Journal of Food and Drug Analysis, Vol. 16, No. 1, 2008, Pages 75-79 藥物食品分析 第十六卷 第一期

Determination of Digitoxin-induced Elevated Hydrogen Peroxide Levels in SK-Hep-1 Cells Using a Chemiluminescent Method

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(Received: January 10, 2007; Accepted: June 3, 2007)

ABSTRACT

Drug-induced intracellular hydrogen peroxide overproduction is proposed as an upstream event in apoptosis signaling. It is therefore important to develop a sensitive method to quantify hydrogen peroxide in biological samples undergoing cell death. We report herein a luminol-based chemiluminescent assay with a suitable detection range of best linearity from 1 to 0.1 μM hydrogen peroxide with a CV range of 5.7% to 21.7% for intraday measurement at the detection range. We hypothesized that digitoxin-treated human hepatoma SK-Hep-1 cells produce hydrogen peroxide to cause cytotoxicity, which was verified by measuring the spent media by our chemiluminescent method in a dose-dependent manner. Taken together, our sensitive luminol-based chemiluminescent assay has potential use in the quantification of trace amounts of digitoxin overdose-induced hydrogen peroxide in biological samples.

Key words: digitoxin, SK-Hep-1 cells, hydrogen peroxide, chemiluminescence

INTRODUCTION

The cardiac glycosides digitoxin (see Figure 1 for structure) and digoxin have been widely used in heart failure treatment for many years.

Recently, the possible use of digitalis as an anti-cancer drug was suggested in medical oncology[(1)]. This is because the IC_{50} values for digitoxin (3-33 nM) in three human cancer cell lines were found to be within or below the concentration range seen in the plasma of patients with cardiac disease receiving this glycoside (20-33 nM). For example, IC_{50} of renal adenocarcinoma cancer cell line (TK-10) was hypersensitive to digitoxin toxicity mediated by apoptosis[(2)].

We had previously observed 2',7'-dichlorofluorescein diacetate staining of digoxin-treated SK-Hep-1 cells, which enabled us to visualize, by significant correlation, the oxidative stress by the emitted fluorescent light (data not shown). It is also known that certain toxins can cause cells to increase their production of H_2O_2; for example, when preosteoclastic cells are exposed to arsenite at high concentrations (10-25 μM), a significant loss of cell viability and a high increase in H_2O_2 production (1.5 μM) is observed[(3)]. Our theory is that digitoxin cytotoxicity in SK-Hep-1 cells is also involved in the overproduction of H_2O_2.

Although H_2O_2 is not a free radical per se, the reaction of H_2O_2 with metal ions, or with other free radicals in the presence of metal ions, can form highly reactive hydroxyl radicals that cause oxidative damage in cells[(4)]. Both the Fenton reaction and the Haber-Weiss reaction below are two well known mechanisms for the conversion of H_2O_2 into OH•.

Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH\bullet$$

$$H_2O_2 + Cu^{+} \rightarrow Cu^{2+} + OH^- + OH\bullet$$

Haber-Weiss reaction:

$$O_2\bullet^- + H_2O_2 \xrightarrow{Fe^{3+} \text{ or } Cu^{2+}} O_2 + OH^- + OH\bullet$$

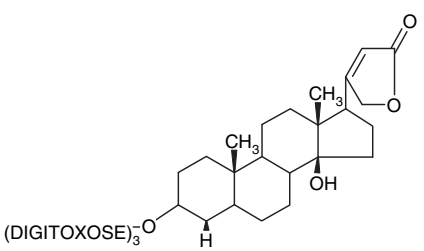

Figure 1. The chemical structure of digitoxin.

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In the field of clinical chemistry, colorimetric detection of H_2O_2 generated through enzymatic reactions is widely used[5]. However, the colorimetric H_2O_2 assay has sensitivity limitations. In order to detect very minute production of H_2O_2 in biological samples, an assay using chemiluminescent readout has been considered in this study. Chemiluminescence (CL) is light emission that is produced in certain oxidation reactions; the light emission arises from the decay of chemically excited intermediates or product molecules to the electronic ground state. The majority of chemiluminescent reactions are oxidation reactions because the production of visible light requires highly energetic reactions. Advantages of CL include sensitivity, speed (signal generated in a few seconds and in some cases stable for several hours), nonhazardous reagents, and simple procedures. Using immunoassay mode of detection for horseradish peroxidase (HRP), the sensitivity of CL readout was 80 times more than that of colorimetry[6]. We therefore used HRP-catalyzed CL generated from luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) to quantify trace amount of H_2O_2 secreted from cells into the media upon the induction of digitoxin.

MATERIALS AND METHODS

I. *Reagents*

Digitoxin, sodium pyruvate, 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sodium bicarbonate, 4-aminoantipyrin, hydrogen peroxide (H_2O_2), *ρ*-hydroxy- benzenesulfonate, luminol (5-amino-2,3-dihydro-1,4-phthalazine), and HRP Type VI-A were from Sigma. DMEM (Dulbecco,s modified Eagle,s medium), penicillin streptomycin, trypsin, and trypan blue were from Gibco. FBS (fetal bovine serum) was from Biological Industries.

II. *Cell Culture*

Poorly differentiated human hepatoma SK-Hep-1 (ATCC, HTB-52) representing malignant adenocarcinoma cells with HBV (+) were obtained from ascites. SK-Hep-1 cells were grown in DMEM medium containing 2.0 mM L-glutamine, 0.1 mM non-essential amino acid solution, 10% FBS, 10 kU/mL penicillin / streptomycin, 3.7 g/L sodium bicarbonate, and 1.0 mM sodium pyruvate at 37°C in an incubator supplied with 5% CO_2. Cells were subcultured every 2 to 3 days by dilution factors of 6 or 7.

III. *Digitoxin Treatment of SK-Hep-1 Cells*

SK-Hep-1 cells (5×10^4 cells/mL) were grown in 24-well plates with 1 mL of medium per well. Thereafter, cells were exposed to serum-free medium for one day, followed by the addition of 5 μL of digitoxin in DMSO and 995 μL of medium containing 10% FBS, to yield a gradient of digitoxin concentrations from 10^{-5} to 10^{-10}M. After two days of drug treatment, cells were washed with PBS before determined for cell viability by MTT assay. The concentration of DMSO in the medium was 0.5%.

IV. *Cell Viability Assay*

Cell growth was assessed by MTT assay. Briefly, cells were seeded to a 24-well plate. After drug treatment, 0.5 mL of MTT solution (5 mg/mL in DMEM) was added to each well and incubated for another 3 hr at 37°C in the dark. The plates were shaken until the crystals dissolved. The resulting DMSO solution containing reduced MTT (0.15 mL per well) was transferred to a 96-well plate and the absorbance at 550 nm was measured in a dual beam microtiter plate reader with a 650 nm reference.

V. *Quantification of H_2O_2-by Chemiluminescent Assay*

In 96-well microtiter plates, 6 μL of sample or H_2O_2 standards added in wells were mixed with 289 μL of reagent containing 100 mM Tris-HCl buffer, (pH 7.5), 10 mM luminol. For the chemiluminescent reaction, 5 μL of 25 kU/L horse radish peroxidase was added to catalyze the reaction in the dark. Light intensity was measured using a luminometer, model VICTOR$^3{}_{TM}$ 1420 MULTI-LABEL COUNTER.

VI. *Preparation of H_2O_2 Standard Curve*

Standard H_2O_2 solutions of concentration, ranging from 10^{-9} M to 10^{-5}M, were added to spent media (2-day-old culture supernatant). The light intensity generated in the luminol-H_2O_2-HRP system was measured as described above.

VII. *Quantification of Hydrogen Peroxide Produced by the Digitoxin Treated Cells*

Two-day-old spent media of digitoxin-treated cells was centrifuged to pellet cell debris and collect supernatant for testing. The concentrations of H_2O_2 produced by the drug-treated cells were measured by the chemiluminescent intensity compared with the H_2O_2 standards, run in parallel.

VIII. *Treatment of Cells by Hydrogen Peroxide*

SK-Hep-1 cells (5×10^4 cells) were grown in 1 mL media in 24-well plates for 24 hr, then changed to serum-free medium to grow for one day. Five microliter of 2×10^{-3}~2×10^{-6} M H_2O_2 and 995 μL of medium containing 10% FBS were added so that the final concentration of H_2O_2 would range from 10^{-8} M to 10^{-5}M. After H_2O_2 exposure for 2 days, cells were washed with PBS once before MTT cell survival analysis.

Journal of Food and Drug Analysis, Vol. 16, No. 1, 2008

RESULTS AND DISCUSSION

Cardiotonic steroids such as digitoxin (Figure 1) are known to be associated with potent cytotoxicity to human tumor cells, the mechanisms by which this effect is produced are not clear. The effect of digitoxin on SK-Hep-1 cells is shown in Figure 2. The IC_{50} value was 100 nM, which is higher than those of digitoxin in renal adenocarcinoma cancer cell lines, as mentioned earlier[2]. This is not surprising since hepatoma cells are known to be highly resistant to therapeutic agents. However, the addition of 10 nM digitoxin, a nontoxic concentration, caused a decrease in cell viability by more than 20%.

The effect of 0.8 μM to 10 μM of exogenous H_2O_2 on the viability of SK-Hep-1 cells is shown in Figure 3. The IC_{50} of H_2O_2 in SK-Hep-1 cells was approximately 1.5 μM. Less than 20% of cells survived in the presence of 2 μM H_2O_2, and less than 10% of cells survived at 4 μM H_2O_2.

We did preliminary tests by colorimetric assay of H_2O_2 in digitoxin-treated cell medium, but failed to detect any increase in hydrogen peroxide after two days of incubation (data not shown). We therefore developed the chemiluminescent assay for this purpose. Since the most extensively studied chemiluminescent reaction is the oxidation of luminol, this compound was used to react with H_2O_2 in the presence of HRP to form either a hydroperoxide or a cyclic endoperoxide intermediate[7]. This intermediate decomposes to produce 3-aminophthalate molecules in an electronically excited state, which decay to the ground state, releasing energy as light in a broad emission (375-550 nm) centered at 425 nm (See Figure 4). A typical standard curve of H_2O_2 in the two day SK-Hep-1 spent media is shown in Figure 5, expressed in terms of log[light emission] vs log[H_2O_2] using in-house developed chemiluminescent reagents. It was noticed that in Figure 5, a nonlinear relationship exists between log [light emission] and exogenous H_2O_2 added in the spent medium, with a best-fit equation of $y = 0.38\ x^2 + 6.26\ x + 28.31$. As shown in Table 1, intraday CV values ranging from 5.7% to 21.7% were obtained by this chemiluminescent analysis of hydrogen peroxide at a detection range of 10^{-6} to 10^{-7} M, where optimal linearity exists. In Table 1, a detailed nonlinear standard curve as mentioned above was demonstrated; for example, the intensity of CL for

Table 1. Intra-run CV values of chemiluminescent assay

H_2O_2 (M)	Mean	SD	CV
10^{-5}	4979976	32272	0.7%
10^{-6}	14087	873	5.7%
10^{-7}	1738	377	21.7%
10^{-8}	750	202	17.3%
10^{-9}	529	115	14.4%

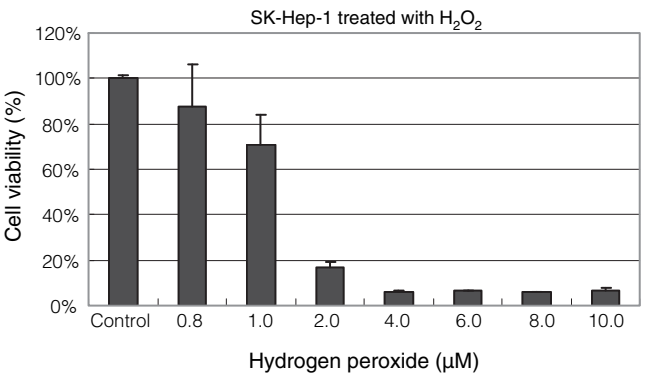

Figure 3. The effect of exogenous hydrogen peroxide on the viability of SK-Hep-1 cells. Each (bar) value represents mean +/- standard deviation (n = 3).

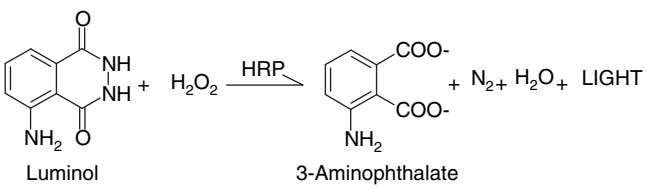

Figure 4. Chemical reaction of the chemiluminescent assay of hydrogen peroxide

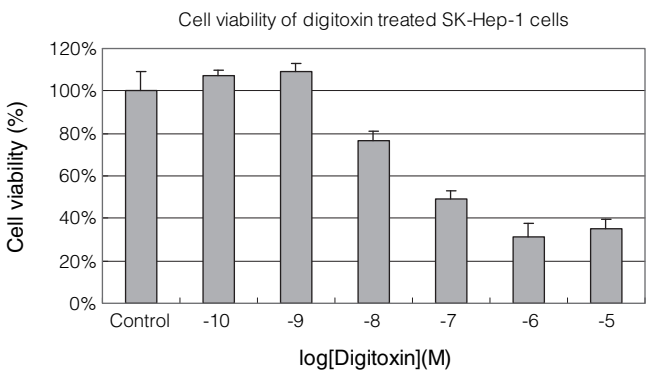

Figure 2. Cell viability of digitoxin-treated SK-Hep-1 cells. Each (bar) value represents mean +/- standard deviation (n = 3).

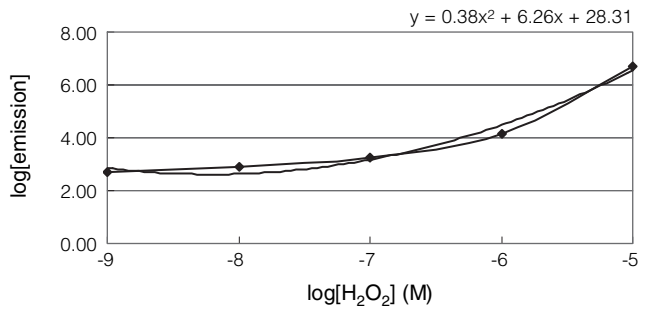

Figure 5. Standard curve of hydrogen peroxide in the two day SK-Hep-1 spent media. Each point value represents triplicate measurement.

100 μM of H_2O_2 is 50 fold higher than that of 10 μM rather than the theoretical 10-fold. Since both inhibitors and enhancers were found in luminol- H_2O_2-HRP CL system[8,9], it is suggested that spent medium may contain limited amount of CL inhibitors.

It had been reported that small amount of H_2O_2 in cell culture may cause apoptosis while high concentration of H_2O_2 may cause necrosis. For example, when human T-lymphoma Jurkat cells are treated with 50 μM H_2O_2, activation of caspase-9 and caspase-3 follows, culminating in apoptotic cell death. Treatment of Jurkat cells with 500 μM H_2O_2 does not lead to caspase activation, and therefore changes the mode of death to necrosis[10].

Digitoxin-induced H_2O_2 production in SK-Hep-1 cells is shown in Figure 6. A linear regression line with $R^2 = 0.9759$ was obtained between the log [digitoxin] and H_2O_2 production ranging from 80 to 280 nM. In a cell-free system, the intensity of the CL signal was found to have no significant difference whether digitoxin (10^{-8} M) was incubated with fresh medium or with medium placed at 37°C in a CO_2 incubator for two days. However, digitoxin concentrations of 10^{-7} M to 10^{-5} M resulted in a CL signal decrease of 10% to 20% (see Figure 7). From these observations, we believe that digitoxin is a weak CL inhibitor and that drug dose-dependent H_2O_2 increase in the presence of cancer cells (Figure 6) must be due to metabolic production upon drug action.

According to the data shown in Figure 6, 10^{-7} M digitoxin will induce H_2O_2 less than 200 nM and this amount of H_2O_2 will cause less than 10% of cell death based on Figure 3. However, the same dosage of digitoxin will induce over 50% cell death as shown in the Figure 2. The difference between the effect of endogenous production and exogenous addition of H_2O_2 may be accountable for these observations. Otherwise, these results suggested that digitoxin-induced cell death may be not only through the H_2O_2 production. The metabolism of H_2O_2 in human liver cells must be quite complicated since there are many enzymes involved in H_2O_2 formation and degradation. The exact mechanism of digitoxin-induced H_2O_2 production in liver cancer cells remains unclear and warrants further investigation. Although this CL method is not suitable to determine the concentration of digitoxin directly, however, it can be applied to monitor digitoxin-induced metabolic product H_2O_2 under toxic overdose situation, i.e., the serum concentration is higher than 59 nM[11].

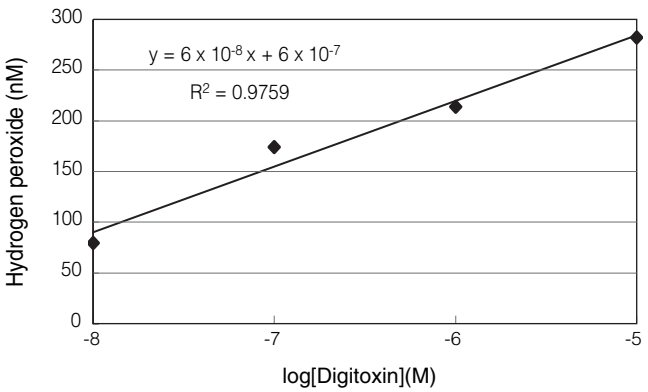

Figure 6. Digitoxin-induced hydrogen peroxide production in SK-Hep-1 cells. Each point value represents triplicate measurement.

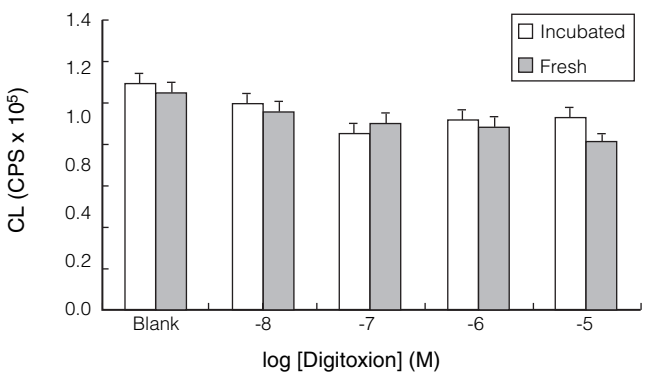

Figure 7. Incubation of digitoxin with medium at cell free system. Digitoxin at various concentrations were incubated with fresh medium (grey bar) or with medium without cells then incubated at 37°C in the CO_2 incubator for 2 days.

ACKNOWLEDGEMENTS

We thank Mr. Ra-Gung Liu for his help on drawing the chemical structures and Mr. Melvin Wei for the help of manuscript preparation. This investigation was supported in part by grant NSC91-2314-B-182- 073 from the National Science Counsel in Taiwan (ROC).

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