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Acetaldehyde stimulates monocyte adhesion in a P-selectin- and TNF α -dependent manner

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Abstract

Objective—The aim of this study was to determine the effects of acetaldehyde on various steps of the monocyte recruitment cascade.

Methods—Human umbilical venous endothelial cells (HUVEC), primary blood monocytes (PBM) and THP-1 monocytes, were treated with acetaldehyde (0.1–0 μ M) for 6 h. Monocyte adherence experiments were performed using 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein-acetoxymethylester labeled PBM or 3 H-thymidine labeled THP-1 cells. HUVEC TNF α mRNA and protein levels were determined by quantitative real-time PCR and immunoassay, respectively, and HUVEC P-selectin and monocyte CCR2 expression were determined by FACS analysis.

Results—Acetaldehyde dose-dependently increased the number of CCR2 positive THP-1 monocytes, with a maximal increase of ~50% observed in the presence of 10 μ M acetaldehyde. There was a significant increase in both the number of P-selectin positive cells and P-selectin receptor density when HUVEC were incubated with acetaldehyde. HUVEC TNF α mRNA expression and secretion were enhanced by acetaldehyde. Moreover, acetaldehyde increased THP-1 and PBM adhesion to HUVEC. Inhibition of P-selectin or TNF α , using antibodies or siRNA-directed gene knockdown, attenuated acetaldehyde-induced monocyte adhesion. In conclusion, acetaldehyde increased the number of CCR2 positive monocytes and stimulated endothelial cell P-selectin and TNF α expression. Moreover, acetaldehyde increased monocyte adhesion to endothelial cells, an effect that was both P-selectin- and TNF α -dependent.

Conclusion—These effects of acetaldehyde may contribute, in part, to the increase in coronary heart disease that is associated with binge patterns of alcohol consumption.

Keywords

Acetaldehyde; TNF α ; P-selectin; Atherosclerosis; Monocytes; Endothelial cells; Binge drinking

1. Introduction

Cardiovascular disease remains the leading cause of death in the Western world. While epidemiologic studies associate moderate alcohol consumption with a reduced incidence of

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cardiovascular disease, the probability of coronary heart disease and cardiovascular mortality increases with both heavier consumption and with abstinence [1,2]. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), a ‘binge’ is a pattern of drinking that corresponds to consuming 5 or more drinks (male), or 4 or more drinks (female), in about 2 h [3]. A number of studies suggest that a binge pattern of drinking may precipitate myocardial ischemia or infarction [4,5] and evidence also exists of an association between binge alcohol consumption and a 2-fold greater mortality after acute myocardial infarction [6]. In addition to its effects on coronary heart disease, an irregular pattern of heavy drinking occasions also appears to have a relationship with other types of cardiovascular death such as stroke and sudden cardiac death [4,7].

The primary step in the metabolism of alcohol is the oxidation of ethanol to acetaldehyde by the enzyme alcohol dehydrogenase. Elevated blood acetaldehyde levels have been demonstrated in both mice and humans following administration of ‘binge’ amounts of alcohol [8–10]. Acetaldehyde has been shown to have multiple cardiovascular effects *in vivo*, including vasodilation, increased heart rate and decreased blood pressure [11]. Despite the fact that there have been numerous studies investigating the neurobehavioral and hepatic effects of acetaldehyde, its role in alcohol’s effect on atherosclerosis remains unclear.

Monocyte recruitment plays a pivotal role in the development of atherosclerosis and involves a sequence of events that include monocyte attraction, tethering and rolling, and firm adhesion. Several studies provide compelling evidence for a direct role of the monocyte chemotactic protein-1 (MCP-1) receptor, CCR2, in monocyte recruitment during atherosclerosis. In particular, the non-redundant role of CCR2 in monocyte recruitment and atherogenesis has been demonstrated in mice with a targeted disruption of the CCR2 gene [12]. P-selectin supports the initial tethering and rolling of monocytes and can be expressed on the cell surface within minutes of cytokine recognition. Inhibition of monocyte rolling on the endothelium in P-selectin knockout mice highlights the critical role of P-selectin in the monocyte adhesion cascade [13]. TNF α has also emerged as an important contributor to the development of atherosclerotic lesions by promoting the expression of adhesion molecules on endothelial cells, leukocyte rolling, the recruitment and activation of inflammatory cells, and the initiation of the inflammatory cascade inside the arterial wall [14,15].

Here we examined the effects of acetaldehyde treatment on various steps of the monocyte recruitment cascade. We investigated the effects of acetaldehyde on endothelial cell adhesion marker expression, monocyte adhesion to endothelial cells, and on monocyte CCR2 receptor expression.

2. Materials and methods

Acetaldehyde was obtained from Sigma (St. Louis, MO). TNF α protein and TNF α antibody were obtained from BD Pharmingen (San Jose, CA). All other reagents were of the highest purity commercially available.

2.1. Endothelial cell isolation and culture

Human umbilical venous endothelial cells (HUVEC) were prepared by established methods as previously described [16]. Cells were grown to confluence in Medium 199 (Gibco) supplemented with 10% heat inactivated FCS (Gibco), penicillin-streptomycin, fungizone and endothelial cell growth factor (BD Biosciences). Cells were assessed for endothelial cell phenotype by morphology, expression of von Willebrand Factor antigen and PECAM. HUVEC between passages 2–5 were used in all experiments.

2.2. Primary blood monocyte isolation

Monocytes were isolated according to the standard blood separation procedure described previously [17]. Blood was drawn from control subjects under a University of Rochester, Institutional Review Board approved protocol. Briefly, peripheral blood monocytes (PBM) were isolated by a Ficoll–Paque™ PLUS (GE Health care Biosciences AB, Uppsala, Sweden), density gradient. T cells were separated by rosetting with sheep red blood cells. Monocytes were isolated from the T cell-depleted peripheral blood mononuclear cells by negative magnetic separation with α CD2, α CD3, α CD19 Dynal magnetic beads (Invitrogen, Carlsbad, CA). A subsequent flow cytometric analysis revealed >90% CD36⁺ cells in MØ samples (data not shown). Culture medium consisted of RPMI-1640 medium supplemented with 15% FCS, 50 IU/ml penicillin, 50 µg/ml gentamicin, 50 µg/ml streptomycin, 2.5 µg/ml fungizone, 4 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM non-essential amino acids and 100 IU/ml Polymyxin B.

2.3. THP-1 cells

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 $\sim 2 \times 10^5$ cells/ml.

2.4. Acetaldehyde treatment

A stock concentration of 100 mM acetaldehyde was made up in PBS and diluted in culture medium to the desired concentration.

2.5. PBM adhesion

Primary blood monocytes (PBM) were resuspended to 5×10^6 /ml and labeled with 2',7'-bis-2-carboxyethyl-5-(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM; Sigma; 10 µg/ml) for 30 min at room temperature in the dark. Dye loading was quenched by adding 10 ml of PBM culture media and centrifuging at 2000 rpm for 5 min, followed by washing. PBM (6×10^5) were added to each well containing control or acetaldehyde-treated HUVEC and incubated for 1 h. Nonadherent monocytes were removed by washing with HBSS. The number of adherent monocytes was determined by fluorescent microscopy and by counting the fluorescence-labeled cells in 4 different microscopic fields using Image J 1.32 software (NIH).

2.6. THP-1 adhesion

Monocyte adherence experiments were carried out essentially as previously described [18]. THP-1 cells in RPMI 1640 medium containing 0.1% fetal bovine serum were labeled with 1 μ Ci of [3 H]thymidine overnight (specific activity 23 Ci/mmol). THP-1 cells (6×10^5) were added to each well containing control, TNF α - or acetaldehyde-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.

2.7. Quantitative real-time RT-PCR

Total RNA was isolated from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' specifications. Total RNA (1–2 μ g) was reverse-transcribed using an iScriptTM cDNA synthesis kit from Bio-Rad (Hercules, CA, USA). The gene-specific oligonucleotide sequences were: TNF α , forward 5-GTCCAGGCTTGTCCTGCTAC-3, reverse 5-CTGAGTCCGTTGAGGGA-GAG-3; P-selectin, forward 5-CAGGATCTCCAGTTCCAAA-3, reverse 5-CTGAGGGCTTAGCAAAGGTG-3; GAPDH, forward 5-CGAGATCCC-TCCAAAATCAA-3, reverse 5-TTCACACCCATGGACGAACAT-3. For the quantitative measurement of mRNA, real-time RT-PCR was performed using the Stratagene MX3005 machine and the SYBR green jumpstart PCR kit (Sigma) as described by the manufacturer.

2.8. Immunoassay for TNF α

Supernatants from treated HUVEC were collected and concentrated using Microcon centrifugal filter devices (Millipore, Billerica, MA). Concentrated samples were analyzed for TNF α protein with a Quantikine human TNF α High Sensitivity Immunoassay according to the manufacturer's instructions (R & D Systems, Minneapolis, MN).

2.9. Fluorescence-activated cell sorting (FACS) analysis

For surface staining, cells were incubated for 30 min on ice with human IgG (MP Biomedicals, Solon, OH) before staining with either an Alexa Fluoro 647 labeled anti-human CCR2 monoclonal antibody (BD Pharmingen), or a Phycoerythrin labeled anti-human ICAM-1, VCAM-1, P-selectin or E-selectin (Santa Cruz Biotechnology) or a matching isotype control at 4 °C for 30 min. Cells were then washed and fixed using 150 μ l Cytofix (BD Pharmingen) for 30 min followed by a 2% FACS Buffer (2% FBS, 0.05% NaN₃, PBS) wash. For intracellular staining, cells were incubated for 30 min on ice in 150 μ l Cytofix/Cytoperm (BD Pharmingen) and washed in Perm/Wash (BD Pharmingen) prior to incubation for 30 min on ice with human 10% (v/v) IgG/Perm/Wash. Cells were then stained with a Phycoerythrin labeled anti-human TNF α monoclonal antibody (R & D Systems) or a matching isotype control at 4 °C for 30 min followed by a Perm/Wash wash. Protein expression was assessed on a Becton Dickinson FACSArray Bioanalyzer System, with 1×10^4 total events collected for each sample. Gating for fluorescence intensity was determined by manually gating in the isotype control cell sample, and maintaining that gating for subsequent samples. Data are represented as percent of positive cells or net mean

fluorescence intensity (MFI) (as calculated by subtraction of isotype control value from that of the positive antibody).

2.10. siRNA transfection

For gene silencing studies, the Gene Pulser Xcell™ system (Bio-Rad, Hercules, CA) was used for transient transfection of HUVEC with gene-specific siRNA. Briefly, 2×10^5 cells were transfected with 2 µg of siRNA targeting TNFα or P-selectin (Ambion, Austin, TX), or 2 µg of each siRNA targeting TNFα and P-selectin or a scrambled negative control siRNA (Ambion) in 75 µl of siRNA electroporation buffer. Following transfection cells were allowed to recover overnight prior to treatment with acetaldehyde.

2.11. Statistics

The data shown are the means $\pm$ S.E.M.; n = number of individual experiments, with a minimum of 3 independent experiments performed. Statistical significance was estimated using the unpaired Student *t* test for the comparison of 2 groups. When more than 2 groups were present, ANOVA (factorial design) was used (Graph Pad Prism; Graph Pad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered significant.

3. Results

3.1. Effect of acetaldehyde on monocyte CCR2 expression

In untreated primary blood monocytes (PBM) approximately 6% of the population were CCR2 positive as determined by FACS analysis. Acetaldehyde treatment (10 µM, 6 h) increased the number of CCR2 positive PBM by 2.5-fold, to approximately 14% (Fig. 1a). In untreated THP-1 monocytes approximately 22% of the population were CCR2 positive. Incubation with acetaldehyde dose-dependently increased the number of CCR2 positive THP-1 monocytes, with a maximal increase of ~50% observed in the presence of 10 µM acetaldehyde (Fig. 1b and c), with no effect on CCR2 receptor density (mean fluorescent intensity). There was no change in either the number of CCR2 positive THP-1 monocytes or CCR2 receptor density when THP-1 monocytes were incubated with TNFα (10 ng/ml, 6 h) (Fig. 1c). There was no effect of acetaldehyde on endothelial cell MCP-1 secretion as determined by ELISA (data not shown).

3.2. Effect of acetaldehyde on HUVEC cell adhesion molecule expression

ICAM-1, VCAM-1, P-selectin and E-selectin expression on endothelial cells were determined by FACS analysis. HUVEC were treated with acetaldehyde (0.1–25 µM, 6 h) or TNFα (10 ng/ml, 6 or 24 h), which was included for control purposes. While there was no effect of TNFα on the expression of ICAM-1, VCAM-1, P-selectin and E-selectin in endothelial cells after 6 h, TNFα treatment for 24 h increased the number of ICAM-1 and VCAM-1 positive cells to >90% and E-selectin positive cells to ~50%, in the absence of any effect on P-selectin expression (Fig. 2a). There was no effect of acetaldehyde (0.1–25 µM, 6 h) on the expression of ICAM-1, VCAM-1 and E-selectin in endothelial cells (data not shown). However, there was a significant increase in the number of P-selectin positive cells (Fig. 2b) and a biphasic increase in P-selectin receptor density (Fig. 2c). In control

(untreated) HUVEC, the average number of cells expressing P-selectin was ~3.5%, which is in agreement with previous reports from the literature.

3.3. Effect of acetaldehyde on endothelial cell TNF α expression

HUVEC were treated with acetaldehyde for 6 h and total RNA was isolated and analyzed by QRT-PCR. Under basal conditions, HUVEC TNF α mRNA levels were virtually undetectable. Acetaldehyde (10 μ M) significantly increased (~2.5-fold) TNF α mRNA expression (Fig. 3a). Analysis by high-sensitivity immunoassay demonstrated that acetaldehyde dose-dependently increased secreted TNF α protein in conditioned media from undetectable levels up to 1.5 pg/ml in the presence of 50 μ M acetaldehyde (Fig. 3b). This dose-dependent increase in TNF α secretion was concurrent with a dose-dependent decrease in HUVEC intracellular TNF α as determined by FACS analysis (Fig. 3c).

3.4. Effect of acetaldehyde on monocyte adhesion to HUVEC

Acetaldehyde (0.1–25 μ M, 6 h) at all concentrations used significantly increased PBM adhesion to HUVEC, when compared to control, untreated cells (Fig. 3d and e); ~250% and 700% increase for 0.1 and 25 μ M acetaldehyde, respectively. When HUVEC were incubated in the presence of TNF α (10 ng/ml, 6 h) there was a 90% increase in THP-1 monocyte adhesion to HUVEC when compared to control, untreated cells (Fig. 3f). Acetaldehyde (0.1–25 μ M, 6 h) at all concentrations used significantly increased THP-1 monocyte adhesion to HUVEC, when compared to control cells (Fig. 3f); ~50% and 75% increase for 0.1 and 25 μ M acetaldehyde, respectively.

3.5. Effect of ethanol on acetaldehyde-induced monocyte adhesion

The acetaldehyde-induced increase in THP-1 monocyte adhesion was completely inhibited by pretreatment of HUVEC with ethanol (10 mM or 25 mM, 2 h) (Fig. 4a).

3.6. Effect of P-selectin blockade on acetaldehyde-induced THP-1 monocyte adhesion

HUVEC were pretreated with an anti-P-selectin antibody (1 μ g/ml, 30 min) or subjected to siRNA-directed P-selectin knockdown prior to the addition of acetaldehyde. Gene silencing of P-selectin with a specific targeted siRNA duplex was confirmed at the mRNA and protein level (by QRT-PCR and FACS analysis, respectively); ~70% and ~90% decrease in P-selectin mRNA and P-selectin positive cells, respectively, when compared with scrambled control transfected cells (Fig. 4b). Treatment with P-selectin antibody or P-selectin siRNA alone had no significant effect on baseline monocyte adhesion. The acetaldehyde-induced increase in monocyte adhesion was significantly inhibited by pretreatment with a P-selectin antibody or by P-selectin siRNA, with ~45% and ~65% inhibition, respectively, observed in the presence of 10 μ M acetaldehyde (Fig. 4c).

3.7. Effect of TNF α blockade on acetaldehyde-induced THP-1 monocyte adhesion

HUVEC were pretreated with an anti-TNF α antibody (2 μ l/ml, 30 min) or subjected to siRNA-directed TNF α knockdown prior to the addition of acetaldehyde. We were unable to confirm gene silencing of TNF α at the mRNA level as basal expression of this gene was already virtually undetectable. However, gene silencing of TNF α with a specific targeted

siRNA duplex was confirmed at the protein level by FACS analysis; ~70% decrease in intracellular TNF α positive cells when compared with scrambled control transfected cells (Fig. 4d). Treatment with TNF α antibody or TNF α siRNA alone had no significant effect on monocyte adhesion in control cells. However, the acetaldehyde-induced increase in monocyte adhesion was significantly inhibited by pretreatment with a TNF α antibody or by TNF α siRNA, with ~60% and ~75% inhibition, respectively, observed in the presence of 10 μ M acetaldehyde (Fig. 4e).

3.8. Effect of dual P-selectin/TNF α siRNA-directed knockdown on acetaldehyde-induced THP-1 monocyte adhesion

HUVEC were subjected to dual siRNA-directed P-selectin/TNF α knockdown prior to the addition of acetaldehyde. Gene silencing of P-selectin/TNF α with specific targeted siRNA duplexes was confirmed at the protein level by FACS analysis; ~50% and ~40% decrease in P-selectin and intracellular TNF α positive cells, respectively, when compared with scrambled control transfected cells (Fig. 4f). Treatment with dual P-selectin/TNF α siRNA alone had no significant effect on monocyte adhesion. The acetaldehyde-induced increase in monocyte adhesion was almost completely inhibited by cotransfection with P-selectin/TNF α siRNA, with ~90% inhibition observed in the presence of 10 μ M acetaldehyde (Fig. 4g).

4. Discussion

The major finding of this *in vitro* study is that acetaldehyde stimulates multiple pathways in the monocyte recruitment cascade. Specifically, acetaldehyde increased the number of CCR2 expressing monocytes and stimulated endothelial cell P-selectin expression and TNF α production. Moreover, acetaldehyde increased monocyte adhesion to endothelial cells, an effect that was significantly inhibited by TNF α or P-selectin blockade. These *in vitro* findings support novel effects of acetaldehyde, the primary metabolite of ethanol, on processes involved in the initiation of atherosclerosis. These effects may be relevant to the increase in cardiovascular mortality associated with binge drinking.

The primary step in the metabolism of alcohol is the oxidation of ethanol to acetaldehyde by the enzyme alcohol dehydrogenase. The elevation of human acetaldehyde levels causes a number of typical effects, such as vasodilation associated with increased skin temperature, increased heart and respiration rate and lowered blood pressure [11]. The concentrations of acetaldehyde used in the current study, 0.1–25 μ M, are physiologically relevant and cover a range of concentrations that are found in the blood following the consumption of moderate and binge amounts of alcohol. Subjects with blood alcohol levels of 15–17 mM, which correlates to moderate alcohol consumption, have corresponding blood acetaldehyde levels of 2–5 μ M [9]. It has been demonstrated that the administration of 2 and 5 g/kg alcohol by oral gavage in mice, achieves blood alcohol concentrations of 46 and 124 mM after 1 h, resulting in blood acetaldehyde levels of 4.3 and 27.6 μ M, respectively [8]. It is of interest to note, in that study, that acetaldehyde levels remain elevated after 12 h despite diminished blood ethanol levels. The effects of acetaldehyde observed in our study were seen after only 6 h of incubation with vascular cells. We used this shorter time in order to more accurately reflect the *in vivo* environment where elevated blood acetaldehyde levels may be observed following binge alcohol consumption.

Seemingly paradoxical effects of acetaldehyde on monocyte-endothelial interactions have been reported. A recent study by Vrsalovic et al. demonstrated that acetaldehyde exhibited similar properties to ethanol in that it impaired monocyte ingestion ability and phagocytic activity, suggesting anti-atherogenic properties [19]. In contrast, Haorah et al., have shown that acetaldehyde possesses pro-atherogenic properties, in that it stimulated monocyte migration across bovine brain microvascular endothelial cells [20]. However, it must be noted that these contrasting anti- and pro-atherogenic effects of acetaldehyde were observed at 3- to 5-fold higher concentrations than used in the present study. Our data demonstrate the novel finding that acetaldehyde stimulates monocyte adhesion to the endothelium at concentrations as low as 0.1 μ M.

In contrast to the effects of its primary metabolite demonstrated here, we have previously reported that ethanol itself (10–100 mM) inhibits interleukin-1 β monocyte adhesion [18]. This concentration range spans blood alcohol levels that would be found in both moderate (0.08% $\approx$ 15 mM) and binge (0.25% $\approx$ 50 mM) drinkers. However, it must be noted that in those *in vitro* experiments there is no significant metabolism of ethanol occurring [21]. Moreover, in the current study we have demonstrated that the acetaldehyde-induced increase in monocyte adhesion is completely inhibited when HUVEC are pretreated with ethanol. It is of interest to note that the concentrations of both the ethanol and acetaldehyde used in our studies were physiologically relevant and based on our findings appear to have anti- and pro-atherogenic properties respectively. This suggests that there may be a delicate interplay between ethanol and its metabolite, acetaldehyde, on pathways involved in atherosclerosis following alcohol consumption.

Monocyte recruitment to the vasculature is dependent on a multistep cascade of adhesive interactions. Monocytes first tether to and roll on vascular endothelium primarily via a selectin-dependent interaction, which is followed by stable adhesion to the endothelial surface. In the absence of P-selectin, both atherosclerotic lesion formation and neointimal growth upon arterial injury were attenuated in apoE and LDL-receptor knockout mice [22,23]. Interestingly, the effects of acetaldehyde on endothelial cell adhesion molecule expression turned out to be very selective. We found that while acetaldehyde had no effect on ICAM-1, VCAM-1 or E-selectin expression, there was a significant increase in both the number of P-selectin positive endothelial cells and P-selectin density. Moreover, blockade of P-selectin, using an antibody or siRNA-directed gene knockdown, significantly inhibited the acetaldehyde-mediated monocyte adhesion to endothelial cells. Thus, this small but significant increase in P-selectin positive endothelial cells, in combination with the observed increase in endothelial cell P-selectin density in the presence of acetaldehyde, may be significant enough to initiate the process of monocyte tethering and rolling.

The absence of any effect of TNF α on endothelial cell P-selectin expression, and the additive inhibitory effect of dual P-selectin/TNF α siRNA knockdown on acetaldehyde-mediated monocyte adhesion when compared to P-selectin or TNF α siRNA knockdown alone, strongly suggest two distinct signaling pathways mediate the effects of acetaldehyde on endothelial cell P-selectin and TNF α expression.

In the current study there was a significant increase in monocyte CCR2 expression following exposure to acetaldehyde. Hayasaki et al. reported in mice that CCR2 deficiency protected the myocardium after myocardial ischemia-reperfusion injury [24]. Moreover, Furukawa et al. reported that neutralization of MCP-1 before, and immediately after arterial injury may be effective in preventing restenosis after angioplasty [25]. These studies provide compelling evidence for a direct role of CCR2 in cardiovascular disease. The fact that acetaldehyde acts to increase monocyte CCR2 receptor expression, as demonstrated in the present study, suggests that this is a potential mechanism by which elevated levels of acetaldehyde achieved with binge drinking episodes may lead to an increase in the occurrence of cardiovascular-related diseases.

A review of the literature to date reveals that the majority of studies investigating acetaldehyde and TNF α have addressed alcoholic liver injury and investigated hepatocellular TNF α production. Hsiang et al. demonstrated that long-term acetaldehyde treatment of HepG2 cells led to a 2-fold increase in TNF α production [26]. However, it has been reported by Nakamura et al. that acetaldehyde treatment suppresses Kupffer cell TNF α release [27]. It has also been shown in primary cell cultures established from inferior turbinate tissue that exposure to acetaldehyde for 4 and 24 h leads to an increase in both TNF α mRNA expression and protein secretion [28]. We obtained similar results in our study using endothelial cells where acetaldehyde treatment led to an increase in both TNF α mRNA expression and protein secretion from endothelial cells with a concurrent decrease in intracellular amounts of TNF α . These results suggest that both *de novo* protein synthesis and the release of intracellular storage pools of TNF α , contribute to the effects of acetaldehyde. Interestingly, TNF α is a well-known activator of monocyte adhesion [29]. The specificity of the acetaldehyde-induced increase in TNF α mediating only the adhesion step in the monocyte recruitment cascade was proven by using TNF α as a control agent in all experiments and by adhesion blockade with an anti-TNF α antibody or siRNA-directed TNF α knockdown. Taken together, these results suggest that the effects of acetaldehyde on monocyte adhesion, may be mediated, at least in part, by TNF α .

In conclusion, this study demonstrates for the first time that physiologically relevant concentrations of acetaldehyde can initiate several key steps involved in monocyte recruitment. Our data demonstrates that acetaldehyde increases monocyte CCR2 and endothelial P-selectin and TNF α expression, and stimulates monocyte adhesion. Based on our findings we suggest a pathway for acetaldehyde-mediated monocyte recruitment that is shown in Fig. 5. Following alcohol consumption, we hypothesize that there is a delicate equilibrium between the effects of ethanol and its metabolite, acetaldehyde, on the vasculature. Further studies are warranted to investigate whether these effects are relevant *in vivo*, and whether these actions of acetaldehyde may underlie, in part, the detrimental effects of binge drinking on cardiovascular disease.

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References

1. Boffetta P, Garfinkel L. Alcohol drinking and mortality among men enrolled in an American Cancer Society prospective study. *Epidemiology*. 1990; 1:342–8. [PubMed: 2078609]
2. Poikolainen K. Alcohol mortality: a review. *Journal of Clinical Epidemiology*. 1995; 48:455–65. [PubMed: 7722599]
3. NIAAA. NIAAA Council approves definition of binge drinking. NIAAA Newsletter. 2004;1.
4. Kauhanen, J.; Kaplan, GA.; Goldberg, DD.; Cohen, RD.; Lakka, TA.; Salonen, JT. *Epidemiology*. Vol. 8. Cambridge, Mass: 1997. Frequent hangovers and cardiovascular mortality in middle-aged men; p. 310-4.
5. McElduff P, Dobson AJ. How much alcohol and how often? Population based case-control study of alcohol consumption and risk of a major coronary event. *BMJ*. 1997; 314:1159–64. Clinical research ed. [PubMed: 9146388]
6. Mukamal KJ, Maclure M, Muller JE, Mittleman MA. Binge drinking and mortality after acute myocardial infarction. *Circulation*. 2005; 112:3839–45. [PubMed: 16365208]
7. Wannamethee G, Shaper AG. Alcohol and sudden cardiac death. *British Heart Journal*. 1992; 68:443–8. [PubMed: 1467026]
8. Isse T, Matsuno K, Oyama T, Kitagawa K, Kawamoto T. Aldehyde dehydrogenase 2 gene targeting mouse lacking enzyme activity shows high acetaldehyde level in blood, brain, and liver after ethanol gavages. *Alcoholism, Clinical and Experimental Research*. 2005; 29:1959–64.
9. Tsukamoto S, Muto T, Nagoya T, Shimamura M, Saito M, Tainaka H. Determinations of ethanol, acetaldehyde and acetate in blood and urine during alcohol oxidation in man. *Alcohol Alcohol*. 1989; 24:101–8. [PubMed: 2719768]
10. Korsten MA, Matsuzaki S, Feinman L, Lieber CS. High blood acetaldehyde levels after ethanol administration. Difference between alcoholic and nonalcoholic subjects. *The New England Journal of Medicine*. 1975; 292:386–9. [PubMed: 1110723]
11. Eriksson CJ. The role of acetaldehyde in the actions of alcohol (update 2000). *Alcoholism, Clinical and Experimental Research*. 2001; 25:15S–32S.
12. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2^{-/-} mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*. 1998; 394:894–7. [PubMed: 9732872]
13. Ley K, Bullard DC, Arbones ML, et al. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *The Journal of Experimental Medicine*. 1995; 181:669–75. [PubMed: 7530761]
14. Norman MU, Lister KJ, Yang YH, Issekutz A, Hickey MJ. TNF regulates leukocyte-endothelial cell interactions and microvascular dysfunction during immune complex-mediated inflammation. *British Journal of Pharmacology*. 2005; 144:265–74. [PubMed: 15655512]
15. Ross R. Atherosclerosis—an inflammatory disease. *The New England Journal of Medicine*. 1999; 340:115–26. [PubMed: 9887164]
16. Redmond EM, Cullen JP, Cahill PA, et al. Endothelial cells inhibit flow-induced smooth muscle cell migration: role of plasminogen activator inhibitor-1. *Circulation*. 2001; 103:597–603. [PubMed: 11157728]
17. Laudanski K, De A, Pellegrini J, Miller-Graziano C. Simultaneous aberrations in Mphi and T cell function adversely affect trauma patients' clinical outcome: a possible faulty IL-13 feedback loop. *Clinical Immunology*. 2006; 118:332–41. [PubMed: 16303333]
18. Cullen JP, Sayeed S, Jin Y, et al. Ethanol inhibits monocyte chemotactic protein-1 expression in interleukin-1 {beta}-activated human endothelial cells. *American Journal of Physiology*. 2005; 289:H1669–1675. [PubMed: 15908470]
19. Vrsalovic M, Vrsalovic MM, Presecki AV, Lukac J. Modulating role of alcohol and acetaldehyde on neutrophil and monocyte functions in vitro. *Journal of Cardiovascular Pharmacology*. 2007; 50:462–5. [PubMed: 18049316]
20. Haorah J, Heilman D, Knipe B, et al. Ethanol-induced activation of myosin light chain kinase leads to dysfunction of tight junctions and blood-brain barrier compromise. *Alcoholism, Clinical and Experimental Research*. 2005; 29:999–1009.

21. Hendrickson RJ, Cahill PA, Sitzmann JV, Redmond EM. Ethanol enhances basal and flow-stimulated nitric oxide synthase activity in vitro by activating an inhibitory guanine nucleotide binding protein. *The Journal of Pharmacology and Experimental Therapeutics*. 1999; 289:1293–300. [PubMed: 10336519]
22. Dong ZM, Brown AA, Wagner DD. Prominent role of P-selectin in the development of advanced atherosclerosis in ApoE-deficient mice. *Circulation*. 2000; 101:2290–5. [PubMed: 10811597]
23. Nageh MF, Sandberg ET, Marotti KR, et al. Deficiency of inflammatory cell adhesion molecules protects against atherosclerosis in mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 1997; 17:1517–20.
24. Hayasaki T, Kaikita K, Okuma T, et al. CC chemokine receptor-2 deficiency attenuates oxidative stress and infarct size caused by myocardial ischemia-reperfusion in mice. *Circulation Journal*. 2006; 70:342–51. [PubMed: 16501303]
25. Furukawa Y, Matsumori A, Ohashi N, et al. Anti-monocyte chemoattractant protein-1/monocyte chemotactic and activating factor antibody inhibits neointimal hyperplasia in injured rat carotid arteries. *Circulation Research*. 1999; 84:306–14. [PubMed: 10024304]
26. Hsiang CY, Wu SL, Cheng SE, Ho TY. Acetaldehyde-induced interleukin-1beta and tumor necrosis factor-alpha production is inhibited by berberine through nuclear factor-kappaB signaling pathway in HepG2 cells. *Journal of Biomedical Science*. 2005; 12:791–801. [PubMed: 16132116]
27. Nakamura Y, Yokoyama H, Higuchi S, Hara S, Kato S, Ishii H. Acetaldehyde accumulation suppresses Kupffer cell release of TNF-Alpha and modifies acute hepatic inflammation in rats. *Journal of Gastroenterology*. 2004; 39:140–7. [PubMed: 15069620]
28. Gosepath J, Brieger J, Muttray A, et al. mRNA induction and cytokine release of inflammatory mediators during in vitro exposure of human nasal respiratory epithelia to acetaldehyde. *Inhalation Toxicology*. 2006; 18:1083–90. [PubMed: 17050345]
29. Schleser S, Ringseis R, Eder K. Conjugated linoleic acids have no effect on TNF alpha-induced adhesion molecule expression, U937 monocyte adhesion, and chemokine release in human aortic endothelial cells. *Atherosclerosis*. 2006; 186:337–44. [PubMed: 16219313]

Fig. 1.

(a) FACS analysis of control and acetaldehyde (10 μ M, 6 h) treated primary blood monocytes (PBM) cells using anti-CCR2 antibody (solid line) or isotype control antibody (dashed line), (representative of an individual experiment). (b) FACS analysis of control and acetaldehyde (10 μ M, 6 h) treated THP-1 cells using anti-CCR2 antibody (solid line) or isotype control antibody (dashed line), (representative of an individual experiment). (c) THP-1 monocytes were incubated with acetaldehyde or TNF α (10 ng/ml) for 6 h and surface CCR2 receptor expression was then determined by FACS analysis. Data are mean $\pm$ S.E.M.; $n = 4$. * $P < 0.05$ vs. control.

Fig. 2.

(a) FACS analysis of control and TNF α (10 ng/ml, 24 h) treated HUVEC using ICAM-1, VCAM-1, E-selectin and P-selectin antibodies (control - solid line or TNF α - dashed line) or isotype control antibody (dotted line). (b) and (c) Effect of acetaldehyde (6 h) on HUVEC P-selectin expression as determined by FACS analysis. (b) Graph with values representing endothelial cells positive for P-selectin, expressed as % of untreated cells and (c) P-selectin mean fluorescent intensity (MFI) expressed as % of untreated cells. Data are mean $\pm$ S.E.M.; $n = 4$. * $P < 0.05$ vs. control.

Fig. 3.

HUVEC were incubated with acetaldehyde for 6 h and (a) total RNA was isolated and quantitative real-time RT-PCR was performed. Analysis of TNF α mRNA expression normalized to GAPDH is shown. (b) Conditioned media was collected and concentrated prior to measurement of TNF α content by high-sensitivity TNF α immunoassay, or (c) cells were permeabilized/fixed and intracellular TNF α content was determined by FACS analysis. Data are mean $\pm$ S.E.M.; $n = 3-4$. $*P < 0.05$ vs. control. (d) Representative pictures of BCECF-AM labeled primary blood monocytes (PBM) attached to control or acetaldehyde-treated HUVEC. Pictures are representative optical fields ($\times 4$ magnification). (e) Effect of acetaldehyde treatment for 6 h on PBM adhesion to HUVEC. Data are mean $\pm$ S.E.M.; $n = 3$. $*P < 0.05$ vs. control. (f) Effect of acetaldehyde and TNF α (10 ng/ml) treatment for 6 h on THP-1 adhesion to HUVEC. Data are mean $\pm$ S.E.M.; $n = 4$. $*P < 0.05$ vs. control.

Fig. 4.

(a) Effect of ethanol (10 or 25 mM) on acetaldehyde-stimulated (6 h) THP-1 adhesion to HUVEC. (b) The effect of P-selectin siRNA transfection on HUVEC P-selectin mRNA (QRT-PCR normalized to GAPDH) and protein expression (FACS analysis: positive cells). (c) Effect of P-selectin antibody (1 $\mu\text{g}/\text{ml}$) or siRNA-directed P-selectin knockdown on acetaldehyde-stimulated (6 h) THP-1 adhesion to HUVEC. (d) The effect of TNF α siRNA transfection on HUVEC TNF α protein expression (FACS analysis: positive cells). (e) Effect of TNF α antibody (2 $\mu\text{g}/\text{ml}$) or siRNA-directed TNF α knockdown on acetaldehyde-stimulated (6 h) THP-1 adhesion to HUVEC. (f) The effect of dual P-selectin/TNF α siRNA transfection on HUVEC P-selectin and TNF α protein expression (FACS analysis: positive cells). (g) Effect of dual siRNA-directed P-selectin/TNF α knockdown on acetaldehyde-stimulated (6 h) THP-1 adhesion to HUVEC. Data are mean $\pm$ S.E.M.; $n = 3-5$. * $P < 0.05$ vs. respective control.

EFFECT OF ACETALDEHYDE ON THE MONOCYTE RECRUITMENT CASCADE

Fig. 5. Suggested pathway for acetaldehyde regulation of the monocyte recruitment cascade. (1) Acetaldehyde stimulates chemoattraction by increasing the expression of the MCP-1 receptor, CCR2, on monocytes. (2) Acetaldehyde promotes tethering/rolling by stimulating the expression of the adhesion molecule, P-selectin, on endothelial cells. (3) Acetaldehyde stimulates monocyte adhesion by increasing the expression and secretion of the inflammatory mediator, $\text{TNF}\alpha$, from endothelial cells.