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Diclofenac induces proteasome and mitochondrial dysfunction in murine cardiomyocytes and hearts

Rajeshwary Ghosh [a,1], Sumanta K. Goswami [b,2], Luis Felipe B.B. Feitoza [a,3], Bruce Hammock [b,4], Aldrin V. Gomes [c,*,5]

[a] Department of Neurobiology, Physiology and Behavior, University of California, Davis, CA 95616, United States
[b] Department of Entomology and Nematology and Comprehensive Cancer Center, University of California, Davis, CA 95616, United States
[c] Department of Neurobiology, Physiology and Behavior, Department of Physiology and Membrane Biology, University of California, Davis, CA 95616, United States

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ABSTRACT

Background: One of the most common nonsteroidal anti-inflammatory drugs (NSAIDs) used worldwide, diclofenac (DIC), has been linked to increased risk of cardiovascular disease (CVD). The molecular mechanism(s) by which DIC causes CVD is unknown.
Methods: Proteasome activities were studied in hearts, livers, and kidneys from male Swiss Webster mice treated with either 100 mg/kg DIC for 18 h (acute treatment) or 10 mg/kg DIC for 28 days (chronic treatment). Cultured H9c2 cells and neonatal cardiomyocytes were also treated with different concentrations of DIC and proteasome function, cell death and ROS generation studied. Isolated mouse heart mitochondria were utilized to determine the effect of DIC on various electron transport chain complex activities.
Results: DIC significantly inhibited the chymotrypsin-like proteasome activity in rat cardiac H9c2 cells, murine neonatal cardiomyocytes, and mouse hearts, but did not affect proteasome subunit expression levels. Proteasome activity was also affected in liver and kidney tissues from DIC treated animals. The levels of polyubiquitinated proteins increased in hearts from DIC treated mice. Importantly, the levels of oxidized proteins increased while the β5i immunoproteasome activity decreased in hearts from DIC treated mice. DIC increased ROS production and cell death in H9c2 cells and neonatal cardiomyocytes while the cardioprotective NSAID, aspirin, had no effect on ROS levels or cell viability. DIC inhibited mitochondrial Complex III, a major source of ROS, and impaired mitochondrial membrane potential suggesting that mitochondria are the major sites of ROS generation.
Conclusion: These results suggest that DIC induces cardiotoxicity by a ROS dependent mechanism involving mitochondrial and proteasome dysfunction.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most important therapeutic interventions for the treatment of a variety of painful conditions including arthritis, osteoarthritis, rheumatoid arthritis, and headaches [1]. Their efficacy in reducing fever and inflammation is well-established [1]. Despite the effective analgesic, anti-inflammatory, and antipyretic effects of these compounds, most NSAIDs are associated with various side effects including increased risk of heart attack, stroke, as well as gastrointestinal, renal and liver problems [2]. Although people taking NSAIDs without any history of cardiovascular diseases (CVD) show increased risk of cardiovascular incidences, this risk is even higher in people with a history of CVD [3]. While various meta-analysis studies and clinical trials have shown the propensity of NSAIDs to increase the risk of CVD and stroke, no mechanism is currently known to be associated with the side effects of the NSAIDs directly on the heart. One possible mechanism that has been related to the side effects of NSAIDs is the inhibition of the cyclooxygenase pathway in activated platelets or endothelial cells leading to a disruption of the homeostasis between the biosynthesis of eicosanoids like thromboxane and prostacyclin [1]. Both non-selective NSAIDs like diclofenac (DIC), ibuprofen, and indomethacin, as well as the cyclooxygenase 2 selective NSAIDs like celecoxib and rofecoxib, lead to decreased prostacyclin levels and increased levels of thromboxane (which promotes thrombosis) in infarcted hearts, which may cause further progression of the disease [4].

* Corresponding author.
E-mail address: avgomes@ucdavis.edu (A.V. Gomes).
[1] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.
[2] This author was responsible for treating the animals with diclofenac.
[3] This author carried out experiments on the hearts, livers and kidneys procured from the diclofenac treated animals.
[4] This author was responsible for providing the animals and diclofenac.
[5] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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DIC is one of the most commonly used NSAIDs [5]. The risk for development of CVD among DIC users is similar to the risk of using rofecoxib, with DIC increasing the risk of heart attack and stroke by approximately 40% [5]. Although DIC is available over the counter at lower doses than normally prescribed (in certain countries such as Indonesia and Denmark), these lower doses still show a 22% increase in the risk of CVD [5]. Apart from increased CVD risk, NSAIDs have also been reported to induce toxicity in the livers and kidneys [6,7]. Hepatotoxicity resulting from DIC use (150 mg/day) within 4–6 months has been associated with elevated levels of aminotransferase, an indicator of liver damage [8]. Hepatotoxicity induced by NSAIDs, such as DIC and mefenamic acid, has been suggested to cause hepatocyte injury by uncoupling mitochondrial oxidative phosphorylation, resulting in decreased ATP synthesis [9].

Some studies suggest that inhibition of COX-2 in cardiomyocytes may contribute to heart failure in patients receiving NSAIDs [10,11] because selective deletion of COX-2 in cardiomyocytes depressed cardiac output and enhanced susceptibility to induced arrhythmogenesis [10]. Recent studies have indicated a COX-independent mechanism of NSAIDs action in different cells like fibroblasts, human gastric adenocarcinoma cells, and endothelial cells [12]. Several studies have reported the generation of reactive oxygen species (ROS) by NSAIDs in different tissues leading to increased cell death [13–15]. However, some NSAIDs like aspirin have been suggested to decrease ROS levels in human endothelial cells [16]. The effect of DIC on ROS levels in cardiac cells, on cardiac myocytes, and the heart has not been previously investigated. Aspirin has been shown to indirectly affect the proteasome [17], and proteasome dysfunction has been associated with various diseases including CVD [18], neurodegenerative diseases [19], and different forms of cancer [20]. Proteasomes are large protein complexes which are part of the ubiquitin proteasome system (UPS). The UPS is responsible for the degradation of normal, damaged, misfolded and oxidized proteins [21]. In the heart, normal cardiac function is maintained by efficient protein degradation of normal, damaged, misfolded, and oxidized proteins to maintain intracellular protein homeostasis. Several studies have revealed the association between altered proteasome function and various pathological conditions of the heart including myocardial ischemia [22], cardiac hypertrophy [23], and atherosclerosis [24].

In our *in vitro* and *in vivo* experiments, DIC was found to be associated with cardiac dysfunction. DIC was a potent inducer of ROS, in contrast to aspirin, which did not induce ROS in cardiomyocytes. DIC was also an effective inhibitor of cardiac mitochondrial complex III, reduced mitochondrial membrane potential, increased oxidized protein levels, and decreased proteasome activity. These adverse changes in protein homeostasis and mitochondrial function likely lead to the observed time and dose dependent DIC induced cardiac cell death.

2. Materials and methods

2.1. Animal studies

Male Swiss Webster mice obtained from Charles River Laboratory and weighing 30–35 g were used for the study. Animal study protocol adheres to the guideline adopted and promulgated by U.S. National Institute of Health. The study protocol was approved by Institutional Animal Care and Use Committee (IACUC) of University of California, Davis. Mice were housed in vivarium and maintained at controlled temperature and humidity. The animals had free access to food and water. Mice were either treated with drinking water or drinking water containing diclofenac. The dose of diclofenac was 10 mg/kg/day, and it was administered for 28 days (chronic treatment group; $n = 4$). In a separate study, the mice were orally administered a single dose of diclofenac at 100 mg/kg (acute treatment group; $n = 4$). At the end of the study the animals were sacrificed using isoflurane anesthesia and hearts, livers and kidneys excised from the mice treated with or without DIC after 18 h and 28 days of treatment. Briefly, the mice were sacrificed and hearts, livers and kidneys were isolated and quickly washed in ice-cold phosphate buffered saline (PBS) twice to get rid of excess blood. The tissues were then frozen and pulverized in liquid nitrogen and care was taken to ensure that all steps were carried out at a very low temperature. The pulverized tissues were then collected in clean microcentrifuge tubes and stored at −80 °C until needed.

2.2. Determination of DIC levels in plasma of treated animals

DIC levels in plasma of treated animals were determined using liquid chromatography–tandem mass spectrometry (LC–MS/MS) as previously described and reported [25].

2.3. Cell culture

Rat cardiac H9c2 cells (H9c2(2–1)), CRL-1446, a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissue) was obtained from American Type Culture Collection (ATCC), Manassas, Virginia) and grown in 75 ml ventilated cell culture flasks (BioLite, Thermo Scientific) in high glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (growth medium, GM), at 37 °C and 5% CO_2. The cells were passaged by trypsinization (0.25% trypsin–EDTA, Gibco BRL) and diluted into culture dishes after confluency of 70–80% was reached.

2.4. Preparation of murine neonatal cardiomyocytes

Neonatal cardiomyocytes were isolated from 1 to 3 day old mice (B6SJLF1 mice from Jackson Laboratories, Sacramento, CA) using the Pierce Primary Cardiomyocyte Isolation Kit (Thermo Scientific). Briefly, hearts from newborn mice were collected in ice cold Hanks' Balanced salt Solution (HBSS). After mincing the hearts with a sterile razor blade, the minced tissues were incubated with the isolation enzymes at 37 °C for 30 min. Following washing with HBSS, the samples were re-suspended in complete media (DMEM containing 10% FBS and 1% Pen-Strep) and plated onto cell culture plates.

2.5. Treatment of cells with DIC

DIC was prepared in DMSO at the required concentrations and frozen in aliquots at −80 °C and used within a week of its preparation. H9c2 and neonatal cardiac cells were treated with DIC at the desired concentrations for different incubation periods ($n = 3$). Care was taken to keep the volume of DMSO below 0.1% in DMEM containing cells to reduce the effects of solvent on the cells. After the desired treatment duration, the cells were harvested in ice-cold 26S buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM DTT, pH 7.5) [26] and sonicated. Centrifugation was carried out at 15,000 ×*g* for 20 min and the supernatant containing proteasomes was collected. Protein concentration of the cell lysate was measured using a nanodrop (2000C, Thermo Scientific) and each sample was diluted to 1 μg/μl to be used for proteasome assays and Western blot analysis.

2.6. 26S proteasome activity assays of H9c2 cells, neonatal cells and tissue lysates

The samples (1 μg/μl) prepared as described under the section "Treatment of cells with DIC" were used for studying either β5 chymotrypsin-like activity, β1 caspase-like activity, or β2 trypsin-like activity. Each assay was carried out using 100 μM ATP with or without a proteasome specific inhibitor: 10 μM bortezomib for β5 activity and 100 μM bortezomib for β1 and β2 activity. The hearts, livers, and kidneys from mice treated with DIC were similarly prepared in 26S buffer. Approximately 20 mg of each pulverized tissue was weighed out and homogenized using a hand-held homogenizer in 26S buffer and centrifuged at 15,000 ×*g* for 20 min at 4 °C. Protein concentration of the supernatant containing the proteasomes was determined and 20 μg of the sample was used for measuring 26S β1, β2 and β5 activity. Specific substrates for each proteasomal β subunits were used for the initiation of the reaction [27–30]. β substrates were purchased from Enzo Life Sciences, NY, USA: β1 substrate Z-LLE-AMC is cleaved after glutamic acid (E) by the β1 catalytic subunit *via* caspase-like activity. Similarly, β2 substrate Boc-LSTR-AMC is cleaved after arginine (R) *via* β2 trypsin-like activity and β5 substrate Suc-LLVY-AMC after tyrosine (Y) *via* β5 chymotrypsin-like activity.

2.7. Immunoproteasome β5i activity

10–20 μg of heart samples from DIC treated animals were used to determine β5i activity. Samples were incubated with immunoproteasome buffer containing 50 mM Tris, 5 mM $MgCl_2$, 20 mM KCl, pH 7.4 and freshly added 2 mM DTT. β5i selective inhibitor, ONX 0914 (20 μM) (Cayman Chemical, MI) was utilized to determine the selectivity of the assay. The reaction was initiated by adding the fluorogenic chymotrypsin-like β5i substrate, ANW-R110 (AAT Bioquest, Inc., CA) and the readings were recorded in a Tecan Infinite M1000 over a duration of 2 h at an excitation of 498 nm and an emission of 520 nm at 37 °C.

2.8. Labeling of proteasome active sites by MV-151

10 μg of rat heart lysate was utilized for proteasome labeling in MV151 homogenization buffer (50 mM Tris, 5 mM $MgCl_2$, 250 mM sucrose, 2 mM ATP and 1 mM DTT, pH 7.5). DIC at different concentrations was added to each tube ($n = 3$) and 20 μM of MG132, a proteasome inhibitor, or no probe (negative controls) were also used. After subsequent mixing, the tubes were incubated for 30 min at 30 °C, and 0.1 μM MV151 added to each tube, and the tubes incubated for 1 h at 30 °C in dark. After the addition of 2× Laemmli sample buffer and subsequent heating, the proteins were separated on a 4–20% stain free gel (Bio-Rad) and the fluorescent signals emerging from the MV151 labeled proteins were visualized using a ChemiDoc MP (Bio-Rad).