Laboratory investigation in normal and pathologic coagulation

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Chapter 4

Ethyl pyruvate exerts combined anti-inflammatory and anticoagulant effects on human monocytic cells.

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Summary

Sepsis is characterized by a concurrent activation of inflammation and coagulation. Recently, recombinant human activated protein C was shown to decrease mortality in patients with severe sepsis presumably due to a combined anti-inflammatory and anticoagulant effect.These promising findings led to a search for other products that influence both the inflammatory and the procoagulant response to severe infection. Ethyl pyruvate (EP) was recently identified as an experimental anti-inflammatory agent during endotoxemia and sepsis. The aim of the present study was to investigate whether EP influences coagulation besides its anti-inflammatory effects. For this we investigated the effects of EP on the expression and function of tissue factor (TF), the principal initiator of coagulation activation in sepsis, in human monocytic (THP-1) cell cultures. EP dose-dependently inhibited the production of tumor necrosis factor (TNF)-α , macrophage inflammatory protein (MIP)-1 α and MIP-1β by lipopolysaccharide (LPS)-stimulated THP-1 cells at mRNA and protein level, thereby confirming its anti-inflammatory properties in this in-vitro system. In addition, EP dose-dependently attenuated the increases in TF mRNA levels, TF-protein-surface expression and cell-surface-associated TF activity in LPS-stimulated THP-1 cells. These results demonstrate for the first time that EP is a compound with combined anti-inflammatory and anticoagulant effects.

Keywords

Tissue factor, ethyl pyruvate, sepsis, lipopolysaccharide, THP-1 monocytic cells

Introduction

Sepsis is associated with concurrent activation of inflammation and coagulation, both of which can contribute to organ damage (1, 2). The recent success of recombinant human activated protein C, a compound with combined anti-inflammatory and anticoagulant properties, to reduce mortality in patients with severe sepsis (3) has triggered renewed interest in products that influence both the inflammatory and the procoagulant response to severe infection. Ethyl pyruvate (EP), an aliphatic ester derived from the endogenous metabolite pyruvic acid, is an experimental anti-inflammatory therapeutic that protects mice against lethal systemic inflammation caused by either endotoxemia or sepsis at least in part by inhibiting the systemic release of both "early" (tumor necrosis factor [TNF] - α) and "late" (high mobility group box 1) cytokines (4). Furthermore, EP reduced organ injury in animals subjected to mesenteric ischemia and reperfusion (5, 6) and hemorrhagic shock (7, 8), among others. We were interested in whether EP would have anticoagulant effects in addition to its anti-inflammatory properties. Tissue factor (TF) is considered to be the principal initiator of coagulation activation during sepsis and systemic inflammation, and monocytes likely are the main source of TF in these pathological circumstances (2). Therefore, the primary aim of the present study was to determine whether EP influences TF cell-surface expression and function in human monocytic cells.

Materials and methods

Cell culture

Suspensions of human monocyte-like THP-1 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 (Bio Whittaker, Verviers, Belgium) with 2 mM L-glutamine and supplemented with FCS, penicillin and streptomycin (GibcoBRL, Life Technologies, Rockville, MD, USA). Cells were washed with medium and grown over night. The next day cells ($5x10^5$ /ml) were incubated with lipopolysaccharide (LPS; from E. coli O111:B4, 0.1 or 1 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) and EP (10, 5 or 1 mM, kindly provided by Critical Therapeutics, Inc., Lexington, MA, USA), freshly prepared in Ringer's Lactated Solution (RLS, Baxter, Deerfield, IL, USA). These EP concentrations were based on previous in-vitro studies

(4, 9–11); these levels can not be related to EP plasma levels since EP rapidly disappears from the circulation after intravenous administration. Supernatants were collected and stored at –80ºC until assayed, and the cell pellets were used for RNA isolation, flow cytometry or a functional coagulation assay (see below).

Multiple ligation-dependent probe amplification

Cells were dissolved in 1 ml Trizol and stored at –80ºC until used for RNA isolation. RNA was isolated and analyzed by multiplex ligation-dependent probe amplification (MLPA) as described before (12).

TNF- α , MIP-1α and MIP-1β measurements

TNF-α protein levels were measured by cytometric bead array multiplex assay (BD Biosciences, San Jose, CA, USA). MIP-1α and MIP-1β protein levels were measured by ELISA according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN, USA).

Flow cytometry

Cells were washed and resuspended (to $2x10^5$/ml) in FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN_3 and 0.35 mM EDTA). Immunostaining for cell-surface TF was performed for 30 minutes at 4ºC using R-phycoerythrin (R-PE)–conjugated mouse monoclonal (IgG1, κ) anti-human TF antibodies (BD Pharmingen, San Diego, CA, USA). After incubation, the cells were washed and resuspended in FACS buffer. The mean channel fluorescence intensity (MFI) of 10,000 events was determined for each sample using a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Cell viability was assessed using annexin V (IQ products, Groningen, The Netherlands) and 7-amino-actinomycin D (7AAD, Pharmingen, San Diego, CA, USA) as recommended by the manufacturers.

Thrombin generation time

Cell-surface TF actitvity was measured by determining the thrombin generation time (TGT). TGT was measured spectrophotometrically by the fibrin polymerization method.

After incubation, cells were washed and resuspended in PBS (to $0.25x10^5$ /ml) and kept at 4ºC. Seventy-five µl of cell suspension samples or TF standard concentrations were added to 100 µl of pooled human citrated plasma (from >150 healthy volunteers). Thrombin generation was initiated by the addition of 75 µl calcium chloride (38 mM). The clotting time was measured spectrophotometrically and expressed as T½ max (time to reach the mid-point of clear to maximum turbid density). TF activity was quantified as TF pg/ml per $1x10^6$ cells measured by reference to a TF (Innovin, Dade Behring, Marburg, Germany) standard curve.

Statistical analysis

All analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Overall differences between groups were calculated using a non-parametric ANOVA test with rank transformed values (Kruskall-Wallis). A p-value <0.05 was considered statistically significant.

Results

EP dose-dependently inhibits LPS-induced cytokine and chemokine production by THP-1 cells

The anti-inflammatory effects of EP have been demonstrated using various cell types *in vitro* (9, 10). We first wished to confirm the anti-inflammatory properties of EP on THP-1 cells. As expected, incubation of THP-1 cells with LPS (0.1 µg/ml) resulted in a transient increase of TNF-α , MIP-1α and MIP-1β expression with peak levels at 1, 1–2 and 2 hours (h), respectively (Fig. 1A, C and E). Maximal mRNA expression preceded peak protein levels for the corresponding cytokines (4, 4–24 and 8–24 h, resp.; Fig. 1B, D and F). EP dose-dependently inhibited these LPS-induced effects with maximal effects achieved at concentrations of 10 mM. LPS (0.1 µg/ml) and/or EP did not influence cell viability as determined by annexin V and 7-AAD staining (data not shown).

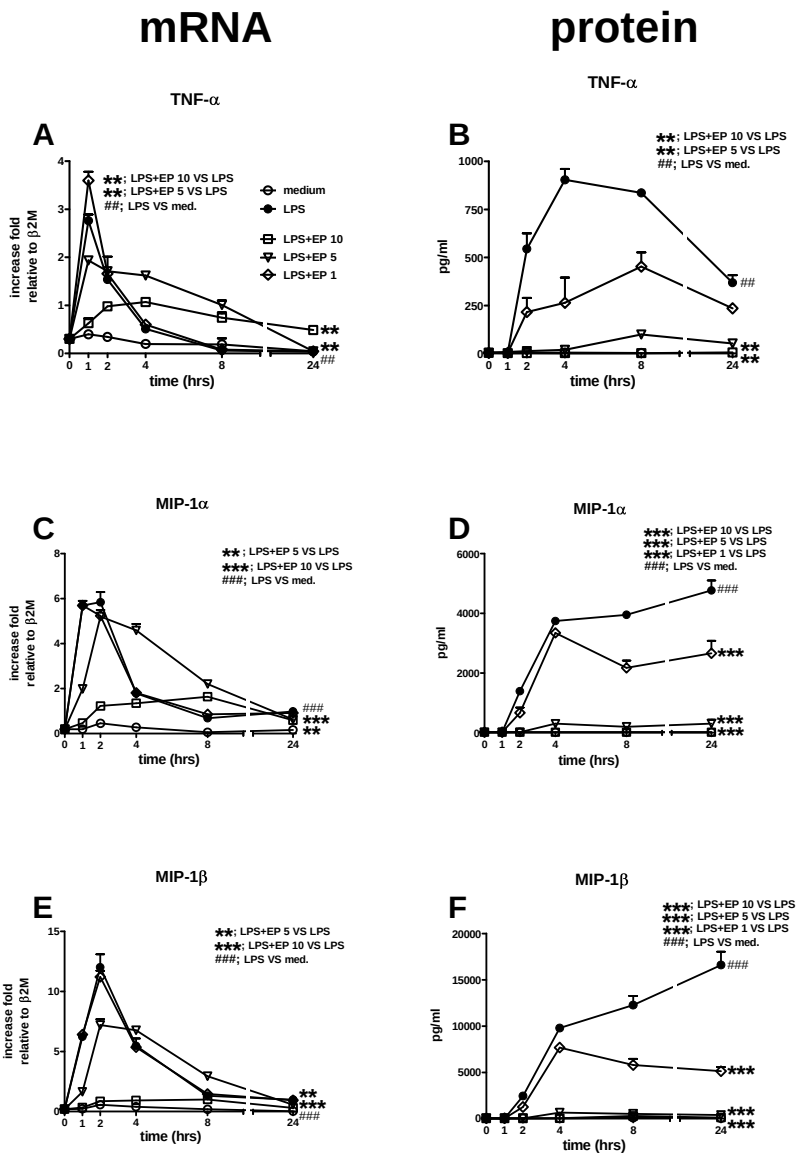

Figure 1: EP dose-dependently inhibits LPS-induced TNF- α , MIP-1α and MIP-1β mRNA and protein levels in human monocytic cells. mRNA (left panels; A, C and E) and protein (right panels; B, D and F) levels of TNF-α , MIP-1α and MIP-1 β (A and B, C and D, E and F, resp.).
THP-1 cells were incubated with LPS (0.1 µg/ml) in the absence or presence of EP (10, 5 or 1 mM) for indicated time periods. Data are means ± SEM (n = 2–4). †: p<0.005 and ¶: p<0.0005 for LPS vs. medium; *: p<0.005 and #: p<0.0005 for LPS vs. LPS+EP 10, 5 or 1 mM.

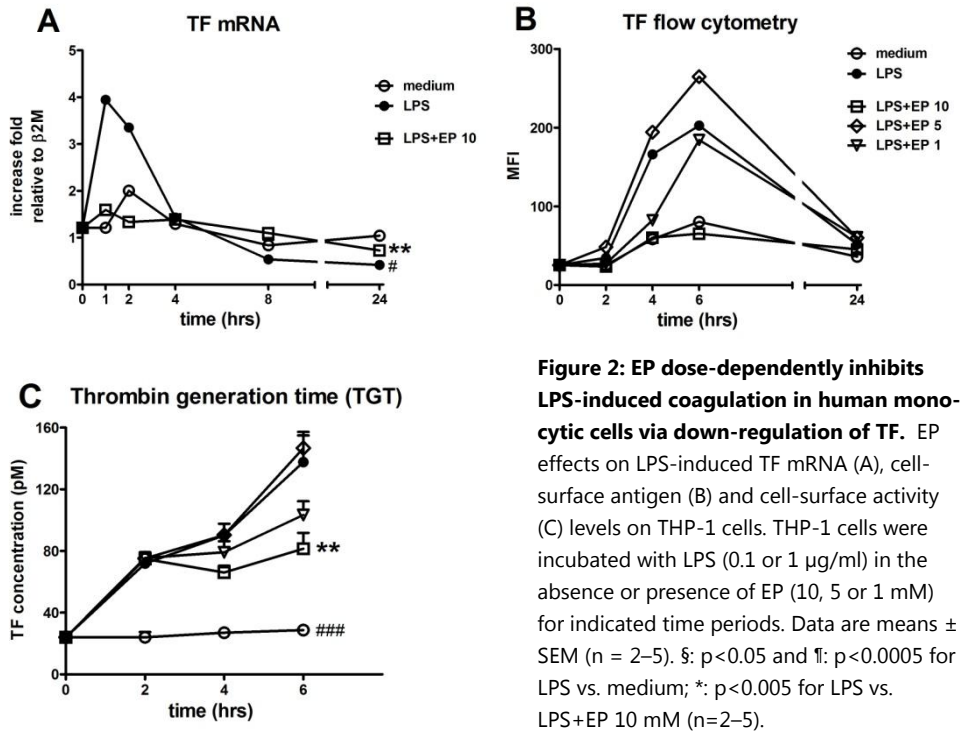

Figure 2: EP dose-dependently inhibits LPS-induced coagulation in human monocytic cells via down-regulation of TF. EP effects on LPS-induced TF mRNA (A), cell-surface antigen (B) and cell-surface activity (C) levels on THP-1 cells. THP-1 cells were incubated with LPS (0.1 or 1 µg/ml) in the absence or presence of EP (10, 5 or 1 mM) for indicated time periods. Data are means ± SEM (n = 2–5). §: $p<0.05$ and ¶: $p<0.0005$ for LPS vs. medium; *: $p<0.005$ for LPS vs. LPS+EP 10 mM (n=2–5).

EP reduces LPS-induced TF mRNA levels in THP-1 cells

LPS (0.1 µg/ml) increased relative TF mRNA levels with peak levels at 1 and 2 h (Fig. 2A). After 4 h, the TF mRNA levels had returned to basal levels. Incubation with medium or EP alone did not induce TF mRNA (Fig. 2A and data not shown). EP inhibited the LPS-induced TF mRNA levels at a dose of 10 mM.

EP dose-dependently decreases LPS-induced TF cellsurface expression

LPS (1 µg/ml) enhanced TF cell-surface expression, becoming apparent after 4 h and peaking after 6 h (Fig. 2B). Incubation with medium or EP alone did not influence TF expression. LPSinduced TF expression was dose-dependently inhibited by EP with maximal effects at a dose of 10 mM.

EP dose-dependently decreases LPS-induced cellsurface procoagulant activity

Finally, EP was studied for its ability to inhibit LPS (1 µg/ml) induced TF activity as measured by the TGT. TF activity of THP-1 cells was enhanced at 2 h and reached peak levels at 6 h in response to LPS (Fig. 2C). Furthermore, the kinetics of enhanced TF activity paralleled those in TF cell-surface protein levels. Medium or EP alone did not affect TF activity. EP dose-dependently attenuated the effects of LPS with a maximal effect at 10 mM.

Discussion

Several studies have documented anti-inflammatory effects of EP, raising considerable interest in this compound as a potential novel therapeutic for conditions characterized by systemic inflammation, including sepsis and ischemia reperfusion injury (4–6). Here we report for the first time that EP also exerts anticoagulant effects: EP dose-dependently inhibited the expression and function of TF, the main initiator of coagulation activation, in human monocytic cell cultures.

THP-1 cells have been used extensively to study TF procoagulant production and activity (13). Since the effects of EP had not been studied in THP-1 cells before, we first showed that EP is capable of inhibiting LPS-induced pro-inflammatory cytokine and chemokine production by these human monocytic cells, thereby confirming and extending previous in-vitro studies with EP in experiments using human Caco-2 enterocyte-like and murine macrophage-like RAW 264.7 cells (9, 10). Likely, these anti-inflammatory effects of EP are at least in part mediated by EP-induced inhibition of NF-κ B signalling by directly targeting the p65 subunit of this transcription factor (11). Next, we established that EP dose-dependently inhibits LPS-induced TF-dependent procoagulant effects in THP-1 cells. Indeed, EP attenuated the effect of LPS on TF mRNA, protein and activity. Of note, the anti-inflammatory effects of EP (inhibition of cytokine and chemokine production) became apparent at lower concentrations (5 mM) than the effect of EP on TF (10 mM), suggesting that the effect of EP on inflammation may be more potent. The apparent discrepancy in our study following LPS treatment between TF mRNA and cell-surface antigen on one hand and TF cell-surface activity on

the other hand deserves comment. Two hours after LPS incubation, TF cell-surface activity was already enhanced (Fig. 2C), while TF cell-surface antigen was barely increased at this time point (Fig. 2B). Furthermore, addition of EP 10 mM to LPS-treated cells leads to a reduction of TF mRNA and cell-surface protein expression to levels comparable with medium incubation alone (Fig. 2A, B), whereas LPS-induced TF cell-surface activity was affected to a lesser extent by incubation with EP 10 mM (Fig. 2C). A possible explanation for these two apparent discrepancies might be that THP-1 cells constitutively express encrypted (functionally inactive) TF (14) which is deencrypted (activated) by LPS, reflected by a rapid increase in TF cell-surface activity (Fig. 2C) without a change in TF cell-surface antigen levels (Fig. 2B). EP might not inhibit this deencryption, reflected by the initial unchanged LPS-induced TF cell-surface activity at 2 h. In addition, LPS induces TF mRNA levels (Fig. 2A), which may lead to de-novo protein synthesis (Fig. 2B), further increasing TF cell-surface expression (Fig. 2C). Likely, this de-novo synthesis is inhibited by EP, as indicated by reduced TF mRNA, cell-surface antigen levels and cell-surface activity at 4–6 h after LPS incubation.

In a recent study, EP treatment was associated with a reduction in TF mRNA levels in kidneys of aged mice with acute renal failure and multiple organ damage due to abdominal sepsis caused by cecal ligation and puncture (15). Considering that TF mRNA levels were determined in whole kidney homogenates, this study left unanswered to what extent the effect of EP on TF mRNA levels was due to inhibition of migration of cells (with increased TF mRNA levels) originating from the blood or to a (more direct) reduction in TF mRNA levels in residential kidney cells. Our present data suggest that EP indeed may directly influence TF expression and function. Further studies are warranted to examine the in-vivo effects of EP on TF expression and coagulation activation in conditions featuring a combined proinflammatory and procoagulant response.

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