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European Journal of Pharmacology 534 (2006) 71–76
www.elsevier.com/locate/ejphar

Proliferation and apoptosis of HeLa cells induced by in vitro stimulation with digitalis ☆

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Received 15 September 2005; received in revised form 12 January 2006; accepted 20 January 2006
Available online 28 February 2006

Abstract

In the HeLa tumor cell line, we studied the characteristics of the dual effect of digitalis compounds on cell growth (proliferation and death). In addition, we explored whether both effects occur by means of the same mechanism. HeLa cell cultures were exposed to increasing concentrations (0.01 nM–10 μM) of ouabain, strophantidin, digoxin, and digoxigenin at 24–96 h intervals. Cell growth in treated cultures was compared with cell growth under nontreated conditions. Additionally, we studied changes in nuclear morphology, as well as in genomic DNA degradation, cytochrome *c* release, and caspase-9 and -3 presence and processing induced by toxic concentrations of digitalis.

Digitalis compounds increased HeLa cell number when exposed to concentrations <10 nM during a 48 h period. Ethacrynic acid (a nonsteroid inhibitor for Na^+/K^+-ATPase) did not induce cell growth at these concentrations. Digitalis concentrations >10 nM induced cell death in a concentration- and exposure period-dependent fashion. Changes in nuclear morphology, DNA fragmentation, mitochondrial cytochrome *c* release, and proteolytic processing of caspases-9 and -3, suggest apoptotic cell death. The IC_{50} for the inducing effect of apoptosis by ouabain at 96 h was 18 nM and corresponds with the IC_{50} for the $Na^{+/}K^+$-ATPase inhibition in HeLa cells.

In conclusion, the dual effect of digitalis compounds on HeLa cells growth is concentration and time-dependent. The apoptosis-inducing effect correlates with inhibition of Na^+/K^+-ATPase. Proliferation does not appear to be mediated through this pathway. The apoptosis-induction pathway is possibly cytochrome *c*-dependent.
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Keywords: Digitalis; Growth cell; Apoptosis; Cytochrome *c*

1. Introduction

Cardiac glycosides are steroid molecules that have different and varied effects; among these are: the positive inotropic effect on heart muscle, and the inductive effect on cell growth, either hypertrophic on heart muscle cells or proliferative on vascular smooth muscle cells (Abramowitz et al., 2003), lymphocytes, and prostate cells (Scheiner-Bobis and Schoner, 2001; Chueh et al., 2001); in addition, there is an antiproliferative effect on different human cell lines, aside from these properties, they posses widely known powerful neuro- and cardiotoxic actions (Ooi and Colucci, 2001).

Taken together, the inotropic and toxic effects of these drugs have been attributed to interaction with Na^+/K^+-ATPase, the enzyme responsible for Na^+, K^+ transport across the cell membrane (Repke and Portius, 1963; Repke et al., 1995).

The HeLa cell line has been used for many years in tumor-cell biologic behavior studies as well as in the evaluation of the

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doi:10.1016/j.ejphar.2006.01.035

mechanism of action of different cytostatic drugs (Velazquez et al., 1998; Lee et al., 2002). This cell line is highly sensitive to the cytotoxic effect of digitalis (Gupta et al., 1989). The mechanisms through which digitalis inhibits cell proliferation and causes cell death in this cell line are unknown, and at present it has not been demonstrated whether digitalis is capable of stimulating HeLa cell growth. The aim of our study was to characterize the double effect of digitalis compounds on cell growth (proliferation and death) and to explore the pathways for inducing both effects on this cell line.

2. Materials and methods

2.1. Cell culture

HeLa cell line was maintained as a monolayer in Dulbecco's minimal essential medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and incubated at 37 °C in a humidified chamber in an atmosphere containing 5% CO_2.

2.2. Digitalis effects on cell growth

Cells were seeded into 96-well dishes at a density of 3.0×10^3 in 100 μl of medium. Cells were treated with different concentrations (0.1 nM–10 μM) of digitalis (ouabain, strophantidin, digoxin, and digoxigenin) for 24, 48, 72, or 96 h. The drugs were dissolved in ethanol (not exceeding 0.1%, v/v). In all assays, we added to the control cell culture the same concentration of ethanol added to treated cultures, which caused no effect on cell growth. Ethacrynic acid was used as a nonsteroidal inhibitor of Na^+/K^+-ATPase. Digitalis effects were assayed measuring the number of adherent cells on microplates (Kueng et al., 1989); in this method, optical density (OD) is linearly related with cell number. Absorbance registered by cells treated with digitalis compounds was compared with that obtained for nontreated cells, which is considered 100% of cell growth (control). IC_{50} values were calculated from concentration–response curves. Data are given as mean ± standard error of the mean (S.E.M). IC_{50} = concentration of glycoside resulting in 50% inhibition of cell growth. Each result described is based on at least three different determinations.

2.3. Histologic examination

To evaluate changes in nuclear morphology, HeLa cells under the effects of toxic ouabain and digoxin concentrations were stained with ethidium bromide as described previously (Ruppova et al., 1999). Cells were visualized in a Zeiss microscope, using epifluorescence for ethidium bromide stain and photographed on Kodak Plus X-Pan film.

2.4. Low molecular weight DNA analysis

Cells (approximately 1×10^6), treated with ouabain 100 nM and nontreated (control) cells, were used for DNA extraction and conventional analysis by agarose gel electrophoresis (Herrmann et al., 1994).

2.5. Cytochrome c release and caspase-9 and -3 detection

HeLa cells (1×10^6) were exposed to ouabain (70 nM) or digoxin (230 nM) for 72 h (these concentrations were calculated from dose–response curves obtained for each digitalis compound); subsequently, cultures were washed twice with cold PBS. In control experiments, cells were incubated with medium with the same ethanol concentration as treated cultures. Total cell extracts were prepared as described by Bandala et al. (2001). Cellular fractions were obtained by selective digitonin permeabilization as reported previously (Takasawa and Tanuma, 2003) with minor modifications. Cells were incubated in on ice-cold extraction buffer (0.2 mg/ml digitonin in PBS) for 5 min. Cells were centrifuged for 5 min at 1000×*g* at 4 °C; the pellet containing mitochondria was lysed in on ice-cold buffer containing [50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40, 0.3 mM Phenylmethanesulfonyl fluoride (PMSF), 10 μg/ml aprotinin] for 5 min. Finally, mitochondria were centrifuged at 10,000×*g* for 10 min at 4 °C, the supernatant was concentrated by precipitation with 5% trichloroacetic acid and washed twice with acetone, and proteins were resuspended in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. Equal amounts of protein were resolved by 10% SDS-PAGE. Polypeptides were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham, UK) and, after blocking, cytochrome *c* was detected using a monoclonal antibody raised against cytochrome *c* (H-104): sc-7159 (Santa Cruz Biotechnology, CA, USA), alpha-tubulin (Oncogene, CA, USA), and caspases-9 and -3 (Santa Cruz Biotechnology, CA, USA). Blots were developed using a horseradish peroxidase-conjugated secondary antibody (Amersham) and visualized using enhanced chemiluminiscence (Amersham), with X-Omat AR films (Kodak, Mexico).

2.6. Substances

Proteinase K (fungal), DMEM and FBS were obtained from GIBCO, while ouabain, strophantidin, digoxin, digoxigenin, ethacrynic acid, RNAase A, and other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.7. Statistical analysis

Statistical analysis of data was performed with the aid of GraphPad Prism v3.0 software. IC_{50} values were calculated by nonlinear regression from dose–response curves. Statistical significance between treatments was evaluated by one-way Analysis of variance (ANOVA), followed by Bonferroni's test. We report mean values ± S.E.M.; differences were identified as significant if the P value was <0.05.

3. Results

3.1. Effect of low concentrations of digitalis compounds on HeLa cells growth

Twenty-four hour exposure to digitalis compounds induced no changes in cellular growth (not shown) as compared with

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