Doxorubicin Increases Oxidative Metabolism in HL-1 Cardiomyocytes as Shown by $^{13}$C Metabolic Flux Analysis

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Doxorubicin (DXR), an anticancer drug, is limited in its use due to severe cardiotoxic effects. These effects are partly caused by disturbed myocardial energy metabolism. We analyzed the effects of therapeutically relevant but nontoxic DXR concentrations for their effects on metabolic fluxes, cell respiration, and intracellular ATP. $^{13}$C isotope labeling studies using [U-$^{13}$C$_6$]glucose, [1,2-$^{13}$C$_2$]glucose, and [U-$^{13}$C$_3$]glutamine were carried out on HL-1 cardiomyocytes exposed to 0.01 and 0.02 $\mu$M DXR and compared with the untreated control. Metabolic fluxes were calculated by integrating production and uptake rates of extracellular metabolites (glucose, lactate, pyruvate, and amino acids) as well as $^{13}$C-labeling in secreted lactate derived from the respective $^{13}$C-labeled substrates into a metabolic network model. The investigated DXR concentrations (0.01 and 0.02 $\mu$M) had no effect on cell viability and beating of the HL-1 cardiomyocytes. Glycolytic fluxes were significantly reduced in treated cells at tested DXR concentrations. Oxidative metabolism was significantly increased (higher glucose oxidation, oxidative decarboxylation, TCA cycle rates, and respiration) suggesting a more efficient use of glucose carbon. These changes were accompanied by decrease of intracellular ATP. We conclude that DXR in nanomolar range significantly changes central carbon metabolism in HL-1 cardiomyocytes, which results in a higher coupling of glycolysis and TCA cycle. The myocytes probably try to compensate for decreased intracellular ATP, which in turn may be the result of a loss of NADH electrons via either formation of reactive oxygen species or electron shunting.

Key Words: systems biology; cytotoxicity; systems toxicology; metabolic flux analysis; HL-1 cardiomyocytes.

Doxorubicin (DXR), an anthracycline antibiotic, is used in first line therapy of many human cancers. However, severe cardiotoxic side effects limit its clinical use (Simuňek et al., 2009). Multiple mechanisms of DXR toxicity in vivo and in vitro have been described (Gewirtz, 1999; Tokarska-Schlattner et al., 2006). These include formation of reactive oxygen species (Bachur et al., 1977; Sinha et al., 1989), disturbances in nitric oxide pathway resulting in protein nitration (Fogli et al., 2004), oxidative damage of DNA (Bates and Winterbourn, 1982; Eliot et al., 1984; Feinstein et al., 1993), lipid peroxidation (Gewirtz, 1999), and apoptosis (Ling et al., 1993; Skladanowski and Konopa, 1993). Yet it is more and more evident that alterations in myocardial energy metabolism, especially due to mitochondrial dysfunction (Berthiaume and Wallace, 2007a), play an important role in the onset of DXR-induced cardiac toxicity. DXR-related acute and chronic cardiotoxicity have been associated with impaired cardiac high-energy phosphate metabolism. The compromising effect of DXR on cardiac energy metabolism is reflected by loss of mitochondrial adenosine-5’-triphosphate (ATP) (Pointon et al., 2010; Vidal et al., 1996). Cardiac substrate usage for the generation of the ATP and its alterations in myocardial diseases have been extensively studied (Ashrafian et al., 2007; Gertz et al., 1988; Mudge et al., 1976; Panchal et al., 2001; Randle et al., 1964; Stanley et al., 1997; Wisneski et al., 1985). It is well accepted that ATP is mainly generated from fatty acid oxidation in the healthy heart (Stanley et al., 2005). Several studies have shown that DXR alters fatty acid and glucose metabolism in the myocardium (Abdel-aleem et al., 1997; Bordoni et al., 1999; Carvalho et al., 2010; Wakasugi et al., 1993). However, the effects of DXR on main energy generating pathways besides tricarboxylic acid (TCA) cycle, such as glycolysis, are contradictory. Recent in vivo and in vitro studies demonstrated increased messenger RNA (mRNA) of several glycolytic enzymes (Berthiaume and Wallace, 2007b) as well as increased glycolytic activity, which is correlated to a decrease in the fatty acid oxidation (Carvalho et al., 2010). In addition, it was recently demonstrated that DXR results in an upregulation of mRNA from TCA cycle relevant enzymes in perfused rat heart (Tokarska-Schlattner et al., 2010). Contradictorily, decreased levels of TCA cycle relevant mRNA has been described in another model (Berthiaume and Wallace, 2007b). Such conflicting data in this regard is most probably related to differences in the applied biological model and experimental setup. Moreover, these differences in observations regarding alterations in
metabolic pathways may be partly due to either acute or chronic effects of DXR.

To our knowledge, holistic fluxome studies on DXR-induced effects on central energy metabolism integrating pathways, such as glycolysis, TCA cycle, and amino acid degradation, have not been carried out at clinically relevant concentrations on myocardial cells. This can be very challenging with commonly used endpoint methods since therapeutic concentrations should not cause acute myocyte dysfunction or cytotoxicity. The plasma kinetics of DRX shows an initial half-life of ~8 min followed by a terminal half-life of about 30 h (Greene et al., 1983). The major exposure occurs during the terminal phase where drug concentrations are generally less than 10^{-7} M. Following a 15 min infusion of DXR, the plasma concentration rapidly declines from 5–0.1μM within 1 h and steadily declines until ~30 h. From total plasma concentration only 20–25% DXR is freely available, i.e., the amount not bound to serum albumin. We assumed that DXR even at nanomolar concentrations exerts an effect on the central metabolism. To assess such effects sensitive methods and cutting edge technology is required. Using 13C metabolic flux analysis, we analyzed the effect of DXR on the central metabolism in an in vitro model (HL-1 cardiomyocytes). The used methodology includes the measurement of a whole set of metabolite uptake and production rates as well as the measurement of 13C enrichment in metabolites derived from 13C-labeled substrates. These data are implemented into a metabolic network model, which allows the estimation of intracellular fluxes over metabolic pathways. This method has been successfully used to study the energy metabolism of mammalian cells in vitro (Forbes et al., 2006; Goudar et al., 2010; Maier et al., 2009; Metallo et al., 2009; Niklas et al., 2011; Strigun et al., 2011) and that of the whole myocardium, as shown earlier (Vo and Palsson, 2006). However, this method has not been yet applied to analyze the effect of DXR on cardiac metabolism. The advantage of 13C-based fluxomic studies over transcriptomic or proteomic approaches is that activities of ATP-relevant pathways can be more reliably determined. Most importantly, specific rates of certain reactions cannot always be directly derived from changes of corresponding genes or proteins, due to the complex regulations of enzyme activities. We investigated the suitability of this method for the description of actual in vivo activities of metabolic pathways.

We furthermore complemented flux determinations with the measurement of cell respiration and intracellular ATP. The HL-1 cardiomyocytes is a murine cardiac cell line (Claycomb et al., 1998; White et al., 2004) which retains a differentiated morphology (Clarke et al., 2006; Fukuda et al., 2005; Sartiani et al., 2002; White et al., 2004). These cells have been used in the study of glucose and fatty acid metabolism (Alfarano et al., 2008; Palanivel et al., 2006) in addition to studying DXR-induced effects (Andersson et al., 2010; Budd et al., 2011). We used a dose-response curve from a lactate dehydrogenase (LDH) release assay to determine the DXR concentrations, which were not cytotoxic and did not inhibit myocyte beating. These concentrations have been reported to be clinically relevant corresponding to serum concentrations in patients undergoing DXR treatment (Greene et al., 1983). These are also significantly lower than the DXR concentrations at which cardiac Troponin T release, an indicator for cardiac damage, has been recently reported in the HL-1 cardiomyocytes (Andersson et al., 2010). The results of the present study are discussed in the context of current knowledge regarding DXR-induced alterations of cardiac energy metabolism.

**MATERIALS AND METHODS**

**Cell culture.** HL-1 cardiomyocytes (Claycomb et al., 1998; White et al., 2004) were kindly provided by Dr Claycomb (Louisiana State University) for research within the framework of European Union STREP-project “INVITROHEART.” The cells were maintained in Claycomb medium (Sigma Chemical), which was supplemented with 2mM glutamine (PAAs Laboratories, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin (PenStrep stock solution; C. C. Pro GmbH, Oberdorf, Germany), 100μM norpinephrine (Sigma Chemical) in 30mM L-ascorbic acid (Sigma Chemical), and 10% fetal bovine serum (FBS) (JRH Biosciences, U.K.). At confluency, the cells were passaged in a split ratio of 1:3. Cells were cultivated in culture flasks (75 cm², Falcon, Germany), which were precoated with a solution of 0.02% (w/vol) gelatin (Applichem, Germany)containing 5 μg/ml fibronectin (Sigma Chemical). The cells were maintained at standard cell culture conditions (37°C, 5% CO₂, and 95% relative humidity) in a cell culture incubator (Memmert GmbH, Schwabach, Germany).

**LDH assay.** A doxorubicin-HCl (DXR) (Sigma Chemical) stock solution (10mM) was prepared in cell culture grade water (PAN Biotech, Germany). Cytotoxicity of DXR on HL-1 cardiomyocytes was assessed using LDH-release assay. 5 × 10⁵ cells/well were seeded in gelatin/fibronectin-coated (see “Cell Culture”) 96-well plates (Greiner, Germany) in 200 μl Claycomb medium (containing 2mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100μM norpinephrine in 30mM L-ascorbic acid, and 10% FBS). After 24 h incubation, the supernatant was removed, and the cells were washed once with Claycomb medium. Fresh supplemented Claycomb medium containing DXR at different concentrations (20, 10, 2.5, 0.6, 0.16, and 0.004μM) was added to the cells in triplicates. The cells were incubated with DXR for 48 h. Finally, LDH assay (Cytotoxicity Detection Kit (LDH); Roche Applied Science, Germany) was carried out according to manufacturer’s instructions.

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Claycomb medium without supplements. The 12 different media containing 0.02, 0.01μM, or no DXR and differently labeled glucose and glutamine substrates were added to the respective wells. Cell free medium served as blank. For each test condition (0.02μM DXR, 0.01μM DXR, untreated control, and cell free medium control), four replicates per 24-well plate were prepared. Replica 1 contained medium 1, replica 2 contained medium 2, replicate 3 contained medium 3, and replicate 4 contained medium 4. Four 24-well plates were prepared in this way.

The plates were incubated under standard cell culture conditions (37°C, 95% relative humidity, and 5% CO₂) in the incubator. After 2, 10, 24, and 34 h of incubation, one plate was removed from the incubator, and the respective supernatants were collected and stored at −20°C until further analysis. The cells of each plate were fixed, and a protein quantification assay, namely, a sulforhodamine B assay (SRB assay), was performed as described previously (Noot et al., 2009). Cell number was estimated using a calibration curve obtained with different cell numbers of HL-1 cardiomyocytes.

Sample preparation for high performance liquid chromatography analysis. Serum proteins, such as albumin, were removed from the samples by microfiltration using Microcon Filter Devices (Millipore, Germany) with an exclusion size of 10 kDa. Subsequently, each filtrate was diluted 1:2 with α-aminobutyric acid solution (400μg/ml in deionized water), which was used as an internal standard for amino acid quantification. Quantification of extracellular glucose, lactate, pyruvate, amino acids, and total fatty acids. Quantification of glucose, lactate, and amino acids was carried out exactly as described recently (Strigun et al., 2011). Quantification of total free fatty acids was performed using a nonesterified fatty acid (NEFA) kit (Wako Chemicals, Germany) for the determination of NEFAs. Three hundred microliter of each culture supernatant was lyophilized and reconstituted in 30 l cell culture grade water. Total free fatty acids in these samples were quantified according to the manufacturer’s instructions. Briefly, 100 μl of the prepared reaction solution were mixed with 10 μl of the sample. After incubation for 10 min at room temperature (RT), the absorbance of the formed dye was measured at 550 nm using a iEMS reader (Labsystems, Finland). To exclude any influence of DXR on the measurement, reconstituted medium without cells and three hundred microliter of each culture supernatant were mixed with 10 μl of the above supplemented Claycomb medium containing 0.01 and 0.02μM DXR was added to the cardiomyocytes. An untreated control was also included. Intracellular ATP was determined after 2 and 34 h of incubation using ATPbiL1Step assay (PerkinElmer). One hundred microliter of lysis solution containing substrate was added to the cells. After 10 min incubation at RT, luminescence was measured using a Lumicount plate reader (Wallac 1420 Multilabel counter Victor3, PerkinElmer). A standard curve with known ATP concentrations was used to estimate the ATP concentration in samples. In parallel, an SRB assay was carried out for the estimation of cell number.

Statistical analysis. All data, except respiration measurement, were compared using one-way ANOVA following Dunnett’s post hoc analysis. Significance of altered cellular respiration was determined via two different methods. The time courses of dissolved oxygen in culture medium of all three conditions (control, 0.01μM DXR, 0.02μM DXR) was compared by repeated measures two-way ANOVA analysis following Bonferroni post hoc test. Secondly, all q02 (3 for each condition [see “Respiration Measurement”]) were compared by one-way ANOVA following Dunnett’s post hoc analysis. One-way and two-way ANOVA as well as post hoc tests were carried out using Prism 5.0.5.0 (GraphPad Software, Inc.).

RESULTS

Cytotoxicity of Doxorubicin

Concentration dependent release of LDH was observed (Fig. 1) after exposure to DXR for 48 h. DXR concentrations lower than 0.16μM did not show a significant release of LDH as compared with the control. However, 0.6μM and higher DXR concentrations led to approximately 1.5- to 1.9-fold higher release of LDH (p < 0.001). Based on these results, two DXR concentrations in an assumed subtoxic range (0.01 and 0.02μM DXR) were considered for the analysis of DXR-induced metabolic changes in HL-1 cardiomyocytes.

Cultivation Profile and Specific Uptake/Production Rates of Metabolites in HL-1 Cardiomyocytes Upon Doxorubicin Treatment

Figure 2A shows the growth profiles of HL-1 cardiomyocytes exposed to 0.01 or 0.02μM DXR and the untreated control.
Microscopically observed beating frequency in both DXR-treated cells and controls were similar throughout the whole experiment. Cell number increased from $1.5 \times 10^5$ cells per well seeding density to $3.61 \times 10^5$ for untreated control, $3.92 \times 10^5$ upon treatment with $0.01 \mu M$ DXR, and $3.77 \times 10^5$ with $0.02 \mu M$ DXR after 24 h preincubation followed by 34 h drug exposure. Although cell growth seemed slightly increased in DXR-treated cells, the actual cell number at each time point was not significantly different from the control cells (Fig. 2A).

Extracellular concentrations of glucose, lactate, and glutamine, the 20 proteinogenic amino acids and total free fatty acids were similar in both controls and treated cells throughout DXR exposure (Figs. 2B–E and Supplementary figure S1). The time course of glucose, lactate, glutamine, and oleic acid are depicted in Figures 2B–E. Despite slight differences of metabolite concentrations at each time point, these alterations were not statistically significant. Specific uptake or production rates of all metabolites were calculated from cell growth (Fig. 2A) as well as metabolite concentrations and are given in Supplementary table S1. Glucose, lactate, and glutamine showed the highest specific uptake/production rates independent of DXR exposure. A trend toward decreased glucose uptake was observed in DXR-treated cells ($300 \text{ fmol/cell/h in untreated control; 288 fmol/cell/h with 0.01\mu M DXR; 262 fmol/cell/h with 0.02\mu M DXR}$). A similar trend was observed for lactate production, which was also slightly decreased in treated cells ($642 \text{ fmol/cell/h in untreated control; 604 fmol/cell/h with 0.01\mu M DXR; 603 fmol/cell/h with 0.02\mu M DXR}$). Glutamine consumption was similar in controls and treated cells (46 fmol/cell/h in untreated control; 45 fmol/cell/h with 0.01\mu M DXR; 38 fmol/cell/h with 0.02\mu M DXR). For the majority of all other metabolites, slight alterations due to DXR exposure were observed. Especially, pyruvate, arginine, lysine, leucine, and isoleucine were consumed in slightly lower amounts as compared with the control.

Influence of Clinically Relevant Concentrations of Doxorubicin on Metabolic Flux Distribution

An enrichment of $^{13}$C in lactate derived from differently $^{13}$C-labeled substrates (see “Experimental setup for labeling studies on HL-1 cardiomyocytes”) was observed (Supplementary table S2–S4). Intracellular fluxes were estimated by implementing extracellular uptake/production rates and the labeling pattern of secreted lactate into a metabolic network model (Supplementary table S6). In Figure 3, simulated lactate mass isotopomer distributions are plotted against respective measured mass isotopomer distribution data for the three test conditions. Simulated mass isotopomer distribution, in this regard, refers to computed values of lactate mass isotopomer distributions, i.e., they represent the output with which lactate labeling theoretically can be expected from the used metabolic network model (Supplementary table S6) at a given set of measured metabolite uptake and production rates. Thus, a good fit in a linear regression between measured and simulated mass distribution indicates that the used metabolic network model is suitable for the applied data and secondly that the computed intracellular fluxes give a robust description of the actual flux distribution in the HL-1 cardiomyocytes. A very good correlation was found as shown by a linear relationship of measured and calculated lactate mass distributions for treated cells and untreated control (linear correlation coefficients $R^2 = 1.00$). The calculated distribution of intracellular steady state fluxes is depicted in Figure 4. The network includes reactions from glycolysis, pentose-phosphate-pathway, TCA cycle, anaplerosis, and amino acid metabolism. The underlying flux values are given in the Supplementary table S5. The calculated intracellular flux distribution revealed significant changes in the metabolism of HL-1 cardiomyocytes upon DXR treatment at the tested concentrations.

Glycolytic flux ($v_1$–$v_4$) was dose dependently decreased in DXR-treated cells to 96% ($0.01\mu M$ DXR; $p < 0.001$) and 87% ($0.02\mu M$ DXR; $p < 0.001$) of the control. Lactate production ($v_21$) was reduced by 17% ($0.01\mu M$ DXR; $p < 0.001$) and by 30% ($0.02 \mu M$ DXR; $p < 0.01$). Alanine production ($v_18$) was increased by 24% in HL-1 cardiomyocytes treated with $0.02\mu M$ DXR ($p < 0.01$).

However, connectivity between glycolysis and TCA cycle was enhanced in DXR-treated cardiomyocytes. Pyruvate
transport into mitochondria (v5) was increased to 197 and 239% of control in cardiomyocytes exposed to 0.01 or 0.02 μM DXR, respectively (p < 0.01). Cytosolic flux from pyruvate to oxaloacetate (v16) was activated upon drug exposure and caused a nearly fourfold increase (p < 0.01) of oxaloacetate import into mitochondria via malate shuttle (v24; p < 0.001) in treated cardiomyocytes. Activity of mitochondrial malic enzyme (v15) producing mitochondrial pyruvate was low in control but increased in cells treated with DXR (p < 0.01). Import of pyruvate into mitochondria was therefore increased via both direct pyruvate transport (v5) and anaplerotic reactions (v15, v16, and v24). TCA cycle fluxes (v11–v14) were significantly increased by 54–72% (p < 0.001) in treated cardiomyocytes compared with the untreated control. TCA cycle was mainly sustained by oxidative decarboxylation of pyruvate into acetyl-CoA (v6) indicating increased glucose oxidation, and glutaminolysis (v27). Upon DXR treatment, oxidative decarboxylation (v6) was significantly increased to 234% (0.01μM DXR, p < 0.001) or 243% (0.02μM DXR, p < 0.05) so that carbons deriving from pyruvate accounted for more than 85% of TCA fluxes compared with only 60% in untreated control. Glutaminolysis (v28) was significantly increased by 74–86% (0.01μM DXR, p < 0.01; 0.02μM DXR, p < 0.05). However, glutamine-derived carbons contributed to the same extent (~20%) to the TCA cycle fluxes in treated cardiomyocytes and untreated control. Whereas the specific rates of the anaplerotic reactions, the TCA cycle fluxes, the major input fluxes into TCA cycle, oxidative decarboxylation, and glutaminolysis, were increased upon DXR exposure, consumption of other amino acids feeding into TCA cycle such as lysine (v35), arginine (v34), branched chain amino acids (v31–v33), and aspartate (v25) was significantly decreased in treated cells (p < 0.05). For valine, isoleucine, arginine, and aspartate, a dose-dependent effect of

**FIG. 2.** Cell number (A), glucose (B), lactate (C), glutamine (D), and oleic acid (E) concentrations versus time in culture medium of untreated HL-1 cardiomyocytes (●) and cells treated with 0.01μM (□) and 0.02μM (△) DXR. Error bars indicate SDs (n = 4). Statistical significance was determined using one-way ANOVA following Dunnett’s post hoc test. The cell number was estimated using an SRB assay.
DXR was observed. Export of citrate via citrate shuttle to produce cytosolic acetyl-CoA and oxaloacetate (v_26) was slightly increased by 7% (0.01 μM DXR; p < 0.01) and 12% (0.02 μM DXR; p < 0.001) as compared with the untreated control.

Influence of DXR on Cellular Respiration

Figure 5A shows the dissolved oxygen for untreated control and cardiomyocytes treated with 0.01 and 0.02 μM DXR in the cultivation media. Dissolved oxygen in the media of both treated and untreated cardiomyocytes decreased due to consumption of oxygen by the cells. Dissolved oxygen in culture medium decreased from 0.19–0.096mM (untreated control), 0.089mM (0.01μM DXR), and 0.085mM (0.02μM DXR). The time course of dissolved oxygen was significantly different after 24 h exposure in the case of cardiomyocytes treated with 0.02 μM DXR (p < 0.05) but not for cardiomyocytes treated with 0.01μM DXR. Figure 5B shows the corresponding specific oxygen uptake rates (qO2), which were derived from the profiles of dissolved oxygen as well as cell growth. The specific oxygen uptake rates were 66.5 fmol/cell/h (untreated control), 78.5 fmol/cell/h (0.01μM DXR; p < 0.05), and 100 fmol/cell/h (0.02μM DXR; p < 0.001).

**Influence of DXR on Intracellular ATP Concentration**

The influence of DXR on intracellular ATP concentration in whole cells extracts was measured at 2 and 34 h after DXR exposure (Fig. 6). After 2 h of incubation, ATP concentration was 16.2 pmol/cell in untreated cells. In cells treated with 0.01 and 0.02μM DXR, ATP concentrations was nonsignificantly decreased to 15.1 pmol/cell and 15.2 pmol/cell respectively. After 34h, ATP concentration was 13.7 pmol/cell in untreated cells. No significant change of ATP concentration was observed in case of cells treated with 0.01μM DXR (13 pmol/cell; p > 0.05). However, in case of cells treated with 0.02μM, a significant decrease of ATP concentration (11.2 pmol/cell; p < 0.05) was observed.

**DISCUSSION**

In the present study, we analyzed the effects of DXR on central energy metabolism in HL-1 cardiomyocytes. The DXR concentrations investigated were clearly not toxic in the LDH-release assay. Furthermore, it was recently shown that these
FIG. 4. Metabolic flux distribution in untreated HL-1 cardiomyocytes and in cells treated with 0.01 and 0.02 μM DXR. Both DXR concentrations showed no significant effect on LDH release (Fig. 1). Cells were incubated for 34 h with 5mM [U-13C6]glucose, 5mM [1,2-13C6]glucose, 5mM unlabeled glucose, and 2mM [U-13C5]glutamine in four parallel experimental setups. Cell number, metabolite concentrations, and 13C enrichment in secreted lactate were measured at specific
concentrations do not result in significant Troponin T release (Andersson et al., 2010). By using $^{13}$C metabolic flux analysis, we found that DXR concentrations as low as 0.01 and 0.02 μM significantly affect glycolysis, TCA cycle, and amino acid catabolism in vitro in HL-1 cardiomyocytes.

Glycolytic rates in DXR-treated cardiomyocytes decreased in a dose-dependent manner. Downregulation of phosphofructokinase at the transcriptional level upon DXR treatment was previously described in neonatal rat cardiomyocytes (Jeyaseelan et al., 1997) and could partly explain decreased glycolytic rates. However, in the work of Jeyaseelan et al., DXR was applied at a concentration of 1 μM, which approximately represents the peak plasma concentration shortly after a bolus administration in cancer patients which declines relatively fast (within 1 h) to the lower nanomolar level (Greene et al., 1983).

DXR has a biphasic pharmacokinetic profile with exposure of tissues without substantial clearance to extracellular free drug concentrations that are identical to plasma free drug exposure. Nowadays, in vivo, DXR is administered by intravenous infusion, and the plasma concentration is much lower than 1 μM. Most in vitro studies on DXR are carried out using high concentrations and do not necessarily reflect the situation in vivo. It is therefore important to investigate effects of DXR in the nanomolar range. Recent in vivo and in vitro transcriptomic studies at clinically relevant concentrations have demonstrated an upregulation of mRNA of several genes in the glycolytic pathway (Berthiaume and Wallace, 2007b; Tokarska-Schlattner et al., 2010), which, at a first view, contradicts the observation of decreased glycolytic fluxes in our study. The level of the corresponding mRNA probably directly correlates with increased enzyme but not necessarily with enzymatic activity. While the upregulation of glycolytic genes in the work of Berthiaume et al. and Tokarska-Schlattner et al. was in good agreement, corresponding data on TCA cycle relevant mRNA from these two studies was conflicting. Contradictory data regarding DXR-induced alterations in the fluxome or transcriptome might be related to different biological models or experimental setups. In addition, it has to be considered that DXR exerts diverse effects on energy metabolism both acutely and chronically. The transcriptomic in vivo study of Berthiaume et al. was carried out on isolated rat hearts upon 5-week chronic subcutaneous DXR treatment, whereby the heart weight was significantly decreased, and the rats were in a poor health (Berthiaume and Wallace, 2007b). In the work of Tokarska-Schlattner et al., rat hearts were perfused with 2 μM DXR for 2 h, and the hearts showed mild cardiac dysfunction. This concentration highly exceeded the concentrations used in our study and the exposure to this relatively high concentration was longer than the observed peak plasma concentration in patients undergoing DXR treatment. Cardiac dysfunction independent of DXR exposure, contributes to increased glycolysis, and glucose oxidation in vivo as shown previously (Lei et al., 2004; Osorio et al., 2002; review: Stanley et al., 2005). As such, care should be taken in the interpretation of results, and cardiac damage should be taken into account. In our study, there was no change in cell viability (LDH and SRB assays) and myocyte

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time intervals. Metabolic fluxes were calculated by implementation of uptake and production rates of glucose, lactate, pyruvate, and amino acids and the enrichment of $^{13}$C in lactate into a metabolic network model (Supplementary table S6). All rates are given as femtomole per cell per hour. The denotations $v_1$–$v_{35}$ represent simplified identifiers of the respective fluxes/reactions (Supplementary table S5). Statistical significance was determined using one-way ANOVA following Dunnett’s post hoc test. Most of the fluxes in DXR-treated cells were significantly different from untreated controls ($p < 0.05$). Dark (red in online version) and light (green in online version) boxes refer to significantly decreased and increased fluxes as compared with controls ($p < 0.05$). A detailed overview of the significance of individual fluxes is given in Supplementary table S5.
contraction/beating. Therefore, the changes in glycolytic fluxes in our study are due to DXR and not due to decreased cardiomyocyte viability and dysfunction.

In our study, the decreased glycolytic activity was accompanied by an increased oxidative decarboxylation (pyruvate → acetyl-CoA), i.e., a higher flux of cytosolic pyruvate into mitochondria to form acetyl-CoA, which points toward an increase of glucose oxidation in DXR-treated cells. Remarkably, the increased oxidative phosphorylation was facilitated by cytosolic pyruvate entering the mitochondrion via two entry routes, first, via direct entry into the mitochondrion through pyruvate transporter and second, via cytosolic and mitochondrial malic enzyme. The anaplerotic entry of pyruvate into mitochondria via malic enzyme might make up for a saturated direct transport via mitochondrial pyruvate transporters and thus facilitate supply of TCA cycle with sufficient carbon. An increased contribution of glucose carbon to mitochondrial acetyl-CoA upon DXR treatment was recently shown in a rat in vivo model (Carvalho et al., 2010), which is in accordance with increased flux of pyruvate into mitochondrial acetyl-CoA, as observed in our study. In the study of Carvalho et al., it was shown that the increased contribution of glucose to acetyl-CoA is related to a decreased contribution of long-chain fatty acids to acetyl-CoA. Decreased fatty acid oxidation has been generally linked to DXR-related cardiac toxicity (Abdel-Aleem et al., 1997; Bordoni et al., 1999; Hong et al., 2002; Iliskovic et al., 1998). However, the uptake of fatty acids in HL-1 cardiomyocytes was relatively low for both untreated controls and DXR-treated cells as compared with the glucose uptake (~0.1%). Comparison of results should be considered with care as most studies on altered fatty acid uptake were carried out either in in vivo models or the applied DXR concentrations (Abdel-Aleem et al., 1997) exceeded the concentrations tested in our study. Nevertheless, it must be noted that the low uptake of fatty acids in HL-1 cardiomyocytes can be partly attributed to the low concentration of fatty acids in the used culture medium. Generally, glucose is the most abundant energy source in most cell culture media. The glucose concentration in the used culture medium was 25mM. In contrast, the total fatty acid concentration in supplemented Claycomb medium mainly derived from 10% FBS, was only ~9μM, which is in the same range as recently reported for culture medium containing 10% FBS (Andersson et al., 2008). In addition, cells cultured in vitro mainly use glucose to generate ATP (Marroquin et al., 2007). However, the fact that fatty acid uptake was relatively low in both DXR-treated and untreated cardiomyocytes points to the fact that the higher contribution of glucose to acetyl-CoA as observed in our considered in vitro model is most probably not related to altered fatty acid uptake.

The consequence of increased oxidative decarboxylation of mitochondrial pyruvate to form acetyl-CoA was significantly increased rates of TCA cycle reactions in DXR-treated cardiomyocytes. This increased oxidative metabolism was in accordance with an increased cell respiration. An upregulation of TCA cycle–related genes upon DXR treatment was recently reported in perfused rat heart (Tokarska-Schlattner et al., 2010) and might explain the increased TCA cycle rates in our study, despite significant differences in the experimental setup. In addition, a recent study (Pointon et al., 2010) using a murine in vivo model showed increased activity of citrate synthase after acute DXR exposure, which further supports the observation in our study. Several other studies on DXR-induced alterations of respiration demonstrated decreased respiration (Bugger et al., 2010; Nicolay and de Kruijff, 1987; Tao et al., 2006). However, these studies were either carried out with much higher concentrations directly on whole cells (Tao et al., 2006) or on isolated mitochondria, which have been shown to be different in their regulatory properties from mitochondria in the cell (Kuznetsov et al., 2008; Picard et al., 2010). Those reported effects, if occurring in vivo, might represent acute effects of DXR toxicity, e.g., when the concentration of DXR is relatively high in plasma within the initial hour of DXR application (Greene et al., 1983).

The increase of TCA cycle activity might be related to an early compensatory mechanism to the onset of functional defects as recently suggested (Tokarska-Schlattner et al., 2010). Functional defects in conjunction with apoptotic events could include loss or depletion of high-energy phosphates. Short-term exposure (2 h) of cells to 0.01 and 0.02μM DXR had no significant effect on cellular ATP concentration in our study. However, at 34 h, ATP concentration decreased at both concentrations of DXR but only significantly at 0.02μM DXR. Recently, decrease of ATP in murine in vivo model upon acute DXR exposure has been described (Pointon et al., 2010). It can be assumed that increased TCA cycle activity as well as respiration is a direct consequence of decreased levels of ATP. By increasing TCA cycle activity, the cells try to compensate for a decrease of intracellular ATP. However, we observed that
increase of oxidative metabolism does not seem sufficient to completely compensate for ATP decrease/depletion.

Besides depletion of ATP due to potential apoptotic events, reduction of DXR itself by mitochondrial reduced form of nicotinamide adenine dinucleotide (NADH) dehydrogenase by complex I in the respiratory chain might partially contribute to increased TCA cycle activity. It is well established that DXR acts as an electron acceptor in reactions involving P450 reductase and NADH dehydrogenase (Doroshow, 1983; Goodman and Hochstein, 1977; Graham et al., 1987). Addition of electrons to the DXR quinone group yields the semiquinone which after interaction with oxygen produces superoxides and other reactive oxygen species in vitro (Bates and Winterbourn, 1982; Doroshow, 1983; Sinha et al., 1989) or results in electron shunting, more likely to occur in vivo as recently suggested (Pointon et al., 2010). Pointon et al. argued that production of reactive oxygen species is more likely to occur in vitro than in vivo due to higher oxygen tension in most in vitro models. Electron shunting in case of DXR may involve electron transfer on DXR by complex I to form the semiquinone and subsequent electron transfer downstream complex I, eventually to cytochrome C, thereby skipping electron transfer through complex II and III (Pointon et al., 2010). Both scenarios would result in an uncoupling of electron transfer and building up of an H+ gradient in the inner mitochondrial membrane and consequently decreased ATP concentration.

In brief, we show for the first time the effect of therapeutically relevant concentrations on the whole central metabolism including glycolysis, TCA cycle, and amino acid degradation in a cardiac cell model by means of 13C metabolic flux analysis. Although 13C flux studies have been carried out for the assessment of DXR’s effect on the metabolism, the estimation of the flux distribution in cardiac cells upon DXR treatment via integration of a whole set of extracellular rates and 13C labeling information into a metabolic network model has not been yet reported to our knowledge, least of all at concentrations as low as 0.01µM. Most importantly, these concentrations had no significant effect on cell growth, beating frequency, and cell morphology. Nevertheless, these tested therapeutically relevant concentrations result in an increased oxidative metabolism in HL-1 cardiomyocytes, i.e., a higher efficiency of glucose usage in TCA cycle. This effect in HL-1 cardiomyocytes is most probably not related to alterations in fatty acid uptake because it was negligible and was not significantly altered by DXR. In fact, changes in metabolic fluxes seem to occur due to decrease of intracellular ATP. The present work therefore complements and partly confirms recent in vivo data. Our results significantly contribute to the understanding of DXR-induced cardiotoxicity, which is more and more highlighted in the context of changes in energy metabolism. Further work including in depth studies of the role of reactive oxygen species in changes of metabolic fluxes would complement the observations in our study. In addition, it would be very interesting to apply 13C metabolic flux analysis to human relevant system such as cardiomyocytes derived from human embryonic stem cells having both atrial and ventricular phenotypes, which would more closely reflect the in vivo situation. This will greatly advance the understanding of mechanism of toxicity of DXR at the metabolic pathways level.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


