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

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Abstract

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

Methods—BDL mice with TNF α knockout (TNF α –/–) and infusion of anti-TNF α antibody were used. Cardiac mRNA and protein expression of NF κ Bp65, *c-Jun*-N-terminal kinases (JNK), p38 mitogen-activated protein kinase (p38MAPK), extracellular-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 TNF α were measured. The effects of TNF α , 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 NF κ Bp65, p38MAPK, iNOS, NOx, anandamide, and plasma TNF α were increased, whereas glutathione, Cu/Zn-SOD, and Mn-SOD were decreased. Cardiac contractility was blunted in BDL mice. Anti-TNF α treatment in BDL mice decreased cardiac anandamide and NOx, reduced expression of NF κ Bp65, p38MAPK, and iNOS, enhanced expression of Cu/Zn-SOD and Mn-SOD, increased reductive glutathione and

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Conflicts of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

restored cardiomyocyte contractility. TNF α -depressed contractility was worsened by UCM707, whereas AM251 improved contractility.

Conclusions—Increased TNF α , acting via NF κ B–iNOS and p38MAPK signaling pathways, plays an important role in the pathogenesis of cardiodepression in BDL mice. TNF α also suppressed contractility by increasing oxidative stress and endocannabinoid activity.

Keywords

Cardiac dysfunction; Endocannabinoids; Tumor necrosis factor-alpha (TNF α); Nuclear factor-B (NF κ B); Inducible nitric oxide synthase (iNOS) 3

Introduction

In chronic liver disease, several factors including bacterial translocation and release of endotoxins stimulate the release of tumor necrosis factor- α (TNF α) and endocannabinoids to depress cardiac contractility [1–5]. Previous studies confirmed that cirrhotic cardiomyopathy is associated with markedly elevated cardiac levels of TNF α and endocannabinoids [5–8]. TNF α signaling is very complex. It activates many intracellular signaling pathways, including nuclear factor κ B (NF κ B) 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 NF κ B activity improves the contractility of cirrhotic hearts [12]. NF κ B 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 TNF α 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 TNF α in the heart of mice with cholestatic fibrosis.

Although evidence has suggested the possible roles of increased TNF α 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 designed to (1) explore the pathophysiological roles of TNF α and its signaling pathways, including NF κ B–iNOS, ERK, JNK, p38MAPK, and endocannabinoids, and (2) clarify the effects of TNF α in cholestasis-induced cardiac dysfunction by using a BDL-induced liver injury model in genetic TNF α -deficient mice, and wild-type mice receiving neutralizing TNF α antibody.

Materials and methods

TNF α 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 TNF α knockout (TNF α –/–, 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 TNF α $-/-$ mice (9 for BDL and 6 for sham-operation) and 53 TNF α $+/+$ (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-TNF α antibody was purchased from BioLegend Inc., (San Diego, CA, USA). UCM707 and AM251 were from Tocris Cookson Ltd. (Elisville, MO, USA). Primary antibodies (NF κ Bp65, 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 TNF α knockout mice (TNF α $-/-$) were used; one group ($n = 9$) was subjected to bile duct ligation, while the other group ($n = 6$) was sham-operated. Four groups of TNF α wild-type (TNF α $+/+$) mice included: sham controls receiving IgG vehicle solution injections (sham-V, $n = 13$), BDL controls receiving vehicle (BDL-V, $n = 16$), sham receiving anti-TNF α antibody (sham-anti-TNF α , $n = 12$), and BDL receiving anti-TNF α antibody (BDL-anti-TNF α , $n = 12$). The rationale for using the anti-TNF α antibody was to neutralize the excessive amount of plasma TNF α in BDL mice. The anti-TNF α antibody 9 μ g 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 μ m). 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 TNF α on cardiomyocyte contractility

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

Acute effects of TNF α and cannabinoid antagonists, and reuptake inhibitors on cardiomyocytes

The acute effects of TNF α 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), TNF α (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 TNF α , TNF α + UCM707, AM251, and TNF α + AM251 for 30 min [18,19].

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

Plasma and hearts were collected from the mice two weeks after surgery. An ELISA assay kit for TNF α 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 μ L) and centrifuged at 4000g for 10 min. The supernatant was measured by commercial available ELISA kits (Cayman Chemical, Ann Arbor, MI, USA). The results were expressed as μ mol/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 μ g/mg.

Western blot analysis

Protein expression of cardiac NF κ Bp65, 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 TNF α , NF κ Bp65, p38MAPK β , p38MAPK α , 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 μ L of reaction mixture containing 10 ng of RNA, 12.5 μ L of 2 $\times$ 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 to control (i.e. sham-V) using the comparative CT method as described in the Applied Biosystems User Bulletin #2.

Measurement of cardiac endocannabinoid levels

Mice were euthanized, their hearts were excised, and the lipids were extracted. Cardiac levels of anandamide (AEA) and 2-archidonoyl glycerol (2-AG) were quantified by liquid chromatography/in line mass spectrometry by using an Agilent 1100 series LC-MSD, equipped with a thermostat autosampler and column compartment [5]. Values are expressed as fmol or pmol per mg of wet tissue.

Statistical analysis

All statistical analyses were performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). The results are expressed as means $\pm$ SEM. An unpaired Student's *t*-test was used to compare the differences between two groups, and for more than two groups, comparisons were analyzed by one-way ANOVA followed by Newman-Keuls *post hoc* test. A *p*-value of <0.05 was considered to be significantly different.

Results

Hepatic and cardiac histology

BDL-V livers compared with those of sham-V mice showed (Fig. 1A), significantly increased collagen deposition and bile duct proliferation in periportal areas (Fig. 1B), organization of fibrotic tissue (Fig. 1C), and mild periportal bridging (Fig. 1D). In contrast, the hearts of BDL-V mice appeared normal and were indistinguishable from sham-V mice on routine light microscopy (not shown).

Chronic effects of pharmacologic/genetic manipulation of TNF α on cardiomyocyte contractility

Compared with sham controls, the maximum systolic and diastolic velocities of cardiomyocytes from BDL mice were significantly reduced. Time to half-peak contractions (TP50) and half-complete relaxations (TR50) were significantly prolonged (Fig. 2). Anti-TNF α antibody significantly improved systolic and relaxation velocities (Fig. 2). Compared with basal conditions, the mean changes in maximal contraction velocity were not significantly different from maximal relaxation velocity as a result of anti-TNF α antibody administration (25.1 ± 2.5 vs. $22.0 \pm 8.9\%$). The results of cardiac function in anti-TNF α -treated BDL mice were similar to those in TNF α knockout mice (BDL-TNF $\alpha^{-/-}$). Neither anti-TNF α antibody nor TNF α knockout affected the contractility/relaxation from the sham groups.

Acute effects of TNF α and cannabinoid antagonists and reuptake inhibitors on cardiomyocytes

TNF α incubation with cardiomyocytes from sham mice significantly decreased systolic and relaxation velocities and prolonged TP50 and TR50. Co-incubation of AM251 (CB1 antagonist) and TNF α with cardiomyocytes from sham mice did not impact the contractile and relaxation velocities (Fig. 3). However, the endocannabinoid reuptake inhibitor UCM707 further enhanced the TNF α depressive effect on contractile and relaxation velocities, whereas AM251 partially improved TNF α -inhibited cardiac function.

Plasma TNF α , cardiac anandamide, 2-AG, NOx, and glutathione (GSH) levels

Compared with sham mice, BDL mice showed higher plasma TNF α , cardiac anandamide, NOx, and lower total and reduced glutathione (Table 1). Anti-TNF α treatment significantly decreased plasma TNF α , cardiac anandamide, NOx, and increased total and reduced glutathione in BDL mice. In BDL-TNF α ^{-/-} mice, cardiac anandamide and NOx, and total and reduced glutathione were lower compared to BDL wild-type mice. However, there was no difference in cardiac 2-AG, NOx, and total and reduced glutathione between sham and sham-anti-TNF α mice (Table 1).

Western blot analysis of signaling pathways

Cardiac NF κ Bp65, p38MAPK, and iNOS were significantly increased and Cu/Zn-SOD was significantly decreased in BDL-V mice (Fig. 4). In anti-TNF α treated BDL and BDL-TNF α ^{-/-} mice, lower NF κ Bp65, p38MAPK, and iNOS; and higher Cu/Zn-SOD protein expression than in BDL-V mice were observed (Fig. 4). However, cardiac JNK expression was not different between any groups of mice (data not shown).

Semi-quantitative RT-PCR of signaling pathways

In contrast to the increase in cardiac *TNF α* mRNA in BDL-V mice, the genotype of the TNF α knockout mice was confirmed by non-detectable cardiac *TNF α* mRNA in BDL-TNF α ^{-/-} mice (Fig. 5A). Compared with the sham group, mRNA expression of cardiac *NF κ Bp65*, *p38MAPK α* , and *p38MAPK β* was significantly increased in BDL-V mice (Fig. 5B–D). Cardiac mRNA transcription of *NF κ Bp65*, *p38MAPK α* , and *p38MAPK β* was significantly lower in anti-TNF α treated BDL and BDL-TNF α ^{-/-} mice compared with the BDL-V group (Fig. 5B–D). Cardiac *ERK1* mRNA expression was not different between BDL-V and sham-V mice (data not shown).

Real-time RT-PCR of signaling pathways

Parallel with the increased cardiac iNOS and Cu/Zn-SOD proteins in BDL-V mice; cardiac *iNOS*, *Cu/Zn-SOD*, and *Mn-SOD* mRNA, measured by real-time RT-PCR, was increased in these animals. Anti-TNF α antibody-treated and TNF α knockout BDL mice showed lower expression of these mRNAs (Fig. 6).

Discussion

The mechanisms underlying cardiac dysfunction in chronic liver disease are only partially known but previous work has suggested a role for increased circulating TNF α and endocannabinoids [8,12]. TNF α , in particular, is thought to be a key mediator of cardiodepression in chronic liver disease [1,4,12,19]. In the current study, pharmacologic/genetic manipulation of TNF α was performed in BDL mice due to availability of genetic TNF α knockout mice as well as anti-TNF α antibody-treated BDL mice. The anti-TNF α antibody we used was able to significantly neutralize the excessive TNF-alpha levels in BDL-V rats. Most importantly, we sought to determine whether TNF α and endo-cannabinoids are involved in the cardiac dysfunction in a murine model of fibro-cholestatic liver disease. In the present study, BDL induced: fibrosis, ductular proliferation, and architectural damage, as previously observed [16,17]; however, the damage stopped short of cirrhosis. In terms of functional depression of cardiac contractility, this BDL mouse model showed similar changes to the chronic BDL rat model which has an established biliary cirrhosis. This suggests that cirrhosis *per se* is not required to induce cardiac dysfunction—the presence of a moderate degree of liver damage may be enough. Moreover, cholestasis by itself can also induce cardiac dysfunction [21–23], and this may contribute to the overall pathogenesis of cirrhotic cardiomyopathy.

The diverse and numerous signaling pathways of TNF α examined in this study make our paper difficult to cleanly interpret but in our opinion, were unavoidable. Focusing on a single pathway would likely have led to a falsely oversimplified conclusion of the signaling pathways by which this key molecule mediates its effects on the cirrhotic heart. The results of the present study support the hypothesis that TNF α , through the NF κ B–iNOS and p38MAPK signaling pathways, plays an important pathogenic role in the development of cholestasis-induced cardiac dysfunction. TNF α elimination, either by genetic deletion or by antibody neutralization, reduced the activities of the NF κ B–iNOS and p38MAPK signaling pathways in BDL mice hearts and improved cardiomyocyte contractility. Isolated cardiomyocytes were used in our study to exclude other confounding effects such as the autonomic nervous system and the peripheral vascular tone.

In the present study, acute incubation of TNF α slowed contraction and relaxation velocities in sham cardiomyocytes to values similar to BDL mice. Moreover, we found that acute and chronic pharmacological inhibition of endogenous TNF α by anti-TNF α antibody and genetic deletion of TNF α , corrected the contraction and relaxation dysfunction in isolated cardiomyocytes from BDL mice.

Activated NF κ B combines with the TNF α promoter to increase TNF α expression and subsequently trigger NF κ B activity [24]. Our previous study had demonstrated that inhibition of the NF κ B activity was associated with a decrease in cardiac TNF α levels and an improvement of cardiomyocyte contractility in cirrhotic rat hearts [12]. The current study showed that over-expression of *TNF α* mRNA levels was accompanied by activation of NF κ B in the BDL mouse heart. In concordance with our previous study, the anti-TNF α -related down-regulation of NF κ B was associated with the improvement of cardiomyocyte contractility in our BDL mice [12].

It has been reported that endotoxemia-induced myocardial dysfunction is caused by the release of cardiodepressants such as NO and cGMP [6,25,26]. We recently showed that the synthesis of NO is increased in the cirrhotic heart [7]. In the present study, the increased plasma level of TNF α was associated with a significant elevation of cardiac NO $_x$ levels in BDL mice. Furthermore, genetic deletion and pharmacological inhibition of endogenous TNF α decreased NO $_x$ levels in BDL mice, and this was accompanied by correction of cardiomyocyte contractile dysfunction. ERK1/2 and JNK had been reported to be involved in the pathogenesis of cardiac 16 hypertrophy [27]. However, there was no difference in cardiac *JNK* and *ERK1* mRNA and protein expression between normal and BDL mice. These results indicated that the JNK and ERK pathways play little or no role in the cardiac dysfunction in our BDL mice. Among four isoforms of p38 (p38 α , p38 β , p38 δ , and p38 γ), p38 α and p38 β are thought to be primarily responsible for regulating inflammation [10,11]. In our current study, mRNA and protein expression of cardiac p38 α and p38 β MAPK were higher in BDL mice than in sham controls. Meanwhile, inhibition of endogenous TNF α suppressed the p38MAPK pathway in BDL mice. Previous studies indicated that activation of p38MAPK, by triggering NF κ B–iNOS signaling pathways, blunts β -adrenergic response to cardiovascular stress, which is the main feature of cirrhotic cardiomyopathy [10,27–29]. In our BDL mice, activation of the p38 pathway might similarly up-regulate the NF κ B–iNOS pathway and contribute to myocardial dysfunction. Our observation that the anti-TNF α -related correction of BDL cardiodepression was associated with the suppression of the NF κ B–iNOS and p38MAPK pathways is consistent with this notion.

TNF α induces oxidative stress in adult rat cardiomyocytes and antioxidant (*N*-acetylcysteine) treatment prevents the depressive effect of TNF α on contraction [30]. Both Cu/Zn-SOD (cytosolic) and Mn-SOD (mitochondrial) are critical determinants in the protection of the heart from oxidative injury [31]. In this study, we estimated the cardiac antioxidative status by

measuring the total, reduced, and oxidized glutathione levels, Cu/Zn-SOD, and Mn-SOD activity. We found that antioxidants were significantly decreased in BDL mice. Moreover, inhibition of endogenous TNF α significantly increased the antioxidants (SOD and glutathione) in BDL hearts.

The mechanism of contractile dysfunction in response to endotoxins and/or oxidative stress is complex. For example, endotoxins and reactive oxygen species *per se* may activate NF κ B and iNOS, and not only through TNF α . This may explain why the pharmacological inhibition of endogenous TNF α in our study only partially restored TNF α -induced cardiomyocyte dysfunction.

We also observed that higher plasma TNF α was accompanied by elevated cardiac levels of anandamide in BDL mice. Moreover, anandamide levels were reduced in anti-TNF α -treated BDL and TNF α knockout mice. However, the detailed mechanisms of local production of endocannabinoids and its interaction with the TNF α –NF κ B pathway remain unclear. In our study, TNF α -related cardiomyocyte dysfunction was aggravated by simultaneous incubation of the endocannabinoid reuptake inhibitor UCM707 and TNF α in sham cardiomyocytes. What are the possible mechanisms for TNF α to release endocannabinoids in the cirrhotic heart? Previous studies found that macrophages, in response to endotoxin, secrete TNF α which further increases oxidative stress and activates the endocannabinoid system [2,3,31,32]. Our results, combined with previous studies, imply that neutralizing TNF α relieves cardiac oxidative stress and subsequently suppresses the local release of endocannabinoids in fibro-cholestatic hearts (Fig. 7).

The activation of cannabinoid CB1 receptors decreases cardiac response to β -adrenergic agonists [5,8]. Parallel to the inhibition of NF κ B activity, CB1 receptor antagonists improve cardiac function in cirrhotic rats [5,8,19]. In order to confirm whether TNF α enhances the release of endocannabinoids, sham cardiomyocytes were incubated with TNF α to mimic TNF α -induced BDL cardiac dysfunction in our study. Although direct incubation of CB1 receptor antagonist (AM251) did not affect the response of cardiomyocytes, concomitant incubation of AM251 and TNF α partially reversed the TNF α -induced blunted response to the β -adrenergic agonist isoprenaline on cardiomyocytes. These experiments suggest that increased local release of endocannabinoids might be partially responsible for the TNF α -induced β -adrenergic hypo-responsiveness in the fibro-cholestatic heart.

In conclusion, increased TNF α , acting via NF κ B–iNOS and p38MAPK signaling pathways, plays an important role in the pathogenesis of cholestasis-induced cardiac dysfunction. Additionally, TNF α -accentuated endocannabinoid action and oxidative stress might also be involved in its negative inotropic effects in fibro-cholestatic hearts. Accordingly, inhibition of TNF α may be potentially therapeutically useful to restore good cardiac contractility in patients with liver disease.

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Fig. 1. Sirius red staining of liver tissue of sham mice (A) and BDL mice (B–D)
Single-head arrow shows periportal bile duct proliferation and collagen deposition. Double-head arrow shows fibrotic nodules.

Fig. 2. Chronic inhibition of TNF α in isolated cardiomyocytes (A) maximal contraction velocity, (B) time from basal length to 50% of peak contraction, (C) maximal relaxation velocity, and (D) time from peak contraction to 50% of basal length in the diastole
 BDL/sham-V: BDL or sham mice receiving vehicle; BDL-anti-TNF α /sham-anti-TNF α : BDL or sham mice receiving chronic anti-TNF α antibody; BDL-TNF α −/−: TNF α knockout BDL mice.

Fig. 3. Effect of acute incubation of TNF α , cannabinoid receptor blocker (AM251), and endocannabinoid reuptake inhibitor (UCM707) on cardiomyocytes
 (A) Maximal contraction velocity, (B) time from basal length to 50% of peak contraction, (C) maximal relaxation velocity, and (D) time from peak contraction to 50% of basal length in diastole in sham-V (sham mice receiving vehicle).

Fig. 4. Cardiac NFκBp65, p38MAPK, and iNOS significantly increased and Cu/Zn-SOD significantly decreased in BDL-V mice

Top: representative Western blot of cardiac (A) NFκBp65, (B) iNOS, (C) p38MAPK, and (D) Cu/Zn-SOD. Bottom: densitometry analysis of Western blot for cardiac NFκBp65, JNK, and P38MAPK (compared to control protein G3PDH). BDL-V: BDL mice receiving vehicle; BDL-anti-TNFα: BDL mice receiving chronic anti-TNFα antibody; BDL-TNFα^{-/-}: TNFα knockout BDL mice.

Fig. 5. Genotype of $TNF\alpha$ knockout mice confirmed by non-detectable cardiac $TNF\alpha$ mRNA in BDL- $TNF\alpha^{-/-}$ mice

Top: representative mRNA levels of (A) $TNF\alpha$, (B) $NF\kappa Bp65$, (C) $p38MPAK\alpha$, and (D) $p38MAPK\beta$ (by semi-quantitative RT-PCR) in all mice. Bottom: densitometry analysis of mRNA levels of $TNF\alpha$, $NF\kappa Bp65$, $p38MPAK\alpha$, and $p38MAPK\beta$ (compared to $G3PDH$). BDL-V: BDL mice receiving vehicle. BDL-anti- $TNF\alpha$: BDL mice receiving chronic anti- $TNF\alpha$ antibody. BDL- $TNF\alpha^{-/-}$: $TNF\alpha$ knockout BDL mice.

Fig. 6. Real-time PCR for mRNA expression (normalized with G3PDH) of (A) iNOS, (B) Cu/Zn-SOD, and (C) Mn-SOD

Data are expressed as percentage change ($\Delta/\%$) and the sham-V is taken as 100%. BDL-V: BDL mice receiving vehicle. BDL-anti-TNF α : BDL mice receiving chronic anti-TNF α antibody. BDL-TNF α -/-: TNF α knockout BDL mice.

Fig. 7. Schematic representation of TNF α -induced cardiac dysfunction

Table 1

Levels of TNF α , endocannabinoids, nitrites/nitrates (NOx), total glutathione, oxidized glutathione, and reduced glutathione.

Test	BDL-V	BDL-anti-TNF α	BDL-anti-TNF α -/-	Sham-V	Sham-anti-TNF α	Sham-TNF α -/-
Plasma TNF α level (pg/ml)	244.19 $\pm$ 21.93*	132.23 $\pm$ 19.73 [†]	–	85.88 $\pm$ 9.74	82.98 $\pm$ 8.51	81.76 $\pm$ 11.21
Cardiac anandamide (fmol/mg)	14.5 $\pm$ 3.6*	7.9 $\pm$ 2.9 [†]	6.4 $\pm$ 3.1 [†]	5.9 $\pm$ 1.9	–	–
Cardiac 2-arachidonoylglycerol (fmol/mg)	0.8 $\pm$ 0.09	1.1 $\pm$ 0.02	1.2 $\pm$ 0.07	0.7 $\pm$ 0.04	–	–
Cardiac NOx (μ mol/mg)	5.71 $\pm$ 0.47*	4.44 $\pm$ 0.54 [†]	4.08 $\pm$ 0.67 [†]	3.65 $\pm$ 0.49	3.70 $\pm$ 0.84	3.91 $\pm$ 0.99
Cardiac total glutathione (tGSH, μ g/mg)	7.25 $\pm$ 0.91*	9.83 $\pm$ 0.83 [†]	10.25 $\pm$ 1.04 [†]	12.73 $\pm$ 1.26	11.6 $\pm$ 1.03	10.9 $\pm$ 2.15
Cardiac oxidized glutathione (GSSG, μ g/mg)	0.68 $\pm$ 0.09	0.84 $\pm$ 0.02	0.85 $\pm$ 0.05	0.90 $\pm$ 0.08	0.84 $\pm$ 0.06	0.96 $\pm$ 0.09
Ratio of cardiac tGSH/GSSG (%)	9.37	8.54	8.29	7.06	7.27	6.99
Reduced cardiac glutathione (rGSH, μ g/mg)	6.56 $\pm$ 1.12*	8.99 $\pm$ 1.25 [†]	9.41 $\pm$ 1.09 [†]	11.83 $\pm$ 1.19	10.75 $\pm$ 1.36	12.05 $\pm$ 2.1

BDL/sham-V: BDL or sham mice receiving vehicle treatment. BDL-anti-TNF α /sham-anti-TNF α : BDL or sham mice receiving chronic anti-TNF α antibody treatment. BDL-TNF α -/-: TNF α knockout BDL mice.

* p < 0.05 vs. sham-V.

[†] p < 0.05 vs. BDL-V.

Table 2

Primer sequences for all genes.

Gene name	Sequences of primers
Tumor necrosis factor-alpha (<i>TNFα</i> , 411 bp)	5'-AGCCCCCAGTCTGTATCCTT-3' (sense); 3'-CTCCCTTTGCAGAACTCAGG-5' (antisense)
Nuclear factor kappa B p65 (<i>NFκBp65</i> , 245 bp)	5'-GGCCTCATCCACATGAACTT-3' (sense); 3'-CACTGTCACCTGGAAGCAGA-5' (antisense)
p38 mitogen-activated protein kinase-beta (<i>p38MAPKβ</i> , 250 bp)	5'-CGTGTACCTGGTGACCCTCT-3' (sense); 3'-AGCCACGTAGCCTGTCATCT-5' (antisense)
p38 mitogen-activated protein kinase-alpha (<i>p38MAPKα</i> , 450 bp)	5'-ACAAGACTGCCTGGGAGCTA-3' (sense); 3'-CCAATGACGTTCTCATGGTG-5' (antisense)
Extracellular-signal-regulated kinase-1 (<i>ERK1</i> , 209 bp)	5'-TCCAAGGGCTACACCAAATC-3' (sense); 3'-TCCAAGGGCTACACCAAATC-5' (antisense)
Inducible nitric oxide synthase (<i>iNOS</i> , 351 bp)	5'-AATGAGGTACTCAGCGTGCT-3' (sense); 3'-TCGCTCCTCGTCCACCTTCT-5' (antisense)
Copper/zinc-superoxide dismutase (<i>Cu/Zn-SOD</i> , 852 bp)	5'-GCGGTGAACCAGTTGTGTTGTC-3' (sense); 3'-CCTCTGGACCCGTTACACTGAC-5' (antisense)
Magnesium-superoxide dismutase (<i>Mn-SOD</i> , 356 bp)	5'-ATGTTACAACCTCAGGTCGCTCTTC-3' (sense); 3'-CCTCTCAACGACCTCCGATAGT-5' (antisense)
Glyceraldehyde-3-phosphate dehydrogenase (<i>G3PDH</i> , 139 bp)	5'-TATCGGACGCCTGGTTAC-3' (sense); 3'-CGTTCAAGTTGCCGTGTC-5' (antisense);