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RESEARCH PAPER

Ethyl pyruvate modulates acute inflammatory reactions in human endothelial cells in relation to the NF-κB pathway

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Background and purpose: Endothelial cell activation plays a critical role in regulating leukocyte recruitment during inflammation and infection. Ethanol (EtOH) reduces host defence systems, including cell adhesion. However, well-known side effects of EtOH limit its clinical use as an anti-inflammatory drug. Instead, ethyl pyruvate (EtP) may represent a better alternative. Here, we compared effects of EtP and EtOH on neutrophil recruitment and activation of human umbilical vein endothelial cells (HUVECs).
Experimental approach: Adhesion of neutrophils to HUVEC monolayers, surface expression of intercellular cell adhesion molecule, E-selectin, vascular cell adhesion molecule, release of interleukin (IL)-8 and granulocyte colony-stimulating factor (G-CSF) from HUVECs were assessed as well as translocation of interleukin-1 receptor-associated kinase (IRAK-1), the nuclear factor-kappa B (NF-κB) subunits p50, p65 and IκB-α. NF-κB activation was analysed with a luciferase reporter plasmid. Cells were stimulated with IL-1β, lipopolysaccharide (LPS) or tumour necrosis factor-α.
Key results: EtP was several-fold more potent than EtOH in reducing adhesion of neutrophils to activated HUVECs, generation of IL-8 or G-CSF and surface expression of the adhesion molecules. This last reaction was decreased by EtP even when added after cytokines or LPS. Translocation of IRAK-1, IκBα and the NF-κB p65 subunit to the HUVEC nucleus was inhibited by EtP for all stimuli, whereas the diminished p50 translocation was stimulus specific. When p65 was constitutively expressed in Cos7 cells, stimulation of an NF-κB-dependent reporter gene was not affected by EtP, suggesting that EtP acted upstream of gene activation.
Conclusions and implications: EtP impedes adhesive, secretory and signalling events typical of the early inflammatory response in endothelial cells, suggesting EtP as a possible treatment for acute inflammatory conditions.
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Keywords: endothelium; ethyl pyruvate; ethanol; alcohol; inflammation; leukocyte adhesion; cytokine; NF-κB

Abbreviations: EtOH, ethanol; EtP, ethyl pyruvate; G-CSF, granulocyte colony-stimulating factor; HUVEC, human umbilical vein endothelial cells; IκB, inhibitor kappa B; ICAM, intercellular cell adhesion molecule; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; PMN, polymorphonuclear neutrophil granulocyte; TNF, tumour necrosis factor; VCAM, vascular cell adhesion molecule

Introduction

Ethanol (EtOH) abuse is associated with pulmonary infections, mainly pneumococcal and by *Klebsiella pneumoniae* (MacGregor, 1986; Jurkovich *et al*., 1993; Fernandez-Sola *et al*., 1995; Moss and Burnham, 2003). Such susceptibility has been shown after chronic alcohol intake as well as after acute alcohol intoxication (Buckley *et al*., 1978; Bagasra *et al*., 1993; Lister *et al*., 1993; Faunce *et al*., 1997; Bagby *et al*., 1998; Zambell *et al*., 2004). Traditionally, this impairment of host defence has been attributed to the effects of EtOH on phagocytic cells, for example, reduced polymorphonuclear neutrophil (PMN) adherence, migration and bactericidal functions.

We have reported that EtOH also reduces the generation of myeloid growth factors and interleukin (IL)-8 in human endothelial and epithelial cells, interactions with neutrophils and nuclear translocation of nuclear factor-kappa B (NF-κB) components (Jonsson and Palmblad, 2001; Johansson

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et al., 2005). These observations extend the spectrum of EtOH effects to the vessel wall as well as to the lung barrier system.

Although these and other findings support the notion that EtOH might be used as an inhibitor of inflammatory reactions, the entry of EtOH into the CNS, with ensuing symptoms of intoxication, severely curtails the possibility of using EtOH in a clinical setting. However, EtOH linked to pyruvate to form the ester, ethyl pyruvate (EtP), has been widely tested over the last few years as an inhibitor of inflammatory reactions *in vitro* and in laboratory animals (see Fink, 2007). EtP is well tolerated and there appear to be no signs of intoxication from EtOH on its use. For instance, EtP afforded strong protection of delayed cerebral ischaemic injury with a wide therapeutic window (Yu *et al*., 2005), improved haemodynamic stability in chronic endotoxemia (Hauser *et al*., 2005), protected against or accelerate recovery from acute renal failure after the renal insult (Miyaji *et al*., 2003) and decreased local and distant organ injury in a murine model of necrotizing pancreatitis (Yang *et al*., 2004). Moreover, delayed treatment with EtP conferred prolonged survival time in a lethal model of endotoxic shock, even if treatment begins after the early tumour necrosis factor (TNF) response (Ulloa *et al*., 2002). Some of the beneficial anti-inflammatory effects of EtP may be due to modification of p65, thereby inhibiting signalling by the NF-κB pathway (Han *et al*., 2005). The long-term effects of EtP suggest good drug stability.

Here, we have exposed human neutrophils (PMN) and umbilical vein endothelial cells (HUVECs) to EtP *in vitro*. We used three well-established proinflammatory agents, lipopolysaccharide (LPS), IL-1β and TNF-α, to stimulate the cells to see if EtP effects on inflammatory markers were agent-specific. In all these assays, EtP provided a substantial reduction of endothelial reactions to the inflammatory stimuli.

Materials and methods

Cell cultures and viability

Human umbilical vein endothelial cells was prepared as described (Jaffe *et al*., 1973). Briefly, HUVECs, obtained from cord veins by treatment with collagenase, were grown until confluence and used as monolayer after a maximum of three passages. The identity of the endothelial cells was established by the presence of Factor VIII-related antigen and CD31.

Cos-7 cells were grown at 37 °C in 5% CO_2 in a 1:1 mixture of high-glucose Dulbecco's modified Eagle's medium and F12 (Ham's) containing 10% fetal bovine serum, 10 IU mL^{-1}/100 μg mL^{-1} penicillin/streptomycin, respectively, and 2 mM L-glutamine.

The EtOH concentrations we have used here (0.5–1 vol vol^{-1} per cent, corresponding to 4–7.9 mg EtOH mL^{-1} or 85–170 mM) were based on our previous and others' work and to permit relevant comparisons (Meagher *et al*., 1982; MacGregor *et al*., 1988; Higashi and Hoek, 1991; Nilsson *et al*., 1991, 1992; Arbabi *et al*., 1999; Jonsson and Palmblad, 2001; Maiya *et al*., 2002). Likewise, the concentrations of EtP were chosen from the work of Sappington *et al*. (2003).

Human umbilical vein endothelial cell viability was assessed before experiments or after incubation. Less than 5% of cells incubated for up to 4 h with cytokines or other chemicals exhibited an altered morphology or uptake of Trypan blue, indicating that membrane integrity was not disrupted under the experimental conditions of this study. Moreover, HUVECs viability was tested by means of a [^{51}Cr] release assay (Bratt and Palmblad, 1997). In our hands, concentrations above10 mM of EtP displayed a clear cytotoxicity; thus, we decided to use 10 mM EtP as the highest, non-toxic concentration. The viability of the HUVEC was >95% after incubation with 170 mM EtOH and IL-1β for 24 h, as measured by the same methods and by annexin V expression (Jonsson and Palmblad, 2001). Exposure of HUVEC monolayers to 10 mM EtP, 10 mM sodium pyruvate (NaP) or 170 mM EtOH caused no detachment of cells, as assessed by regular microscopic inspections of cell layers or by assessment of platelet/endothelial cell adhesion molecule or vascular endothelial cadherin expression, as detailed below.

Measurement of cytokine production

After incubation of HUVECs with 10 mM pyruvate 70–170 mM of EtOH or 2.5–10 mM of EtP for 10 min (Jonsson and Palmblad, 2001) and then with various agonists for 4 h (a time point found to be optimal based on our previous findings), culture supernatants were harvested. The concentrations of cytokines were determined by Quantikine assays (R&D Systems Inc., Minneapolis, MN, USA). At the concentrations used in this study, neither EtOH nor EtP had an effect on the cytokine assays. We have previously reported that >90% of granulocyte colony-stimulating factor (G-CSF) and IL-8 were secreted into the extracellular space (Jonsson and Palmblad, 2001). To compensate for differences in cytokine production among donors of HUVECs, we expressed cytokine concentrations relative to those determined for stimulated cells. At the concentrations used in this study, EtP, NaP or EtOH had no effect on the cytokine assays.

PMN adherence

The donation of blood was in accordance with the Helsinki declaration and approved by the Ethical Committee of the Karolinska Institutet. The assays have been described previously (Jonsson and Palmblad, 2001). PMN were obtained from healthy donors by a one-step discontinuous Percoll gradient centrifugation as described (Ringertz *et al*., 1985). The purified neutrophils (>95% purity and viability) were stained with the fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (Lerner *et al*., 1993). Adherence of PMN was assessed either as adherence to a plastic surface after stimulation with the chemotactic formyl tripeptide fMet-Leu-Phe, or as adherence to LPS or cytokine-stimulated HUVEC monolayers (Jonsson and Palmblad, 2001). In the first assay, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester-labelled PMN were added to albumin-coated 96-well polystyrene microtitre plates. After treatment with EtP, EtOH or NaP at various concentrations (or buffer) for 10 min,

fMet-Leu-Phe (at 0.1 μ mM, or buffer) was added. The process was stopped after 15 min, as described below. In the second assay, HUVEC monolayers in 96-well polystyrene microtitre plates (in Hanks' balanced salt solution (HBSS) with 1% fetal calf serum) were treated with EtP, EtOH, NaP or buffer for 10 min before 100 ng of LPS per mL, 100 ng of TNF-α per mL or 5 U of IL-1β per mL in HBSS with 1% fetal calf serum were added. After incubation at 37 °C for 4 h, cells were washed, then 2×10^5 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester-stained PMN were added to each well and allowed to adhere for 15 min. Common to the two protocols, non-adherent PMN were then removed and adherent PMN lysed. Fluorescence was determined in a microtitre plate fluorimeter. Test results were expressed as the percentage of adherent cells in relation to added cells. The evaluation of the adherence assay with regard to possible interfering factors, for example, PMN aggregation and so on, has been described previously (Heimburger and Palmblad, 1996). We have also reported previously the essential role of intercellular cell adhesion molecule (ICAM)-1 for PMN binding to stimulated endothelial cells, in that a blocking antibody to ICAM-1 totally abolished PMN adhesion (Palmblad and Lerner, 1992).

Detection of endothelial adhesion molecules

The cell surface expression of adhesion molecules, E-selectin, vascular cell adhesion molecule (VCAM-1) and ICAM-1 as well as platelet/endothelial cell adhesion molecule (CD31) and V-cadherin on HUVEC monolayer was examined by a modified cellular enzyme-linked immunosorbent assay (Jonsson and Palmblad, 2001). After treatment with EtP, EtOH or NaP for 10 min and stimulation with 100 ng of LPS per mL, 100 ng of TNF-α per mL or 5 U of IL-1β per mL for 4 h, HUVECs were fixed with 2% paraformaldehyde and incubated for 1 h at room temperature with 5% non-fat dried milk in phosphate-buffered saline. Adhesion molecules were labelled with specific monoclonal antibodies ($1\,\mu g\,mL^{-1}$) for 2 h and then for 1 h with horseradish peroxidase-conjugated secondary antibodies. The cells were washed and immune complexes were then detected by incubation with TMB peroxidase substrate (Bio-Rad, Hercules, CA, USA) for 15 min, after which the reaction was terminated with sulphuric acid and absorbance was measured at 450 nm. Data are expressed in arbitrary units and as a percentage of the level of adhesion molecule expression apparent in stimulated cells.

Translocation of NF-κB and IRAK-1

The translocation of inhibitor κ B-α and the NF-κB subunits p65 and p50, and of interleukin-1 receptor-associated kinase (IRAK-1) from the cytosol to the nucleus was assessed by immunohistochemistry (Jonsson and Palmblad, 2001). In short, confluent EC monolayer on glass coverslips were incubated with EtOH, EtP or buffer alone for 10 min and stimulated with the indicated agonists for 1 h (for NF-κB) or 15 min (for IRAK-1), then fixed, permeabilized and incubated with antibodies. Immune complexes were detected with the Vectastain ABC kit and DAB substrate for peroxidase (Vector, Burlingame, CA, USA). The cells were examined with an Olympus microscope, and micrographs were scanned with a Jandel SigmaScan Pro instrument for densitometric assessment of the ratio of staining between nucleus and cytoplasm. We analysed $\geqslant 5$ cells on each micrograph, that is, a total of >50 cells. To safeguard against cell detachment, we used EtOH at 85 mM and EtP at 5 mM.

Reporter plasmids and expression vector

The plasmid $3 \times$ NF-κB(IC)tk-LUC (Johansson *et al*., 2005) contains three copies of the NF-κB binding site from the human ICAM-1 promoter in front of a minimal thymidine kinase promoter (-105 to $+52$) fused to the luciferase (LUC) reporter gene. The p65 expression plasmid was as described by Liden *et al*. (2003).

Transfection

FuGENE (Roche, Mannheim, Germany) reagent was used in all transfections according to the manufacturer's instructions. The p65 expression plasmid (Liden *et al*., 1997) and the reporter gene plasmids were used at a concentration of 50 ng per well and 200 ng per well, respectively. The plasmid cytomegalovirus-galactosidase was used as an internal control for variation in transfection efficiency at a concentration of 50 ng per well. In brief, 30 000 cells per well were seeded in 24-well plates 24 h before transfection. Twenty hours after transfection, cells were exposed to treatment, 2.5–10 mM EtP or 40–170 mM EtOH, for 4 h and the cell medium was collected and the cell extract was prepared for measuring luciferase activities. Cos-7 cell viability, which remained over 95%, was examined with microscopy of cell layers and by assessment of galactosidase.

Statistical analyses

Data are presented as mean ± s.e.mean for the indicated number of separate experiments. Each experiment was performed at least in duplicate and each assay at least in triplicate. Differences between the groups were assessed by ANOVA. When the ANOVA test was significant, a Newman–Keul test was performed between the groups. All statistical analysis was performed by STATISTICA (data analysis software system), version 7. www.statsoft.com. Differences were considered statistically significant if $P<0.05$ and results are reported accordingly with an asterisk (*).

Materials

Chemicals and antibodies were obtained as follows: E-selectin, ICAM-1, VCAM-1, platelet/endothelial cell adhesion molecule, vascular endothelial cadherin (BD Pharmingen, San Diego, CA, USA), P65, P50 and IκB-α (Oncogene Research Products, Cambridge, MA, USA), IRAK-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). IL-1β (Roche); fetal bovine serum, HEPES, penicillin, streptomycin, Dulbecco's modified Eagle's medium, Ham's F-12 medium, phosphate-buffered saline, sodium pyruvate and Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, NY, USA); ethanol (Kemetyl, Stockholm, Sweden), acetone and

methanol (Apoteksbolaget, Stockholm, Sweden); and polystyrene plates (96- and 6-well) and other tissue culture plastic material (TPP, Trasadingen, Switzerland). All other chemicals were obtained from Sigma Chemicals (St Louis, MO, USA).

Results

Adhesion of neutrophils to a plastic surface or to HUVECs

Quiescent PMN showed a 2.5 ± 0.7% adherence to the albumin-coated plastic surface. When stimulated with fMet-Leu-Phe, adherence increased 7.6-fold, to 19 ± 4%. When cells were pretreated with EtP there was a pronounced decrease of adherence (Figure 1a). This effect was dose-dependent and, when 10 mM of EtP was used, only half of fMet-Leu-Phe stimulated PMN adherence persisted. Furthermore, when PMN were pretreated with 170 mM of EtOH (that is, 1%), cells showed a decrease of adherence (Figure 1a). Sodium pyruvate (NaP, 10 mM) used as a control for the pyruvate moiety of EtP, exhibited no significant effect on PMN adherence to the plastic surface (Figure 1a). Spontaneous adherence of PMN was not affected by EtP, EtOH or NaP.

The adhesion of PMN to quiescent HUVECs ranged from 1 to 5% of added PMN. No change of this spontaneous adhesion was observed when EtP, EtOH or NaP was added to HUVECs (data not shown). After stimulation of HUVECs with 100 ng of LPS per mL for 4 h, the adhesiveness for PMN was enhanced, with an 11-fold increase compared with controls (from 2.1 ± 0.3 to 23.5 ± 3.2% of added PMN; $n = 18$), as described earlier (Jonsson and Palmblad, 2001). Likewise, 5 U of IL-1β per mL enhanced adherence 15-fold (to 31.8 ± 4.1%; $n = 12$), whereas 100 ng of TNF-α per mL conferred a 14-fold increase (to 30.1 ± 3.4%; $n = 11$).

When HUVEC monolayers were treated with 2.5, 5 or 10 mM EtP and then stimulated with LPS, IL-1β or TNF-α, substantial reductions in binding of PMN to HUVECs were noted for all three agonists. As shown in Figure 1b, the LPS response was blunted and became close to that of quiescent HUVECs at the highest dose of EtP. The responses to IL-1β were similarly reduced (Figure 1c), and those to TNF-α were diminished with 23, 76 and 80% of the values for controls, respectively. When HUVEC monolayers were treated with EtOH and then stimulated with LPS, significant decreases in binding of PMN to HUVECs were observed. The attenuation conferred by 85 mM was 10%, and by 170 mM EtOH was 28% of the LPS control treated with buffer alone (Figure 1b). The reaction to EtOH or NaP in IL-1β or TNF-α activated cells was negligible (Figure 1 and data not shown, respectively). The effect of EtP resembles the effect of an ICAM-1-blocking antibody (Palmblad and Lerner, 1992).

Thus, EtP over this concentration range consistently diminished the stimulated adherence of PMN to plastic and HUVECs, whereas effects of EtOH were more variable.

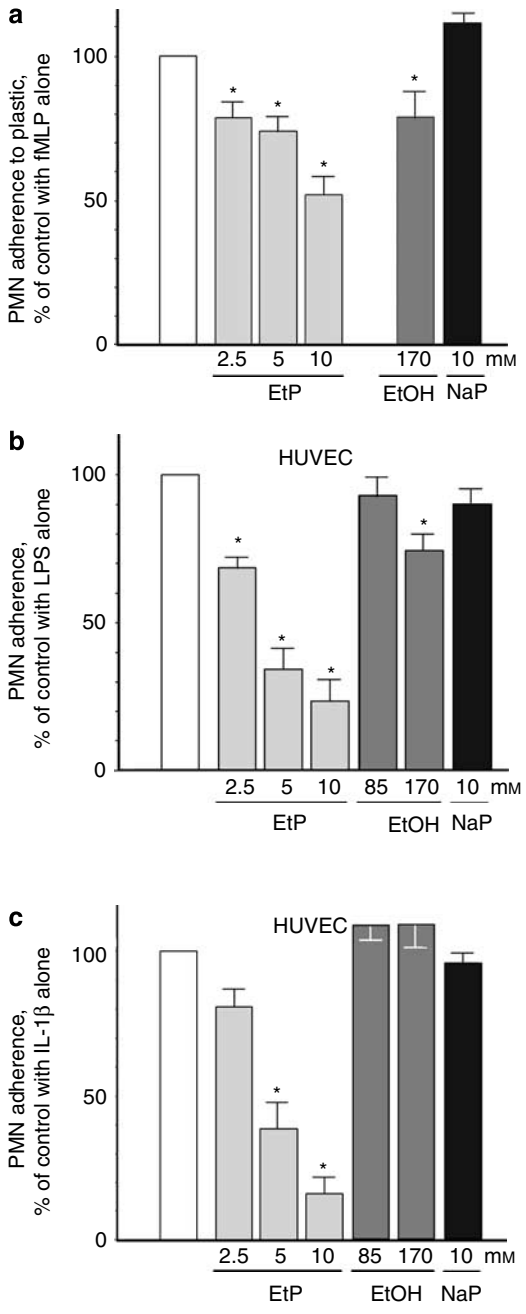

Figure 1 Effect of ethyl pyruvate (EtP), ethanol (EtOH) or sodium pyruvate (NaP) on adhesion of neutrophils to plastic (**a**) or human umbilical vein endothelial cells (HUVECs) (**b** and **c**). (Panel **a**) Polymorphonuclear neutrophil granulocyte (PMN), added to an albumin-coated plastic surface were treated with the drugs (as indicated) and then stimulated with 0.1 μM of fMet-Leu-Phe (in continued absence or presence of the EtP, EtOH or NaP). After 15 min, plates were analysed for adherent PMN. (Panels **b** and **c**) HUVECs were treated with EtP, EtOH or NaP (in concentrations indicated) and then stimulated for 4 h with 100 ng of LPS per mL (**b**) or 5 U of IL-1β per mL (**c**) (in continued absence or presence of EtP, EtOH or NaP). PMN was added and adherence analysed after 15 min. The data are means ± s.e.mean ($n = 6$–36). Asterisks above columns indicate a P-value of < 0.05 for comparisons with cells stimulated with agonists only.

Adhesion molecule expression

ICAM-1. In HUVECs, activation with LPS, IL-1β or TNF-α for 4 h upregulated ICAM-1 expression in accordance with previous reports (Jonsson and Palmblad, 2001). TNF-α conferred the most extensive response (a sixfold upregulation), followed by IL-1β and LPS (five- and threefold, respectively).

When HUVECs had been treated with EtP before addition of cytokines or LPS, ICAM-1 expression was significantly affected. EtP reduced the expression dose-dependently for LPS, IL-1β and TNF-α responses, where only 56, 47 and 51%, respectively, remained detectable on the cell surface when 10 mM of EtP had been used (Figure 2a shows results for IL-1β).

E-selectin and VCAM-1. The surface expression of E-selectin was upregulated 8-, 12- and 17-fold and the expression of VCAM was upregulated 6-, 11- and 12-fold with LPS, IL-1β or TNF-α (respectively) in HUVECs.

Treatment with EtP before incubations with LPS, IL-1β or TNF-α resulted in a dose-dependent reduction of E-selectin expression that was decreased to 46, 40 and 46% by 10 mM EtP, respectively. Similarly, VCAM-1 surface expression was inhibited with the most pronounced effect noted for 10 mM EtP (to approximately 65% of the control values for all three stimuli). Also, 2.5 and 5 mM of EtP induced reductions with 20 and 40%, respectively. Figures 2b and c show results for EtP on the stimulation of these adhesion molecules by IL-1β. Note that EtOH or NaP did not influence expression of the adhesion molecules (Figures 2a–c).

As controls for the specificity of effects on surface adhesion molecules, we also tested platelet/endothelial cell adhesion molecule (CD31) and vascular endothelial cadherin expressions, but they were not influenced by LPS, EtP or EtOH treatments (data not shown).

We also tested the possibility that EtP (or EtOH) might interfere with an agonist-generated response that already had been initiated. For this reason HUVECs were stimulated for 2 h with IL-1β, TNF-α or LPS, then EtP (or EtOH) was added and remained in contact with the cells for the remaining 2 h period. EtP caused significant reductions of ICAM-1, E-selectin and VCAM-1 expression but these were not a large as when EtP was given before proinflammatory stimulation (Figure 2d shows results for VCAM-1). Likewise, EtOH reduced E-selectin and VCAM-1 expressions in this model of delayed administration of the agents, but did not affect ICAM-1 (Figure 2d and data not shown). NaP did not influence any responses.

Cytokine release

We have previously reported that stimulated HUVECs produce large amounts of several cytokines, including IL-8, G-CSF and granulocyte macrophage colony-stimulating factor, and that EtOH reduced the production of these cytokines to 20–30% of that in LPS-stimulated HUVECs (Jonsson and Palmblad, 2001). Now, we expand these findings with effects of EtP and NaP on IL-8 and G-CSF release.

IL-8 secretion. In HUVECs, 100 ng of LPS per mL, 5 U of IL-1β per mL as well as 100 mg mL^{-1} TNF-α caused 13- and 7-fold increases of the release of this chemokine (from 1.7 ± 0.5 to 13.3 ± 0.2, 20.7 ± 0.3 and 12.2 ± 0.1 pg mL^{-1},

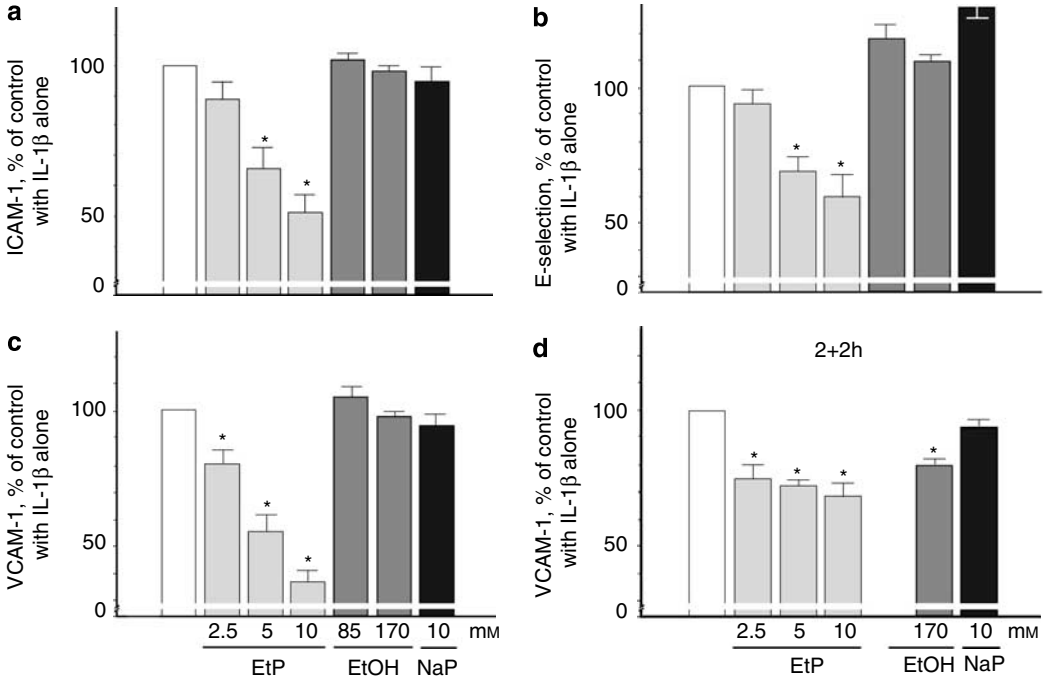

Figure 2 Effect of ethyl pyruvate (EtP), ethanol (EtOH) or sodium pyruvate (NaP) on surface expression of intercellular cell adhesion molecule (ICAM)-1 (panel **a**), E-selectin (panel **b**) or vascular cell adhesion molecule (VCAM)-1 (panels **c** and **d**) in human umbilical vein endothelial cells (HUVECs), induced by interleukin (IL)-1β. (Panels **a**–**c**) show the results when cells were treated with EtP, EtOH or NaP (in concentrations indicated) and then stimulated with 5 U of IL-1β per mL (in continued absence or presence of EtP, EtOH or NaP) for 4 h. (Panel **d**) shows results when HUVECs were stimulated for 2 h with IL-1β, then EtP, EtOH or NaP was added, remaining for the next 2 h. After a total of 4 h of incubation, antibodies were added and epitope abundance assayed. The data are means ± s.e.mean ($n = 14$–15). Asterisks above columns indicate a P-value of < 0.05 for comparisons with cells stimulated with agonists only.

respectively). In experiments on LPS-mediated IL-8 release, treatment with 5 mM EtP caused a reduction of 30%, and 10 mM EtP a 57% reduction of IL-8 (data not shown). Likewise, EtOH reduced the LPS-induced release of IL-8 by 40% (data not shown). When IL-1β was used as stimulus, 5 mM EtP caused a 36% reduction and 10 mM of EtP impaired the IL-8 secretion by 80% (Figure 3a). Treatment with 5 and 10 mM EtP also caused 54 and 84% reductions of TNF-α-mediated IL-8 release, respectively. EtOH or NaP did not diminish IL-1β or TNF-α-induced IL-8 release (Figure 3a and data not shown).

G-CSF secretion. Lipopolysaccharides caused a 37-fold increase of the release of this growth factor (to $135 \pm 51\,pg\,mL^{-1}$). EtP dose-dependently inhibited LPS-stimulated G-CSF secretion with 62, 85 and 94% reduction, for 2.5, 5 or 10 mM EtP, respectively. As reported previously, EtOH was also a potent antagonist for this response (Jonsson and Palmblad, 2001).

Interleukin-1β induced a massive, more than 200-fold, increment of G-CSF secretion (from 3.6 to $741 \pm 129\,pg\,mL^{-1}$). Exposure of HUVECs to EtP before incubation with IL-1β resulted in a dose-dependent inhibition of stimulated G-CSF release, as shown in Figure 3b. At the highest dose (10 mM) EtP almost totally abolished the release of this cytokine (Figure 3b). EtOH (170 mM) also reduced G-CSF release stimulated by IL-1β (Figure 3b).

A total of 100 ng of TNF-α per mL induced considerably lower level of G-CSF secretion: a twofold increase of G-CSF release into the supernatants was observed after 4 h of incubation of cells (to $8 \pm 1\,pg\,mL^{-1}$). A total of 5 and 10 mM EtP reduced the G-CSF release by 44 and 76%, respectively, but EtOH did not diminish G-CSF release in response to this stimulus. Figures 3a and b also shows that NaP produced no significant changes in either IL-8 or G-CSF release.

To find out if EtP effects could be mimicked by the simultaneous addition of EtOH and NaP, we tested this combination on adhesion of PMN and expression of adhesion molecules. However, there was no significant difference between cells treated with the combination and those treated with EtOH or NaP alone (data not shown).

To see if effects of EtP were easily reversible we washed HUVECs after they had been treated with EtP and agonists for 2 h; subsequently cells were left untreated for another 2 h. There was no significant change in PMN adhesion or expression of adhesion molecules after the washing, suggesting that these EtP effects persisted for at least the 2 h period (data not shown).

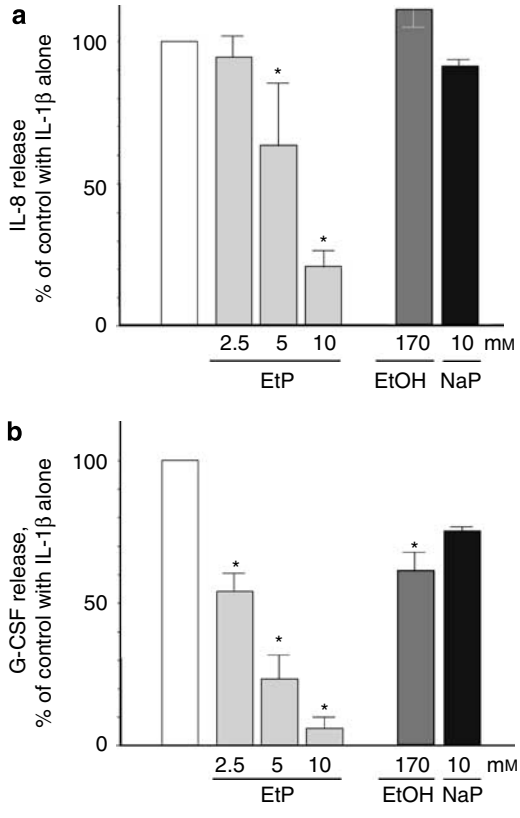

Figure 3 Effect of ethyl pyruvate (EtP), ethanol (EtOH) or sodium pyruvate (NaP) on secretion of interleukin (IL)-8 (panel **a**) or granulocyte colony-stimulating factor (G-CSF) (panel **b**) by human umbilical vein endothelial cells (HUVECs), induced by IL-1β. Cells were treated with EtP, EtOH or NaP (in concentrations indicated) and then stimulated with 5 U of IL-1β per mL (in continued absence or presence of EtP, EtOH or NaP). After 4 h of subsequent incubation, supernatants were analysed for the cytokines. The data are means ± s.e.mean ($n = 12$–40). Asterisks above columns indicate a *P*-value of < 0.05 for comparisons with cells stimulated with agonists only.

Signalling systems

Here, we assessed a common pathway for signals generated by binding of LPS, IL-1β and TNF-α to their receptors, namely nuclear translocation of IRAK-1 and the p65 and p50 subunits of the NF-κB complex in HUVECs. Translocation of IRAK-1 from the cytosol to the nucleus after stimulation with IL-1β, also described by Böl *et al*. (2000), was induced after 15 min incubation with LPS and IL-β. EtP (5 mM) and EtOH (85 mM) treatment inhibited the LPS- and the IL-1β-induced translocation of IRAK-1 (Figure 4a). However, IRAK-1 translocation was not induced with TNF-α and thus no effects were seen with EtP or EtOH treatment, as assessed by the staining technique and densitometric analyses.

As reported previously, LPS induced a significant translocation of p65 from the cytosol to the nucleus of HUVECs after 1 h that was inhibited by EtOH by 46% when added before LPS stimulation (Jonsson and Palmblad, 2001). Here, this effect was corroborated and extended to EtP, which also significantly prevented p65 translocation induced by LPS in HUVECs (Figure 4b). In contrast, translocation induced with IL-1β or TNF-α was not significantly blocked by EtP or EtOH treatment (data not shown). When we examined the effects on phosphorylated p65, we found that EtP treatment diminished the LPS-mediated total amount of phosphorylated p65 in HUVECs.

We also examined the effect of EtP and EtOH on translocation of NF-κB subunit p50, from the HUVEC cytoplasm to the nucleus, observing that exposure to EtP conferred a decreased amount of p50 in nucleus compared with cells stimulated with IL-1β only, whereas EtOH caused no significant reduction (Figure 4c). Furthermore, treatment of cells with EtP or EtOH decreased p50 translocation elicited

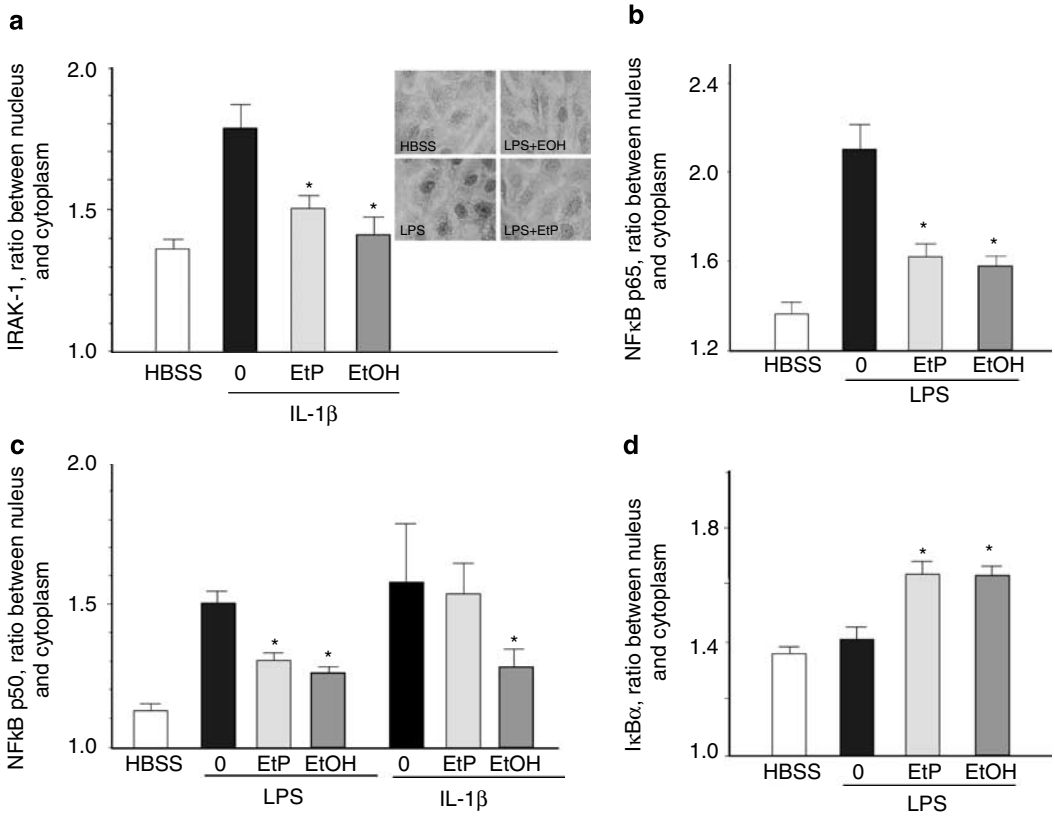

Figure 4 Effect of ethyl pyruvate (EtP; 5 mM), ethanol (EtOH; 85 mM) on translocations of interleukin-1 receptor-associated kinase (IRAK)-1 (**a**) and of nuclear factor-kappa B (NF-κB) subcomponents p65 and p50 (**b** and **c**) and of inhibitor kappa B (IκB)α (**d**) from the cytoplasm to the nucleus in human umbilical vein endothelial cells (HUVECs). (Panel **a**) shows effects on IRAK-1 after stimulation with interleukin (IL)-1β for 15 min. The micrographs insert show results of lipopolysaccharide (LPS) stimulation. (Panel **b**) shows effects on p65 after stimulation with LPS for 60 min. (Panel **c**) shows effects on p50 after stimulation with IL-1β for 60 min or with LPS for 60 min. (Panel **d**) shows the level of IκBα in nucleus after stimulation with EtOH or EtP and LPS for 45 min. The data are means ± s.e.mean ($n = 5$–10). Asterisks above columns indicate a *P*-value of < 0.05 for comparisons with cells stimulated with agonists only.

by LPS (Figure 4c). EtP or EtOH had no effect on TNF-α-induced p50 translocation.

Finally, we analysed IκBα in HUVECs stimulated with LPS for 15–45 min. This inhibitory factor is believed to be phosphorylated, ubiquitinated and degraded upon cell activation and release of NF-κB. We observed staining for IκBα in the nucleus and in the cytosol 15 min after stimulation with LPS. Cellular staining then declined during the ensuing 30–45 min, presumably reflecting degradation of IκBα. Exposure of HUVECs to EtP or EtOH before LPS for 45 min increased IκBα staining in the nucleus (Figure 4d).

To analyse if the inhibition was due to reduced transcriptional activation by NF-κB of NF-κB-target genes, we transfected Cos-7 cells (because HUVECs were not consistently transfected with the plasmids used here) with one plasmid constitutively expressing p65 and one plasmid containing three copies of the NF-κB-binding site coupled to the luciferase (LUC) reporter gene before treating with EtP, EtOH or vehicle. Treatment of the transfected Cos-7 cells with EtP or EtOH did not affect the activity of the luciferase reporter gene compared with untreated controls. These data suggest that the ability of EtP or EtOH to inhibit adhesion molecule expression or cytokine release is not mediated by a direct inhibition of transcriptional activation by p65.

Discussion

This study shows that EtP is a highly efficient molecule in disrupting the adherence of, and between, neutrophils and endothelial cells. The effect occurs at concentrations that are 10–20 times lower than the maximal EtOH concentration tested here. Effects were much more robust with EtP, involved more systems and, hence, are probably more comprehensive. Other studies have found EtP to be safe, well tolerated and suitable for further development to be used in clinical settings (Fink, 2007). A phase 2 study of the drug in coronary by-pass operated patients was recently reported (but discontinued before reaching the set end point; ClinicalTrials.gov Identifier: NCT001076669). Of note, effects of EtP (and to some extent also of EtOH) can be observed when added some hours after a LPS or cytokine response has been initiated (Figure 2d), which mimics a clinical setting.

Our rationale for studying neutrophils and endothelial cells is that these are among the first to be engaged in an inflammatory reaction. Several lines of evidence demonstrate that disruption of delivery of neutrophils to an inflammatory lesion, particularly if this is of non-infectious nature, reduces the organ and tissue damage and restores

organ function more rapidly. That has been shown with a number of agents, including steroids, antibodies against adhesion molecules of these cells, a glycoprotein interfering with selectins and so on. Although concerns have been expressed that, as neutrophils are necessary for host defence against intruding micro-organisms, impairment of delivery of the phagocytes to tissues might confer a risk for enhancing infection. However, in a bacterial meningitis model, hindrance of PMN delivery was in fact associated with better survival (Granert *et al*., 1994). So far, studies with EtP have corroborated this view and in none of the published animal studies were mortality higher in the EtP-treated groups; instead it was lower (Fink, 2007; cf. Su *et al*., 2008). Likewise, EtOH reduced arthritis manifestations in a rodent model (Jonsson *et al*., 2007).

Reduced release of G-CSF from endothelial cells and TNF-α from monocytic cells (van Zoelen *et al*., 2006) and in bronchoalveolar lavage fluid (van Zoelen *et al*., 2007) might raise concerns that EtP could interfere with myeloid cell proliferation and maturation, particularly if administered for a prolonged period of time. Such reactions have not been reported so far but the studies have probably not been designed to address this issue specifically.

Three main conclusions can be drawn from our study. The first is that EtOH and EtP displayed overall rather similar effects (albeit that EtP was more potent on a molar basis). Given these similarities, it is likely that the major target for both drugs is located downstream of surface receptors (*vide infra*). Nonetheless, some effects were clearly different. One involved ICAM-1, VCAM-1 and E-selectin, another IL-8 and G-CSF, whereas EtOH did not or just marginally affected surface expressions or extracellular releases, but EtP did so efficiently. We do not know if this is a fundamental difference that might be related to effects on targets specific for that response or merely reflects dose–response relationships beyond our concentration ranges. As EtP or EtOH did not influence the ability of p65 to stimulate transcription from an NF-κB-dependent reporter gene, it is likely that the effects of EtP and EtOH are mediated by effects upstream of transcriptional initiation.

The second point is that NaP was without effects, suggesting that the ethyl moiety of the EtP molecule is essential for the effects observed. The molecule has to be delivered intact as the combination of EtOH and NaP did not reproduce effects of EtP alone.

The third point is that EtOH and EtP effects did differ depending on the inflammatory agonist used. Thus, effects elicited by LPS were clearly susceptible to EtOH, whereas effects by IL-1β and TNF-α were far less influenced. In contrast, EtP effects were observed for all agonists. Examples of this are adhesion of neutrophils to HUVEC and release of IL-8 from HUVECs. This suggests specific targets for EtP and EtOH in the signalling pathways.

Recently, Han *et al*. (2005) showed in RAW264.7 cells that EtP interacts with the NF-κB system by directly targeting p65 at Cys38, inhibiting DNA-binding, but EtP failed to affect IκBα or IκBβ degradation after LPS stimulation (Han *et al*., 2005). Here, we have extended those results by measurements of p65 nuclear translocation (which was inhibited by EtP in LPS-exposed cells), p50 translocation (which was inhibited by EtP in LPS- and IL-1β-exposed cells and expression of IκBα in the nucleus (finding that EtP increased its expression). Furthermore, the p65 interaction with the κB-site and gene activation was not affected by EtP. These data support the view that the site of action of EtP or EtOH in inhibiting the responses we describe, is not on the p65 DNA binding and subsequent transcription activation, but upstream in the ligand-receptor activation pathway.

Thus, it is clear that EtOH and EtP have effects on release, translocation and degradation of components of the NF-κB system that are complex and which may be stimulus, time and cell specific.

Saeed *et al*. (2004) reported that EtOH treatment *in vitro* blocked adhesion and adhesion molecule expression by human microvascular endothelial cells for neutrophils and monocytes after 5–20 h of TNF-α stimulation. Here, we did not find an effect of EtOH for neutrophil adhesion or adhesion molecules after LPS and IL-1β stimulation. One difference between these studies is that we studied adhesion molecules at a time point relating to maximal neutrophil adhesion to endothelial cells and emigration in to tissues, as well as for the peak of E-selectin and VCAM-1 expression with LPS and IL-1β stimulation, that is, 4 h. Microvascular endothelial cells and HUVECs might also differ phenotypically with respect to this reactivity.

In conclusion, our results on EtOH provide clues to the mechanisms for reduced neutrophil and endothelial cell activation and recruitment seen in infected alcoholics. Moreover, our data suggest that EtP might be a novel anti-inflammatory agent that should be tested in various clinical settings where very rapid effects are needed, a situation that might apply to severe sepsis.

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Conflict of interest

The authors state no conflict of interest.

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