Exp Oncol 2006
28, 1, 86–88

Experimental ONCOLOGY

PROTECTIVE EFFECT OF ANTIOXIDANT ENZYMES AGAINST DRUG CYTOTOXICITY IN MCF-7 CELLS

A. Ozkan, K. Fiskin*
Akdeniz University, Art-Science Faculty, Biology Department, Antalya, Turkey

Aim: **To evaluate protective effect of antioxidant enzymes against epirubicin-HCI (EPI) cytotoxicity *in vitro*. *Materials and Methods:* Viability of MCF-7 cells treated with EPI was measured using the MTT test. Glutathione (GSH), protein content and enzymatic activity were measured spectrophotometrically. NADPH — dependent cytochrome P-450 reductase (NADPH-CYP-450) and glutathione S-transferase pi (GST-pi) expression in MCF-7 cells were determined by Western blot analysis. *Results*: The IC50 values of EPI in MCF-7 cells were 1.0, 0.7 and 0.5 ng/ml respectively for 24, 48 and 72 h applications. Simultaneously enzymatic activity of glutathione S-transferase, glutathione peroxidase, GSH and expression of GST-pi, NADPH-CYP-450 reductase were increased in EPI (1 ng/ml) — treated cells at the end of the 24 h incubation. Addition of superoxide dismutase, catalase and GSH decreased cytotoxicity of EPI. *Conclusion:* We hypothesized that the production of reactive oxygen species and hydrogen peroxide as result of EPI treatment can cause cytotoxicity in MCF-7 cells and antioxidant enzymes protect the cells against this process.**
Key Words: **MCF-7, epirubicin-HCI, antioxidants.**

Anthracycline antibiotics are widely used in chemotherapy of oncological patients. The anthracycline analogue epirubicin-HCI (EPI) is potent cytotoxic compound, but causes lower cardiac injury than doxorubicin derivatives [1]. The proposed mechanism for cytotoxic effect of EPI involves the formation of intracellular free radicals caused by the quinone group of anthracyclines. Quinones can undergo either one-electron reduction to yield the semiquinone free radical or two-electron reduction directly to the hydroquinone [2]. Most semiquinones are readily re-oxidised in aerobic conditions and can enter redox cycles with molecular oxygen, forming various reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen. They were shown to induce programmed cell death upon exogenous addition. Apoptosis is induced by depletion of cellular antioxidants and can be reversed upon action of exogenous antioxidants such as catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) etc [3]. The enzymatic activities of the glutathione reductase (GSSG-Rx), glutathione peroxidase (GSH-Px), CAT protect the cells from the effects of ROS generated during the one-electron reduction of quinones.

Received: September 21, 2005.
*Correspondence: Fax: 90 242 2278911
E-mail: aysunozkan95@yahoo.com
aozkan@akdeniz.edu.tr

Abbreviations used: CAT – catalase; CYP – cytochrome; DMEM – Dulbecco's modified Eagle's medium; ECL – electrochemiluminescence; FCS – fetal calf serum; GSH – glutathione reduced form; GSSG – glutathione oxidized form; GSSG-Rx – glutathione reductase; GSH-Px – glutathione peroxidase; GST – glutathione S-transferase; GST-pi – glutathione S-transferase pi; IC50 – the concentration of a drug that inhibits cell growth by 50%; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH-CYP450 reductase – NADPH-dependent cytochrome P450 reductase; OD – optic density; PPMSF – phenylmethylsulfonylfluoride; ROS – reactive oxygen species; SOD – superoxide dismutase; TBE – Tris-boric acid EDTA; TE – Tris-EDTA.

On the other hand, many tumors and tumor cell lines have high levels of glutathione S-transferase pi (GST-pi), which, at least in certain cases, appears to correlate with survival and/or acquired resistance to cytotoxic drugs. One of the important enzymes in detoxification mechanism is NADPH-cytochrome P-450 reductase, which is essential for the reconstitution of xenobiotic metabolic activity in the system [4, 5].

In a number of works the protective effect of some antioxidant enzymes against oxidative stress *in vitro* was shown [6–9]. Antioxidants and detoxification mechanisms that include glutathione, glutathione peroxidase and GST, can contribute to the drug resistance of various tumors.

The present work was aimed on evaluation of protective effect of antioxidant enzymes against EPI cytotoxicity *in vitro*.

MCF-7 cell line. MCF-7 line was purchased from American Type Culture Collection (Rockville, MD). Cells were routinely cultured in Dulbecco's Modified Eagle's Medium supplemented with 10 % fetal calf serum, 1% antibiotic-antimycotic solution in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT assay. After trypsinization of subconfluent cultures, the viable, trypan blue-excluding cells were counted in a haemocytometer (average cell number in squares x 10^4 x dilution factor = cell number/ml). MCF-7 cells (500 cells/well, monolayer) were plated in a 96-well plate. The next day the cells were treated with EPI (Farmitalia Carlo Erba, Italy) at the concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 ng/ml for 24, 48 and 72 h. The cytotoxicity of EPI on MCF-7 cells was determined by the MTT assay [10]. The absorbance of the reaction solution at 570 nm was recorded. The absorbance at 630 nm was used as a reference. The viability (%) was calculated by the formula $(A_{570\,nm}\text{-}A_{630nm})_{sample}/(A_{570\,nm}\text{-}A_{630nm})_{control}$ x 100, and IC50 value of EPI was calculated.

Enzymatic assays. In a separate experiments preincubation of MCF-7 cells (plated at a density

5–10 x 10^5 cell/100 mm dishes) with GSH (10 mM), SOD (100 μg/ml), CAT (50 units/ml) and SOD + CAT for 30 min before addition of EPI was performed. After 24 h incubation, cells were scraped from the plates, centrifuged (400 g for 10 min), washed with PBS and sonicated (3 x 15 s) in 50 mM potassium phosphate pH 7.2, containing 1 mM PMSF (Sigma) and 1 μg/ml leupeptin (Sigma) and centrifuged at 150,000 g for 1 h. The supernatant was used for the enzymatic assays. All experiments were repeated eight times.

Glutathione transferase (GST) was determined according to Habig et al. [11] using 1-chloro-2,4-dinitrophenol as a substrate. One unit of enzyme activity results in the binding of one μmole GSH/min.

GSH-Px was determined according to Flohe and Gunzler (1984) with tert-butyl hydroperoxidase as substrate [12]. One unit of enzyme activity results in the oxidation of 1 μmol GSH/min.

Total GSH content was determined by spectrophotometry following the reduction of 5.5-dithiobis (2-nitrobenzoic) acid by NADPH in the presence of GSSG-Rx [13]. GSH content is expressed as nmol/mg protein.

Protein was determined by the method [14] with bovine serum albumin as a standard.

Western blotting. The cells treated as described above were detached with trypsin, washed with PBS, lysed with lysis buffer RIPA (Tris-HCl, NaCl, NP40, Na-deoxycholate, EDTA + protease inhibitor, Sigma P8340), centrifuged 150,000 g for 1 h, and the amount of protein was determined [14]. Proteins were divided and visualized by standard Western blot analysis [4] using primary antibodies for GST-pi and NADPH-CYP-450 reductase (Pharmingen, 1 : 1000) and goat polyclonal antibodies (sc-8091 1 : 100, Santa Cruz Biotechnology); goat anti mouse IgG-HRP (sc-2001, 1:100) and donkey antigoat IgG (sc-2033, 1 : 1000) as secondary antibodies; and ECL system (ECL™, Amersham).

Statistical analysis. Data were analysed statistically by Minitab Release 13.0 program [15]. Comparisons between groups were made by the ANOVA GLM procedure (General Linear Model).

As we have shown (Fig. 1), the IC50 values of EPI in MCF-7 cells were 1.0, 0.7 and 0.5 ng/ml. for 24 h, 48 and 72 h incubation, respectively, and these values were different statistically ($P < 0.05$). Preincubation of MCF-7 cells with SOD, CAT and SOD + CAT resulted in the decreased cytotoxicity of EPI (IC50) ($P < 0.05$).

It was shown also that, 24 h incubation of MCF-7 cells with 1.0 ng/ml EPI upon preincubation with antioxidants (see Materials and Methods) resulted in statistically significant changes in the levels of GSH-PX and GST activities, content of GSH, GST-pi and NADPH-CYP-450 reductase expression in MCF-7 cells ($P < 0.01$). EPI treated MCF-7 cells have 4-fold higher GSH-Px activity, 3-fold higher GST and 3-fold higher GSH content than these in control cells. There was no significant difference in the levels of GSH-Px activity between EPI + SOD + CAT, EPI + SOD, EPI + CAT treated and control cells, whilstl GST activities in all groups were different statistically from that in the control. Preincubation of MCF-7 cells with SOD + CAT prevented generation of hydrogen peroxide and reactive oxygen radicals in the cells. Data on the effect of EPI on enzymes activities and GSH amount in MCF-7 cells are presented in Table 2.

It was shown by Western blot analysis that in EPI treated MCF-7 cells significantly higher expression of NADPH-CYP-450 reductase and GST-pi was registered compared to that in untreated cells (Fig. 2 and 3).

Table 1. Cytotoxicity of EPI (1.0 ng/ml) in MCF-7 cells preincubated with SOD, CAT and SOD + CAT

Treatment	Cytotoxicity (%)		
	24 h	48 h	72 h
EPI (IC50)	51.10 ± 2.3	65.00 ± 1.4	88.90 ± 1.6
SOD + Cat + EPI	5.0 ± 1.1*	7.1 ± 0.7*	6.40 ± 0.5*
SOD + EPI	21.01 ± 1.2*	29.11 ±1.3*	33.20 ±2.1*
CAT + EPI	30.01 ± 1.3*	33.99 ± 2.1*	37.87±2.3*
GSH + EPI	35.00 ± 1.4*	46.31 ±1.6*	51.31 ±1.8 *

*The data statistically different ($P < 0.001$) from the control (EPI(IC50). Values are mean ± SE for 8 experiments

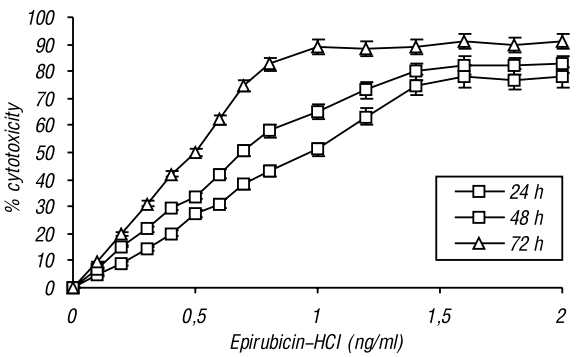

Fig. 1. Dose- and time-dependent cytotoxicity of EPI in MCF-7 cells

Table 2. Effect of 1.0 ng/ml EPI on enzymes activity and glutathione content in MCF-7 cells

Treatment	GSH-Px (mU/mg protein)	GST (mU/mg protein)	GSH (nmol/mg protein)
Control	10 ± 1.5	63 ± 1.5	40 ± 2.1
EPI + SOD + CAT	41 ± 2.4 *	190 ± 2.1*	124 ±2.3*
EPI + SOD	15 ± 1.1*	160 ± 1.9*	60 ± 3.0 *
EPI + CAT	21 ± 1.2*	165 ± 2.1 *	63 ± 3.0 *
EPI + GSH	19 ± 1.1*	162 ± 2.5 *	62 ± 3.0 *
	35 ± 2.1*	185 ± 1.9 *	55 ± 3.0 *

* The data are statistically different from control ($P < 0.001$).
Values are mean ± SE for 8 experiments.

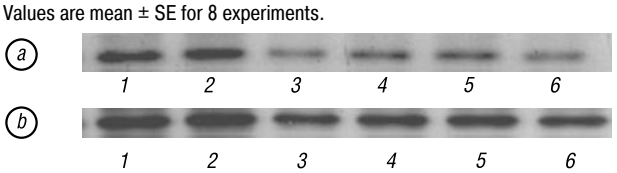

Fig. 2. Western blot analysis of NADPH-CYP-450 *(a)* and GST-pi *(b)* expression in MCF-7 cells treated with: EPI + GSH (lane *1*); EPI (IC50) (positive control) (lane *2*); EPI + CAT (lane *3*); EPI + SOD (lane *4*); EPI +SOD + CAT (lane *5*); untreated MCF-7 cells (negative control) (lane *6*)

Taken together, our results demonstrated that GSH, GSH-Px, GST, GST-pi, CAT, NADPH-CYP-450 and SOD may protect MCF-7 cells from EPI-dependent cytotoxicity.

We can conclude that EPI may generate ROS as other anthracyclines. An increase in the activity of GST and GSH-Px, amount of GSH and expression of GST-pi and NADPH-CYP4-50 reductase in MCF-7 cells treated with EPI seems to be a major effect of detoxification. Moreover, GSH, SOD and CAT are included in the de-

fence mechanism of MCF-7 cells against the hydrogen peroxide and free radicals produced by EPI.

ACKNOWLEDGMENTS

This work was supported by Akdeniz University Scientific Research Projects Unit. We thank Prof. Lester Packer (UNESCO, University of California), Prof. Enrique Cadenas (University of Southern California) and Prof. Ayala Hochman (Tel Aviv University).

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ЗАЩИТНЫЙ ЭФФЕКТ АНТИОКСИДАНТНЫХ ФЕРМЕНТОВ ПРИ ДЕЙСТВИИ ЭПИРУБИЦИНА НА КЛЕТКИ ЛИНИИ MCF-7

Цель: **оценить защитный эффект антиоксидантных ферментов против цитотоксичности эпирубицина-HCI (EPI) *in vitro*. *Материалы и методы:* жизнеспособность клеток линии MCF-7, обработанных EPI, оценивали при помощи MTT-метода. Содержание глутатиона (GSH), концентрацию белка и ферментативную активность измеряли спектрофотометрически. Экспрессию NADPH-зависимой цитохром P-450 редуктазы (NADPH-CYP-450) и глутатион-S-трансферазы-pi в клетках линии MCF-7 определяли при помощи Вестерн блот-анализа. *Результаты:* значения IC50 для клеток MCF-7, обработанных EPI, составили 1,0, 0,7 и 0,5 нг/мл при 24, 48 и 72 ч инкубации соответственно. Через 24 ч инкубации клеток с 1 нг/мл EPI отмечали повышение ферментативной активности глутатион-S-трансферазы и глутатион-пероксидазы, а также уровней экспрессии GST-pi и NADPH-CYP-450 редуктазы. Прединкубация клеток с супероксиддисмутазой, каталазой и GSH приводила к снижению цитотоксичности EPI. *Выводы:* образование свободных радикалов кислорода и перекиси водорода при действии EPI на клетки линии MCF-7 приводит к цитотоксическому эффекту, в снижении которого участвуют антиоксидантные ферменты.**

***Ключевые слова*: клетки линии MCF-7, эпирубицин, антиоксидант.**