Chronic ethanol mediated decrease in cAMP primes macrophages to enhanced LPS-inducible NF-κB activity and TNF expression: relevance to alcoholic liver disease

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ABSTRACT

Increased plasma and hepatic TNF-α activity has been implicated in the pathogenesis of alcoholic liver disease. We previously reported that monocytes from alcoholic patients show enhanced constitutive as well as LPS-inducible NF-κB activation and TNF-α production. Studies in monocytes have shown that cAMP plays an important role in regulating TNFα expression, and elevation of cellular cAMP suppresses TNF-α production. The effects of chronic ethanol exposure on the cellular levels of cAMP as well as TNF expression in monocytes were examined in vitro as well as in rat primary hepatic Kupffer cells obtained from a clinically relevant enteral alcohol feeding model of alcoholic liver disease (ALD). Chronic ethanol exposure significantly decreased cellular cAMP levels in both LPS stimulated and un-stimulated monocytes. Consistent with the decrease in cAMP levels, ethanol led to an increase in LPS-inducible TNF-α production by affecting NF-κB activation and induction of TNF mRNA expression, without any change in the TNF mRNA stability. Enhancement of cellular cAMP with dibutyryl cyclic AMP (dbcAMP) abrogated LPS mediated TNF-α expression in ethanol treated cells. Importantly, cAMP did not affect LPS-inducible NF-κB activation but significantly decreased its transcriptional activity. Taken together, these data strongly suggest that ethanol can synergize with LPS to up-regulate the induction of TNF gene expression and consequent TNF overproduction by decreasing the cellular cAMP levels in monocytes/macrophages. Further, these data also support the notion that cAMP elevating agents could constitute an effective therapeutic approach in attenuating or preventing the progression of liver disease in alcoholic patients.

KEYWORDS: alcohol, Kupffer cells, dbcAMP
INTRODUCTION

Inflammatory cytokines, particularly TNF-α, plays a significant role in the pathogenesis of liver injury and the clinical/biochemical abnormalities of alcoholic hepatitis (AH) (12, 22-24). We first reported dysregulated TNF metabolism in AH over 15 years ago with the observation that cultured monocytes (which produce the overwhelming majority of systemic circulating TNF and are a surrogate marker for Kupffer cells) from AH patients spontaneously produced TNF and produced significantly more TNF in response to an LPS stimulus (22). Further, we and others have shown that there are increased serum levels of TNF, increased monocyte TNF production, and hepatic immunohistochemical staining for TNF in AH that frequently correlate with disease severity and mortality (3, 8, 16, 18, 21, 22, 24, 30). Circulating monocytes play an important role in the response to inflammatory agents, particularly to agents entering the circulation that are derived from gut bacteria, such as bacterial endotoxin. This is relevant since patients with AH commonly have endotoxemia (2, 4, 5, 30). LPS is a potent stimulus for monocyte TNF production, and LPS activates the oxidative stress–sensitive transcription factor NF-κB, which modulates the synthesis of several adhesion molecules and inflammatory cytokines including TNF (10, 26, 39). Concomitant with these human findings, studies in rats, mice, and cultured cells evaluated the role of cytokines (especially TNF) in experimental models of liver disease (21). Perhaps the most compelling data documenting the critical role of TNF in the development of alcoholic liver disease (ALD) is provided by the experiments which successfully used anti-TNF antibody to prevent liver injury in alcohol-fed rats (14) and the observation that mice lacking the TNF-type I receptor do not develop alcoholic liver injury (40).
Although the role of TNF as a critical inflammatory cytokine in the progression of ALD has been well established, the mechanism by which ethanol enhances TNF expression, particularly in monocytes/macrophages, is only beginning to be understood (17, 41, 43). Increases in cellular cAMP levels by different types of cAMP enhancers has invariably been found to result in the suppression of TNF production stimulated either *in vitro* or *in vivo* with LPS in monocytes/macrophages of both human and murine origin (44). Various studies have documented that depending on the cell type, suppression of LPS-inducible TNF production by cAMP can occur in both NF-κB dependent as well as independent mechanisms (28, 31, 33, 36, 38). In relation to the effects of chronic ethanol exposure, it has been observed that lymphocytes from alcoholics in comparison to non-alcoholics have greatly diminished levels of both basal and receptor-stimulated cAMP levels (6, 27). Although, cAMP is the most widely known second messenger that down-regulates TNF production, its role in the ethanol mediated enhancement of LPS-inducible TNF production by monocytes/macrophages has not been investigated. It is also not known whether elevation of cellular cAMP in monocytes/macrophages would attenuate/inhibit the synergistic enhancement in TNF-α expression caused by ethanol and LPS.

In the present study we assessed the effects of chronic ethanol exposure on monocytes/macrophages in relation to basal and LPS-stimulated cAMP levels and TNF production. Using a non-degradable, cell permeable cAMP analogue (dbcAMP) we further evaluated the effect of increased cellular cAMP levels on LPS-inducible TNF expression in monocytes/macrophages exposed chronically to ethanol. Our data reveal that chronic ethanol exposure leads to a decline in basal and stimulatable cAMP levels
which play a major role in the enhancement of LPS-inducible TNF production by monocytes/macrophages. Further, an increase in cellular cAMP levels leads to the inhibition of the synergistic enhancement in TNF-α expression caused by ethanol and LPS. The attenuation of TNF expression occurs at the transcriptional level and involves inhibition of NF-κB activity, the major regulator of TNF transcription in monocytes/macrophages.

**MATERIALS AND METHODS**

*Materials.* RAW 264.7 murine monocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA). LPS (Escherichia coli 0111:B4) was purchased from Difco Laboratories (Detroit, MI). Before use, LPS was dissolved in sterile, pyrogen-free water, sonicated, and diluted with sterilized phosphate-buffered saline (PBS). Penicillin, streptomycin, DMEM media, fetal bovine serum was purchased from Invitrogen (Grand Island, NY); murine TNF-α ELISA kit was from BioSource International (Camarillo, CA) and cAMP Immunoassay kit was from R&D Systems, Inc. (Minneapolis, MN). Dibutyryl cyclic AMP was from Sigma, and p65 antibody was from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). Cell proliferation reagent WST-1 was purchased from Roche Applied Science (Roche Diagnostics GmbH, Germany).

*Animal model.* The rat model of alcoholic liver disease we used has been previously described in detail (35). Increased gut permeability leading to enhanced circulating levels of LPS and TNF expression by Kupffer cells in this model is a major cause of liver injury (7, 14, 15).
Briefly, male Wistar rats weighing 350-375 g were implanted with long-term gastrostomy catheters to enable continuous intragastric infusion of the high-fat diet plus ethanol. Ethanol was gradually increased from 8 to 12.5 g x kg\(^{-1}\) x day\(^{-1}\) during a 6-wk experimental period. All required vitamins and minerals were included at the recommended concentrations by using AIN-76A vitamin and salt mixtures (Dyets, Bethlehem, PA) (9). Controls received intragastric infusion of a high-fat diet plus isocaloric dextrose solution. This regimen was continued for 6 wk, the durations that result in fatty liver only or fatty liver, focal centrilobular steatonecrosis, mononuclear cell infiltration, and activation of Kupffer cells, respectively. After 6 wk of ethanol administration, the animals were killed; a sample of liver was obtained for histopathological analysis; and the remainder of the liver was rapidly excised, washed with ice-cold potassium chloride, cut into small pieces, transferred to plastic vials to be frozen in liquid nitrogen, and stored at -80°C. The animal protocol used was approved by the Institutional Animal Care and Use Committee of the University of Southern California, and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals [DHHS publication no. (NIH) 86-23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

**Kupffer cell isolation.** Kupffer cells were isolated control and ethanol treated animals by in situ sequential digestion of the liver with pronase and collagenase and arabinogalactan gradient ultracentrifugation as previously described (15). Kupffer cell viability was tested by the trypan blue exclusion test and always exceeded 97%. The adherence purification method was performed to raise the purity of Kupffer cells >96% as determined by phagocytosis of 1 µm latex beads. Briefly, freshly isolated cells were seeded at 50 x 10\(^6\)
cells/10-cm dish and were incubated for 3 h with DMEM (GIBCO-BRL, Grand Island, NY) containing 10% FBS (GIBCO-BRL), supplemented with 2 mM L-glutamine (Biochrom KG), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Biochrom KG). Nonadherent cells were removed 3 h after seeding, and fresh culture medium with 5% FBS was added and replaced every day until the experiments began. Cells were grown in a humidified 5% CO₂-37°C atmosphere and were used 2-3 days after seeding.

**Cell culture.** RAW 264.7 cells were cultured in DMEM containing 10% (vol/vol) fetal bovine serum, 10 U/ml penicillin, and 10 µg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells were chronically exposed to 25 mM ethanol for a period of 6 weeks.

**Cell proliferation assay.** Cell proliferation and viability assay was measured in control as well as ethanol treated cells by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases according to the manufacturer’s instructions.

**TNF-α and cyclic AMP assay.** TNF-α in conditioned medium and cyclic AMP concentrations in cell lysates were quantified using ELISA kit in accordance with the manufacturer’s instructions. All assays were run in triplicate.

**RNA isolation and real-time PCR analysis.** RT-PCR assays were used to assess TNF-α mRNA levels in RAW 264.7 and Kupffer cells. Total RNA was isolated from cells using TRIZOL (Invitrogen, Carlsbad, CA, USA). For real-time PCR, the first-strand cDNA was synthesized using TaqMan Reverse transcription reagents (Applied Biosystems). The reverse transcription was carried out using 1x Taqman RT buffer, 5.5mM MgCl₂, 500 mM of each dNTP, 2.5 mM random hexamer, 8 U of RNase inhibitor
and 25 U of Multiscribe Reverse Transcriptase with 200 ng of total RNA. The RT conditions were 10 min at 25°C, 30 min at 48°C and 5 min at 95°C. Reactions in which the enzyme or RNA were omitted were used as negative controls. Real-time PCR was performed with an ABI prism 7000 sequence detection system and SYBR green I dye reagents. The specific primers were designed for mouse and rat GAPDH and TNF-α using Primer3 software program. The following primers were used in real-time PCR:

- mGAPDH-FP: 5’ TTGTGGAAGGGCTCATGACC 3’
- mGAPDH-RP: 5’ TCTTCTGGGTGGCAGTGATG 3’
- mTNF-α-FP: 5’ GAAGTTCCAATGGGCTCC 3’
- mTNF-α-RP: 5’ GTGAGGGTCTGGGCCATAGA 3’
- rGAPDH-LP: 5’ TGACAGCAAGAAGGTGGTGA 3’
- rGAPDH-RP: 5’ CGGCATCAAAGGTGGAAG 3’
- rTNF-α-LP: 5’ GCTCCCTCTCATCAGTTCCA 3’
- rTNF-α-RP: 5’ TTGGTGTTTCTACGACG 3’

The parameter Ct (threshold cycle) was defined as the fraction cycle number at which the fluorescence passed the threshold. The relative gene expression of TNF-α was analyzed using $2^{\Delta\Delta Ct}$ method (19) by normalizing with GAPDH gene expression in all the experiments.

*Nuclear protein extraction.* Cells were washed twice with PBS and lysed by hypotonic shock in cytoplasmic extraction buffer (10mM HEPES pH 7.8, 1.5mM MgCl$_2$, 0.5mM DTT, 0.1% Nonidet P-40, 0.5µM PMSF), and 1µg/ml each of the protease inhibitors aprotinin, leupeptin and leucinethiol. The cells were collected by scraping and the nuclei were separated from the cytosolic proteins by a low speed centrifugation at
1500 x g for 5 min. The supernatant (cytoplasmic extract) was stored at –70°C. The nuclear pellet was resuspended in nuclear extraction buffer (20mM HEPES pH 7.8, 520mM NaCl, 1.5mM MgCl₂, 0.1mM EDTA, 25% glycerol, 0.5mM DTT, 0.1% Nonidet P-40, 0.5µM PMSF), and 1µg/ml each of the protease inhibitors aprotinin, leupeptin and leucinethiol and incubated on ice for 60 min. The samples were centrifuged at 100,000 x g for 15 min and the supernatant containing nuclear proteins was stored in small aliquots at –70°C, until further use. The protein concentration in nuclear extracts was measured using the BioRad Dye Reagent (Bio-Rad, Hercules, CA) in accordance with the manufacturer’s protocol.

*Electrophoretic mobility shift assay (EMSA).* Double stranded oligonucleotide containing the binding site for NF-κB (5’-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3’, Promega, Madison, WI) was end-labeled with (³²P) dATP using T4 polynucleotide kinase (GibcoBRL, Grand Island, NY). Nuclear proteins (5µg) were incubated in binding buffer (50mM Tris pH 7.5, 500mM NaCl, 5mM DTT, 5mM EDTA, 20%(v/v) glycerol and 0.4mg/ml sonicated salmon sperm DNA) with 0.3ng of ³²P-labeled probe for 20 min. at room temperature. The resultant DNA/protein complexes were separated by electrophoresis on a 5% nondenaturing polyacrylamide gel in 0.5X TBE (50mM Tris pH 8.0, 45mM borate, 5mM EDTA). The gels were dried and analyzed by autoradiography for ~ 18h. The specificity of the reaction was evaluated by incubating the nuclear extract with a 10 fold molar excess of unlabeled wild type (W) and mutant (M) oligonucleotides.

*Western blot analysis.* Nuclear proteins (30 µg) were analyzed by SDS-polyacrylamide gel electrophoresis using a Bio-Rad (Hercules, CA) electrophoresis system, followed by immunoblotting according to the manufacturer’s instructions.
Immunoreactive bands were visualized using the enhanced chemiluminescence light detection reagents (Amersham, Arlington Heights, IL).

Plasmids and transfections. NF-κB-Luciferase reporter construct was obtained from BD Biosciences/Clontech (Palo Alto, CA). Transfections were carried out using FuGene according to instructions from the manufacturer (Roche Diagnostics, Indianapolis, IN). To control for differences in transfection efficiency, transfected cells were scraped and re-plated after 24 hours at a density of 1x10^6 cells/well in 24 well plates and treated for 6 hours.

Luciferase assay. Luciferase activity was measured using Luciferase Assay Reagent (Promega, Madison, WI). After treatments, total cell lysates were prepared in reporter lysis buffer (Promega). Luciferase enzymatic activity was measured in a TD 20/20 Luminometer using a specific substrate provided by Promega.

Statistical analysis. All experiments were repeated at least three times. Data were presented as means± SD for the indicated number of independently performed experiments. Student’s t-test was used for the determination of statistical significance. P<0.05 was considered significant.

RESULTS

Effect of chronic ethanol exposure on cellular cAMP levels. Chronic ethanol exposure decreases cellular cAMP levels in monocytes: Studies in monocytes have shown that cAMP plays a significant role in regulating TNF-α expression and elevation of cellular cAMP suppresses TNF-α production. Hence, the effect of chronic ethanol exposure on the cellular levels of cAMP as well as TNF expression in monocytes was examined. Mouse monocytic cells (RAW 264.7) were continuously maintained in ethanol (25 mM) supplemented medium which was changed after every 12 h over a
period of 6 weeks. The concentration of ethanol chosen to treat the cells reflects the blood alcohol level that can be achieved during human alcohol consumption (30) and was routinely measured over the study interval using the Sigma Diagnostics Alcohol Reagent (Sigma Diagnostics, St. Louis, MO). Typically, under the experimental conditions of ethanol treatment, a reduction in ethanol concentration by approximately 12-15% occurred over the incubation period of 12 hours and a minor reduction in the doubling time of the cells was observed only in the first week. The effects of the long term exposure of ethanol on the cell viability and proliferative response were evaluated by the WST-1 cell proliferation and viability assay. After 6 weeks of ethanol exposure control and ethanol-treated cells were plated in 96 well plates (2 x 10^4/well) and cell proliferation reagent WST-1 was added (1:10 final dilution). Absorbance (A_{440}) was measured at different time points. There was no detectable effect of long term ethanol exposure (6 wks) on either the proliferation or viability of the monocytic cells (Fig. 1).

Intracellular cAMP levels were evaluated in cell lysates obtained from monocytes with or without chronic ethanol treatment and subsequent LPS stimulation (100 ng/ml; 3 h). As has been reported earlier, LPS stimulation led to an increase in the intracellular cAMP levels. However, chronic ethanol exposure led to a considerable decrease in cellular cAMP levels in both basal and LPS stimulated monocytes (Fig. 2A). We extended these observations to demonstrate a similar effect of chronic ethanol exposure in rat primary hepatic Kupffer cells obtained from a clinically relevant enteral alcohol feeding model of ALD. As compared to the control pair-fed animals, ethanol-fed animals have increased gut permeability and measurable circulating levels of endotoxin. Consequently, Kupffer cells isolated from these animals are exposed and sensitized to
gut-derived LPS. Cellular cAMP levels were measured in Kupffer cells obtained from pair-fed (control, n=4) and alcohol fed (n=4) animals. cAMP levels in Kupffer cells from alcohol fed animals (similar to LPS-treated, ethanol-exposed RAW cells) were significantly lower compared to control animals (Fig. 2B) demonstrating that alcohol down-regulated cellular cAMP levels both in vitro and in vivo.

*Increase in cellular cAMP levels inhibits ethanol-mediated augmentation of TNF expression.* The effect of chronic ethanol exposure and decreased cellular cAMP levels on LPS-inducible TNF-α expression was assessed. After six weeks of continuous ethanol exposure cells were plated into 24-well cell culture plate at 0.6x10^6 cells/well and stimulated with LPS (100 ng/ml) for 18 h. Supernatants were collected and analyzed for TNF production by ELISA. Although ethanol had a minimal effect on the spontaneous release of TNF, LPS-inducible TNF production was found to be significantly increased in ethanol-treated cells (Fig. 3). Further, the effect of increased cellular cAMP levels on TNF expression by ethanol treated cells was examined. Enhancement in cellular cAMP levels was achieved by exogenous supplementation of nondegradable cAMP (dbcAMP). Pretreatment of ethanol treated monocytes with dbcAMP (100 -1000 µM) effectively inhibited the synergistic enhancement in the TNF-α expression caused by ethanol and LPS in a dose dependent manner (Fig. 4).

*Effect of chronic alcohol exposure and cellular cAMP levels on LPS-induced TNF gene transcription in monocytes.* To address the mechanism involved in the effects of chronic ethanol exposure and cellular cAMP levels on TNF production, steady state levels of TNF mRNA were quantified. Real time PCR analysis of total RNA obtained from appropriately treated cells showed that the LPS-inducible steady state TNF mRNA
levels were significantly higher in ethanol treated monocytes as compared to control cells, corroborating our earlier findings and work done by others (Fig. 5). Further, this synergistic enhancement in the TNF mRNA levels caused by ethanol and LPS was significantly attenuated by dbcAMP at each time point evaluated (Fig. 6). Also, in correspondence with decreased cellular cAMP levels, and in agreement with earlier reports, hepatic Kupffer cells obtained from rats chronically fed alcohol (which have been exposed to gut-derived LPS) showed a substantial increase (four fold) in the steady state TNF mRNA levels (Fig. 7).

The effect of ethanol on the LPS-inducible steady state TNF mRNA expression in monocytes could be due to an increase in the synthesis of TNF mRNA or an increase in the stability of TNF mRNA. Hence, the half-life ($t_{1/2}$) of LPS-induced TNF mRNA was measured in control and ethanol treated cells. Cells were stimulated with LPS (100 ng/ml) for 60 minutes followed by actinomycin D (1 µg/ml) for 2 hours. RNA was harvested at 0, 30, 60 and 120 minutes after actinomycin D (Act D) addition and analyzed for TNF mRNA expression. The data obtained showed that the chronic ethanol exposure did not alter the stability of the LPS-induced TNF mRNA. The $t_{1/2}$ of LPS-induced TNF mRNA was approximately 24 minutes in both control (ethanol untreated) monocytes as well as in monocytes chronically exposed to ethanol (Fig. 8). Also, the pretreatment with dbcAMP which abolished the enhancement in the LPS-inducible steady state TNF mRNA expression in ethanol treated monocytes, had no effect on the $t_{1/2}$ of LPS-induced TNF mRNA (data not shown).

*cAMP does not decrease LPS-inducible NF-κB nuclear translocation and DNA binding, but attenuates transcriptional activity of NF-κB.* LPS stimulation of RAW
264.7 and Kupffer cells is known to induce TNF-α mRNA expression through a rapid and transient NF-κB activation leading to the induction of the TNF-α promoter. To assess whether cAMP mediated inhibitory effect on LPS-stimulated TNF-α mRNA expression in ethanol primed RAW cells was due to a decrease in NF-κB activation, nuclear levels of p65 and NF-κB DNA binding activity was examined in the nuclear proteins extracted from RAW cells pretreated with dbcAMP (500 µM for 15 minutes) followed by stimulation with LPS (100 ng/ml) for 30 min. Immunoblot analysis showed that ethanol treated cells had increased nuclear levels of LPS stimulatable p65 as compared to cells not treated with ethanol (Fig. 9A, lanes 2 and 5). These results further extend and corroborate our earlier finding that PBMCs from patients with alcoholic hepatitis (AH) have enhanced LPS-inducible NF-κB activation and TNF-α expression. Notably, pretreatment of cells with dbcAMP which effectively inhibited the synergistic enhancement in the TNF-α expression caused by ethanol did not decrease the nuclear levels of LPS-inducible p65 (Fig. 9A, lanes 3 and 6), on the contrary, nuclear p65 levels were slightly increased.

EMSA was performed to assess the effect of ethanol and dbcAMP on NF-κB DNA binding to the consensus κB sequence. Nuclear extracts were isolated from ethanol treated monocytes without or with dbcAMP followed by LPS treatment. Correspondent to the p65 nuclear levels, dbcAMP did not have any effect on the ethanol and LPS mediated NF-κB DNA binding (Fig. 9B).

\textit{cAMP downregulates LPS-stimulated NF-κB transcriptional activity.} We then examined the effect of dbcAMP on the LPS-stimulated NF-κB transcriptional activity by performing transient transfection in RAW 264.7 cells with a luciferase reporter construct
under the control of an NF-κB promoter containing 3 tandem repeats of the κB sequence (κB-luc). LPS stimulation for 6 hours of ethanol treated cells increased the NF-κB promoter activity approximately fourfold. dbcAMP pretreatment significantly attenuated the increases in LPS-stimulated NF-κB promoter activity (decreased by 43%) (Fig. 10).

Taken together, these data indicate that cAMP has minimal to no effect on the LPS-inducible signaling components leading to NF-κB activation, however, it can significantly decrease gene expression mediated by NF-κB dependent trans-activation. Further, the data also suggests that a decrease in LPS-inducible cAMP levels caused by chronic ethanol exposure plays a major role in the priming of monocytes to produce higher levels of TNF-α and enhancement of cAMP levels can effectively block this NF-κB mediated increase in TNF expression caused by ethanol.
DISCUSSION

The results from this study strongly suggest that decreases in cellular cAMP caused by ethanol play a critical role in the priming of monocytes/macrophages leading to enhanced LPS-inducible TNF expression. Several previous reports clearly demonstrate that cAMP exerts an inhibitory effect upon LPS-inducible TNF expression in human and murine monocytes/macrophages as well as rat Kupffer cells (reviewed in 44). Hence, the decreased cellular levels of cAMP observed in monocytes chronically exposed to ethanol are highly likely to contribute to the ethanol mediated enhancement of LPS stimulated TNF expression. The role of decreased cellular cAMP levels in ethanol treated monocytes was further confirmed by increasing the cellular cAMP levels using a cAMP analogue, which inhibited the enhanced TNF production caused by the synergism of ethanol and LPS. These data suggest that enhanced TNF production observed in our previous studies on PBMCs from alcoholic patients, as well as Kupffer cells in a clinically relevant model of ALD could be due to decreased cellular cAMP levels caused by chronic ethanol consumption. Since increased TNF expression plays an important role in the evolution of clinical and experimental ALD, its down-regulation via cAMP elevating agents may constitute an effective therapeutic approach.

In contrast with a published report that ethanol stabilizes TNF mRNA leading to an enhancement in TNF production (17), we did not observe any changes in TNF mRNA half-life in monocytes subjected to prolonged ethanol treatment. In our study the decrease in TNF expression caused by the elevation of cellular cAMP was found to reduce the steady state levels of TNF mRNA expression without affecting TNF mRNA half life. Additionally, the increase in ethanol mediated expression of LPS-inducible
TNF was observed only after a prolonged ethanol exposure (25 mM) for 6 weeks as opposed to the reported 48 h exposure leading to TNF mRNA stability (17). Indeed, in agreement with earlier work investigating the effects of acute alcohol (20, 34, 41, 42), we consistently found a decrease in LPS-inducible TNF expression after 48 h of ethanol exposure (data not shown).

Numerous studies have demonstrated the critical role of NF-κB in the LPS-inducible transcriptional induction of the TNF gene (1, 10, 26). Our previous work has demonstrated that overproduction of TNF observed in PBMCs of alcoholic patients is associated with increased NF-κB activation and elevated TNF mRNA levels (11). Since increased cellular cAMP levels significantly decreased LPS-inducible TNF mRNA expression in untreated as well as ethanol treated monocytes, the effect of cAMP on LPS induced NF-κB activation and activity was evaluated. We found that cAMP had no effect on LPS-inducible NF-κB activation (nuclear translocation) as indicated by the nuclear p65 levels and DNA binding in ethanol treated cells. However, functional studies in ethanol treated monocytes demonstrated that LPS-inducible transcriptional activation of the NF-κB reporter plasmid was significantly inhibited by elevated levels of cellular cAMP. These data and our previous observations suggest that chronic ethanol exposure may not only lead to an increase in LPS-inducible NF-κB activation but further enhance the functional activity of nuclear NF-κB-Rel complexes due to decreased cellular cAMP levels, leading to the overproduction of TNF by “ethanol primed” monocytes. This inference is also supported by several observations in different cell types including monocytes which show that dbcAMP represses NF-κB mediated transcription and TNF mRNA expression without affecting NFκB activation/DNA binding activity (28, 31, 33, 35–37).
The exact mechanisms by which cAMP levels regulate NF-κB activity are unclear. Recent studies suggest a role for the redox regulator thioredoxin (TRX) in modulating NF-κB activity in various cell types. Interestingly, TRX appears to play dual and opposite roles in regulating NF-κB dependent gene expression, depending on its subcellular localization. Increased cytoplasmic TRX inhibited NF-κB activation by interfering with IKK and blocking degradation of I-κB in HeLa cells and transformed human keratinocytes (13, 25). On the other hand, over expression of nuclear targeted TRX enhanced NF-κB activity by increasing its DNA binding in keratinocytes in response to UV irradiation (13). In our system, we find that elevation in cellular cAMP levels do not affect NF-κB activation and nuclear translocation, since we observe no differences in nuclear p65 level in ethanol treated monocytes with dbcAMP. Further, no significant differences were seen in the NF-κB DNA binding activity under conditions of elevated cellular cAMP levels. Hence, it appears that the TRX system may not play a significant role in the cAMP regulation of LPS-inducible NF-κB in ethanol primed monocytes. However, additional studies to examine the effect of changes in cAMP on the levels and cellular localization of TRX are needed to make definitive conclusions.

Importantly, in contrast to NF-κB binding sites, an AP-1/CRE-like site in the proximal promoter region has been demonstrated to play an essential role in the cAMP-mediated down-regulation of TNF gene transcription in murine macrophages (29). Hence, it is likely that decreased levels of cAMP caused by chronic ethanol exposure could (i) increase the NF-κB dependent transactivation of the TNF promoter and (ii) decrease AP-1/CRE mediated suppression of the TNF promoter activity, ultimately leading to overproduction of TNF.
Taken together, these data demonstrate that chronic ethanol exposure can decrease cellular cAMP levels in monocytes/macrophages and synergize with LPS to up-regulate the induction of TNF gene leading to excessive TNF production. Indeed, we postulate that the observed decrease in cAMP plays a critical role in the “priming” of monocytes/macrophages/Kupffer cells for enhanced LPS stimulated TNF production following chronic ethanol exposure. Furthermore, since enhanced TNF expression plays a significant role in the evolution of clinical and experimental ALD, its down-regulation via cAMP elevating agents could constitute an effective therapeutic approach in attenuating or preventing the progression of liver disease in alcoholic patients.

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GRANTS

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DISCLOSURES

None
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FIGURE LEGENDS:

Fig. 1. Long-term (chronic) alcohol exposure of RAW cells does not affect their proliferation response or viability. A colorimetric assay for the quantification of cell proliferation and viability using WST-1 reagent was performed on control cells and cells exposed to ethanol (25 mM) for 6 weeks. Data are presented as means and standard deviation.

Fig. 2. Chronic alcohol exposure significantly attenuates intracellular cAMP levels in macrophages. (A) After 6 week-alcohol exposure (25 mM), cells were stimulated with LPS (100 ng/ml) and collected after 3 hours for cAMP measurements. Data are representative of three separate experiments. *P<0.01 compared with un-stimulated control and ** P<0.01 compared with LPS-stimulated control. (B) Kupffer cells from control (n=4) and alcohol fed (n=4) rats were isolated and lysed, and cellular cAMP levels were measured. *P<0.01 compared with control . Obtained cAMP values were normalized by protein content. Data are presented as means and standard deviations.

Fig. 3. Long-term (chronic) alcohol exposure significantly increases LPS-inducible TNF expression in RAW cells. Cells were exposed to 25 mM alcohol for 6 weeks and stimulated with LPS (100 ng/ml) for 18 hours. TNF-α was quantified in supernatant using ELISA and normalized by protein values. Data are presented as means and standard deviations. *P<0.05 compared with LPS-stimulated control.
Fig. 4. Elevation of intracellular cAMP levels leads to abrogation of LPS- and ethanol-mediated TNF production in RAW cells. 15 minutes before LPS (100 ng/ml) stimulation, cells were pretreated with increasing concentrations of cAMP analogue, dibutyryl cAMP (db, 100-1000 µM). Supernatants were collected after 18 hours, TNF was quantified by ELISA. Data are presented as means and standard deviations and are representative of at least three separate experiments. *P<0.05, ** and *** P<0.01 compared with LPS-stimulated ethanol-treated. # and ##P<0.05 compared with LPS-stimulated control cells.

Fig. 5. Chronic alcohol exposure of RAW cells markedly up-regulates LPS inducible TNF-α mRNA expression. Cells were stimulated with LPS (100 ng/ml) and collected in 2 hours for total RNA isolation. TNF-α mRNA was quantified using real time PCR. Data are representative of three separate experiments. *P<0.01 compared with LPS-stimulated control.

Fig. 6. Elevation of intracellular cAMP levels significantly attenuates LPS-inducible TNF-α mRNA expression. Alcohol-exposed cells were pretreated with dbcAMP (500 µM) for 15 minutes and further stimulated with LPS (100 ng/ml) for 2, 3 and 4 hours for RNA extraction. TNF-α mRNA was quantified using real time PCR. Data are representative of three separate experiments. *P<0.05 compared with LPS-stimulated at 2h, **P<0.05 compared with LPS-stimulated at 3h, and ***P<0.001 compared with LPS-stimulated at 4h.
Fig. 7. Rat primary Kupffer cells obtained from a clinically relevant enteral alcohol feeding model of ALD exhibit a significant up-regulation of TNF message. RNA was extracted from Kupffer cells isolated from control (n=4) and alcohol fed rats (n=4) and TNF mRNA expression was measured using real time PCR. *P<0.01.

Fig. 8. Chronic ethanol exposure does not alter the TNF mRNA half-life. Cells chronically exposed to ethanol were treated with LPS (100 ng/ml) for 1 h, followed by actinomycin D (Act D, 1µg/ml) and total mRNA was isolated at indicated times. The remainder TNF mRNA at each time point was quantified using real time PCR.

Fig. 9. (A) Augmentation of LPS-inducible nuclear levels of p65 caused by ethanol is not affected by dbcAMP. Control and ethanol-treated cells were pretreated with dbcAMP (500µM) for 15 minutes and stimulated with LPS (100 ng/ml) for 30 min. Nuclear extracts were analyzed by Western Blotting for p65. (B) Increase in intracellular cAMP levels of alcohol-exposed cells on NF-κB binding. Nuclear extracts were prepared from ethanol-treated cells stimulated for 30 minutes with LPS (100 ng/ml) with and without dibutyryl cAMP (dbcAMP, 500 µM). EMSA was performed to assess NF-κB binding. dbcAMP had no influence on LPS-inducible NF-κB activation. W – competition assay using unlabeled wild type NF-κB, and M – competition assay using mutant NF-κB oligonucleotides.

Fig. 10. Increase in intracellular cAMP levels of alcohol-exposed cells significantly attenuated NF-κB activity in response to LPS. Cells were transfected with a luciferase
reporter construct containing the NF-κB -responsive IκB promoter as described in Methods. The transfected cells pretreated with dbcAMP (500 µM) for 15 minutes, were stimulated with LPS (100 ng/ml) for 6 hours. Cytoplasmic extracts were prepared and equal amounts were assayed for luciferase activity. *P<0.01 compared with LPS-stimulated without dbcAMP.
Fig. 1

[Graph showing absorbance (A440) over time after addition of WST-1 reagent (h) for Ctrl and EtOH conditions.]

Absorbance (A440) vs. Time after addition of WST-1 reagent (h)
Fig. 2

A

UT LPS

Control

EtOH

B

Control Alcohol-Fed

cAMP, pmol/mg protein

Control

Alcohol-Fed

* * *
Fig. 5

![Graph showing TNF mRNA (fold change) with and without LPS and EtOH treatment.]

Fig. 6

![Graph showing TNF mRNA (fold change) with and without dbcAMP and LPS treatment.]

Fig. 7

![Graph showing TNF mRNA (fold change) for Control and Alcohol-fed groups.](image)

Fig. 8

![Graph showing TNF mRNA (percent remaining) over time after addition of Act D for Control and EtOH groups.](image)
**Fig. 9**

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**NF-kB**

W M
Fig. 10

![Bar graph showing luc activity/mg protein for EtOH and EtOH+LPS conditions with and without dbcAMP.](image)

- **EtOH**
  - Without dbcAMP
  - With dbcAMP

- **EtOH+LPS**
  - Without dbcAMP
  - With dbcAMP

* indicates a significant difference.