NOTE  Immunology

Ethyl Pyruvate Downregulates Tumor Necrosis Factor Alpha and Interleukin (IL)-6 and Upregulates IL-10 in Lipopolysaccharide-Stimulated Canine Peripheral Blood Mononuclear Cells

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ABSTRACT. We investigated the anti-inflammatory effects of ethyl pyruvate (EP) on LPS-stimulated canine PBMCs in vitro. We found that EP treatment inhibited the mRNA expressions of proinflammatory cytokines (TNF-α and IL-6), but induced mRNA expression of anti-inflammatory cytokines (IL-10). ELISA measurements revealed that EP also effectively downregulates the LPS-induced increase in proinflammatory cytokine release, while upregulating anti-inflammatory cytokine release. These data indicate that EP could be an effective anti-inflammatory agent in dogs.

Key words: canine peripheral blood mononuclear cells, cytokine, ethyl pyruvate, mRNA.

Ethyl pyruvate (EP) is a simple aliphatic ester derived from the endogenous metabolite pyruvic acid and has been shown to exhibit antioxidant [5] and anti-inflammatory [1] properties. While EP is known to inhibit activation of p28 mitogen-activated protein kinase and NF-κB in murine macrophages [10], its anti-inflammatory mechanism remains unclear.

We hypothesized that lipopolysaccharide (LPS) treatment of canine PBMCs would induce proinflammatory cytokine expression and that EP would attenuate this increase at either the mRNA or protein level. To address this hypothesis, we selected TNF-α and IL-6 as representative proinflammatory cytokines that are upregulated after LPS administration. In addition, we selected IL-10 as an anti-inflammatory cytokine.

Blood was collected from six healthy beagles and peripheral blood mononuclear cells (PBMCs) were separated using Histopaque® 1077 (Sigma, St. Louis, MO, U.S.A.). The isolated PBMCs were seeded into 24-well Nunclon® plates (NUNC, Roskilde, Denmark) in a final volume of 2 × 10⁶ cells/ml, supplemented with RPMI-1640 (Hyclone®, Thermo Fisher Scientific Inc., Logan, UT, U.S.A.) medium, 10% heat-inactivated FBS (fetal bovine serum, Hyclone®, Thermo Fisher Scientific Inc., Logan, UT, U.S.A.) medium, 100 U/ml penicillin G and 10 μg/ml streptomycin (Sigma) at 37°C in 5% CO₂ and incubated overnight. The next day, the mononuclear cells were exposed to 100 ng/ml of LPS (Escherichia coli serotype O111: B4, Sigma) and simultaneously treated with various concentrations of EP (Sigma) (5 d 10 mM each) based on previous in vitro studies [9]. The cell pellets were used for RNA isolation, and the cell-free supernatants were stored at −80°C until they were analyzed. Samples were harvested after 3, 6, 12 and 24 hr of cultivation. Unstimulated cells and cells in 10 mM ethyl pyruvate were used as controls. All procedures used in this study were approved by local animal welfare authorities (CBU2008-021).

For the quantification of cytokine gene expression, SYBR® green real-time PCR was used. Total RNA was extracted using an RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany), and subsequent DNase digestion was performed using RNase-free DNase (RQ1 RNase-free DNase, Promega, Madison, WI, U.S.A.). Five hundred nanograms to one microgram of total RNA was reverse transcribed in a 20 μl reaction volume containing random hexamers, dNTP mixture, reverse transcriptase, recombinant RNase inhibitor, DTT and 5 × First-Strand Buffer. Then, 1 μl of E. coli RNase H was added and incubated at 37°C for 20 min. All materials were purchased from Invitrogen Corporation (SuperScript™ III, Invitrogen, Carlsbad, CA, U.S.A.). PCR was performed in a 20 μl reaction volume containing 300 nM of each primer, 1 μl of cDNA and 10 μl of 2 × iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, U.S.A.) using the CFX384 Real-Time PCR detection system (Bio-Rad) and the following steps: an initial i-Taq™ DNA polymerase activation step at 95°C for 3 min, 40 cycles of denaturation for 15 sec at 95°C, annealing for 30 sec at 62°C for GAPDH and IL-6 to 62°C for TNF-α, extension. Data collection and analysis were performed using the CFX Manager™ Software version 1.0 (Bio-Rad). The primers utilized in this study are summarized in Table 1. The optimal annealing temperature (Tₐ) for all assays ranged from 60°C for GAPDH and IL-6 to 62°C for TNF-α and IL-10. Primer efficiency calculations [E (%) = (10(ΔCt/ΔCt)−1) × 100, S=slope] for all of the standard lines were between 95.7 and 102.4%. Relative quantification was analyzed using the −2ΔΔCt method as previously described [4]. Tests were performed in duplicate.

Our quantification of cytokine mRNA abundance demonstrated that EP inhibits TNF-α and IL-6 mRNA synthesis and promotes IL-10 mRNA expression on LPS stimulation,
As illustrated in Fig. 1. Although we could not statistically confirm that gene expression changes, similar patterns were always observed in our experiments. Lipopolysaccharide treatment produced a 150-, 1500- and 8-fold increase in TNF-α, IL-6 and IL-10 mRNA, respectively, at 3 to 6 hr. However, EP markedly inhibited the expressions of the proinflammatory cytokines, TNF-α and IL-6, in a dose-dependent manner at 3 and 6 hr. In addition, the expression of IL-10 mRNA was increased at 24 hr following treatment with EP. To evaluate the levels of these cytokines at 24 hr following treatment with EP, ELISA was used. The results of this study support the potential application of EP for anti-inflammatory treatment in dogs. To the best of our best knowledge, this is the first report on the anti-inflammatory effects of EP in canine PBMC cultures. Because EP is not associated with toxicity, the therapeutically achievable and safe dose of EP treatment should be studied further in vivo.

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REFERENCES


Table 1. Primers used for the SYBR® green real-time PCR assay

<table>
<thead>
<tr>
<th>Target gene</th>
<th>GenBank accession No.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AB038240</td>
<td>678: 59 58 TCCCCACCCCCAAATGATC</td>
<td>789: 58 50 TCCTGTCTCATCATTCTTGG</td>
<td>92</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM 01003224</td>
<td>726: 59 50 CCAACGACCCCTTTGATCA</td>
<td>808: 59 55 CCAGCAGCTGACCCCTTAAATT</td>
<td>83</td>
</tr>
<tr>
<td>IL-6</td>
<td>U12234</td>
<td>10: 59 50 CCCACAGGAAAGAAGAGA</td>
<td>77: 58 50 CTTGTGAGGAGGAGTCTAGCG</td>
<td>68</td>
</tr>
<tr>
<td>IL-10</td>
<td>U33843</td>
<td>245: 59 55 CAAACCGCTGCGGAGATGAT</td>
<td>322: 59 52 CTTGAGTCTGCGTCTCGT</td>
<td>78</td>
</tr>
</tbody>
</table>

Primers were designed using the Primer Express® Software (version 3.0, Applied Biosystems, Foster City, CA, U.S.A.) and several criteria were applied as follows: amplify a template region of 75–150 base pair (bp); maintain a Tm between 50 and 65°C; use an Ta above the Tm; avoid targets with long (>4) repeats of single bases; maintain a GC content of 50–60%; avoid repeats of Gs and Cs longer than 3 bases; place Gs and Cs on ends of primers and avoid primer-dimer formation. To evaluate the secondary structure of the product, the DNA mfold server of Dr. Michael Zuker (http://mfold.bioinfo.rpi.edu) was used. Ta, annealing temperature; Tm, melting temperature.

a) Positions of genes are given according to accession number.

b) Theoretical melting temperature calculated by Primer Express® Software.
Fig. 1. Relative mRNA expressions and cytokine levels after ethyl pyruvate treatment of LPS-stimulated canine PBMCs. The relative mRNA expressions of TNF-α (a) and IL-6 (b) were decreased by treatment with ethyl pyruvate, while IL-10 mRNA (c) was increased. All mRNA values were normalized to the GAPDH signal in each lane, and the mRNA content of the calibrator (non-stimulated sample) was arbitrarily set at 1.0. ELISA measurements demonstrated that EP also inhibited TNF-α (d) and IL-6 (e) expressions and induced IL-10 (f) production. The data are presented at the mean ± SEM.