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Original Contribution

Involvement of ferritin heavy chain in the preventive effect of metformin against doxorubicin-induced cardiotoxicity

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ABSTRACT

Doxorubicin is a wide-spectrum chemotherapeutic agent, although a cumulative dose may cause cardiac damage and lead to heart failure. Doxorubicin cardiotoxicity is dependent on the intracellular iron pool and manifests itself by increasing oxidative stress. Our group has recently shown the ability of metformin, an oral antidiabetic with cardiovascular benefits, to protect cardiomyocytes from doxorubicin-induced damage. This work aimed to study whether metformin is able to modulate the expression of ferritin, the major intracellular iron storage protein, in cardiomyocytes and whether it is involved in their protection. The addition of metformin to adult mouse cardiomyocytes (HL-1 cell line) induced both gene and protein expression of the ferritin heavy chain (FHC) in a time-dependent manner. The silencing of FHC expression with siRNAs inhibited the ability of metformin to protect cardiomyocytes from doxorubicin-induced damage, in terms of the percentage of cell viability, the levels of reactive oxygen species, and the activity of antioxidant enzymes (catalase, glutathione peroxidase, and superoxide dismutase). In addition, metformin induced the activation of NF-κB in HL-1 cells, whereas preincubation with SN50, an inhibitor of NF-κB, blocked the upregulation of the FHC and the protective effect mediated by metformin. Taken together, these results provide new knowledge on the protective actions of metformin against doxorubicin-induced cardiotoxicity by identifying FHC and NF-κB as the major mediators of this beneficial effect.

Doxorubicin is an anthracycline chemotherapeutic agent with potent wide-spectrum anticancer activity, but whose benefits are limited because of its high cardiotoxicity [8,42]. Cardiac damage by doxorubicin manifests itself as histopathological changes that lead to myofibrillar disarray and loss, dilated cardiomyopathy, with subsequent development of left-ventricular contractile dysfunction and congestive heart failure. Recently, the use of doxorubicin has increased in oncological protocols despite the above-mentioned risk of heart failure, which runs at 26% for accumulated doses of 550 mg/m^2 [43,52]. Whereas dose-limitation strategies have failed to completely eliminate the risk of cardiotoxicity, pharmacological cardioprotection seems to be an effective option. To date, only dexrazoxane has been approved as a preventive strategy against doxorubicin cardiotoxicity [19], so that it is necessary to look for new agents able to decrease doxorubicin cardiotoxicity without attenuating its antitumor activity [23,40,50].

Doxorubicin-induced cell damage is due to an increase in the generation of reactive oxygen species (ROS)[1], such as H_2O_2, $HO^{\bullet}$, and $ONOO^{\bullet}$ [4,47], and a reduction in antioxidant activity [24], which, together, trigger cardiomyocyte death [11,29,33]. In this cardiotoxicity, the intracellular labile iron pool plays a key role [40,51]; increased labile iron enhances the cardiotoxic effects of doxorubicin, whereas some iron chelators, such as dexrazoxane, can prevent it [23,51]. The extracellular iron, which circulates bound to transferrin, enters the cardiomyocyte through the transferrin receptor and is released into the cytoplasm for use in the cellular metabolism. Iron is an essential trace element in proper cell functioning, and both its deficit and its excess generate oxidative stress and profound cellular toxicity [40,48]. To avoid this toxic effect, the excess of iron is stored bound to ferritin, the protein responsible for intracellular iron storage. Ferritin is a protein composed of two subunits, termed ferritin light chain (FLC) and ferritin heavy chain (FHC), the latter being responsible for binding to iron and sequestration in the ferritin mineral core [46]. Through its iron-chelating action, FHC can reduce doxorubicin-induced

Abbreviations: BSA, bovine serum albumin; CAT, catalase; CFDA-SE, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester; DCF, 2′,7′-dichlorofluorescin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′, N′*-tetraacetic acid; ERK, extracellular-signal-regulated kinase; GPx, glutathione peroxidase; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; ROS, reactive oxygen species; SOD, superoxide dismutase; TNFα, tumor necrosis factor-α

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cardiotoxicity; for example, FHC knockout mice are more susceptible to damage induced by doxorubicin and have increased mortality after chronic doxorubicin treatment compared with wild-type mice [32]. Other authors have reported a doxorubicin-induced increase in ROS, which "turn on" FHC upregulation as a defensive mechanism to compete with the binding of doxorubicin with iron [9]. All the above suggests that those agents able to induce the expression of FHC might also prevent the cardiotoxic effect of doxorubicin and, importantly, be suitable for use in clinical practice, in which they would prevent adverse effects among oncologic patients receiving these therapies.

Metformin is an antihyperglycemic biguanide widely used in the treatment 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] and mortality in diabetic patients with heart failure [13,37,39]. Their antioxidant activity in cardiomyocytes has recently been evaluated. Metformin, via AMP-activated protein kinase (AMPK) activation, reduces the generation of ROS in animal models of heart failure [18,38] and protects cardiomyocytes from the oxidative stress induced by H_2O_2 or TNFα [25,38]. Our group has recently demonstrated the ability of metformin to prevent oxidative stress and doxorubicin-induced cell death in the cardiomyocyte [3], although the mechanisms involved remain unclear.

The aim of this study was to assess whether metformin is able to modulate the expression of ferritin in the cardiomyocyte and whether this modulation is involved in the preventive action against doxorubicin-induced damage.

Materials and methods

Reagents

Doxorubicin, metformin, Claycomb medium, fetal bovine serum, protease inhibitor cocktail, L-glutamine and penicillin–streptomycin mixture, (±)-norepinephrine(+)-bitartrate salt, phenylmethanesulfonyl fluoride (PMSF), and other biochemicals were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA). The enhanced chemiluminescence (ECL) immunoblot detection reagents and prestained molecular weight markers were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The NF-κB SN50 inhibitory peptide was obtained from Enzo Life Sciences (Farmingdale, NY, 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 [3]. 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 doxorubicin for the indicated treatment times. The dosage of doxorubicin was selected according to previous assays with concentrations ranging from 0.5 to 10 μM; the selected dose of 5 μM reproduces the plasma peak concentration reached by standard infusions in patients [3,16]. The metformin concentration selected was 4 mM, which represents the physiological dose [35]. 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. For nuclear isolation, cardiac myocytes were pelleted and incubated for 10 min on ice in a buffer containing 10 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 1 mM PMSF, and 1% phosphatase inhibitor cocktail (v/v) followed by the addition of Igepal CA-630 to a final concentration of 0.5% (v/v). Cells were homogenized for 15 s until completely lysed, and nuclei were collected by centrifugation (6000*g*, 15 min). The supernatant containing cytoplasmic proteins was aliquotted and stored at −80 °C for further analysis. The pelleted nuclei were washed once with the buffer described above and then resuspended in a buffer containing 10 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.4 M KCl, 1 mM DTT, 20% glycerol (v/v), 1 mM PMSF, and 1% phosphatase inhibitor cocktail (v/v). The nuclei were mixed vigorously on a shaking platform for 30 min at 4 °C, followed by centrifugation at 12,000*g* for 15 min and at 4 °C. The supernatant was aliquotted and stored at −80 °C for further study. The protein content of the supernatants was determined by the bicinchoninic acid method.

MTT assay

Cell viability was determined by a colorimetric assay using MTT as previously described [3]. Briefly, cells at $\sim 15 \times 10^3$/well were seeded in quadruplicate in 24-well plates and grown at 37 °C for 4 days in complete culture medium. Subconfluent cultures were subjected to the indicated treatment. Culture medium from treated or untreated cells was aspirated and cells were washed twice with prewarmed PBS. The redox potential of live cells was evaluated after incubation with 300 μl/well 1 mg/ml MTT for 1 h in the CO_2 incubator. In the mitochondria of living cells, yellow MTT undergoes a reductive conversion to formazan, giving a purple color. The formazan precipitate was dissolved by adding 250 μl/well dimethyl sulfoxide and shaking the plate for 5 min at room temperature. Absorbance of the samples was read at 570 nm and the background value at 690 nm was subtracted. Because doxorubicin could interfere with the interpretation of results (Supplementary Fig. 1A), HL-1 cells treated with doxorubicin alone were used to subtract the intrinsic absorbance value of doxorubicin. Cell viability was expressed as percentage MTT viability of untreated cells.

CFDA-SE assay

HL-1 cells were grown in 25-cm^2 flasks at 37 °C for 4 days in complete culture medium (2×10^6 cells per flask). After the indicated treatment, HL-1 cells were loaded with the CFDA-SE fluorescent probe (2 μM). The decrease in fluorescence was used to calculate the loss of cell viability. The fluorescence of the cells was monitored with a FACSort flow cytofluorimeter (Becton–Dickinson, San Jose, CA, USA) equipped with a 15-mW argon laser, tuned to 488 nm, and standard sets of filters for green (FL1) and red (FL3) fluorescence. HL-1 cells treated with doxorubicin alone were used to adjust the voltage of the photomultipliers and the autofluorescence of doxorubicin was subtracted from each measurement before analysis (Supplementary Fig. 1B). Propidium iodide (PI) was added to all the samples before data collection to identify dead cells. The flow-cytometry results presented as dot plots are representative of four experiments performed under selected assay conditions. Cell viability was calculated as the percentage of fluorescence compared with the control.

Quantitative real-time PCR

For the isolation of total RNA the QuickPrep Extraction kit (Amersham) was used and first-strand cDNA was synthesized with the GeneAmp RNA PCR kit (Roche, Branchburg, NJ, USA), as per the manufacturer's instructions. Real-time PCR was carried out as described previously [3]. The gene studied was FHC, and