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Nfe2 expression in the hyperthyroid rat heart receiving doxorubicin

Ekspresja *Nfe2* w sercach szczurów z hipertyreozą otrzymujących doksorubicynę

INTRODUCTION

Doxorubicin (DOX) belongs to chemical group of anthracyclines and has been widely used in antitumor therapy for over 40 years. However, its use is limited by the risk of dilated cardiotoxicity which may become evident months or even years after completed chemotherapy. Most evidence has suggested that the initial cause of DOX cardiotoxicity is reactive oxygen species (ROS) and consequently oxidative stress. In cells, mostly from NADPH, one electron is transferred by flavoenzymes to DOX molecule. The created DOX-radical (DOX*) spontaneously give back an electron to O_2. As a results DOX* goes back to parent compound (DOX) and superoxide anionoradical (O_2*-) is a second product of this reaction [11]. That cycle may be repeated many times and may result in oxidative stress which is a phenomenon during which the cell antioxidant system cannot neutralize arising ROS amount. In the last decade the attractive hypothesis was proposed allowing us to understand why serious cardiotoxic effects might appear many years after DOX excretion from an organism [7, 8, 9]. According to the hypothesis DOX cause mitochondrial DNA (mtDNA) oxidative damage which results in a disorder in mitochondrial electrons transfer. In that case, apart from four electron reduction of oxygen, one, two or three electron reduction occur leading to ROS formation. ROS are responsible for the next oxidative mtDNA injury and respiratory dysfunction of cardiomyocytes.

As it was mentioned above, NADPH is the source of electron for reduction of DOX and consequently oxidative stress generation. Additionally, seemingly paradoxically NADPH is a key

compound for regeneration of glutathione – main cell antioxidant. The major sources of NADPH are reactions catalysed by glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase and malic enzyme. Expression of genes responsible for the synthesis of these enzymes are controlled by iodothyronine hormones [3, 10]. These facts lead to an assumption that the impact of anthracyclines on oxidative stress may differ in hyperthyroid and euthyroid individuals. In these studies first of all we tested the hypothesis that symptoms of oxidative stress may be evident long time since last dose of DOX and if hypothyreosis may change cardiomyocytes antioxidative response in rats which receive DOX.

In a cellular adaptive process against oxidative stress, the transcription factor Nrf2 (*Nfe2*) plays a crucial role [6]. Nrf2 (nuclear factor erythroid 2-related factor; *Nfe2*) binds to antioxidant response elements (AREs) and activates AREs-related gens. As the results, the encode enzymes that directly inactivate oxidants [5], increase the level of glutathione synthesis and regeneration by activation of gene encoding glutathione reductase [12], stimulate NADPH synthesis [4,14] by activation genes encoding cellular NADPH-regenerating enzymes (glucose 6-phospate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme [6].

MATERIAL AND METHOD

The experiment was approved by Local Bioethical Commission of Medical University of Lublin. Male Wistar rats strain, having a range in body weight of 160 to 195 was purchased from Breeding Rats Brwinów/Warsaw. Rats were maintained in stable life conditions at 22˚C with a 12-h light/dark cycle and given standardized granulated fodder LSM. The experiment was carried out at the Central Animals Unit of Medical University of Lublin being under supervision of Veterinary Inspectorate in Pulawy.

The rats were administered i.p. DOX (Ebewe Arzneimittel Ges. M.B.H., Austria) and/or thyroxin in drinking water. The animals were randomly divided into six groups: control; 2T4 – 2mg thyroxin/l of drinking water; 4T4 – 4mg thyroxin/l of drinking water; DOX – doxorubicin 1.5 mg/kg; 2T4+DOX – thyroxin 2.0 mg/l and doxorubicin 1.5 mg/kg; 4T4+DOX – thyroxin 4 mg/l and doxorubicin 1.5 mg/kg. Rats from groups: DOX, 2T4+DOX and 4T4+DOX were administered i.p. 1.5 mg/kg of doxorubicin once a week for twelve weeks. In addition, the animals from groups: 2T4+DOX and 4T4+DOX were given thyroxin in drinking water, concentration respectively: 2mg/ml and 4mg/l. Administration of thyroxin begun a week before the first dose of doxorubicin and finished three weeks after the last doxorubicin dose.

Heart ventricles were obtained from animals which were anesthetized with 60 mg/kg/body weight of sodium pentobarbital (Morbital; Biowet, Puławy). To avoid the contribution of the red blood cells remaining in the heart to the measured parameters, the heart was washed with saline then dried and placed in liquid nitrogen and stored at −75˚C until analysis. Tissue samples were homogenized in 20 mM phosphate buffer (pH 7.4; proportions: 0.5g of tissue and 2 cm^3 of buffer) with protease inhibitor cocktail (Sigma-Aldrich, USA) in homogenizer with Teflon piston (5 minutes at 4.000 rpm). Then, homogenates were centrifuged at 14.000 rpm at 4˚C for 20 minutes.

NADPH concentration was determined by the spectophotometric method described in commercial kit (Bio Vision, USA).The obtained myocardium samples (20 mg) were homogenized in extraction

buffer provided by the manufacturer and then incubated at 60ºC in order to decompose NADP particles. Final readings were made using Power Wave Microplate Spectrophotometer (Bio-Tek, USA).

Glutathione concentration (total) and GSH/GSSG ratio were assessed in heart mitochondria by the spectophotometric method described in commercial kit (Bio Vision, USA). Mitochondrial fraction was separated using Sigma-Aldrich (USA) kit.

Total RNA was isolated from frozen tissues with Tri Reagent (Ambion) according to manufacturer instruction. The obtained RNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with RNase inhibitor. Real-time assays were performed on 7500 Fast system (Applied Biosystems). Primers and probes were designed and synthesized by Applied Biosystems. Each PCR reaction contained cDNA template, TaqMan® Gene Expression Assay and Master Mix (Applied Biosystems). GAPDH was used as an internal control for relative quantification. All real-time PCR experiments were performed in triplicates and the average CT for the triplicates was used in all subsequent analysis. Thermal profile was: 95ºC – 20 sec and 40 cycles: 95ºC – 3 sec 60ºC – 30 sec. (bez 50ºC – 2 min).

Relative quantification of RNA expression was calculated with the 2-ΔΔCt method. The ranges of the RQ values (relative quantities) were calculated by use of the equation: $RQ = 2^{-\Delta\Delta Ct}$.

The obtained results were expressed as mean ± SD and statistically analyzed with STATISTICA 5.0 software. The statistical significance of differences between control and other groups was evaluated either by t-Student test or U Mann-Whitney test and group to group comparisons were made by one-way ANOVA. A value of $P \leq 0.05$ was considered as statistically significant.

RESULTS

The mean value of mRNA amount in heart of the rat receiving DOX were 2-times higher than control, but there was no statistical difference in *Nfe2* expression in heart of rats after doxorubicin administrations (group of DOX) versus control (Fig. 1). The value of SD in this group indicates a broad range of individual reactions for DOX. The highest expression of mRNA for *Nfe2* was observed in group of DOX+4T4 and the change of value was significant, comparing to the control. However, there was no significant difference comparing DOX+4T4 vs. DOX.

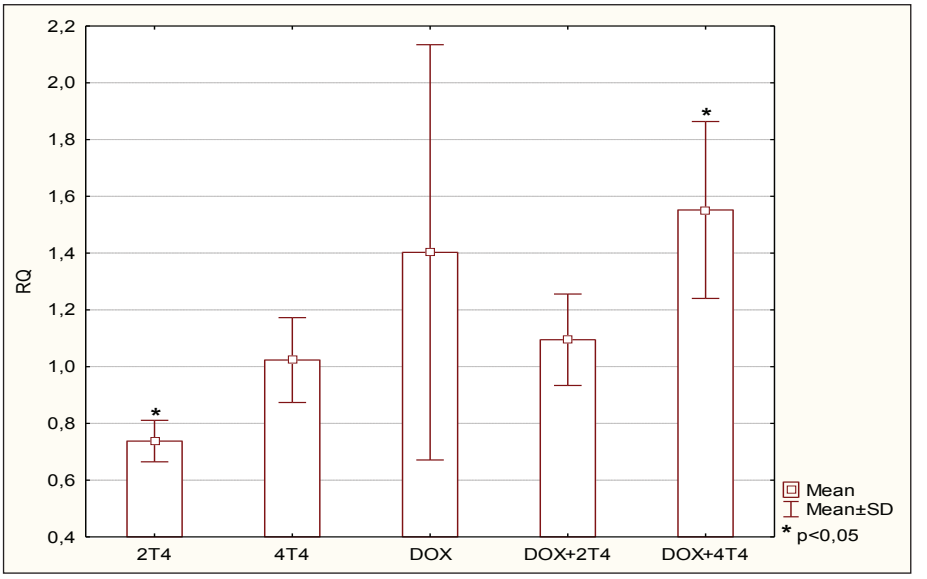

Fig. 1. *Nfe2* expression (represents as RQ); ANOVA – p>0.05

Additionally we measured heart concentration of NADPH (Table 1). There were no statistical differences in NADPH concentrations in heart between controls and all studied groups and DOX+4T4 vs. DOX. Moreover, any changes in total glutathione and GSH/GSSG ratio of mitochondrial fraction of heart were observed comparing back to the control (Table 2 and Table 3 respectively).

Table 1. Statistical characteristics of NADPH concentration of heart (unit of method – pmol/well)

Group	N	M	Me	Min	Max	SD	p	Analysis of variance
Control	6	164.90	166.37	136.08	188.24	20.641	-	-
2T4	6	143.53	143.14	128.04	162.16	11.536	0.05121	p=0.07248
4T4	6	156.86	158.33	135.69	170.00	11.451	0.42362	
DOX	6	179.12	176.08	128.63	218.82	31.332	0.37522	
2T4+DOX	6	164.87	158.82	130.59	204.12	26.446	0.99814	
4T4+DOX	6	173.79	177.06	140.39	206.67	22.086	0.48786	

Table 2. Statistical characteristics of total glutathione in heart mitochondria [µM]

Group	N	M	Me	Min	Max	SD	p
Control	6	0.274	0.274	0.246	0.297	0.016	-
DOX	5	0.256	0.256	0.28	0.286	0.020	0.14348

Table 3. Statistical characteristic of GSH/GSSG ratio in heart mitochondria

Group	N	M	Me	Min	Max	SD	p
Control	6	2.090	2.132	1.829	2.277	0.1764	-
DOX	5	1.987	2.045	1.819	2.092	0.1144	0.29481

DISCUSSION

In mitochondria DOX passes one-electron reduction. The donor of an electron is predominantly NADPH. It is the first step to reactive oxygen species (ROS) production leading to oxidative stress. NADPH also seemingly paradoxically takes part in anti-oxidative defense of cell. It is indispensable in reduced glutathione (GSH) regeneration process as an opposition to GSH oxidation by ROS. The equilibrium of the key cell redox buffer – reduced glutathione/oxidized glutathione (GSH/GSSG) is controlled by glutathione reductase using NADPH as cofactor. From this reason a drop in NADPH concentration may cause progress in oxidative stress damages in mitochondria. Mitochondria are especially sensitive to oxidative damages. Despite the presence of various antioxidant enzymes, mitochondria even in physiological conditions appear to be the most powerful intracellular source of ROS. According to one estimation, the steady-state concentration of superoxide anionoradical (O_2*-) in the mitochondrial matrix is approximately five- to tenfold higher than that in the cytosol or nucleus [2]. The concentration of ROS in mitochondria is rising at presence of DOX [1]. Mitochondria are not only a major source of ROS in both normal and non-physiological conditions, they are also a sensitive target for damaging effect of ROS. Especially mtDNA is exceptionally susceptible to attack by ROS owing to its close proximity to electron transport chain, the major locus of ROS production, and the lack of protective histones [13]. mtDNA, therefore, represents a critical cellular target for oxidative damage that could lead even to lethal cell injury through the loss of electron transport, mitochondrial membrane potential and ATP synthesis [13].

In our study there was no statistical difference in *Nfe2* heart expression of rats from DOX group, but it might by stressed that particularly rats have extremely different response on DOX (SD many times higher versus SD of control group). However, we have observed changes in NADPH concentration of heart, as well as mitochondrial total glutathione concentration and GSH/GSSG ratio. It might be evidenced that at tested point in time the changes do not pass the border of adaptive processes. It seems that three weeks after last dose of DOX, there is no chance for directing ROS production and similarly it is too early for long term disorders in mtDNA leading to changes in electron transport chain resulting in ROS generation.

The blood concentrations of T3 and T4 in rats of group 2T4 and 4T4 were significantly higher than seen in the control (unpublished data). That fact proves that the concentration used in our study was chosen properly. Significantly higher expression versus control of *Nfe2* was observed in group of 4T4+DOX. That may indicate an interaction between DOX and T4 diet supplementation. However, detailed analysis indicated no statistical difference in *Nfe2* expression in DOX+4T4 comparing to DOX. It is worth underlining that *Nfe2* and T4 regulate the expression of the same genes encoding glucose 6-phospate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme responsible for NADPH synthesis.

CONCLUSIONS

1. After three weeks since last doxorubicin administration there were no significant signs of oxidative stress in the heart.

2. In our study conditions there was no interaction of doxorubicin and thyroxin referring to oxidative stress in the heart.

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SUMMARY

The most evidence suggests that the major cause of doxorubicin (DOX) cardiotoxicity is reactive oxygen species and consequently oxidative stress. The accumulation of oxidative damages leading to mitochondrial dysfunction results in contractility disorders. In a cell adaptive process against oxidative stress, the transcription factor Nrf2 (*Nfe2*) plays a crucial role by stimulating expression of genes controlling synthesis of enzymes taking part in oxidative defence and red-ox equilibrium, e.g. glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase and malic enzyme.

Expression of genes responsible for the synthesis of these enzymes is controlled by iodothyronine hormones. These facts lead to an assumption that the impact of anthracyclines on oxidative stress may differ in hyperthyroid and euthyroid individuals. In these studies first of all we tested the hypothesis that symptoms of oxidative stress may be evident long time since last dose of DOX and if hypothyreosis may change myocardium antioxidative response in rats which received DOX. There was no statistical difference in *Nfe2* expression in hearts of rats after doxorubicin administrations (group of DOX) versus controls. In this group there were no statistical differences in cardiac NADPH and total mitochondrial glutathione concentrations and GSH/GSSG ratio comparing to the controls. Moreover, any changes in all tested parameters were observed between DOX vs. DOX+4T4. We concluded that after three weeks since last doxorubicin administration there were no significant signs of oxidative stress in the heart, and that in our study conditions there was no interaction of DOX and thyroxin referring to oxidative stress in the myocardium.

Keywords: Doxorubicin, thyroxin, hyperthyreosis, *Nfe2* (Nrf2) expression, NADPH, glutathione, oxidative stress.

STRESZCZENIE

Większość dowodów wskazuje, iż główną przyczyną kardiotoksyczności doksorubicyny (DOX) są reaktywne formy tlenu i w konsekwencji stres oksydacyjny. Kumulacja uszkodzeń oksydacyjnych prowadzi do dysfunkcji mitochondriów powodując zaburzenia kurczliwości. W procesie adaptacji komórki do stresu oksydacyjnego, czynnik transkrypcyjny Nrf2 (*Nfe2*) odgrywa ważną rolę ze względu na stymulację genów odpowiedzialnych za syntezę enzymów biorących udział w obronie antyoksydacyjnej i równowadze red-oks: np. dehydrogenaza glucoso-6-fosforanowa, dehydrogenaza fosfoglukonianu i enzym jabłczanowy. Ekspresja genów odpowiedzialnych za syntezę tych enzymów jest także kontrolowana przez hormony jodotyroninowe. Fakty te skłaniają do przypuszczeń, że wpływ antracyklin na stres oksydacyjny może się różnić u osobników z eutyreozą i hipertyreozą. W przeprowadzonych badaniach przede wszystkim zweryfikowano hipotezę, iż cechy świadczące o stresie oksydacyjnym mogą być zauważalne w odległym czasie od podania ostatniej dawki doksorubicyny. Ponadto oceniono czy hipertyreoza może zmieniać odpowiedź antyoksydacyjną mięśnia sercowego szczurów otrzymujących doksorubicynę. Nie stwierdzono znamiennych różnic w ekspresji *Nfe2* w sercach szczurów otrzymujących DOX w odniesieniu do kontroli. W grupie tej nie stwierdzono również istotnych różnic w stężeniu NADPH oraz stężeniu mitochondrialnego glutationu całkowitego oraz ilorazu GSH/GSSG w porównaniu do kontroli. Wykazano brak znamiennych różnić we wszystkich badanych parametrach między grupami DOX vs. DOX+4T4. Przeprowadzone badania wykazały, że trzy tygodnie od podania ostatniej dawki DOX nie dochodzi do zmian wskazujących na występowanie stresu oksydacyjnego w sercach szczurów. Ponadto w warunkach przeprowadzonego doświadczenia nie stwierdzono znamiennych interakcji DOX i tyroksyny w odniesieniu do stresu oksydacyjnego w mięśniu sercowym.

Słowa kluczowe: doksorubicyna, tyroksyna, hipertyreoza, ekspresja *Nfe2* (Nrf2), NADPH, glutation, stres oksydacyjny