Ethanol inhibits monocyte chemotactic protein-1 expression in interleukin-1β-activated human endothelial cells

John P. Cullen,1,* Shariq Sayeed,1* Ying Jin,1 Nicholas G. Theodorakis,1 James V. Sitzmann,1 Paul A. Cahill,2 and Eileen M. Redmond1
1Department of Surgery, University of Rochester Medical Center, Rochester, New York; and 2Vascular Health Research Centre, Dublin City University, Dublin, Ireland

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CARDIOVASCULAR DISEASE REMAINS the leading cause of death in the Western world. Although chronic alcohol abuse is associated with increased morbidity and mortality, epidemiologic studies associate moderate alcohol consumption with a reduced incidence of cardiovascular disease (14, 18, 32). Most of the benefit of alcohol appears to be conferred by a reduction in atherosclerotic risk (12, 36). However, the precise cellular mechanisms whereby ethanol (EtOH) elicits its cardioprotective effects are not yet fully understood. In addition to favorable effects on plasma lipoprotein levels, potentially beneficial effects of EtOH on platelets and on vascular endothelial cells and smooth muscle cells (SMC) have been recently reported (6, 10, 11, 24, 26).

Monocyte chemotactic protein-1 (MCP-1) is a member of the C-C chemokine family and a potent chemoattractant for monocytes (27). The involvement of MCP-1 in the pathogenesis of atherosclerosis has been widely investigated, and much evidence exists to support its central role in the process.

Migration of monocytes across the endothelial barrier is a significant early event in the formation of atherosclerotic lesions. Several studies provide compelling evidence for a direct role of MCP-1 in monocyte recruitment during atherosclerosis. In response to several atherogenic stimulants such as oxidized LDL, PDGF, and IL-1β, MCP-1 is induced in endothelial cells, SMC, and monocytes (31, 33). The expression of MCP-1 and other chemokines is thought to be regulated primarily at the level of gene transcription, although contributions by post-translational mechanisms have also been reported (35). The promoter region of the MCP-1 gene contains binding sites for the redox-responsive transcription factors NF-κB and AP-1 (16). MCP-1 mediates its biological activity mainly through interaction with an MCP-1 receptor, CCR2 (also known as C-C chemokine receptor) on the surface of its target cells, which include monocytes. This receptor belongs to the superfamily of G protein-coupled receptors with seven transmembrane domains. The important role of CCR2 in atherogenesis has been demonstrated in studies using gene knockout animal models; there was a marked decrease in atherosclerotic lesion formation in apolipoprotein E (apoE)-null mice that lacked CCR2 (4), and increased CCR2 expression is evident in patients with hypercholesterolemia (9). Several agents, including homocysteine and oxidized LDL, have been shown to affect CCR2 expression (37).

Although moderate alcohol consumption is correlated negatively with cardiovascular risk, the effect of EtOH on MCP-1 and its CCR2 receptor in the context of vascular disease and the molecular mechanisms involved are unknown. Here, we examined the effect of EtOH treatment on chemokine-induced MCP-1 expression in human endothelial cells in culture and on CCR2 receptor binding on THP-1 monocytes. We demonstrate that although EtOH has no effect on monocyte CCR2 binding activity it inhibits IL-1β-stimulated endothelial MCP-1 expression by decreasing MCP-1 mRNA stability, NF-κB and AP-1 binding activity, and MCP-1 gene transcription.

MATERIALS AND METHODS

Endothelial cell isolation and culture. Endothelial cells from human umbilical veins (HUVEC) were prepared by established methods as previously described (23). Briefly, HUVEC were harvested from human umbilical veins by adding 0.1% collagenase (GIBCO Laboratories, Grand Island, NY) for 30 min. The cells were grown to confluence in medium 199 (GIBCO) supplemented with 10% heat-inactivated FCS (Flow Laboratories, McLean, VA), penicillin-strept-

* J. P. Cullen and S. Sayeed contributed equally to this work.

Address for reprint requests and other correspondence: E. M. Redmond, Univ. of Rochester Medical Center, Dept. of Surgery, Box SURG, 601 Elmwood Ave., Rochester, NY, 14642-8410 (e-mail: eileen_redmond@urmc.rochester.edu).

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tobramycin (GIBCO), fungizone (GIBCO), and endothelial cell growth factor. Cells were assessed for endothelial cell phenotype by morphology and expression of von Willebrand factor antigen and platelet endothelial cell adhesion molecule. Endothelial cells between passages 2 and 5 were used in all experiments. THP-1 cells, a human monocytic cell line, were obtained from American Type Culture Collection (ATCC, Manassas, VA). Growth medium consisted of RPMI 1640, 10% FCS, 0.05 mM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, and 1.5 g/l bicarbonate. To avoid differentiation, cultures were maintained at a density of ~2 × 10^5 cells/ml.

EtOH treatment. Two hundred-proof EtOH (Pharmco Products, Brookfield, CT) was diluted and added to HUVEC medium to achieve desired concentrations. Cultures (70–80% confluent) were pretreated with or without EtOH for 2 h before stimulation with IL-1β (1 ng/ml), in the absence or presence of EtOH, for various times (1–24 h). To avoid evaporation of EtOH, petri dishes were immediately sealed in Parafilm. Cell viability was evaluated by Trypan blue exclusion assay and by comparing gross morphology to that of control cells. Actual levels of EtOH in culture medium over time were determined with a diagnostic kit from Sigma.

ELISA for MCP-1. Supernatants from treated HUVEC were collected and analyzed for MCP-1 protein concentration with a Chemikine human MCP-1 sandwich ELISA kit according to the manufacturer’s instructions (Chemicon International, Temecula, CA). 125I-labeled MCP-1 binding to THP-1 cells. For binding assays THP-1 cells were washed in PBS before being resuspended in binding buffer (RPMI 1640, 0.5% BSA, 25 mM HEPES, pH 7.4) at 1 × 10^6 cells/200 μl. Cells were incubated for 90 min at 25°C with 0.02–0.4 nM 125I-labeled MCP-1 (specific activity 2,200 Ci/mmol) in the absence or presence of 100 nM unlabeled MCP-1. The reaction was terminated by filtration of the reaction mixture through a GF/B filter (presoaked in 0.03% polyethyleneimine) with a Brandel cell harvester (Brandel Biomedical Research and Development Laboratories, Gaithersburg, MD). Radioactivity on the washed filters was counted in a gamma counter. Specific 125I-MCP-1 binding was determined by subtracting the nonspecific binding estimated in the presence of 100 nM unlabeled MCP-1 from total binding. To determine the exact binding affinity (Kd) and the total maximum number of binding sites (Bmax), the data were analyzed with Prism software (GraphPad).

Monocyte adhesion. Monocyte adherence experiments were carried out essentially as described by Chang et al. (5). THP-1 cells in RPMI 1640 medium containing 0.1% fetal bovine serum were labeled with 1 μCi of [3H]thymidine overnight (specific activity 23 Ci/mmol). THP-1 cells (6 × 10^5) were added to each well containing control or IL-1β-treated HUVEC and incubated for 1 h. Nonadherent monocytes were removed by washing with HBSS, HUVEC with adherent THP-1 cells were lysed with 1 M NaOH, and the radioactivity was counted by a scintillation counter.

Northern blot analysis. Total RNA was isolated from HUVEC with TRIzol reagent (GIBCO-BRL). Aliquots (10 μg) of the total RNA samples were separated on formaldehyde-agarose gels. The RNAs were transferred and UV cross-linked to nylon membranes and hybridized with 32P-labeled cDNA probes for human MCP-1 and GAPDH (ATCC) that were prepared by random priming. Transcripts were quantitated by NIH Image 1.60 and normalized with GAPDH levels for equal loading.

Electrophoretic mobility shift assay. EMSA was carried out essentially as described previously (5). To extract nuclear protein, HUVEC were washed and scraped in PBS. After centrifugation, the cell pellet was resuspended in buffer A (in mM: 10 KCl, 0.1 EDTA, 1 DTT, and 1 PMSF). The cells were lysed by adding 10% NP-40 and then centrifuged to obtain nuclear pellets. The nuclear pellets were extracted in buffer B (in mM: 20 HEPES, 1 EDTA, 1 DTT, 1 mM PMSF with 0.4 M NaCl), vigorously agitated, and then centrifuged. The supernatant containing the nuclear proteins was used for the EMSA or stored at −70°C for later use. Oligonucleotides corresponding to AP-1 (5′-CGCTTGATGACTGCCG-GAA-3′, Promega) and consensus NF-κB (5′-AGTTGAAGGTATTCCAGGC-3′, Promega) binding sites were end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. Nuclear extracts, normalized for protein content (10 μg), were incubated with 0.1 ng of 32P-labeled DNA for 15 min at room temperature in a final volume of binding buffer of 25 μl containing 1 μg of poly(dI-dC). The mixtures were electrophoretically separated on 6% nondenaturing polyacrylamide gels under high ionic strength. Gels were dried and imaged by autoradiography.

Nuclear run-on assay. Nuclear run-on transcription analysis was performed as described previously (29). HUVEC were washed with cold PBS and lysed with 0.5% NP-40 solution. The nuclei were isolated by centrifugation and resuspended in a 40% glycerol buffer. The nuclei were incubated with [α-32P]UTP at room temperature for 30 min. The run-on RNA was purified by treating the reaction mixture with DNase I and proteinase K followed by precipitation with EtOH. The labeled RNA was hybridized to 10 μg of linearized, denatured plasmid DNA blotted on membrane in 50% (vol/vol) formamide, 0.3 M NaCl, 0.03 M sodium citrate, 0.01% Ficoll, 0.01% polyvinylpyrrolidone, 0.01% bovine serum albumin, and 0.1% SDS-100 μg of salmon sperm DNA per milliliter (hybridization buffer) at 42°C for 24 h. The same number of counts of RNA was hybridized to each filter.

Statistics. Data are means ± SE; n is the number of individual experiments, with a minimum of three independent experiments performed. Statistical significance was estimated with the following analysis: unpaired Student’s t-test for comparison of two groups and Wilcoxon signed-rank test for the densitometric data. A value of P < 0.05 was considered significant.
Concentration was the same in the absence or presence of production in a time-dependent manner (Fig. 1).

Treatment of HUVEC with IL-1 in basal conditions, HUVEC MCP-1 levels were undetectable. MCP-1 was measured in medium samples by ELISA. Under basal conditions, IL-1 

**RESULTS**

**Effect of EtOH on IL-1β-stimulated MCP-1 secretion.** MCP-1 was measured in medium samples by ELISA. Under basal conditions, HUVEC MCP-1 levels were undetectable. Treatment of HUVEC with IL-1β (1 ng/ml) induced MCP-1 production in a time-dependent manner (Fig. 1A). The induction of MCP-1 by IL-1β was evident as early as 1 h after treatment. At 24 h after IL-1β treatment, MCP-1 levels were generally 700–900 pg/ml. The phorbol ester PMA (10 ng/ml) also potently induced MCP-1 secretion in HUVEC (Fig. 1A) and was included for comparison purposes. EtOH (1–200 mM) dose-dependently inhibited IL-1β-stimulated MCP-1 secretion, with significant inhibition (~25%) observed at 100 mM and ~90% inhibition at 200 mM (Fig. 1B). The IC50 (i.e., the concentration required to cause 50% inhibition of maximal response) for EtOH was 50 mM. EtOH at all concentrations used had no significant effect on HUVEC viability as assessed by Trypan blue exclusion (data not shown). The EtOH concentration in cell culture plates over the experimental period (up to 24 h) was >90% of the original concentration. The concentration was the same in the absence or presence of endothelial cells, suggesting that no significant metabolism of EtOH was occurring.

**Effect of EtOH on IL-1β-stimulated THP-1 monocyte adhesion to HUVEC.** There was a significant increase in the number of THP-1 monocytes adherent to IL-1β-activated HUVEC compared with control HUVEC (Fig. 2). EtOH pretreatment dose-dependently decreased IL-1β-activated monocyte adhesion to HUVEC: ~25% and 50% inhibition for 10 mM and 100 mM EtOH, respectively (Fig. 2).

**EtOH and CCR2 receptor binding.** CCR2 receptor surface expression on whole THP-1 cells was determined by 125I-MCP-1 binding analysis as described in MATERIALS AND METHODS. 125I-MCP-1 bound to control THP-1 cells (Fig. 3) with an equilibrium binding constant (Kd) of 0.125 nM and Bmax of 3.52 fmol/106 cells. Nonspecific binding was ~5–20% of total binding. There was no significant effect of EtOH treatment (10–100 mM, 24 h) on 125I-MCP-1 binding to THP-1 cells (Fig. 3).

**Effect of EtOH on IL-1β-stimulated MCP-1 mRNA levels.** HUVEC were pretreated with or without EtOH (1–100 mM) and then stimulated with IL-1β, in the absence or presence of EtOH, for various periods of time (1–5 h). Total RNA was isolated from HUVEC and used in Northern blot analysis. After exposure of HUVEC to IL-1β there was a time-dependent increase in the level of MCP-1 mRNA (Fig. 2) compared with untreated cells. EtOH pretreatment significantly inhibited IL-1β-induced MCP-1 mRNA expression (Fig. 4). Densitometric analysis of autoradiographic bands showed 36% and 57% reduction for 20 and 100 mM EtOH, respectively, at 2 h (Fig. 4). The inhibitory effect of EtOH (100 mM) on MCP-1 mRNA expression was quantitatively similar to that of 3-morpholinosydnonimine (SIM-1, 0.3 mM; 58–65% inhibition), a NO donor previously reported to inhibit MCP-1 (Fig. 4). EtOH decreases MCP-1 mRNA stability. MCP-1 mRNA expression can be regulated at the transcriptional or posttranscriptional level. To study whether the inhibitory effect of EtOH on MCP-1 gene expression is caused by a decrease in mRNA stability, HUVEC were treated with or without EtOH (50 mM) and IL-1β followed by an incubation with actinomycin D (10 μg/ml) to prevent further synthesis of MCP-1 mRNA. Total RNA was extracted at different times. As indicated in Fig. 5, EtOH significantly increased MCP-1 mRNA degradation rate in actinomycin D-treated cells; estimated half-life of the MCP-1 transcript was ~9–10 h vs. 3–4 h in EtOH-treated cells. This indicates that the inhibitory effect of
EtOH on MCP-1 is caused, in part, by decreasing the stability of MCP-1 mRNA.

Effect of EtOH on MCP-1 gene transcription. To determine the effect of EtOH at the level of transcriptional regulation, nuclear run-on studies were performed with IL-1β stimulation. In vitro transcription studies indicated that there is minimal transcriptional activity of the MCP-1 gene in HUVEC under basal conditions grown in standard culture conditions. Treatment with IL-1β significantly increased MCP-1 mRNA transcription after only 30 min relative to tubulin gene transcription. There was an inhibitory effect of concomitant administration of EtOH (20 and 100 mM) on MCP-1 transcription (Fig. 6).

Effect of EtOH on transcription factor binding activities. Binding of the transcription factors NF-κB and AP-1 in the MCP-1 promoter region has been implicated in MCP-1 gene induction by a variety of agents. Thus, nuclear extracts from HUVEC were isolated, and EMSA was performed with oligonucleotides containing the putative NF-κB and AP-1 binding sites, respectively. As shown in Fig. 7, exposure of HUVEC to IL-1β for 60 min induced activation of both NF-κB and AP-1. Stimulation of NF-κB activity induced by IL-1β was reduced (~25% and 50% inhibition for 20 and 100 mM EtOH, respectively) in cells that were also exposed to EtOH treatment (Fig. 7). AP-1 binding activity was also reduced by EtOH treatment (Fig. 7). In contrast, EtOH had no effect on binding of the transcription factors SP-1 and OCT-1 (data not shown).

DISCUSSION

The major finding of this in vitro study is that EtOH, at physiologically relevant concentrations, inhibits the endothelial production of the proatherogenic chemokine MCP-1 by decreasing gene transcription and mRNA stability.

Several studies provide evidence for a key role of MCP-1 in cardiovascular disease. The role of MCP-1 in human disease has been implicated by immunohistochemical studies of atherosclerotic plaques (38). Direct evidence for the atherogenic potential of macrophage MCP-1 expression was derived from a study in apoE-knockout mice (1), a well-characterized model of atherogenesis. Analysis of atherosclerotic lesions in apoE mice overexpressing a murine MCP-1 transgene, when compared with the response of control apoE-knockout mice, revealed enhanced lipid staining, increased oxidized lipid content, and increased immunostaining for macrophage cell surface markers (1). In another study, mice lacking the CCR2 receptor were crossed with apoE-knockout mice and fed a Western diet. These mice displayed a decrease in lesion size and a decrease in the number of macrophages in these lesions (4), supporting the hypothesis that MCP-1 is important in...
monocyte recruitment during atherosclerosis and that the process of monocyte recruitment is a major determinant of lesion size and complexity. In addition, MCP-1 can induce chemotaxis of endothelial cells and can act as a direct mediator of angiogenesis (25), a process believed to be maladaptive in the atherosclerotic plaque. There is also growing evidence that chemokines, and MCP-1 in particular, may play an important pathogenic role in other cardiovascular diseases such as myocardial ischemia (20) and congestive heart failure (2).

Despite the inverse relationship between moderate alcohol consumption and the risk of coronary heart disease and the evidence for a prominent role of MCP-1 in the pathogenesis of cardiovascular disease, only a few studies have investigated the effect of EtOH on MCP-1 expression in this context. A review of the literature to date reveals that the majority of studies investigating alcohol and MCP-1 have addressed alcoholic liver injury and investigated Kupffer cell chemokine production after alcohol intoxication (see, e.g., Refs. 3, 19, 22). Szabo

Fig. 6. Nuclear run-on analysis of the effect of EtOH on IL-1β-mediated MCP-1 gene expression. HUVEC were stimulated with IL-1β in the absence or presence of EtOH. After 30 min nuclei were isolated, and nuclear run-on assays were performed with MCP-1, GAPDH, and β-tubulin cDNA as described in MATERIALS AND METHODS. A: hybridization bands were quantified, with GAPDH and β-tubulin serving as internal controls for standardization. B: normalized graph with values representing means ± SE of 3 separate experiments. *P < 0.05 vs. IL-1β treatment alone.

Fig. 7. EMSA analysis of the effect of EtOH on IL-1β-mediated NF-κB and AP-1 DNA binding activity. HUVEC were stimulated with IL-1β in the absence or presence of EtOH. After 60 min nuclear protein was isolated and subjected to EMSA with 32P-labeled DNA probes containing either the NF-κB or AP-1 binding sites as described in MATERIALS AND METHODS. A: top, representative blot showing dose-dependent inhibition by EtOH of IL-1β-stimulated NF-κB/DNA binding; bottom, normalized graph with values representing means ± SE of 3 separate experiments. B: top, representative blot showing inhibition by EtOH of IL-1β-stimulated AP-1/DNA binding; bottom, normalized graph with values representing means ± SE of 4 separate experiments. *P < 0.05 vs. IL-1β treatment alone.
et al. (30) reported that acute alcohol consumption attenuated the production of monocyte MCP-1 in response to subsequent bacterial stimulation in humans by inhibiting NF-κB binding. In addition, Feng et al. (8) demonstrated in a balloon injury rabbit model that long-term consumption of red wine inhibited MCP-1 expression and reduced neointimal hyperplasia, and Estruch et al. (7) reported that daily red wine consumption decreased MCP-1 in monocytes. The authors did not determine whether this effect was mediated by the phenolic compounds or the EtOH present in the red wine and provided no information on the mechanism(s) underlying the inhibitory effect. Hypercholesterolemic rabbits that underwent balloon angioplasty and daily alcohol feeding for 10 wk exhibited an increase in lumen size, a decrease in the number of foam cells in the neointima, and reduced MCP-1 and PDGF levels compared with controls (17). Again, the molecular mechanisms underlying these effects were not addressed in that study.

MCP-1 is produced by monocytes, endothelial cells, and SMC in response to various stimuli. The level of vascular endothelial cell and SMC expression of MCP-1 has been correlated with serum MCP-1 concentration (15). MCP-1 inhibitors, acting by decreasing either agonist production or receptor expression, represent potentially innovative pharmacological tools for the prevention of cardiovascular-related diseases. Although EtOH had no effect on CCR2 receptor binding activity in monocytes, our data demonstrate a dose-dependent inhibition of IL-1β stimulated endothelial cell MCP-1 by EtOH concomitant with inhibition of monocyte adhesion to activated endothelial cells. Although the possibility exists that EtOH may also alter IL-1β binding to its receptor, resulting in decreased activation and a decreased MCP-1 response, our data support an effect of EtOH at the level of MCP-1 mRNA. As evidenced by an increased MCP-1 mRNA degradation rate in actinomycin D-treated cells, EtOH decreased the stability of MCP-1 mRNA in HUVEC. Of interest, it was reported previously that EtOH stabilizes LPS-induced TNF-α mRNA in Kupffer cells (13). Thus EtOH does not appear to cause a general destabilization of induced mRNA. With nuclear run-on analysis we demonstrated that IL-1β-stimulated MCP-1 gene transcription was inhibited by EtOH. Moreover, EMSA indicated reduced binding of the transcription factors NF-κB and AP-1, which were previously implicated in the induction of MCP-1 (16), after EtOH treatment. Given the evidence for a key role of MCP-1 in atherogenesis, inhibition by EtOH of endothelial cell MCP-1 production in vivo would be expected to result in decreased recruitment, adhesion, and transeンド혈루의 migration of monocytes at sites of arterial injury and thus decreased atherosclerosis.

An additional mechanism for the proatherogenic effect of MCP-1 has recently emerged, namely, MCP-1 directly inducing SMC proliferation by differential activation of NF-κB and AP-1 (34). Interestingly, we showed previously (10, 26) that EtOH inhibits SMC proliferation by modulating the activity and expression of cell cycle regulatory molecules. The inhibitory effect of EtOH on MCP-1 reported here thus represents a second, albeit indirect, mechanism whereby EtOH may affect SMC proliferation and thus negatively affect atherogenesis. Moreover, several studies have established a relationship between NO and chemokine production by demonstrating that NO can downregulate MCP-1 production in the endothelial cell or that increased MCP-1 production is coupled with endothelial nitric oxide synthase (eNOS) downregulation (39). In light of this, our previous study (11) demonstrating a stimulatory effect of EtOH on eNOS suggests a possible synergistic mechanism whereby EtOH could inhibit MCP-1 indirectly via stimulation of NOS activity.

“Moderate” alcohol consumption is generally considered to be in the range of one to three drinks per day (14, 18, 32), giving rise to blood alcohol levels of ~5–25 mM. A blood alcohol level (BAL) of 0.1 g% is approximately equivalent to 25 mM EtOH. In alcoholics, BAL can reach in excess of 100 mM. Thus the concentrations of EtOH used in our study (1–100 mM) are physiologically relevant. Significant effects of EtOH on MCP-1 were demonstrated at concentrations as low as 1 mM. Whereas a J-shaped relationship exists between alcohol and total mortality, several studies have demonstrated a strong dose-response relationship between alcohol and cardiovascular disease (21). Of note, our data demonstrate that EtOH inhibits IL-1β-induced MCP-1 production in a dose-dependent manner.

The importance of our data and their relevance to the in vivo efficacy of EtOH remain speculative. However, EtOH’s inhibition of endothelial MCP-1 at transcriptional and posttranscriptional levels may represent a novel mechanism contributing to its apparent cardiovascular protective effects as supported by epidemiologic studies.

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