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Original Contributions

Ferritin heavy chain as main mediator of preventive effect of metformin against mitochondrial damage induced by doxorubicin in cardiomyocytes

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ABSTRACT

The efficacy of doxorubicin (DOX) as an antitumor agent is greatly limited by the induction of cardiomyopathy, which results from mitochondrial dysfunction and iron-catalyzed oxidative stress in the cardiomyocyte. Metformin (MET) has been seen to have a protective effect against the oxidative stress induced by DOX in cardiomyocytes through its modulation of ferritin heavy chain (FHC), the main iron-storage protein. This study aimed to assess the involvement of FHC as a pivotal molecule in the mitochondrial protection offered by MET against DOX cardiotoxicity. The addition of DOX to adult mouse cardiomyocytes (HL-1 cell line) increased the cytosolic and mitochondrial free iron pools in a time-dependent manner. Simultaneously, DOX inhibited complex I activity and ATP generation and induced the loss of mitochondrial membrane potential. The mitochondrial dysfunction induced by DOX was associated with the release of cytochrome *c* to the cytosol, the activation of caspase 3, and DNA fragmentation. The loss of iron homeostasis, mitochondrial dysfunction, and apoptosis induced by DOX were prevented by treatment with MET 24 h before the addition of DOX. The involvement of FHC and NF-κB was determined through siRNA-mediated knockdown. Interestingly, the presilencing of FHC or NF-κB with specific siRNAs blocked the protective effect induced by MET against DOX cardiotoxicity. These findings were confirmed in isolated primary neonatal rat cardiomyocytes. In conclusion, these results deepen our knowledge of the protective action of MET against DOX-induced cardiotoxicity and suggest that therapeutic strategies based on FHC modulation could protect cardiomyocytes from the mitochondrial damage induced by DOX by restoring iron homeostasis.

Anthracyclines such as doxorubicin (DOX) are widely used as antitumor drugs. However, a major limitation to their use is the development of cardiomyopathy at high cumulative doses, which finally leads to congestive heart failure [32,35]. DOX cardiotoxicity typically occurs at an average total dose of 500 mg/m^2, although recent studies have shown that such cardiotoxicity may appear at doses as low as 300 mg/m^2 [40,46]. In addition, chemotherapy-induced cardiomyopathy is becoming more prevalent because heart failure in DOX-treated patients can go undetected for between 4 and 20 years after the cessation of treatment and long-term cancer survivors are increasing in numbers [43]. Because dose-limitation strategies have failed to eliminate the risk of DOX cardiotoxicity, considerable research has focused on the search for new drugs able to prevent the cardiotoxic effects of DOX without interfering with its ability to kill cancerous cells [19,26,53]. Nevertheless, to date, only the iron chelator dexrazoxane has been approved as a preventive strategy for DOX cardiotoxicity [19].

Mitochondria are believed to be the main target of the cardiotoxicity induced by DOX, which causes morphological and

Abbreviations: ΔΨm, mitochondrial membrane potential; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DOX, doxorubicin; EDTA, ethylenediaminetetraacetic acid; FHC, ferritin heavy chain; HBSS, Hanks' balanced salt solution; MET, metformin; MPTP, mitochondrial permeability transition pore; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; RPA, rhodamine B–[(1,10-phenanthrolin-5-yl)aminocarbonyl]benzyl ester; RPAC, rhodamine B–4-[(phenanthren-9-yl)aminocarbonyl]benzyl ester; TNFα, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

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functional alterations in the mitochondrial structure of the heart [38,50]. The primary effect of DOX on mitochondrial performance is its interference with the respiratory chain and oxidative phosphorylation, leading to increased oxidative stress, the depletion of cellular reducing equivalents, and the inhibition of ATP synthesis [16,47]. The altered redox status is believed to cause the induction of the mitochondrial permeability transition pore and the complete loss of mitochondrial integrity and function [30]. If taken to an extreme, this loss of mitochondrial plasticity may lead to the release of signals that mediate cardiomyocyte apoptosis, leading to progressive cardiomyocyte death and the development of congestive heart failure. Although the precise mechanisms involved in DOX-induced mitochondrial dysfunction are not completely understood, iron-mediated free radicals are thought to be primarily responsible [32,42]. The redox state of iron can be converted between the iron(II) and the iron(III) states by interaction with DOX, generating toxic reactive oxygen species (ROS). Iron is an essential trace element for proper cell functioning, but an excess generates oxidative stress and profound cellular toxicity [42,51]. To avoid this toxic effect, any excess of intracellular iron is sequestered by the ferritin heavy chain (FHC), the subunit of ferritin responsible for intracellular iron storage [48]. In cardiomyocytes, the DOX-induced increase in ROS has been associated with FHC upregulation, which seems to be a physiological defensive response to compete with the binding of DOX with iron and so reduce its cardiotoxicity [9]. In this sense, FHC-knockout mice are more susceptible to damage induced by DOX and have increased mortality after chronic DOX treatment compared with wild-type mice [34]. Therefore, iron homeostasis and the modulation of FHC seem to play a key role in DOX-induced mitochondrial dysfunction in the cardiomyocyte.

Metformin (MET) is an oral antihyperglycemic biguanide, widely used for the management of type 2 diabetes, which has also been seen to reduce the risk of all-cause mortality and myocardial infarction in patients with type 2 diabetes [1,2]. It has also been seen to reduce mortality in diabetic patients with heart failure [13,41]. MET has an important antioxidant activity in cardiomyocytes, reducing the generation of ROS in animal models of heart failure [18,39] and protecting cardiomyocytes from the oxidative stress induced by H_2O_2 or TNFα [27,39]. Our group has recently reported the ability of MET to prevent oxidative stress and cell death induced by DOX in cardiomyocytes [4]. In addition, we identified NF-κB-mediated FHC upregulation as the main mechanism responsible for the protective effect of MET against DOX-induced cardiotoxicity [5]. The modulation of FHC induced by MET suggests a role for the biguanide in preserving cardiac iron homeostasis and therefore in preserving mitochondrial function. A recent study in a rat model of DOX-induced cardiotoxicity suggested that the protective role of MET involves reversing the ultrastructural deterioration of mitochondria induced by the anthracycline and restoring DOX-induced energy starvation [6]. However, the mechanisms involved in this protective role of MET remain unclear. In light of all the above, this study aimed to assess the protective role of MET against the DOX-induced loss of iron homeostasis and mitochondrial dysfunction in cardiomyocytes and the involvement of NF-κB and FHC in this effect.

Materials and methods

Reagents

DOX, MET, Claycomb medium, fetal bovine serum, protease inhibitor cocktail, L-glutamine and penicillin–streptomycin mixture, (±)-norepinephrine (+)-bitartrate salt, phenylmethylsulfonyl fluoride (PMSF), and other biochemicals were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA). The ECL immunoblot detection reagents and prestained molecular weight markers were from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Cell culture, treatments, and preparation of cell extracts

HL-1 cells are a cardiac muscle cell line derived from the AT-1 mouse atrial cardiomyocyte tumor lineage, which contracts and retains phenotypic characteristics of the adult cardiomyocyte. HL-1 cells were a kind gift from Dr. W.C. Claycomb (Louisiana State University Medical Center, New Orleans, LA, USA). Cells were maintained in exponential growth phase as previously described [5]. All the experiments were conducted in the absence of fetal bovine serum and antibiotics. For the induction of cardiotoxicity, plated cells were exposed to 5 μM DOX for the indicated treatment times. The dosage of DOX was selected according to previous assays [4,5] and reproduces the plasma peak concentration reached by standard infusions in patients [15]. The MET concentration selected was 4 mM, which represents the physiological dose [36]. For cell extracts, cells were harvested with trypsin/EDTA, washed with phosphate-buffered saline (PBS), and solubilized in 10 mM Tris–HCl, pH 7.4, 1% Triton X-100 (v/v) and 0.1 mM PMSF. Samples were centrifuged at 10,000 *g* for 20 min at 4 °C. The supernatant was aliquotted and stored at −80 °C for further study. The isolation of cytosolic and mitochondrial samples was based on a previously described method for selective permeabilization of the plasma membrane [44]. Briefly, cells were collected by centrifugation, washed twice in ice-cold PBS, and resuspended at $\sim 4 \times 10^4$ cells/μl in ice-cold medium for permeabilization: 75 mM KCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, and 250 mM sucrose, pH 7.4. The protease inhibitor cocktail at 4 μl/1 $\times 10^6$ cells and an amount of digitonin to give 600 μg/ml were added. The incubation was maintained for 5 min in an ice bath. Cells were centrifuged at 10,000 *g* for 10 min and at 4 °C and the supernatant was stored at −80 °C in the bank of samples. The pellet was resuspended in a volume of solubilization buffer equal to the volume of medium previously used for permeabilization. The addition of protease inhibitor cocktail at 4 μl/1 $\times 10^6$ cells and a brief sonication (4–5 s with 30-s intervals) at ice-water temperature were followed by a new centrifugation step at 10,000 *g*. The resulting supernatant was aliquotted and frozen at −80 °C. The protein content of the supernatants was determined by the bicinchoninic acid method.

Primary neonatal rat cardiomyocyte isolation

A primary culture of neonatal rat cardiomyocytes was isolated using the neonatal cardiomyocyte isolation system (NCIS) purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA). Briefly, primary cardiomyocyte cultures were prepared from ventricles of 3-day-old Wistar rats. The hearts from 6 to 10 rats were excised, the ventricles pooled, and the ventricular cells isolated according to the NCIS protocol. The pups were killed by decapitation with sterile scissors, and their beating hearts were surgically removed and immediately placed in ice-cold Hanks' balanced salt solution (HBSS). Hearts were minced and digested overnight by trypsinization at 4 °C. The following morning, the tissue preparation was digested for 45 min with collagenase, slowly shaking at 3 rpm at 37 °C. Cells were then dispersed by trituration, filtered through a cell strainer, sedimented, and centrifuged at 1000 rpm for 5 min, and the supernatant was removed. Pellets were resuspended in Dulbecco's modified Eagle's medium low-glucose culture medium supplemented with 5% fetal bovine serum, 10% horse serum, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. After fibroblast separation, neonatal rat cardiomyocytes were counted, seeded, and subjected to the specific treatment as indicated.