Cardiac Glycosides Decrease Prostate Specific Antigen Expression by Down-Regulation of Prostate Derived Ets Factor

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Abbreviations and Acronyms

ATPase = adenosine triphosphatase

BCA = bicinchoninic acid

CD-FCS = dextran coated charcoal FCS

ETS = E 26

FCS = fetal calf serum

LDH = lactate dehydrogenase

MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

PCR = polymerase chain reaction

PDEF = prostate derived Ets factor

PSA = prostate specific antigen

RPMI-PRF = RPMI 1640 phenol red-free

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Purpose: While cardiac glycosides are the mainstay of congestive heart failure treatment, early studies showed that pharmacological doses of cardiac glycosides inhibited prostate cancer cell line proliferation. We evaluated the mechanisms of cardiac glycosides, including digoxin, digitoxin and ouabain (Sigma®), on prostate specific antigen gene expression in vitro.

Materials and Methods: We cultured LNCaP cells (ATCC®) and used them to determine the effect of cardiac glycosides on prostate derived Ets factor and prostate specific antigen expression. We determined prostate derived Ets factor and prostate specific antigen expression by reverse transcription-polymerase chain reaction, immunoblot, transient gene expression assay or enzyme-linked immunosorbent assay.

Results: Noncytotoxic doses (100 nM) of cardiac glycosides for 24 hours inhibited prostate specific antigen secretion by LNCaP cells. Reverse transcriptase-polymerase chain reaction and immunoblot revealed that cardiac glycosides significantly down-regulated prostate specific antigen and prostate derived Ets factor expression. Transient gene expression assays showed that prostate derived Ets factor over expression enhanced prostate specific antigen promoter activity. However, prostate specific antigen and prostate derived Ets factor gene promoter activity was attenuated when LNCaP cells were treated with 100 nM cardiac glycosides. When LNCaP cells were treated with 25 nM digitoxin or digoxin for 60 hours, prostate specific antigen secretion decreased by 30%.

Conclusions: Results suggest that cardiac glycoside inhibition of prostate specific antigen gene expression may be caused by the down-regulation of prostate derived Ets factor gene expression. When cells were chronically treated with digoxin or digitoxin at concentrations close to or at therapeutic plasma levels, prostate specific antigen secretion decreased. This phenomenon merits further study to determine whether it occurs in men on cardiac glycoside therapy.

Key Words: prostate; prostatic neoplasms; prostate-specific antigen; gene expression; SPDEF protein, human

PROSTATE specific antigen assay and digital rectal examination are the standards of prostate cancer screening. Several strong risk factors for prostate cancer include patient age, ethnic group, and family history of prostate cancer.[1] Using PSA, prostate volume and digital rectal examination physicians can determine the patient risk group, and estimate the underlying risk of prostate cancer and its aggressiveness.[2]

While digitalis-like steroids and related agents are the mainstay of congestive heart failure treatment,[3] early studies showed that pharmaco-

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logical doses of cardiac glycosides inhibited prostate cancer cell line proliferation and promoted apoptosis by the ability to induce sustained Ca^{+2} increases in cells.[4,5] Except for the growth effect of cardiac glycosides on the human prostate, previous in vitro study suggested that ouabain induces an increased tension response in human prostate tissue due to noradrenaline release via an effect on the Na^{+} dependent Ca^{+2} influx system.[6]

PDEF, also termed SPDEF/PSE, is a member of the Ets family of transcription factors that is expressed in abundance in prostate tissue.[7] In situ hybridization on prostate tissue frozen sections revealed diffuse strong expression restricted to luminal epithelial cells.[8] PDEF bound with high affinity to DNA containing sequences in 2 of the 11 putative ETS binding sites in the PSA promoter/enhancer region.[7] Multiplex gene expression analysis showed that the ability of cardiac glycosides to induce apoptosis in PC-3 human prostate carcinoma cells correlated with their inhibition of prostate target gene expression, including PDEF.[9] Recent studies indicated that phytoestrogen compounds block PSA gene expression via PDEF down-regulation in prostate carcinoma LNCaP cells.[10]

To our knowledge no group to date has examined in detail how in elderly patients long-term cardiac glycoside therapy may affect the results of clinical test, such as PSA, to detect prostate cancer. We provide the first evidence that cardiac glycosides at nontoxic doses down-regulate PDEF gene expression, which decreases PSA gene expression in vitro.

MATERIALS AND METHODS

Materials, and Cell Lines and Culture

We used the prostate cancer cell line LNCaP, and digoxin, digitoxin and ouabain. Stock solutions (10 mM) were made of all drugs by dissolving each in 100% ethanol. We used the BCA protein assay kit (Pierce, Rockford, Illinois). Steroids were removed from FCS (HyClone®) by treatment with dextran coated charcoal (Sigma) (1 gm/500 ml FCS), resulting in CD-FCS. Since the structure of cardiac glycosides is similar to that of steroid hormones and the androgen decreased β1-subunit of $Na^{+}K^{+}$-ATPase of LNCaP cells,[11] experiments in which cells were treated with cardiac glycosides in RPMI-PRF medium (Invitrogen™) were supplemented with CD-FCS to prevent the effect of exogenous steroid hormone in FCS.

Cell Proliferation Assays

We seeded 5,000 cells in each well of a 96-well plate with RPMI 1640 medium (Invitrogen) and 10% FCS, and incubated them for 48 hours. Medium was then changed to RPMI-PRF medium with 5% CD-FCS and various concentrations of digoxin, digitoxin or ouabain, as indicated, for an additional 48 hours. We measured cell proliferation rate in response to drugs using the CellTiter 96® AQueous One Solution cell proliferation MTS assay.

Reverse Transcriptase-PCR

Total RNA was isolated with TRIzol® reagent and cDNA was synthesized using the SuperScript® III preamplification system according to manufacturer instructions. The PDEF primers (5′-GACCAGTGAGGAGAGCTGGACCGA-3′ and 5′-TGACCTTGGGCTCTGGAAGGTCAG-3′) were used to amplify sequences specific to human PDEF mRNA. Sequences of primers for PSA and β-actin were previously described.[12] PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Western Blot

Cells were treated with digoxin, digitoxin or ouabain for 24 hours and then lysed with lysing buffer composed of 62.5 mM tris (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol and 7 M urea. Equal amounts of protein (20 μg) were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel and assayed by enhanced chemiluminescence, as described by the manufacturer (Amersham Biosciences™). Blotting membranes were probed with polyclonal PSA antiserum (Dako, Glostrup, Denmark) (1:200), diluted β-actin antiserum (C11, Santa Cruz Biotechnology, Santa Cruz, California) (1:1,000) or diluted PDEF antiserum (1:5,000). Rabbit anti-human PDEF serum was prepared at our laboratory, as described previously.[12] Immunoblot of the whole LNCaP cell extract revealed 1 major band at 37 kDa and another weak band at 50 kDa, which represented the glycosylated form of PDEF. We analyzed the intensity of different bands using GeneTools of ChemiGenius (Syngene, Cambridge, United Kingdom).

PSA Enzyme-Linked Immunosorbent Assay

LNCaP cells were incubated with 1 ml RPMI-PRF medium with 5% CD-FCS and digoxin, digitoxin or ouabain as indicated in a 6-well plate (2×10^5 cells per well) for 24 hours. After incubation the conditioned medium supernatant from each well and cell pellets were collected for PSA enzyme-linked immunosorbent assay, as previously described.[13] The PSA level in each sample was adjusted by the protein concentration in the whole cell extract, which was measured using the BCA protein assay kit.

LDH Assay

LNCaP cells were incubated with RPMI-PRF medium with 5% CD-FCS and 100 nM digoxin, digitoxin or ouabain for 24 hours. LDH activity was measured at 30C as the amount of pyruvate consumed by continuously monitoring the decrease in absorbance due to nicotinamide adenine dinucleotide oxidation at 339 nm. LDH enzymatic activity was adjusted by the protein concentration of the cytosolic extracts, which were determined by BCA protein assay, as previously described.[14]

Reporter Vector Constructs

The reporter vectors pPSAH −41 to −5874), pPSABHE (−4801 to −3933 and −41 to −589), pPSAKH (−41 to −1557) and pPSABH (−41 to −589) containing the 5′-flanking region of the human PSA gene were cloned by 5′-deletion or PCR, as previously described.[15] DNA fragments (−1 to −3280) of the promoter/enhancer of the