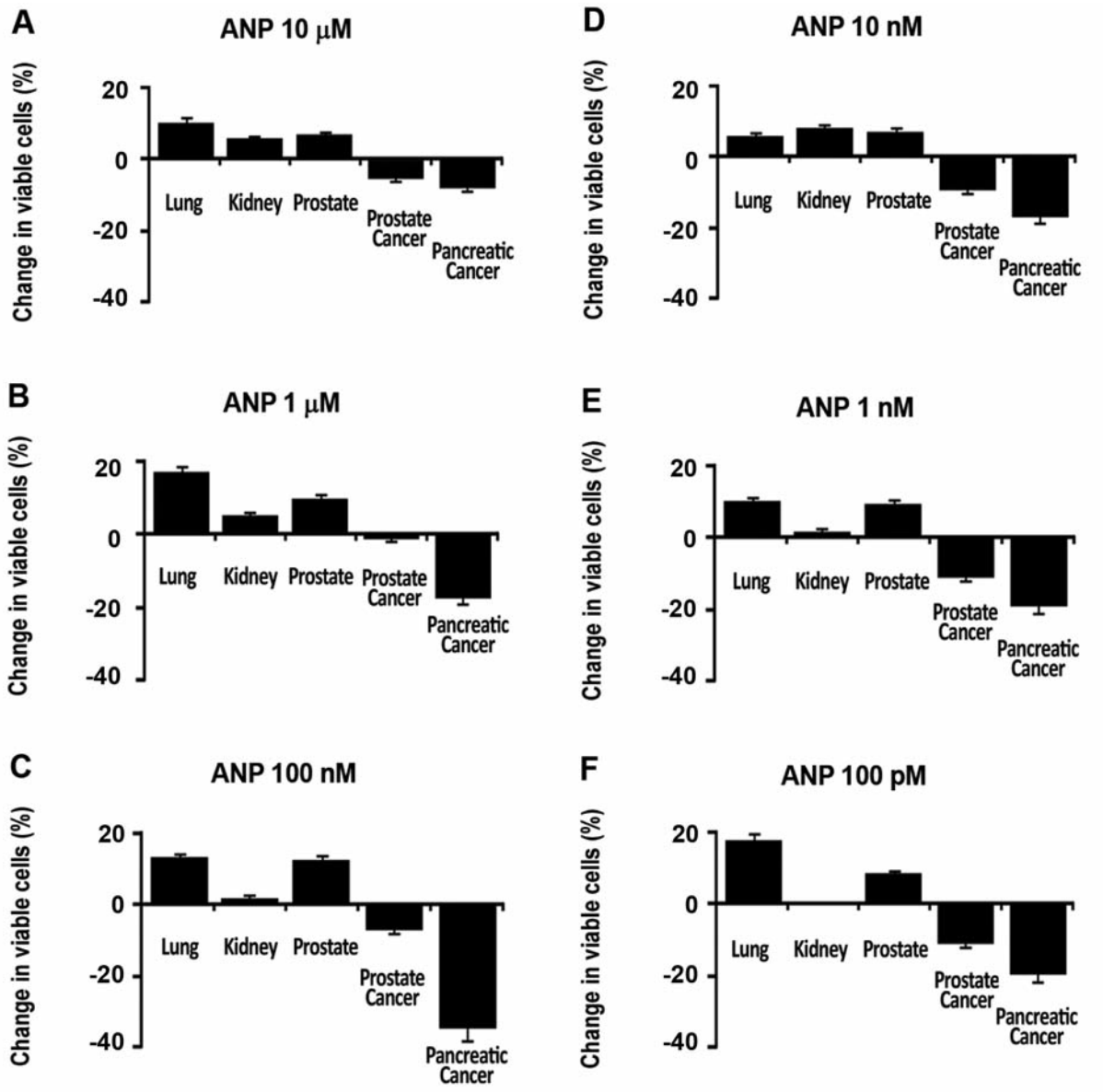

Figure 3. *Dose response of atrial natriuretic peptide (ANP) causing death of human prostate and pancreatic adenocarcinoma cells but not of normal prostate, kidney, and lung cells when exposed to ANP for 2 hours at concentrations shown. p>0.05 for normal cells and p<0.02 for pancreatic cancer and p<0.05 for prostate cancer at all concentrations (except for 1 μmol/l and 10 μmol/l for prostate cancer) for change in % of viable cells when evaluated by ANOVA.*

cells with 100 pmol/l to 10 μmol/l of LANP when exposed to this peptide hormone for two hours as can be seen in Figure 1 A-F. Likewise, there was no death of human kidney cells with any concentration of vessel dilator for two hours (Figure 2 A-F). With vessel dilator, the percentage of human kidney cells increased by 2% (with 100 pmol/l) to 14% with the highest concentration (10 μmol/l) of vessel dilator. ANP for two hours did not cause death of any human kidney cells at the various concentrations used in dose–response curves (Figure 3 A-F). In the cell death assay for kidney cells, there was no evidence of cell death secondary to ANP, with values of 0% (100 pmol/l; Figure 3 F) to 10% increase in cell numbers at 10 nmol/l of ANP (Figure 3 D). Kaliuretic peptide for two hours did not cause death of normal kidney cells at any of the concentrations used in dose–response studies (Figure 4 A-F). The normal kidney cells increased in number by 6% (at 100 pmol/l; Figure 4 F) to 10% (at 10 μmol/l; Figure 4 A) with kaliuretic peptide. Thus, there was no evidence of death of normal kidney cells secondary to any of the cardiac hormones.

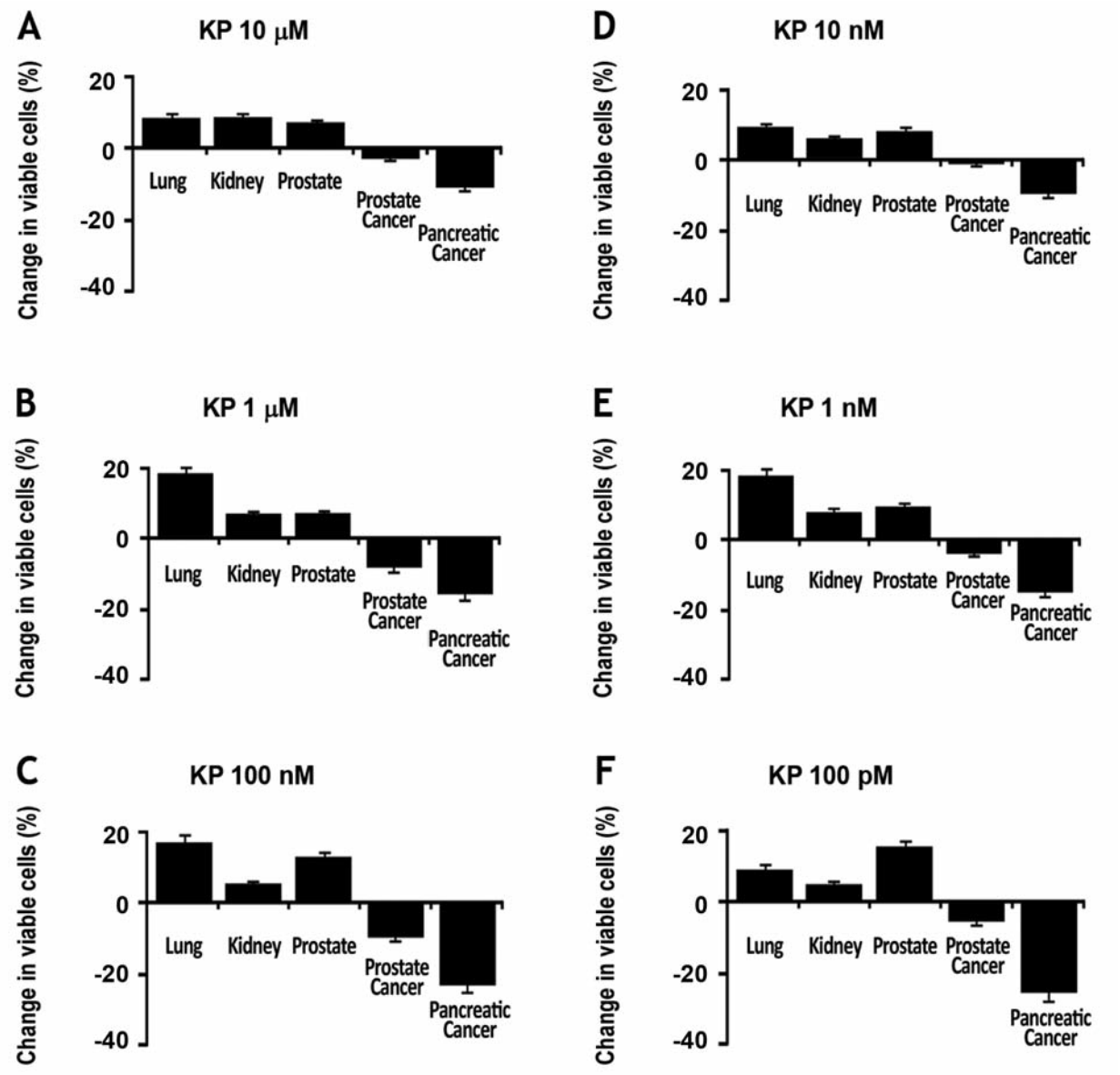

Figure 4. *Kaliuretic peptide causes death of human pancreatic and prostate adenocarcinoma cells but not of normal prostate, lung, and kidney cells when exposed to kaliuretic peptide for 2 hours at concentrations shown. p>0.05 for normal cells and p<0.01 for pancreatic cancer and p<0.02 for prostate cancer at all concentrations (except 10 nmol/l in the prostate cancer cells) for change in % of viable cells when evaluated by ANOVA.*

No cell death of human lung cells secondary to cardiac hormones. LANP did not cause cell death of normal human lung cells (Figure 1 A-F) when they were exposed to LANP for two hours. There was a 1% increase in cell numbers at 1 μmol/l (Figure 1B) to a maximal 13% increase (at 1 nmol/l; Figure 1E) of healthy human lung cells with LANP. Vessel dilator did not cause death of normal human lung cells (Figure 2 A-F). There was no cell death in the vessel dilator dose–response curves when cells were exposed to vessel dilator for 2 hours. Rather, the number of healthy cells increased by 9% (1 nmol/; Figure 2 D) to 20% (1 μmol/l; Figure 2 A) when incubated with vessel dilator for two hours. ANP, likewise, did not cause death of healthy human lung cells (Figure 3 A-F). Human lung cells increased by 2% (100 pmol/l; Figure 3 F) to 10% (10 nmol/l; Figure 3 D) when incubated with ANP for two hours. Kaliuretic peptide when evaluated in dose–response curves did cause death of normal human lung cells (Figure 4 A-F). There was either no change (1 nmol/l; Figure 4 E) or a 10% increase in human lung cells (1 μmol/l; Figure 4 A) with kaliuretic peptide for

two hours. Thus, there was no evidence of cell death of normal human lung cells at any concentration of the respective cardiac hormones.

Discussion

The four cardiac hormones synthesized by the same gene (6), which have anticancer effects *in vitro* (8, 9) and *in vivo* (7), caused cell death of human pancreatic and human prostate adenocarcinoma cells. It is important to note that the concentrations which caused cell death of the pancreatic and prostate cancer cells are the same concentrations which eliminate up to 80% of human pancreatic cancers in athymic mice (7), two-thirds of human breast cancers in athymic mice (13), and eliminate up to 86% of human small-cell lung carcinomas growing in mice (14). Their ability to cause death of a significant percentage of these respective cancer cells would appear to contribute to their ability to eliminate these cancers *in vivo* (7). It is important to note that up to 36% of the cancer cells died with only two hours of exposure to the respective cardiac hormones. With longer exposure, more death of the respective cancer cells may occur as these cardiac hormones are known to eliminate human pancreatic adenocarcinomas growing in athymic mice in up to 80% of the mice when treated for four weeks *via* subcutaneous pumps (7).

Each concentration of the respective cardiac hormones caused more cell death in the human pancreatic adenocarcinoma cells *versus* the human prostate adenocarcinoma cells (Figures 1-4). Thus, there was a difference between the two types of cancer with respect to the amount of cell death induced by the respective cardiac hormones with two hours of their exposure. The reason for this is unknown at present. Also unknown at present is whether exposure for longer periods of time (*i.e.* days) might allow for an equal number of prostate and pancreatic cancer cells to undergo cell death. Treatment with the cardiac hormones to eliminate cancers *in vivo* is for weeks (7) rather than for two hours as in the present *in vitro* study.

A very important finding of the present investigation is that the cardiac hormones did not cause death of normal cells at the same concentration that caused death of human pancreatic and prostate adenocarcinoma cells (Figures 1-4). These findings suggest that normal cells, *i.e.* lung, kidney and prostate in the present investigation, are spared cell death at concentrations that cause anticancer effects (7-9) and cell death of pancreatic and prostate cancer cells.

Although the whole mechanism of how the observed death of prostate and pancreatic cancer cells secondary to the cardiac hormones leads to elimination of these cancer cells, it is known that part of their mechanism of elimination of cancer cells is a strong inhibition of multiple targets in the Ras-MEK 1/2-ERK 1/2 kinase cascade within the cancer cells (15-20). Thus, the cardiac hormones of the present investigation inhibit up to 95% of the activity of Ras (15, 16), up to 98% of the activity (phosphorylation) of MEK 1/2 kinases (17, 18), and up to 96% of the phosphorylation of ERK 1/2 kinases in prostate and pancreatic cancer cells (19, 20). In correlation with the findings of the present investigation, the cardiac hormones do not inhibit ERK 1/2 kinases in healthy non-cancerous cells (21). Thus, the cell death of cancer cells but not healthy human cells at the cellular level appears to be related to the cardiac hormones' inhibition of the Ras-MEK 1/2-ERK 1/2 kinases in cancer cells but not in healthy cells.

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