Fas Receptor Activation by Endogenous Opioids Is A New Mechanism for Cardiomyopathy in Cirrhotic Rats

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Background: Cirrhosis, a common consequence of chronic liver inflammation is associated with various cardiovascular dysfunctions which are called cirrhotic cardiomyopathy (CC). Among the various possible causes of CC, apoptosis is considered to have a pivotal role. **Objectives:** To explore the contribution of endogenous opioids in the apoptosis process in a rat model of CC. **Material and methods:** Four genes were selected to cover both intrinsic and extrinsic pathways of apoptosis. Cardiac samples from 4 groups of rats were evaluated. Two groups were cirrhotic through bile duct ligation (BDL) receiving either naltrexone (BDL-naltrexone) or saline (BDL-saline), two others were normal rats as sham groups receiving either naltrexone (sham-naltrexone) or saline (sham-saline). Expression level of BCL2, Caspase3, Fas and FasL was explored in all groups using reverse transcriptase real-time PCR. **Results:** BDL-saline group showed significant over-expression of BCL2, caspase3 and Fas. BCL2 expression was 1.44 ($P < 0.001$) and caspase3 was 1.35 ($P < 0.001$) times higher than sham-saline group, Fas was also overexpressed 1.3 ($P < 0.001$) times higher than BDL-naltrexone group and 1.91 ($P < 0.001$) compared to sham-naltrexone group. Caspase3 expression was 1.35 ($P < 0.001$) folds higher than sham-naltrexone group. The expression pattern of FasL revealed no statistically significant change among study groups. **Conclusion:** Fas molecule enrollment during CC is a novel finding. Fas molecule is activated during cirrhosis through elevated levels of endogenous opioids. This pathway is one of the leading causes of CC. Our findings also demonstrated the protective role of naltrexone as opioids antagonist on cardiomyocytes in a rat model of CC. (J CLIN EXP HEPATOL 2017;7:107–114)

Apoptosis, or programmed cell death is a type of cell death which is not modulated by inflammation and is triggered either by the death receptor (extrinsic pathway through Fas ligand) or mitochondria (intrinsic pathway). It is known that changes in apoptosis pathway are responsible for some important medical diseases including some lymphoma subtypes, cardiomyopathies and cirrhosis.¹ Cirrhosis is a common consequence of chronic liver inflammation characterized by replacement of liver tissue by fibrous scar and regenerative tissue, leading to a progressive loss of liver function.² Cardiovascular dysfunction is a major complication of cirrhosis which includes

increased cardiac output, decreased arterial pressure and total peripheral resistance, systolic incompetence, diastolic dysfunction, and electrophysiological abnormalities in the absence of any known cardiac disease. These abnormalities are named cirrhotic cardiomyopathy (CC).^{3–5} Abnormalities in β -adrenergic signaling pathway, altered cardiomyocyte membrane fluidity and several mediators such as nitric oxide (NO) and carbon monoxide are involved in the pathogenesis of CC.³

Recently, the contribution of endogenous opioids system in impaired cardiac contractility in CC is demonstrated. Additionally, inhibiting opioid system has pharmacologically corrected bradycardia and reduced chronotropic and inotropic response to both α and β -adrenergic agonists in cholestatic rats.^{6,7} Moreover, endogenous opioids can induce apoptosis in hepatocytes during liver cholestasis, and reducing liver antioxidant defense system is hypothesized as the main mechanism through which increased level of opioid system leads to hepatocytes damage and apoptosis.⁸ Previously we tried to evaluate the effect of opioids on cardiomyocyte apoptosis in a rat model of CC.⁹ We found increased amount of apoptotic cells in

Keywords: cirrhotic cardiomyopathy, apoptosis, opioid, Fas

Received: 29.06.2016; Accepted: 12.10.2016; Available online: 17 October 2016

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Abbreviations: BDL: bile duct ligation; CC: cirrhotic cardiomyopathy; H&E: Hematoxylin and Eosin; MAPKs: mitogen-activated protein kinases; NO: nitric oxide; qRT-PCR: Real-Time Reverse Transcription PCR

<http://dx.doi.org/10.1016/j.jceh.2016.10.002>

bile duct ligated rats by immunohistochemical method which was decreased using naltrexone as an opioid antagonist.

In order to confirm our previous findings, and to find the main apoptotic pathway (intrinsic versus extrinsic) involved in the process, this time we tried to evaluate the effect of endogenous opioids in molecular level. So, here we evaluated the nitrogen-kept samples of the same formalin-kept specimens of our previous study to evaluate the molecular pathways of apoptosis. We tried to identify the molecular pathways through which apoptosis was mediated by opioids in CC.

MATERIALS AND METHODS

Specimens

We previously⁹ showed that opioid blockade using naltrexone can reduce apoptosis in CC. Briefly, we created a rat model of cirrhosis by ligating rats' bile ducts (BDL) for 4 weeks—during which cirrhosis took place according to various studies in literature.^{10–12} We examined four groups of rats each containing six rats treated as following: BDL-naltrexone (group A): six rats which underwent bile duct ligation (BDL) and then treated by subcutaneous naltrexone injection for 29 ± 1 days. Sham-naltrexone (group B): six rats which treated by naltrexone as like as the first group without BDL. BDL-saline (group C): six rats which underwent BDL and treated by normal saline for the same time-period as group A. Sham-saline (group D): six rats treated by normal saline for 29 ± 1 days. After treatment, the rats were sacrificed and samples from various organs were obtained both in formalin solution and liquid nitrogen for further histologic and molecular studies, respectively. Formalin-kept ventricular samples were evaluated in our previous study. Presence of cirrhosis was documented using pathologic evaluation which showed marked fibrosis and nodule formation within liver samples of BDL rats (10), [Figure 1](#). Cardiomyopathy was confirmed by evaluating left ventricular wall thickness which significantly decreased LV wall thickness was observed in BDL rats, [Table 1](#). Pathologic evaluation to confirm cardiomyopathy was also performed using Hematoxylin and Eosin (H&E)

Figure 1 Showing fibrotic bundles and nodule formation with cholestasis in a cirrhotic liver of bile duct ligated rat.

and Masson's trichrome staining methods which showed attenuated, stretched and irregular cardiomyocytes in H&E staining and presence of focal fibrosis in Masson's trichrome staining which are evidences of cardiomyopathy,¹³ [Figure 2a](#) and [b](#). Finally, we found increased amount of apoptotic cells in bile duct ligated rats by immunohistochemical method. We also found that endogenous opioids blockade using naltrexone had decreased apoptosis density in BDL rats ([Figures 3a](#) and [b](#) and [4](#)). In the present study the liquid nitrogen-kept samples from our previous study were examined for molecular analysis. We analyzed the expression levels of FASL, FAS (extrinsic apoptotic pathway genes), BCL2 (mitochondrial apoptotic pathway gene) and caspase3 (active executioner) by qRT-PCR in four above mentioned experimental groups, A, B, C and D. Expression level of these 4 genes was compared in the studied groups.

RNA Extraction and cDNA Synthesis

Rat ventricular myocardium total RNA was extracted using TRIzol reagent (Invitrogen, Inc.) following the manufacturer's instructions. Quantity and quality assessments were determined by NanoDrop_ND-1000 UV-Vis

Table 1 Left Ventricular Thickness in Studied Groups.

Groups	Subgroups	Left ventricular thickness (mm)	P value
BDL	Naltrexone (group A)	2.75 ± 0.14	0.1
	Saline (group C)	2.26 ± 0.26	
Sham	Naltrexone (group B)	3.8 ± 0.2	0.3
	Saline (group D)	3.6 ± 0.1	
BDL	A + C	2.5 ± 0.16	<0.001
	B + D	3.75 ± 0.1	

BDL: bile duct ligation.

P value under 5% is considered as significant. The differences between groups A and B, A and D, C and B and C and D are also significant.

Figure 2 (a) Mason's trichrome stain showing collagen bundles within cardiac sample of bile duct ligated (BDL) rat. (b) Showing no fibrotic bundle in a normal control (sham group) cardiac tissue.

Spectrophotometer (Nano-Drop® Technologies). 1000 ng total RNA was subjected for cDNA synthesis using AccuPower® RT Pre Mix (Bioneer, Korea).

Real Time Quantitative RT-PCR

Gene expression was investigated with the Real-Time Reverse Transcription PCR (qRT-PCR), using SYBR GreenI TAKARA in duplicate and the Rotor-Gene™ 6000 (Corbett Life Science™, Germany) under following conditions: 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, elongation at 72 °C for 20 s and 60–95 °C, ramp 1 °C/s intervals (melt temperature curve). The specificity of the PCR products was confirmed by gel analysis, and products were shown to consist of only a single band. Real-time PCR was carried out in a volume of 25 µl containing: 12.5 µl PCR Master

Figure 3 (a) Showing apoptotic cells (arrow) in immunohistochemical (IHC) staining in cardiac tissue. (b) Showing IHC staining of a control cardiac tissue.

Mix, 2 µl of cDNA which had been diluted 5 times (200 ng/µl), 1 µl mixed forward and reverse primers (10 pmol/µl) and 9.5 µl nuclease-free water. To determine the maximum efficiency of each primer pairs, serial dilution of mixed primers (mixed forward and reverse concentration = 10 pmol/µl) by 0.5, 1, 1.5 and 2 µl was performed and 0.5 µl for each primer was considered as the best efficiency. Using gradient PCR prior to qPCR, optimum annealing temperatures were determined.

Considering the importance of proper housekeeping gene(s) which is assumed to be invariant between the compared experimental groups for normalization of expression of target genes, here we used two housekeeping genes, POLR2A and RPL32 to evaluate the expression of our studied genes in rat myocardium.¹⁴ It is expected that by using 2 reference genes, the relatively large errors of conventional use of a single gene for normalization will be minimized. The sequences of primers are listed in Table 2.

Figure 4 Shows mean number of apoptotic cells in different groups. Specifically stained slides for apoptosis were analyzed by counting apoptotic cells in ten representative high power fields. There were a markedly increased number of apoptotic cells after bile duct ligation. The amount of apoptotic cells also showed decrease after naltrexone injection in BDL rats. BDL: bile duct ligation, ntx: naltrexone.

Statistical Analysis

Relative expression data were analyzed by LinReg 11 (to determine the individual amplification efficiency and cycle threshold for each reaction tube) and REST 2009 which uses geometric mean of multiple selected housekeeping genes (to calculate expression levels by analyzing output of data obtained from LinReg 11).¹⁵

RESULTS

In this study, we analyzed the expression levels of FASL, FAS, BCL2, and caspase3 by qRT-PCR in four experimental groups, A (BDL-naltrexone), B (sham-naltrexone),

C (BDL-saline), and D (sham-saline). Results are summarized in [Tables 3 and 4](#).

Fas Expression Analysis

Expression analysis within our two main groups which had BDL including groups C and A showed that rats categorized in group C (receiving normal saline) had significant over-expression of Fas molecule. Fas was overexpressed 1.3 times higher than group A ($P < 0.001$), [Figure 5](#). Also group C showed significant over expression of Fas which was 1.91 times ($P < 0.001$) higher than group B. No significant difference was seen in expression level of Fas between other studied groups.

Bcl2 Expression Analysis

Our results showed that Bcl2 had over-expression in group C, 1.44 times higher, in comparison to group D (control group) ($P < 0.001$). No significant difference was seen in expression level of Bcl2 between other studied groups.

Caspase 3 Expression Analysis

Expression analysis showed 1.3 folds upregulation of caspase3 in group C compared to group B ($P < 0.001$) and 1.35 folds in group C compared to group D ($P < 0.001$). There was no significant difference between expression levels of caspase3 in other studied groups.

FasL Expression Analysis

Expression analysis revealed no difference in expression level of FasL in the studied groups. An example of melting curves of studied genes is shown in [Figure 6](#).

DISCUSSION

In the current study, we showed that endogenous opioids activated Fas mediated apoptosis pathway in

Table 2 Summary of the Selected Genes Used for qPCR, and Amplicon Characteristic.

Primers		Sequences 5 → 3	Amplicon length (bp)	T _m (°C)
BCL2	F	AGAGCGTCAACAGGGAGATG	185 bp	60
	R	GAGCAGCGTCTTCAGAGACAG		
Caspase 3	F	TGGCCCTGAAATACGAAGTC	235	60
	R	AGTTTCGGCTTTCCAGTCAG		
FAS	F	ACCTGGTGACCCTGAATCTG	231	60
	R	TGATACCAGCACTGGAGCAG		
FASLG	F	ACTCCGTGAGTTCACCAACC	234	60
	R	ACGAAGTACAACCCAGCCTC		
POLR2A	F	CAAGATGCAGGAGGAGGAAG	232	60
	R	CATCAGGCTCACACCATCTG		
RPL32	F	AGAAGTTCATCAGGCACCAG	202	60
	R	AGCTCCTTGACATTGTGGAC		

Table 3 Results of the Gene Expression Analysis for BCL2, CASPASE3, FAS and FASL in Four Studied Groups.

Studied groups	BCL2	CASPASE3	FAS	FASL
C sample/A control				
AGR	1.14	1.12	1.3	1.15
SE	±1.0775	±0.95233	±0.46782	±1.08102
P value	0.42	0.60	<0.001	0.24
B sample/D control				
AGR	1.24	1.04	1.15	1.02
SE	±0.612	±0.661	±0.595	±0.501
P value	0.26	0.74	0.11	0.74
C sample/D control				
AGR	1.44	1.35	6.24	1.1
SE	±0.709	±0.887	±0.691	±0.657
P value	<0.001	0.001	0.183	0.215
A sample/D control				
AGR	1.21	0.74	1.94	1.13
SE	±0.372	±0.391	0.421	±0.478
P value	0.68	0.66	0.578	0.768
C sample/B control				
AGR	1.00	1.3	1.91	1.05
SE	±0.51523	±0.40088	±0.59248	±1.33365
P value	0.91	<0.001	0.024	0.269

AGR absolute gene regulation, indicating the fold change in gene expression, SE standard error. A P value <0.05 was considered statistically significant. Groups A, B, C and D indicate rats which are BDL-naltrexone, sham-naltrexone, BDL-saline and sham-saline, respectively.

Table 4 Real Time Analysis of the BCL2, Caspase3, Fas and FasL Gene Expression in the Studied Groups A, B, C and D. The Significant Differences Are Highlighted. P Value < 0.05 Is Considered As Significant.

	BCL2	Caspase3	FAS	FASL
C s/A c	1.14	1.12	1.3*	1.15
B s/D c	1.24	1.04	1.15	1.02
C s/D c	1.44*	1.35*	1.05	1.1
A s/D c	1.21	0.74	0.83	1.13
C s/B c	1.00	1.3*	1.91*	1.05

*P value <0.05 was considered statistically significant.

Groups A, B, C and D indicate rats which are BDL-naltrexone, sham-naltrexone, BDL-saline and sham-saline, respectively.

cardiomyocytes during cirrhosis. Previous studies have demonstrated various pathways which are involved in pathogenesis of CC but there are rare studies focusing on the role of endogenous opioids during CC. Enrollment of endogenous opioids in CC through apoptosis pathways is a novel finding.

According to the literature, apoptosis plays a pivotal role in cardiomyopathy. Roughly it has two main initial pathways, intrinsic and extrinsic, which can be inhibited or activated through several molecules. If the initial pathways do not be inactivated, the final phase of apoptosis which is called executive phase would be started.

Through various studies which were performed to explore the underlying mechanisms of CC, mitogen-activated protein kinases (MAPKs)—proteins involved with

growth, proliferation, differentiation, and apoptosis,¹⁶ overproduction of NO,¹⁷⁻¹⁹ etc. are mentioned as possible involved molecules.

As there are no other studies exploring the relation among apoptosis, endogenous opioids and CC, we tried to focus on this possible relationship in this study. Previously we have shown elevated levels of endogenous opioids during cirrhosis.¹² Here, we tried to evaluate their effect on apoptosis of cardiomyocytes during cirrhosis in molecular level by examining expression level of Fas and FasL molecules as mainstays of extrinsic pathway, Bcl2 for intrinsic pathway and caspase3 for execution phase.

Fas (CD95)/FasL pathway is the major complex of extrinsic apoptosis pathway which is mostly activated via T-cytotoxic lymphocytes and is roughly an indicator

Figure 5 showing real time graph of Fas molecule gene expression in groups C and A. It shows that Fas molecule has statistically significant over expression in group C (BDL-saline) comparing to group A (BDL-naltrexone), $P < 0.001$. P value < 0.05 is considered as significant. BDL: bile duct ligation.

Figure 6 An example of melting curves of studied genes.

of an underlying inflammatory process of an apoptotic phenomenon.²⁰ It was shown previously that endogenous opioid blockade inhibited Fas mediated hepatitis in mice. The role of Fas/FasL pathway was also mentioned in ischemia reperfusion cardiac injury.^{21,22} It was shown that endogenous opioids specially those acting through Mu-opioid receptor could induce apoptosis through activation of Fas molecule.^{23,24} Our results showed overexpression of Fas molecule in BDL rats while naltrexone injection had decreased its expression level. We found 1.9 times higher expression level of Fas molecule in BDL-saline rats (group C) compared to normal rats, but only 1.3 times higher

expression level in the same group comparing to BDL rats receiving naltrexone (group A). These results showed the inhibitory effect of naltrexone on Fas molecule expression.

Bcl2 is another key molecule involved in intrinsic apoptosis pathway which has an anti-apoptotic function.^{20,25,26} No difference was found in Bcl2 expression level between BDL-saline and BDL-naltrexone groups. But, we found a light over expression of Bcl2 gene in BDL-sham group in comparison to sham-saline group. As we did not find any difference between two BDL groups receiving either naltrexone or saline and to best of our knowledge there are no data showing the relation between Bcl2 and

opioid system, and considering the fact that there are many interconnections between two main apoptotic pathways, this finding can be interpreted as a compensatory phenomenon which is not directly related to opioid system.

Caspase family includes molecules involved in final phase of apoptosis, executive phase. Each intrinsic and extrinsic pathway finally reaches a final executioner complex composed of some of caspase family molecules including caspase 3 and 6.²⁷⁻³⁰ Comparing two groups of cirrhotic rats, no difference was found in expression level of caspase3, but increased expression level was observed comparing BDL-saline group with both control groups. As we also found over expression of Fas molecule in BDL rats, we concluded that over expression of Caspase3 was due to higher expression and activation of Fas.

Naltrexone, a nonspecific opioid receptor antagonist with strong affinity to both mu and kappa subtypes, is proposed as a treatment of pruritus in cirrhosis.¹² Previous studies also have shown anti-inflammatory and also pro-angiogenic effects of naltrexone.^{12,18}

Many pathophysiological aspects of cirrhosis are still unclear and many studies have explored the role of opioids during cirrhosis. Considering the effects of endogenous opioids on various pathways including apoptosis, angiogenesis, inflammation, etc., in the course of cirrhosis further investigation of opioids role during cirrhosis may lead to better management of the disease. Our study has some limitations. We studied a small number of molecules involved in apoptosis pathway. Although we tried to examine some of the most important interacting molecules but apoptosis is a very complex pathway with complex interactions between involving molecules with also some unknown aspects. Therefore, examining some parts of this complex pathway with small number of molecules may not be a representative of the whole process. Considering these limitations, further studies on other aspects of the apoptosis pathway is suggested.

In summary, we found Fas overexpression in cirrhotic rats which was attenuated by naltrexone administration. Since no differences were found between expression levels of FasL molecule in different groups, it was concluded that changes in expression level of Fas molecule were not an inflammatory process but were due to endogenous opioids, and Fas molecule was activated by elevated levels of endogenous opioids during cirrhosis. Additionally, we showed apoptosis in cardiac samples of cirrhotic rats, and along with overexpression of Fas molecule we suggested that CC can be induced through extrinsic apoptosis pathway (through Fas molecule) activation via endogenous opioids. But it should be noted that according to mild changes in Fas molecule expression, this pathway may be a supplementary rather than the main pathway in occurring CC. We also demonstrated the promoting role of naltrexone by inhibiting endogenous opioids in a progressive condition like CC.

CONFLICTS OF INTEREST

The authors have none to declare.

ACKNOWLEDGMENT

This study was supported by research grants of Tehran University of Medical Sciences.

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