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IMPACT OF ALTERED METHYLATION IN CYTOKINE SIGNALING AND PROTEASOME FUNCTION IN ALCOHOL AND VIRAL-MEDIATED DISEASES

Kusum K. Kharbanda^{1,2,*}, Fawzia Bardag-Gorce³, Shirish Barve⁴, Patricia E. Molina⁵, and Natalia A. Osna^{1,2}

Kusum K. Kharbanda: kharbanda@unmc.edu; Fawzia Bardag-Gorce: fgorce@labiomed.org; Shirish Barve: shirish.barve@louisville.edu; Patricia E. Molina: pmolin@lsuhsc.edu; Natalia A. Osna: nosna@unmc.edu

¹Research Service, Veterans Affairs Nebraska-Western Iowa Health Care System, Omaha, Nebraska, 68105, USA; Phone: 1-402-995-3752; Fax: +1-402-449-0604

²Department of Internal Medicine, University of Nebraska Medical Center, Omaha, Nebraska, 68198, USA; Phone: 1-402-995-3735; Fax: +1-402-449-0604

³Department of Pathology, Los Angeles Biomedical Research Institute, Harbor UCLA Medical Center, 1124 W Carson St., Torrance, CA, 90502, USA; Phone: +1-310-222-1846; Fax: +1-310-222-3614

⁴Department of Medicine and Alcohol Research Center, University of Louisville, Louisville, KY 40202, USA; Phone: +1-502-852-5245; Fax: +1-502-852-8927

⁵Department of Physiology and Alcohol and Drug Abuse Center of Excellence, Department of Physiology, Louisiana State University Health Sciences Center, 1901 Perdido Street, Medical Education Building, New Orleans, LA 70112; Phone: 504-568-6187; Fax: 504-568-6158

Abstract

Data from several laboratories have shown that ethanol feeding impairs many essential methylation reactions that contribute to alcoholic liver disease (ALD). Ethanol is also a co-morbid factor in the severity of hepatitis C virus-induced liver injury. The presence of viral proteins further exacerbates the methylation defects to disrupt multiple pathways that promote the pathogenesis of liver disease. This review is a compilation of presentations that linked the methylation reaction defects with proteasome inhibition, decreased antigen presentation, and impaired interferon (IFN) signaling in the hepatocytes and dysregulated TNF α expression in macrophages. Two therapeutic modalities, betaine and S-adenosylmethionine (SAM), can correct methylation defects to attenuate many ethanol-induced liver changes, as well as improve IFN signaling pathways, thereby overcoming viral treatment resistance.

Ethanol administration leads to many adverse functional and structural changes in humans and experimental animals, and disrupts metabolic processes. Liver is one of the tissues most susceptible to the toxic effects of ethanol. Many pathways have been postulated and studied in search of potential mechanisms involved in the pathogenesis of alcoholic liver disease (ALD). In particular, the interaction of ethanol with nutrients and/or their metabolism has been considered to significantly contribute to the generation of the pathology observed in alcoholic liver injury.

*Corresponding author: Kusum K. Kharbanda, Ph.D., Research Service, Veterans Affairs, Nebraska-Western Iowa Health Care System, Omaha, Nebraska, 68105, USA Tel.: +1-402-995-3752; Fax: 1-402-449-0604; kharbanda@unmc.edu.

Using various animal models and human studies, over the years, alcohol researchers have established that multiple steps in the methionine metabolic cycle in the liver are affected by ethanol (for reviews see (Kharbanda, 2009; Kharbanda and Barak, 2005). As a consequence of all the ethanol-induced alterations in methionine metabolic pathways, a major change that occurs in the liver is alteration in the S-adenosylmethionine (SAM):S-adenosylhomocysteine (SAH) ratio which occurs due to increases in hepatocellular SAH levels and may or may not be accompanied by decreases in hepatic SAM levels. Since the ratio of SAM to SAH is an important metabolic indicator for cellular methylation status, a decrease in SAM: SAH ratio in the presence of increased SAH is associated with impaired activity of many SAM-dependent methyltransferases (Clarke and Banfield, 2001), causing profound functional consequences.

This is particularly relevant in the liver as more than 85% of all SAM-dependent transmethylation reactions take place in this organ (Clarke and Banfield, 2001). As these methyltransferases have a broad range of cellular functions (Clarke and Banfield, 2001), there is a myriad of detrimental consequences that can arise due to impaired activity of many critical SAM-dependent methyltransferase in this organ. Particularly, by inhibiting specific methyltransferases, ethanol impairs the generation of methylated molecules that serve several important biological functions. In addition, the ethanol-induced DNA methylation and histone methylation changes can affect the expression of several susceptible genes (Pal-Bhadra et al., 2007; Shukla and Aroor, 2006; Shukla et al., 2008) and epigenetic memory as well as altered cytokine signaling.

Methylation defects and TNF regulation

Dysregulated TNF metabolism in alcoholic hepatitis (AH) was first reported almost two decades ago with the seminal finding that cultured monocytes from patients with AH spontaneously produced TNF, and this production was increased in response to an LPS stimulus (McClain and Cohen, 1989). Increased serum levels of TNF, increased monocyte TNF production, and hepatic immunohistochemical staining for TNF in AH frequently correlates with disease severity and mortality (Bird et al., 1990; Felver et al., 1990; McClain et al., 1999; Ohlinger et al., 1993). Further, the LPS-inducible TNF expression in ethanol primed monocytes/macrophages (Gobejishvili et al., 2006) has been mechanistically linked to the decrease in cellular cAMP.

Intracellular levels of cAMP and cGMP are regulated by the rate of degradation via a large family of phosphodiesterases (PDEs). There are 11 different members belonging to the mammalian class I PDE super family (PDE1–PDE11) (Conti and Beavo, 2007; Houslay). Of the cAMP-specific PDEs, the PDE4 family is widely expressed and represents a large complex family, with four genes (PDE4A/B/C/D) encoding more than 20 distinct PDE4 isoforms that exhibit distinct cellular functions for temporal and compartmentalized dynamics of cAMP levels (Conti and Beavo, 2007; Houslay). In monocytes/macrophages that predominantly express isoforms of PDE4 A, B, and D, it has been established that PDE4B is involved in LPS-induced signaling mediated by TLR-4 (a transmembrane LPS receptor), and is essential for LPS-induced TNF expression (Jin and Conti, 2002; Jin et al., 2005). Studies have shown that chronic ethanol exposure leads to a significant increase in the LPS-inducible PDE4B expression and activity in both monocytes and macrophages (Gobejishvili et al., 2008). These data strongly suggested that augmentation of PDE4B expression and activity induced by ethanol play a critical role in priming the monocytes/macrophages to mount an exaggerated inflammatory response to endotoxin.

The contribution of methylation in the production of the inflammatory cytokine, TNF, was revealed when SAM (a methyl donor) supplementation could significantly attenuate LPS-

induced TNF expression in monocytes/macrophages and Kupffer cells (Song et al., 2005; Veal et al., 2004; Watson et al., 1999). Although the mechanisms of action of SAM have not been fully elucidated, earlier work indicated that the ability of SAM to decrease TNF production in LPS-stimulated monocytes/macrophages probably involves increased cellular cAMP (Song et al., 2005). Further investigations revealed that the potential mechanism for the anti-inflammatory effect of SAM occurred by ameliorating the LPS-induced up-regulation of PDE4B2 mRNA and protein expression to ultimately increase cellular cAMP levels (Gobejishvili et al., 2011).

Since histone methylation plays a distinctive role in the regulation of both transcriptional activation and repression of genes (Kouzarides, 2002) and a causal link between promoter-associated histone 3 lysine 9 trimethylation (H3K9Me3) and transcriptional repression has been established (Hublitz et al., 2009), the potential epigenetic mechanisms underpinning SAM's anti-inflammatory mechanisms was examined. SAM treatment induced transcriptionally repressive histone methylation modifications leading to an increase in H3K9 trimethylation on the PDE4B2 intronic promoter region (Gobejishvili et al., 2011). As illustrated in Figure 1, SAM treatment led to an increase in the trimethylation of the lysine 9 on the H3 histones located within the -700 bp upstream of the transcriptional start site (TSS), as well as those located within +400 bp downstream of the TSS in the 5'-untranslated region. H3K9 trimethylation and transcriptional repression has been shown to occur via specific histone methyl transferases in a SAM-dependent manner (Wang et al., 2003). These data strongly suggest that SAM treatment could be affecting H3K9-specific histone methyl transferases, increasing the promoter-associated H3K9 trimethylation and consequent suppression of the LPS-induced PDE4B2 mRNA expression.

The SAM-mediated decrease in LPS-inducible PDE4B2 expression resulted in an increase in cellular cAMP levels and activation of cAMP-dependent protein kinase A (PKA), which plays an inhibitory role in LPS-induced TNF production. These results demonstrate that inhibition of LPS-induced PDE4B2 up-regulation and increased cAMP-dependent PKA activation are significant mechanisms contributing to the anti-TNF effect of SAM. Moreover, these data also suggest that SAM may be used as an effective PDE4B inhibitor in the treatment of chronic inflammatory disorders in which TNF expression plays a significant pathogenic role.

Methylation defects, proteasome dysfunction and HCV virus persistence

Ethanol is also a co-morbid factor in the severity of virus-induced organ injury. Among the roughly 4 million Americans infected with the hepatitis C virus (HCV), chronic alcohol use has long been known to dramatically accelerate the progression of liver disease. Recent analysis has shown that long-term alcohol exposure increases the risk of death from HCV more than 8-fold over viral infection alone (Safdar and Schiff, 2004). Despite its importance as one of the strongest independent predictors of both cirrhosis and death, very little is known about the nature of the interaction between alcohol and HCV pathogenesis. With the yearly cost of treating the HCV epidemic in the US approaching \$5 billion (Szabo et al., 2006; Vidali et al., 2008), there is a critical need to determine the mechanism linking the two most important causes of liver disease in the US population. Armed with this information, we can then work to reverse the interaction and thereby slow or halt the progression to end stage liver disease and death.

Virally-infected hepatocytes are eliminated by cytotoxic-T cells following recognition of viral antigens on the cell surface in the context of MHC-I molecules. Antigen processing is the most upstream step in antigen presentation that results in the processing of proteins to the size of 8–10 amino acids for MHC class I folding.

Proteasome is a multi-catalytic enzyme responsible for cleavage of intracellular proteins and peptides. The main function of proteasome is to process peptides for antigen presentation as well as degraded, oxidatively modified proteins and signal transduction factors. Proteasome exists in equilibrium of two particles, 20S proteasome and 26S proteasome. Proteins covalently bound by ubiquitin, a small 8.5-kDa protein, are degraded by the 26S proteasome. This process requires ATP. Proteins subjected to degradation have multiple ubiquitin molecules covalently attached to generate polyubiquitin chain binding to internal lysine (lysine 48) residue. In contrast, 20S proteasome degrades non-ubiquitylated proteins, most of which are oxidatively modified (reviewed by Donohue, Cederbaum, French, Barve, Gao and Osna, 2007, Osna and Donohue, 2007). This process is ATP-independent.

As revealed from previous studies (Bardag-Gorce, 2009; Bardag-Gorce et al., 2006; Donohue et al., 2006; Osna and Donohue, 2007), proteasome activity in the liver is suppressed by ethanol metabolism. This suppression of proteasome function in hepatocytes and ethanol-metabolizing hepatoma cells results in decreased cleavage of antigenic peptides, causing a reduction in peptide processing and subsequent presentation of MHC class I-peptide complexes on the cell surface (Osna and Donohue, 2007; Osna et al., 2009). Suppression of proteasome function is oxidative stress dependent. However, additional mechanisms also modulate proteasome function. Specifically, the preservation of proteasome function by SAM supplementation in the ethanol diet suggested that methionine metabolic pathway reactions also regulate proteasome activity (Bardag-Gorce et al., 2007; Li et al., 2008).

Proteasome activity was markedly inhibited when hepatocytes or CYP2E1-expressing human hepatoma Huh7 (Huh7CYP) cells were exposed to tubercidin, a specific methylation inhibitor that preferentially causes SAH accumulation without oxidative stress induction (Osna et al., 2010). When the same cells were incubated in the buffer containing SAM and SAH at the ratio of 2.5 (corresponding to the ratios observed in the livers of ethanol-fed rodents), proteasome activity was also inhibited, indicating that the cellular methylation potential regulates proteasome activity (Osna et al., 2010). To exclude the possibility of epigenetic changes mediating the effects of tubercidin, proteasome activity was determined in the liver cytosols prepared from chow-fed mice that were incubated *in vitro* for 4h in the presence of varying SAM to SAH ratios (which was achieved by altering the relative concentration of these two metabolites in the incubating medium). A decrease in proteasome activity corresponding to the decrease in the SAM: SAH ratio was observed. Similar results were obtained when purified 20S cytosolic proteasome was exposed to differential SAM:SAH ratios in the buffer. These results suggested that in addition to methylation-dependent epigenetic regulation, proteasome activity can also be regulated directly by changes in SAM:SAH ratios. Importantly, tubercidin, had no effect on activity of purified proteasome in the absence of SAM and/or SAH in the buffer (Osna et al, 2010).

Proteomics studies revealed that the lysine and arginine residues on proteasome are methylated (Gomes et al., 2009). Examination of methylation of these residues on the proteasome at varying SAM:SAH ratios demonstrated a reduction of methyl lysine expression with lower SAM:SAH ratios (Osna et al., 2010). This suggests that alterations in SAM:SAH ratios could regulate proteasome function by affecting lysine methyltransferase activity and the subsequent lysine methylation of the proteasome subunit. Figure 2 depicts the methylation regulation of proteasome functions in the context of antigen presentation.

The sensitivity of IFN-dependent immunoproteasome to defective methylation reactions is higher than constitutive proteasome, suggesting that IFN signaling may also be altered by impaired methylation. In the literature, arginine methylation of signal transducer and activator of transcription 1 (STAT1, a major signaling pathway component activated by

IFNs) protects activated STAT1 from attaching to the pathway's inhibitor, protein inhibitor of activated STAT1 (PIAS1) (Mowen et al., 2001). The complex formation between PIAS1 and STAT1, in turn, blocks the attachment of activated STAT1 to DNA, thereby disrupting IFN signaling at the downstream level of gene activation (O'Shea et al., 2002). Studies further provided evidence that tubercidin suppressed not only attachment of activated STAT1 to DNA, but also upstream STAT1 phosphorylation, indicating that impaired methylation of STAT1 limits its ability to be activated by IFNs (Osna, 2008). Furthermore, in liver cells, ethanol also suppressed both STAT1 phosphorylation (Osna et al., 2005) and the attachment of STAT1 to DNA, and these effects could be partially prevented by co-treatment with SAM or betaine (Osna, 2008).

When proteasome-mediated antigen processing and presentation on liver cells was investigated using a SIINFEKL-H2Kb model (Osna et al., 2009), tubercidin substantially reduced the display of the peptide-MHC class I complex on the cell surface (unpublished observations). This was attributed to both compromised proteasome function and defective IFN signaling.

Another consequence of this proteasome dysfunction is the accumulation of oxidized, damaged and ubiquitinated proteins in the liver (Bardag-Gorce, 2010; Bardag-Gorce et al., 2010; Donohue et al., 2007). The aggregation of the ubiquitinated proteins results in the formation of Mallory-Denk body (MDB) in the liver of alcoholic patients (Bardag-Gorce et al., 2007; Zatloukal et al., 2007). Recently, an association between MDBs and immunoproteasome formation was established that also negatively correlated with 26S proteasome function (Bardag-Gorce, 2010; Bardag-Gorce et al., 2010). SAM supplementation prevented the immunoproteasome formation and allowed the recovery of 26S proteasome activity to suppress MDBs formation (Oliva et al., 2010a). The mechanisms of protection by SAM included the decrease in induction of pro-inflammatory cytokines (Oliva et al., 2010a; Yang et al., 2008) as well as TLR 2/4 signaling (Bardag-Gorce, 2010; Bardag-Gorce et al., 2010), which may in turn reduce $\text{INF}\gamma$ and $\text{TNF}\alpha$ -dependent immunoproteasome formation (Bjork, 1976). These results suggest that immunoproteasome serves as a target of SAM-elicited regulation of the cytokine network. While it has been shown that the proteasome function is regulated by methylation potential (Osna et al., 2010), the converse that proteasome regulates methionine metabolic pathway was also investigated.

Proteasome regulation of the methionine metabolic pathway in the liver

Inhibiting proteasome activity in rats by administering an ip injection of proteasome inhibitor PS-341 at a small dose of 0.25 mg/kg body weight revealed a significant down-regulation in the gene expression of several methyltransferases (i.e. betaine-homocysteine methyltransferase (BHMT), glycine N-methyltransferase (GNMT), methionine adenosyltransferase 1A (MAT1A) as illustrated in Figure 3 as well as a reduced protein, DNA and histone methylation (Oliva et al., 2009b). These results indicated that in addition to proteasome's role in the controlling transcriptional mechanisms via degradation of RNA polymerase II (Kinyamu and Archer, 2007; Szutorisz et al., 2006) or via stabilizing transcription factors (Oliva et al., 2010b), it could act as a co-activator of the transcriptional machinery of the target genes, BHMT, GNMT, and MAT1A. Data also suggests that the suppression in the expression of the target genes by proteasome inhibitor is independent of $\text{NF}\kappa\text{B}$ activation (Ou et al., 2007).

Additionally, the treatment with proteasome inhibitor PS-341 to the rats fed ethanol (Bardag-Gorce et al., 2011) suppressed liver steatosis (Oliva et al., 2010b), up-regulated the anti-oxidative response (Bardag-Gorce et al., 2011), and reduced inflammation by stabilizing $\text{I}\kappa\text{B}$ and lowering $\text{NF}\kappa\text{B}$ activation (Bardag-Gorce, 2011). In addition, a transient

induction of ER stress by PS-341 treatment activated an adaptive cytoprotective mechanism, including the upregulation of chaperone proteins GRP78/GRP94, heat shock proteins, and the major regulator of antioxidant response elements, ATF4 (Bardag-Gorce et al., 2011). The key factor for beneficial cytoprotective effects of PS-341 is its small, non-toxic dose and the kinetics in proteasome activity changes. Since PS-341 has a life span of about 9 to 15 hrs, it is cleared by 24 to 48 hours post-treatment. At 72 hrs post-treatment, the activity of the proteasome rebounds and is even higher when compared to controls (Bardag-Gorce et al., 2011), which possibly helps to degrade the misfolded proteins and lowers ER stress.

Recent studies revealed that betaine supplementation can restore proteasome function suppressed by chronic ethanol feeding (Osna et al., 2010). In attempt to further study this interaction, we simultaneously treated animals with the proteasome inhibitor PS-341 and SAM. The results showed the methyl donor, SAM, corrected hypomethylation and the decrease in methyltransferases caused by PS-341 treatment (French et al., 2010; Oliva et al., 2009a; Oliva et al., 2009b). Furthermore, the transient downregulation of BHMT, GNMT, and MAT1a was significantly corrected and upregulated when SAM was given to the animal simultaneously with PS-341. While these studies refute previous studies implicating SAM downregulation of BHMT (Ou et al., 2007), they reveal anew mechanism by which methyl donors could be used in the treatment of ALD.

It is quite possible that the response of proteasome to ethanol and hypomethylation conditions depends on the type of proteasome subunits expressed in the organs. Normally, liver cells possess a mixed proteasome population where immunoproteasome coexists with constitutive proteasome. However, if only constitutive proteasome is present, such as in the cells that do not metabolize ethanol like the muscles, the effects of ethanol exposure/ impaired methylation may be different from those observed on mixed proteasome populations.

It is evident that impaired methylation reactions/DNA methylation modulate proteasome function in ethanol-metabolizing liver cells. However, whether methylation defects also play a role in regulating proteasome function in ethanol-non-metabolizing cells with or without virus infection is not known. These aspects are under investigation in Patricia Molina's laboratory, who is examining proteasome activity and the inflammatory/oxidative and anti-anabolic milieu in skeletal muscles using a chronic alcohol-fed simian immunodeficiency virus-infected macaque model (Molina et al., 2006).

CONCLUSIONS AND SUMMARY

To summarize, alcohol-induced alterations in methionine metabolic pathway impair methylation potential and can disrupt normal proteasome function and cytokine signaling. This can be especially relevant for virally infected cells such as hepatocytes that can metabolize ethanol to disrupt antigen processing in the context of MHC Class I and INF signaling that will ultimately lead to persistence of viral infection and progressive liver injury. Betaine or SAM treatment can reverse proteasome inhibition and interferon signaling by correcting the ethanol-induced alterations in methylation potential. Controlled proteasome inhibition could also be used as a therapy for ALD on account of up-regulation of the cellular anti-oxidant capability. In addition, inhibition of LPS-induced PDE4B2 up-regulation and increased cAMP-dependent PKA activation appears to be a significant mechanism contributing to the anti-TNF effect of SAM. These data also suggest that SAM may be used as an effective PDE4B inhibitor in the treatment of chronic inflammatory disorders in which TNF expression plays a significant pathogenic role. In conclusion, betaine and SAM treatment, can correct methylation defects to attenuate many ethanol-

induced liver changes, as well as improve IFN signaling pathways to overcome viral treatment resistance.

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Fig. 1. Effect of ethanol and SAM treatment on TNF promoter-associated histone H3 lysine 9 (H3K9) methylation

Ethanol along with LPS leads to a decrease in H3K9 trimethylation, enhancing H3K9 acetylation and LPS-inducible TNF transcription. On the other hand, SAM treatment counters the effects of ethanol and LPS, leading to an increase in H3K9 trimethylation, a decrease in H3K9 acetylation and reduced TNF transcription.

Fig. 2. Regulation of proteasome activity by the hepatic methylation status

Proteasome activity is regulated by hepatocellular SAM:SAH ratio. At a normal SAM:SAH ratio (range of 3 – 5), lysine residues on the 20S proteasome subunit (presumably, β -subunits) are methylated that maintain proteasome function for generation of peptides for normal MHC class I-restricted antigen presentation. *In vivo* or *in vitro* exposure to ethanol decreases the hepatocellular SAM:SAH ratio (range of between 1 – 2.5) and causes reduced methylation of lysine residues thereby lowering the proteasome activity. This ultimately results in the decreased peptide generation and suppressed antigen presentation.

Fig. 3. Effect of proteasome inhibition on methionine metabolic pathway

Four main participants of the methionine metabolic pathway are methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine. Methionine adenosyltransferase (MAT) converts methionine to SAM, which then serves as a methyl-group donor substrate for many diverse methyltransferase-catalyzed reactions. The other product of these reactions is SAH, which is hydrolyzed to adenosine and homocysteine by S-adenosylhomocysteine hydrolase (SAHH). The methionine cycle is completed when homocysteine is remethylated back to methionine by B₁₂-dependent methionine synthase (MS) using 5-methyltetrahydrofolate (5-methyl-THF) as a substrate. Homocysteine can also be remethylated to methionine by an alternate pathway in the liver via betainehomocysteine methyltransferase (BHMT)-catalyzed reactions with the formation of dimethylglycine (DMG). The methyltransferase, phosphatidylethanolamine methyltransferase (PEMT) catalyzes the synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE) and Glycine–N methyltransferase (GNMT) catalyzes the synthesis of sarcosine from glycine. Low level of proteasome inhibition down regulates several enzymes of the methionine metabolic pathway, thus reducing trans-methylation reactions.