Research Article

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Blunted cardiac response to hemorrhage in cirrhotic rats is mediated by local macrophage-released endocannabinoids

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Background & Aims: Cirrhosis is associated with blunted cardiovascular response to stimuli such as hemorrhage, but the mechanism remains unclear. We aimed to clarify the role of endocannabinoids in blunted hemorrhage response in cirrhotic rats.

Methods: Cirrhosis was induced by bile duct ligation (BDL). Hemodynamics were measured. Cannabinoid receptor-1 (CB1) antagonist, AM251, and macrophage inhibitor gadolinium chloride ($GdCl_3$) were administered. Myocardial levels of anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) were measured and resident monocytes and macrophages quantified by immunohistochemistry. Isolated cardiomyocyte contractility was measured before and after incubation with monocytes from BDL and sham controls.

Results: Hemorrhage significantly decreased arterial pressure and left ventricular dP/dT. After hemorrhage, these changes quickly reversed in controls, but were severely prolonged in BDL rats. Chronic AM251 treatment restored this impaired response. AEA and 2-AG levels were increased in BDL hearts and further increased after hemorrhage. Sham hearts showed virtually no monocytes or macrophages before or after hemorrhage, whereas BDL hearts had significantly more white blood cells which further increased after hemorrhage. $GdCl_3$ treatment significantly reduced cardiac endocannabinoid levels both at baseline and after hemorrhage. This treatment also restored cardiovascular response to hemorrhage in BDL rats but did not affect sham controls. Monocytes isolated from BDL rats more potently inhibited cardiomyocyte contractility than sham control monocytes.

Conclusions: The cirrhotic heart showed increased monocyte recruitment and endocannabinoid levels. CB1 blockade or $GdCl_3$ treatment restored blunted cardiovascular response to hemorrhage. Endocannabinoids released by monocytes blunt cardiac response to hemorrhage. Preventing monocyte recruitment or blocking endocannabinoid signaling may improve cardiovascular homeostasis in cirrhosis.

Introduction

In cirrhotic patients, cardiac output is increased at baseline; however, ventricular contractile response to pharmacological, surgical or physiological stimuli is blunted. The attenuated cardiac contractile responsiveness in the face of increased baseline cardiac output is known as cirrhotic cardiomyopathy [1].

Cardiovascular dysfunction is responsible for 7–15% of mortality after liver transplantation in cirrhotic patients [2]. Moreover, hemorrhagic events like rupture of esophageal varices are a significant cause of morbidity and mortality in cirrhotic patients and impaired cardiac response could result in poorer outcomes [3].

Exogenous cannabinoids have been known for centuries with substances like hashish and marijuana. Endogenous cannabinoids are lipid-like substances and include arachidonoyl ethanolamide or anandamide, and 2-arachidonoylglycerol [4].

We previously demonstrated the increased endocannabinoid tone in blunted cardiac contractility in a cirrhotic rat model. Furthermore, we found an increase in local cardiac production of endocannabinoids in cirrhotic rats [5].

Circulating macrophages/monocytes are an important source of endocannabinoids, the production of which is increased in various forms of shock [6,7] and liver cirrhosis [8,9] through the action of elevated plasma levels of bacterial endotoxin [8,10]. Activated macrophages depress myocardial contractility through increased adherence to myocardiocytes and the release of soluble mediators [11,12]. The present study was to investigate the possible role of macrophage-derived endocannabinoids in cardiac dysfunction in cirrhotic rats in an *in vivo* setting and in particular, their role in altered cardiac response to hemorrhage.

Keywords: Cirrhosis; Cardiomyopathy; Endocannabinoids; AM251; Hemorrhage; CB-1; Monocytes; Macrophages.
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Abbreviations: BDL, bile duct ligation; CB1, Cannabinoid receptor-1; $GdCl_3$, gadolinium chloride; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; ESPVR, end-systolic pressure-volume relationship.

Materials and methods

Animal model

The study protocols were approved by the Animal Care Committee of the University of Calgary, Faculty of Medicine, under the guidelines of the Canadian Council on Animal Care. Male Sprague–Dawley rats (Charles River, St Laurent, QC, Canada) weighing 200–250 g were used. Rats had free access to rat chow and water and were maintained in a 12-hour light/dark cycle. Common bile duct ligation (BDL) was performed to induce cirrhosis as described previously [13]. Briefly, the common bile duct was exposed by a midline abdominal incision under pentobarbital anesthesia. The duct was doubly-ligated and sectioned between the ligatures. Sham operated rats were treated in the same manner without BDL. All studies were performed 4 weeks after BDL or sham operation.

Hemodynamic indices

A left ventricular pressure-volume loop was constructed by placing a microtip pressure–volume catheter (SPR-839; Millar Instruments, Houston, TX) in the left ventricle via the right carotid artery as described previously [14]. Measurement of mean arterial pressure and drug administration were performed by placing PE-50 and PE-10 polyethylene cannulae into the left femoral artery and right jugular vein, respectively. After stabilization for 30 min, the signals were continuously recorded at a sampling rate of 1000 Hz using an ARIA pressure–volume conductance system (Millar Instruments) coupled to a Powerlab/4SP A/D converter (AD Instruments, Mountain View, CA), and then stored and displayed on a computer. All pressure–volume loop data were analyzed with a locally developed data acquisition program (CVWorks1.2; Advanced Measurements Inc., Calgary, AB, Canada). Although theoretically this setup could be calibrated for absolute volume measurement using standard saline-filled cylindrical holes, in our setting volume measurements were not reproducible between different animals. However, relative volume measured in each animal was reliable which allowed us to calculate percent changes of the volume-related indices for each rat in a single experiment, but absolute values of these indices in different animals could not be compared with each other.

End-systolic pressure-volume relationship (ESPVR) was calculated by gradually decreasing the preload by applying pressure on the inferior vena cava and calculating the slope of the line that passes through the end-systolic points of the PV loop. This intervention was performed by placing a saline-soaked cotton tip applicator on the inferior vena cava through an abdominal incision. Changes in the slope of the ESPVR are well-accepted as a relatively preload- and afterload-independent measure of contractility in a single experiment [15]. Again because of the involvement of volume measurement in this index, it was not possible to compare the results among different animals.

Controlled hemorrhage was produced by withdrawal of blood through the femoral artery (2 ml/kg/min × 3 min) by a syringe connected to a motorized withdrawal pump.

Drugs

The cannabinoid receptor-1 (CB1) antagonist, AM251 (Sigma Chemicals), was administered either acutely (3 mg/kg, iv) in the experiments involving ventricular pressure-volume studies and measurement of changes in ESPVR or chronically (3 mg/kg/day, SC × 2 days) in the hemorrhage studies. The CB-2 receptor antagonist AM630 (Sigma) was administered acutely (3 mg/kg iv) in ventricular pressure-volume experiments. Gadolinium chloride (12 mg/kg iv daily × 2 days, Sigma) was used to deplete monocytes and macrophages from cardiac tissue.

Monocyte isolation and effect on cardiomyocyte contractility

Heparinized blood (15–30 unit/ml) 5 ml was carefully layered over 5 ml Histopaque-1077 solution (sigma) and centrifuged at 400 g for 20 min. The mononuclear fraction (a white layer at the interface) was aspirated, resuspended in 2 ml of phosphate-buffered saline (PBS), and centrifuged at 800 g for 20 min. The resulting pellet was resuspended in 0.5 ml PBS and used for subsequent experiments. Unloaded cell shortening was quantified using a video sarcomere detector (Ionoptix, Milton, MA, USA). After recording the baseline contractility of cardiomyocytes, 30 µl of monocyte suspension was directly applied to the recording chamber which contained 300 µl Tyrode solution and cardiomyocytes. Ten minutes after incubation, the contractility was recorded in the same cell.

Quantification of endocannabinoids in cardiac tissue

The heart was rapidly removed from sacrificed rats. Left ventricular tissue was divided into roughly 100 mg pieces and frozen in liquid nitrogen. Following pulverization of the frozen sample under liquid nitrogen, the samples were homogenized in 0.5 ml of an ice-cold solution of methanol/Tris buffer (50 mM, pH 8.0), 1:1, containing 7 ng per 100 mg tissue weight of d4-anandamide, synthesized as described [16]. To each homogenate, 2 ml of ice-cold chloroform/methanol (1:1) and 0.5 ml of 50 mM Tris buffer, pH 8.0, was added. The homogenate was centrifuged at 4 °C (500 × g for 2 min), the chloroform phase was recovered and transferred to a borosilicate tube, and the water phase was extracted two more times with ice-cold chloroform. The combined extract was evaporated to dryness at 32 °C under a stream of nitrogen. The dried residue was reconstituted in 110 µl of chloroform, and 2 ml of ice-cold acetone was added. The precipitated proteins were removed by centrifugation (1800 × g, 10 min), and the clear supernatant was removed and evaporated to dryness. The dry residues were reconstituted in 50 µl of ice-cold methanol, of which 35 µl was used for analysis by liquid chromatography/in line mass spectrometry, by using an Agilent 1100 series LC-MSD, equipped with a thermostated autosampler and column compartment. Liquid chromatographic separation of endocannabinoids was achieved by using a guard column (Discovery HS C18, 2 cm × 4.0 mm, 3 µm, 120A) and analytical column (Discovery HS C18, 7.5 cm × 4.6 mm, 3 µm) at 32 °C with a mobile phase of methanol/water/acetic acid (85:15:0.1, vol/vol/vol) at a flow of 1 ml/min for 12 min followed by 8 min of methanol/acetic acid (100:0.1, vol/vol). The MSD (model LS) was set for atmospheric pressure chemical ionization, positive polarity, and selected ion monitoring to monitor ions m/z 348 for AEA, 352 for d4-AEA, and 379 for 2-arachidonoylglycerol (2-AG). The spray chamber settings were as follows: vaporizer, 400 °C; gas temperature, 350 °C; drying gas, 5.0 L/min; and nitrogen was used as the nebulizing gas with a pressure of 60 psig. Calibration curves were produced by using synthetic anandamide and 2-AG (Cayman Chemical, Ann Arbor, MI). The amounts of AEA and 2-AG in the samples were determined by using inverse linear regression of standard curves. Values are expressed as fmol per mg wet tissue.

Immunohistochemistry

The removed heart was retrogradely perfused with ice-cold saline followed by ice-cold 4% paraformaldehyde. After the hearts were embedded in paraffin, transverse sections were cut and stored for immunohistochemistry. 5 µm sections of the heart were deparaffinized, and treated with 0.3% hydrogen peroxide in methyl alcohol for 30 min to block endogenous peroxidases. After three washes in phosphate buffer, the sections were exposed to normal goat serum, and then incubated with a monocyte/macrophage-specific primary antibody (mouse anti-rat anti-CD68 antibody also known as ED1) diluted in PBS for 1 hour at room temperature. After washing, the sections were sequentially treated with biotinylated goat anti-mouse immunoglobulin and avidin-biotin peroxidase complex; developed with diaminobenzidine-hydrogen peroxide solution and finally counterstained with hematoxylin. Quantification of CD68-positive cells was performed by calculating the average number of cells per high power field (20×).

Statistical analysis

The results are expressed as mean ± SE. Student's *t* test was used to compare the differences between two groups and multiple comparisons for three or more groups were analyzed by one-way or two-way ANOVA, followed by a Newman-Keuls post hoc test, where appropriate. A p value <0.05 was considered to be significantly different.

Results

Effects of CB1 blockade on PV loop and ESPV relationship

Fig. 1 shows an example of left ventricular pressure-volume loop in a BDL rat before and after administration of AM251. This treatment resulted in a significant increase in left ventricular end-systolic pressure and a significant decrease in left ventricular end-diastolic volume in the BDL group. However, this treatment did not change pressure-volume loop in sham control animals (Fig. 1A). AM251 administration also resulted in a significant