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S-adenosylmethionine prevents the up regulation of Toll-like receptor (TLR) signaling caused by chronic ethanol feeding in rats ☆

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ABSTRACT

Toll-like receptors (TLR) play a role in mediating the proinflammatory response, fibrogenesis and carcinogenesis in chronic liver diseases such as alcoholic liver disease, non-alcoholic liver disease, hepatitis C and hepatocellular carcinoma. This is true in experimental models of these diseases. For this reason, we investigated the TLR proinflammatory response in the chronic intragastric tube feeding rat model of alcohol liver disease. The methyl donor S-adenosylmethionine was also fed to prevent the gene expression changes induced by ethanol. Ethanol feeding tended to increase the up regulation of the gene expression of TLR2 and TLR4. SAMe feeding prevented this. TLR4 and MyD88 protein levels were significantly increased by ethanol and this was prevented by SAMe. This is the first report where ethanol feeding induced TLR2 and SAMe prevented the induction by ethanol. CD34, FOS, interferon responsive factor 1 (IRF-1), Jun, TLR 1,2,3,4,6 and 7 and Traf-6 were found to be up regulated as seen by microarray analysis where rats were sacrificed at high blood alcohol levels compared to pair fed controls. Il-6, IL-10 and IFNγ were also up regulated by high blood levels of ethanol. The gene expression of CD14, MyD88 and TNFR1SF1 were not up regulated by ethanol but were down regulated by SAMe. The gene expression of IL-1R1 and IRF1 tended to be up regulated by ethanol and this was prevented by feeding SAMe. The results suggest that SAMe, fed chronically prevents the activation of TLR pathways caused by ethanol. In this way the proinflammatory response, fibrogenesis, cirrhosis and hepatocellular carcinoma formation due to alcohol liver disease could be prevented by SAMe.

Introduction

What is the mechanism by which alcohol abuse increases the risk of hepatocellular carcinoma in patients with hepatitis C, hepatitis B, non-alcoholic and alcoholic liver disease, hemochromatosis and α1-antitrypsin deficiency? Evidence is emerging that the synergism is due to the activation of a common pathway in which the Toll-like receptor (TLR) signaling induces proinflammatory cytokine production through NFκB up regulation of growth factors by activation of activator protein 1 (Mandrekar et al., 2009; Rodrigues et al., 2008). The presence of toxins in the body (like lipopolysaccharides and alcohol) induces an inflammatory reaction in the liver. LPS recognition by Toll-like receptor 4 (TLR4) on macrophages and other cell types in the liver and activation of downstream signaling pathways culminating in the activation of transcription factors such as NFκB and AP-1 lead to increase inflammatory cytokine production in alcoholic liver diseases. In the model of chronic ethanol feeding by intragastric cannula, we observed the mRNA expression increase of the TLR genes and the TLR signaling pathway, in rat livers obtained after one month of ethanol feeding. Increased activation of the TLR4 signaling pathway by ethanol, which was dependent on TRIF and not the MyD88 pathway, had been reported earlier (Hansen et al., 1994; Hritz et al., 2008; Uesugi et al., 2001).

In the refeeding drug-primed mouse model, we observed that the activation of the TLR pathway, when used with the drug DDC, was prevented by SAMe (Bardag-Gorce et al., 2010c). Based on that previous observation, we postulated that SAMe fed with ethanol would prevent TLR up regulation (Bardag-Gorce et al., 2010c). In a prior study with ethanol using the intragastric cannula feeding model, we observed that the expression of a large number of genes were induced by high blood ethanol levels but were barely changed at low blood alcohol levels (BAL) through the BAL cycle (Bardag-Gorce et al., 2006). Similar results were observed when rats were sacrificed after being fed SAMe (Bardag-Gorce et al., 2010a). However, from the microarray gene mining, it was observed that numerous changes in the expression of cytokines and TLR were prevented when SAMe was fed with ethanol. Specifically, IL-1-2, Tnfrsf6, IL-1R1, CxC14, Ccl6, TLR4, TLR2, CxC12, Tgfβr3 and TNF were all up regulated by ethanol when sacrificed at low blood ethanol levels. This was prevented when SAMe was added to the diet.

To further investigate this phenomenon, PCR arrays (SA Biosciences) on mRNA of the same rat livers studied above were done to further explore changes in cytokines and TLR pathways. The changes

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noted were confirmed partially by qRT-PCR. The results of this present study have been reported in part in an abstract (Oliva et al., 2010).

Methods

Animals

Male rats were fed ethanol with or without SAMe intragastrically for 1 month. Results from the animals used have been reported previously. A portion of the fast frozen livers stored at −80 °C were used in the present study to further characterize the effects of ethanol and SAMe on the expression of cytokines and the TLR pathway using PCR arrays (SA Biosciences) n = 1. The treatment of the animals has been published (Bardag-Gorce et al., 2010a). One male rat fed ethanol intragastrically for 1 month was sacrificed at the peak blood alcohol level, in order to generate hypoxia and select proteins for gene expression analysis. A pair-fed control was also sacrificed at the same time. The livers from both animals were subjected to a microplate array specific for the TLR signaling pathway (SA Bioscience a Qiagen company).

Quantitative real-time RT-PCR assay

Total liver RNAs were extracted with Trizol Plus RNA Purification kit (Invitrogen, Carlsbad, CA). Synthesis of cDNAs was performed with 5 μg total RNA, and 50 ng random hexamer primers using Super-SriptIII RNase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR primers were designed with the Primer Express software (Applied Biosystems, Foster City, CA).

Gene	Identification	Forward	Reverse
CD14	NM_021744.1	CCGGGAACTGACTCTTGAAAAC	CATCCAGAAGCGGCGAAA
Keap-1	NM_057152.1	CAGAACAAGCCATGCCTTCTT	TCTGGTCTTCCACAAGGTCCTT
TLR2	NM_198769.2	GCAGTGAGTGGTGCAAGTATGAA	CGCGTCATTGTTCTCGTCAA
TLR3	NM_198791.1	GCACTTTCTCCGGGCTGAA	GTCGCGGAGGCTGTTGTAG
TLR4	NM_019178.1	GGTGTGAAATTGAGACAATTGAAGAC	GTTTCCTGTCAGTACCAAGGTTGA
TLR9	NM_198131.1	TCCTCCAGAAACTGGATGTCAGT	TCTACCGCCAGAGCAAAGAAG
IL1R1	NM_013123.3	CCCATATCAGCGGACAAGGA	TGGCGGGAACAAACCAAA
IRF1	M34253.1	CCCAAGACTTGGAAGGCAAA	TGGTCCTTCACTTCCTCGATGT
MyD88	NM_198130.1	GCCAGCGAGCTCATTGAGA	TTTGCAGGTAATCGTCAGAAACA
TNRF1 SF1	NM_153629.1	TGCTGCTGACATGACAAAGGTA	GCAGGTTCAGCTTACTATTCATCCA

Western blot analysis

Proteins (50 μg) from liquid nitrogen frozen stored livers and nuclear and histone extracts were separated by SDS-PAGE gels and transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1 h in 25 mM Tris–HCl (pH 8.3), 192 mM glycine and 20% methanol. The membranes were stained using primary antibodies against TLR4 (BioVision, Mountain View, CA) and MyD88 (Thermo Scientific, Worcester MA). Appropriate species polyclonal and monoclonal HRP-conjugated antibodies were used as the secondary antibodies. The membranes were subjected to chemiluminescence detection using luminol, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistics

Data were obtained from at least three separate experiments. Bars represent mean values ± SEM. P values were determined by one-way ANOVA and Student–Newman Keuls for multiple group comparisons (Sigma-Stat software, San Francisco, CA). Statistical significance is set at $p = <0.05$.

Results

PCR microplate array analysis specific for the rat TLR signaling pathway was performed (SA Biosciences) on a rat fed ethanol for 1 month intragastrically and sacrificed at the peak blood alcohol level. It was compared with a pair fed control fed isocaloric glucose. The results are shown in Table 1 (n = 1). This PCR array analysis led to the study of the key player genes in the TLR signaling pathway such as TLR 2, 4, 3, 9 and the associated proteins CD14, and MyD88. The levels of

Table 1
Data mining analysis of TLR signaling pathway using specific PCR microarray (BAL peak vs. Control).

Description	Gene Name	FC
CD14 molecule	CD14	2.82
Cd80 molecule	Cd80	1.9
CD86 molecule	CD86	1.86
C-type lectin domain family 4, member e	Clec4e	26.53
Eukaryotic translation initiation factor 2-alpha kinase 2	Eif2ak2	1.68
FBJ osteosarcoma oncogene	FOS	17.38
Hypoxanthine phosphoribosyltransferase 1	Hprtl	1.22
Heat shock 70kD protein 1A	Hspa1a	1.98
Interferon-alpha 1	Ifna1	2.49
Interferon gamma	Ifng	2.02
Interferon regulatory factor 1	IRF1	2.53
Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	Ikbkb	2.28
Interleukin 10	Il10	4.08
Interleukin 1 receptor, type I	IL1R1	3.22
Interleukin 2	Il2	2.18
Interleukin 6	Il6	5.09
Interleukin-1 receptor-associated kinase 1	Irak1	1.21
Interleukin-1 receptor-associated kinase 2	Irak2	1.94
Jun oncogene	Jun	3.86
Lymphotoxin alpha (TNF superfamily, member 1)	Lta	1.31
Lymphocyte antigen 96	Ly96	1.54
Mitogen activated protein kinase kinase 3	Map2k3	1.79
Mitogen activated protein kinase kinase 4	Map2k4	1.5
Mitogen activated protein kinase kinase kinase 7	Map3k7	2.12
Mitogen-activated protein kinase 8 interacting protein 3	Mapk8ip3	1.86
Mitogen-activated protein kinase 9	Mapk9	2.15
Myeloid differentiation primary response gene 88	MyD88	1.24
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Nfkbia	1.67
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	Nfkbib	1.8
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	Nfkbil1	3.09
Nuclear factor related to kappa B binding protein	Nfrkb	1.8
Nuclear receptor subfamily 2. group C, member 2	Nr2c2	1.69
Pellino 1	Peli1	1.93
Peptidoglycan recognition protein 1	Pglyrp1	3.25
Peroxisome proliferator activated receptor alpha	Ppara	1.6
Prostaglandin-endoperoxide synthase 2	Ptgs2	2.18
V-rel reticuloendotheliosis viral oncogene homo log (avian)	Rel	1.63
V-rel reticuloendotheliosis viral oncogene homolog A (avian)	Rela	2.09
Receptor-interacting serine-threonine kinase 2	Ripk2	1.31
Ring finger protein 138	Rnf138	1.64
Sterile alpha and TIR motif containing 1	Sarm1	5.31
Toll-like receptor adaptor molecule 2	Ticam2	3.53
Toll-like receptor 1	TLR1	3.73
Toll-like receptor 2	TLR2	2.8
Toll-like receptor 3	TLR3	3.27
Toll-like receptor 4	TLR4	1.4
Toll-like receptor 5	TLR5	1.45
Toll-like receptor 6	TLR6	4.85
Toll-like receptor 7	TLR7	1.85
Toll-like receptor 9	TLR9	1.33
Toll interacting protein	Tollip	1.8
Tumor necrosis factor (TNF superfamily, member 2)	Tnt	1.2
Tumor necrosis factor receptor superfamily, member la	TNFRSF1A	2.47
TNFAIP3 interacting protein 2	Tnip2	1.86
TNFRSF lA-associated via death domain	Tradd	1.53
Tnf receptor-associated factor 6	Traf6	2.26