Research Article

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Mechanisms of TNFα-induced cardiac dysfunction in cholestatic bile duct-ligated mice: Interaction between TNFα and endocannabinoids

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Background & Aims: Chronic liver disease is associated with endotoxemia, oxidative stress, increased endocannabinoids and decreased cardiac responsiveness. Endocannabinoids activate the tumor necrosis factor-alpha (TNFα)–nuclear factor κB (NFκB) pathway. However, how they interact with each other remains obscure. We therefore aimed to clarify the relationship between the TNFα–NFκB pathway and endocannabinoids in the pathogenesis of cardiodepression of cholestatic bile duct ligated (BDL) mice.

Methods: BDL mice with TNFα knockout (TNFα−/−) and infusion of anti-TNFα antibody were used. Cardiac mRNA and protein expression of NFκBp65, *c-Jun*-N-terminal kinases (JNK), p38 mitogen-activated protein kinase (p38MAPK), extracelullar-signal-regulated kinase (ERK), inducible nitric oxide synthase (iNOS), Copper/Zinc and Magnesium-superoxide dismutase (Cu/Zn- and Mn-SOD), cardiac anandamide, 2-arachidonoylglycerol (2-AG), nitric oxide (NOx) and glutathione, and plasma TNFα were measured. The effects of TNFα, cannabinoid receptor (CB1) antagonist AM251 and the endocannabinoid reuptake inhibitor UCM707, on the contractility of isolated cardiomyocytes, were assessed.

Results: In BDL mice, cardiac mRNA and protein expression of NFκBp65, p38MAPK, iNOS, NOx, anandamide, and plasma TNFα were increased, whereas glutathione, Cu/Zn-SOD, and Mn-SOD were decreased. Cardiac contractility was blunted in BDL mice. Anti-TNFα treatment in BDL mice decreased cardiac anandamide and NOx, reduced expression of NFκBp65, p38MAPK, and iNOS, enhanced expression of Cu/Zn-SOD and Mn-SOD, increased reductive glutathione and restored cardiomyocyte contractility. TNFα-depressed contractility was worsened by UCM707, whereas AM251 improved contractility.

Conclusions: Increased TNFα, acting via NFκB–iNOS and p38MAPK signaling pathways, plays an important role in the pathogenesis of cardiodepression in BDL mice. TNFα also suppressed contractility by increasing oxidative stress and endocannabinoid activity.

Introduction

In chronic liver disease, several factors including bacterial translocation and release of endotoxins stimulate the release of tumor necrosis factor-α (TNFα) and endocannabinoids to depress cardiac contractility [1–5]. Previous studies confirmed that cirrhotic cardiomyopathy is associated with markedly elevated cardiac levels of TNFα and endocannabinoids [5–8]. TNFα signaling is very complex. It activates many intracellular signaling pathways, including nuclear factor κB (NFκB) and three mitogen-activated protein (MAP) pathways, which include the extracellular-signal-regulated kinases (ERK), *c-Jun* N-terminal kinase (JNK), and p38 MAP kinase (p38MAPK) [9].

JNK and ERK are cardioprotective factors, while p38MAPK has negative effects on heart contractility [10,11]. Our recent study showed that inhibition of the NFκB activity improves the contractility of cirrhotic hearts [12]. NFκB activates transcription of inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO) and subsequently cGMP [9,13]. We previously showed that the iNOS–NO–cGMP pathway plays an important role in the development of cirrhotic cardiomyopathy [6].

It is known that TNFα increases endocannabinoid synthesis in macrophages [2]. However, the pathogenic mechanisms of increased endocannabinoids in the cholestatic heart have not been studied yet. We hypothesized that there are additive or synergistic effects on cardiac inhibition between endocannabinoids and TNFα in the heart of mice with cholestatic fibrosis.

Although evidence has suggested the possible roles of increased TNFα and endocannabinoids in the cirrhotic heart [5,8], the exact cellular mechanism of these factors in the development of cholestasis-induced cardiac dysfunction is not yet completely understood. The present study was therefore

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designed to (1) explore the pathophysiological roles of TNFα and its signaling pathways, including NFκB–iNOS, ERK, JNK, p38MAPK, and endocannabinoids, and (2) clarify the effects of TNFα in cholestasis-induced cardiac dysfunction by using a BDL-induced liver injury model in genetic TNFα-deficient mice, and wild-type mice receiving neutralizing TNFα antibody.

Materials and methods

TNFα gene knockout mice

The 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 22–24 g TNFα knockout (TNFα−/−, C57BL/6J-TNG tm1GK1) mice and age-matched C57BL/6J wild-type (WT) controls were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). The animals were maintained on a 12-h light/dark cycle under controlled temperature (18–21 °C) and humidity and they had free access to food and water. Mice were divided randomly into sham-operated control groups (sham) and bile duct ligation (BDL) groups. In total, 15 TNFα−/− mice (9 for BDL and 6 for sham-operation) and 53 TNFα+/+ (wild-type) mice (28 for BDL and 25 for sham-operation) were used.

Surgical procedures

Bile duct ligation was performed under sterile conditions as described previously [15]. Sham animals underwent the same surgery except bile duct ligation and section. Animals were studied two weeks after BDL or sham surgery. Previous studies showed that 4–6 weeks of BDL fail to induce cirrhosis in mice [16,17]. In our pilot studies, even 8 weeks of BDL failed to induce cirrhosis and markedly increased the mortality rates; thus the 2-week period was chosen for this study.

Chemical reagents

Anti-TNFα antibody was purchased from BioLegend Inc., (San Diego, CA, USA). UCM707 and AM251 were from Tocris Cookson Ltd. (Elisville, MO, USA). Primary antibodies (NFκBp65, JNK, p38MAPK, iNOS, Cu/Zn-SOD, and G3PDH) and secondary antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Other reagents were purchased from Sigma, Bio-Rad (Hercules, CA, USA), or Fisher Scientific (Pittsburgh, PA, USA).

Experimental groups

A total of six groups were studied. Two groups of TNFα knockout mice (TNFα−/−) were used; one group ($n = 9$) was subjected to bile duct ligation, while the other group ($n = 6$) was sham-operated. Four groups of TNFα wild-type (TNFα+/+) mice included: sham controls receiving IgG vehicle solution injections (sham-V, $n = 13$), BDL controls receiving vehicle (BDL-V, $n = 16$), sham receiving anti-TNFα antibody (sham-anti-TNFα, $n = 12$), and BDL receiving anti-TNFα antibody (BDL-anti-TNFα, $n = 12$). The rationale for using the anti-TNFα antibody was to neutralize the excessive amount of plasma TNFα in BDL mice. The anti-TNFα antibody 9 μg was injected i.p. every 4 days after surgery, for two weeks [14]. The same dose of mouse IgG (Sigma, Chemical) was given to BDL-V and sham-V mice serving as controls.

Hepatic fibrogenesis determination

Liver tissue was immediately fixed with 10% formalin in phosphate buffered saline (PBS). Samples were later embedded in paraffin and sectioned (3 μm). For the assessment of hepatic fibrosis, sections were mounted on glass slides and deparaffinised, then immersed for 10 s in saturated aqueous picric acid containing 0.1% Sirius Red F3BA (Polysciences Inc., Warrington, PA, USA), which selectively binds to collagenous proteins.

Cardiomyocyte contractility

Ventricular myocytes were isolated from murine hearts using the methods described previously [15]. Cell contraction and relaxation were assessed using a video sarcomere detector (IonOptix Corporation, Milton, MA, USA). Briefly, cardiomyocytes were placed in a Warner chamber mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan) and superfused (~1 ml/min at 25 °C) with a standard Tyrode solution and continuously gassed with 100% O_2. The cells within the recording chamber were continuously field stimulated at a rate of 1 Hz, and the contractile response to this stimulation was taken as baseline value. The cardiomyocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera. Myocytes were allowed to equilibrate to this stimulus for a minimum of 5 min before isoproterenol (10^{-5} M) stimulation. Calibrated output of the video sarcomere detector was digitized for off-line analysis. Peak cell shortening, maximal contraction, and relaxation velocities, time from basal length to 50% of peak contraction, and time from peak contraction to 50% of basal length in the diastolic phase were recorded and analysed.

Chronic effects of pharmacologic/genetic manipulation of TNFα on cardiomyocyte contractility

Isolated cardiomyocytes from BDL-V, sham-V, BDL-anti-TNFα, sham-anti-TNFα, BDL-TNFα−/−, and sham-TNFα−/− mice were measured for cell contraction and relaxation function ($n = 15$ in each group).

Acute effects of TNFα and cannabinoid antagonists, and reuptake inhibitors on cardiomyocytes

The acute effects of TNFα and the cannabinoid CB1 receptor antagonist AM251, and the reuptake inhibitor UCM707 on contractility were examined. Cardiomyocytes were incubated in storage solution (Tyrode solution containing 1.1 mM CaCl2 and bovine serum albumin 50 mg/ml, pH 7.4, at 30 °C) with vehicle (mouse IgG), TNFα (200 pg/ml), UCM707 (3×10^{-6} M), AM251 (10^{-6} M). In sham-V mice, cell contraction, and relaxation were measured after acute incubation with TNFα, TNFα + UCM707, AM251, and TNFα + AM251 for 30 min [18,19].

Measurement of plasma TNFα, cardiac nitrite/nitrate (NOx), and glutathione (GSH)

Plasma and hearts were collected from the mice two weeks after surgery. An ELISA assay kit for TNFα was purchased from Biosource (Camarillo, CA, USA). The results were expressed as pg/ml.

For cardiac nitrite/nitrate (NOx) measurement, hearts (50 mg) were homogenized in phosphate buffer saline (200 μL) and centrifuged at 4000*g* for 10 min. The supernatant was measured by commercial available ELISA kits (Cayman Chemical, Ann Arbor, MI, USA). The results were expressed as μmol/mg.

For glutathione detection, an ELISA kit was purchased from BioVision (Mountain View, CA, USA). The reduced cardiac glutathione (GSH) level was calculated as the difference between total glutathione and oxidized glutathione (GSSG). The results were expressed as μg/mg.

Western blot analysis

Protein expression of cardiac NFκBp65, JNK, p38MAPK, iNOS, and Cu/Zn-SOD were quantified as previously described [15,18,20]. G3PDH (glyceraldehydes-3-phosphate dehydrogenase) expression was used as an internal control.

Semi-quantitative RT-PCR of signaling pathways

Cardiac *TNFα*, NFκB*p65*, *p38MAPKβ*, *p38MAPKα*, and *ERK1* mRNA transcription was measured by semi-quantitative RT-PCR as in previous studies [15,18,20]. *G3PDH* served as an internal control. Primers synthesized by Gibco-BRL Life Technologies (Burlington, ON, Canada) are listed in Table 2.

Real-time RT-PCR of signaling pathways

Further quantification of the *iNOS*, *Cu/Zn-SOD*, and *Mn-SOD* mRNA levels were done by SYBR Green two-step real-time PCR. Sequences of the primers for *iNOS* and *G3PDH* are listed in Table 2. Real-time PCR reactions were carried out in a final volume of 25 μl of reaction mixture containing 10 ng of RNA, 12.5 μl of 2× SYBR Green Master Mix (Stratagene), 75 nM of each specific gene primer, and H2O. The samples were run in triplicate, and the mean value was used as the final expression value. A negative control without RNA template was run. Data were analyzed according to the relative standard curve method. CT values obtained for *iNOS* were first normalized with that of *G3PDH* prior to analysis. Fold-change in *iNOS*, *Cu/Zn-SOD*, and *Mn-SOD* mRNA was then calculated relative