Study of the metabolic doxorubicine-induced alterations in H9C2 cells and the effect of preincubation with dexrazoxane, a cardioprotective agent

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Introduction

Currently, doxorubicin anti-cancer treatment is limited by its irreversible cardiotoxicity *(1)*. The gravity of the toxicity depends on cumulated doxorubicin doses and can range from subclinical myopathy to patient's death *(2)*. The main toxicological mechanism is the production of reactive oxygen species (ROS), which lead to an oxidative stress impairing mitochondrial function and membrane integrity. It results in cardiomyocytes death by apoptosis and necrosis, clinically expressed by a progressive heart failure *(3)*. Nowadays, the main strategy to deal with this cardiotoxicity is the co-administration of dexrazoxane, a cardioprotective agent that acts by reducing ROS production through iron chelation. However, this strategy shows limited efficacy and there is a need for new cardioprotective strategies *(1)*. The goal of this research is to studying doxorubicin-induced metabolic alterations and the possible protective role of dexrazoxane. This strategy should help in highlighting possible new targets to counteract doxorubicin cardiotoxicity.

Material and methods

Cell culture, exposure and samples collection

Rat cardiomyoblasts H9C2 (2-1) (ECACC 88092904) were cultured in DMEM according to ECACC guidelines. For all exposure procedures, cells were first seeded with a density of 30.000 cells/cm^2 and were kept growing during 48 hours before any exposure. For metabonomic investigations, cells were randomly assigned into 4 groups (n=6) : a control group (CTR), a 0,3 µM doxorubicin-exposed group (DOX), a 3 µM dexrazoxane-exposed group (DEX) a group pre-incubated with 3 µM of dexrazoxane during 30 min before 0,3 µM doxorubicin exposure (DEX-DOX). After 24 hours of incubation, culture medium was collected and stored at -80°C. Then, cells were washed with D-PBS and collected in 6 ml of cold methanol by scrapping and quickly frozen in liquid nitrogen before storage at -80°C.

Samples preparation for ^{1}H-NMR, spectra acquisition and treatment

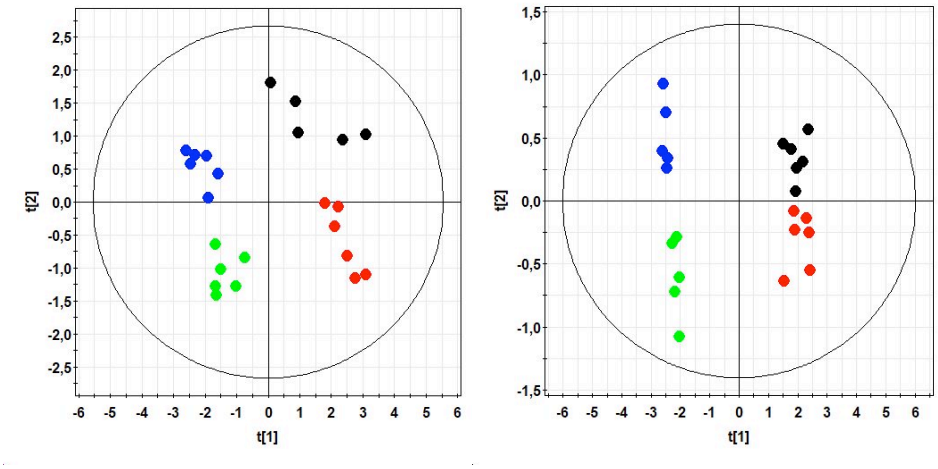
A chloroform-methanol-water extraction was carried out with collected cells to extract intracellular metabolites. Briefly, cells were lysed by sonication and the addition of the 3 solvents allowing separation of hydrophilic and lipophilic intracellular metabolites. The methanol-water phase was collected and evaporated with a speed vacuum. Polar metabolites and culture media were mixed to phosphate buffer and TSP (external reference necessary for spectral calibration) into 5 mm NMR tubes. ^{1}H-NMR spectra were acquired by a Bruker Avance 500,16 Mhz spectrometer with a 5 mm PABBO BB- probe and a NOESYPRESTAT-1D sequence (256 scans). For each spectrum, a baseline correction, a phase correction and a TSP reference calibration to 0,00 ppm were carried out. Spectral area from 0,08 to 10 ppm was subdivised into sub-regions of 0,04 ppm wide. Each subregion was then integrated. The water peak (4,20 to 5,32 ppm), was deleted and each subregion integral was normalized to spectrum total area.

Multivariate data analysis, metabolites identification and statistical tests

Data were analysed by projection to latent structure discriminant analysis (PLS-DA) modeling with 4 defined classes corresponding to the 4 exposure groups. R^2_{cum} and Q^2_{cum} parameters, p-value of CV-ANOVA were determined. Variables with a VIP value > 0,8 were selected as most discriminant variables. Corresponding metabolites were identified with several databasis (« in house » databasis and HMDB) and by the use of Chenomix Profiler software. Statistical signifiance of identified metabolites was determined by integrating the ^{1}H-NMR peaks of each metabolite. Integrals were normalized to spectral total area. For normal and homoscedastic variables, signifiance was determined using a one-way ANOVA. For non-normal or heteroscedastic variables, signifiance was determined using Dunn test.

Results

A PLS-DA modeling was performed on both cell extract and medium spectra to highlight discriminant metabolites between the four exposure groups. Scores plots (Fig. 1) of both cell extracts and culture media show a clear separation between the 4 groups, indicating 4 different metabolic profiles.



A **B**

*Figure 1. **A** : Scores plot of PLS-DA performed on cellular extracts. Parameters : R^2_{cum} = 0,59 Q^2_{cum} = 0,54, Hotteling's T2 = 0,95, P-value (CV-ANOVA) < 0,05. **B** : Scores plot of PLS-DA performed on culture media. Parameters : R^2_{cum} = 0,59 Q^2_{cum} = 0,52, Hotteling's T2 = 0,95, P-value (CV-ANOVA) < 0,05. Legend : CTR group (•), DOX group (•), DEX group (•), DEX-DOX group (•).*

Single comparisons between CTR group and DOX or DEX group were carried out to highlight metabolic changes induced by doxorubicin or dexrazoxane exposure. A single comparison was also made between DOX group and DEX-DOX group to highligh metabolic changes due to dexrazoxane pre-incubation. Metabolic changes are shown in table 1.

Metabolites	Cell extracts			Culture media		
	DOX	DEX	DEX-DOX	DOX	DEX	DEX-DOX
Alanine	=	↓ (3,91)**	↓ (1,67)	-	-	-
Glucose	-	-	-	↓ (6,62)	↓ (2,38)	↓ (2,68)
Glutamate	=	↑ (1,05)**	↑ (1,38)	-	-	-
Glutamine	↑ (2,69)**	=	↓ (1,72)	↑ (2,49)***	=	↓ (0,13) *
Glycerophospho-choline	=	↑ (3,28)***	↑ (1,32)***	-	-	-
Glycine	=	↑ (1,23)*	↑ (2,17)*	-	-	-
Guanidoacetate	=	↑ (1,05)**	=	-	-	-
Isoleucine	↑ (1,09)	=	=	↑ (1,07)	↓ (2,19)	↑ (1,24)

Lactate	↑ (5,38)*	↓ (5,75)***	↓ (5,63)***	↑ (7,03)	↓ (11,74)***	↓ (12,00)***
Leucine	↑ (1,09)	=	↑ (1,02)	-	-	-
Methionine	↑ (5,06)	=	↓ (1,58)	-	-	-
Phosphocholine	=	↑ (3,28)***	↑ (1,32)***	-	-	-
Phosphocreatine	↑ (0,93)*	=	↑ (1,21)***	-	-	-
Proline	↑ (1,13)	↑ (1,52)	↓ (1,61)	-	-	-
Pyruvate	-	-	-	↓ (0,80)	↓ (0,17)**	=
Serine	=	↑ (8,82)***	↑ (8,79)***	=	↑ (3,91)***	↑ (2,71)***
Succinate	-	-	-	↑ (0,81)	↑ (2,66)**	↑ (2,80)*
Taurine	↑ (5,38)***	↓ (4,15)***	↓ (4,90)***	-	-	-
UDP-glucose	↓ (0,88)	↑ (4,95)*	↑ (1,44)	-	-	-
Valine	-	-	-	↑ (1,27)	↓ (1,60)	-

*Table 1. Identified discriminant metabolites. Metabolite concentration changes in DOX group and DEX group compared to CTR group and changes in DEX-DOX group compared to DOX group are indicated by arrows. VIP values are indicated between brackets. One way ANOVA or Dunn test : * p-value < 0,05, ** p-value < 0,01 *** p-value < 0,001.*

Discussion

The metabonomic study highlighted some metabolic alterations due to doxorubicin exposure : a switch from mitochondrial aerobic energy metabolism to cytosolic anaerobic metabolism (increase of lactate, phosphocreatine and glutamine production and secretion, decrease of UDP-glucose level), a cell response to oxidative stress by an increased intracellular taurine level *(4)*, modification of amino acids metabolism. The metabonomic study also highlighted metabolic effects of dexrazoxane pre-incubation : recovery of mitochondrial aerobic metabolism (decrease in lactate, glutamine levels and increase in UDP-glucose level), activation of choline metabolism (phosphocholine, glycerophosphocholine and serine increased levels, activation of creatine phosphorylation and an increased secretion of succinate. Interestingly, choline metabolism activation may be linked to cell survival and growth pathways as well as succinate secretion may promote proliferation pathways by simulating GPR91 receptor *(5)*. Thus, choline metabolism and GPR91 could be potential targets for improving cardioprotection during doxorubicin exposure.

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