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.

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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 p38 mitogen-activated protein kinase and NF- κ B 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 2×10^6 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.) and 100 U/ml penicillin G and 10 μ g/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. Unstimu-

lated 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 μ l reaction volume containing random hexamers, dNTP mixture, reverse transcriptase, recombinant RNase inhibitor, DTT and 5 $\times$ First-Strand Buffer. Then, 1 μ l 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 μ l reaction volume containing 300 nM of each primer, 1 μ l of cDNA and 10 μ l of 2 $\times$ 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 and 15 sec at the annealing temperature and then 15 sec at 72°C for 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 (T_a) 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 (%) = $(10^{(1/S)} - 1) \times 100$, S=slope] for all of the standard lines were between 95.7 and 102.4%. Relative quantification was analyzed using the $-2^{\Delta\Delta CT}$ 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,

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

Target gene	GenBank accession No.	Forward primer			Forward sequences	Reverse primer			Reverse sequences	Amplicon size (bp)
		Start ^{a)}	T _m ^{b)} (°C)	GC (%)		Start ^{a)}	T _m ^{b)} (°C)	GC (%)		
GAPDH	AB038240	698	59	58	TCCCCACCCCAATGTATC	789	58	50	TGCCTGCTTCACTACCTTCTTG	92
TNF-α	NM001003224	726	59	50	CCAAACCGACCCTTTGATCA	808	59	55	CCAGCCCTGAGCCCTTAATT	83
IL-6	U12234	10	59	50	CCCACCAGGAACGAAAGAGA	77	58	50	CTTGTGGAGAGGGAGTTCATAGC	68
IL-10	U33843	245	59	55	CAAGCCCTGTCGGAGATGAT	322	59	52	CTTGATGTCTGGGTCTGTGGTT	78

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 T_m between 50 and 65°C; use an T_a above the T_m; avoid templates 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. T_a, annealing temperature; T_m, melting temperature.

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

b) Theoretical melting temperature calculated by Primer Express® Software.

as illustrated in Fig. 1. Although we could not statistically confirm that gene expression changes, similar patterns were always observed in our experiment. 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 10 mM EP.

Because endotoxin can alter both the rate of transcription and the rate of translation for cytokines [3, 6], we also determined the cytokine concentrations in response to LPS and EP. To evaluate the cytokine changes after LPS infusion, plasma TNF- α , IL-6 and IL-10 were measured using sandwich enzyme-linked immunosorbent assays (ELISA). The ELISAs were based on commercially available matched antibody pairs (DuoSet ELISA Development Kit, R&D Systems, Minneapolis, MN, U.S.A.). Assay concentrations were calculated using the SoftMax® Pro software (ver. 5.3, Molecular Devices Corp., Boston, MA, U.S.A.) from the absorbance difference (A450-A540) measured with a microplate spectrophotometer (VersaMax™, Molecular Devices Corp., Boston, MA, U.S.A.). The detection limits of each cytokine were 15.6 pg/ml for TNF- α , 62.5 pg/ml for IL-6 and 31.3 pg/ml for IL-10, respectively. All tests were performed in duplicate.

While LPS increased the proinflammatory cytokines in the supernatant of canine cultured mononuclear cells, EP markedly inhibited the production of these proinflammatory cytokines in a dose-dependent manner. On the other hand, the IL-10 levels increased in the EP-treated group (Fig. 1), while all of the cytokines in the control group (before LPS stimulation and only EP-treated) were below the detection limit.

Here, we observed that EP inhibited TNF- α and IL-6 mRNA expressions and induced IL-10 mRNA and protein synthesis in canine mononuclear cell cultures when EP treatment was simultaneously performed with LPS stimulation. This study indicates that the experimental pharmacological

agent EP can modulate the inflammatory response by down-regulating proinflammatory cytokines and upregulating anti-inflammatory cytokines after LPS stimulation in canine PBMC cultures. Though the biochemical basis for the anti-inflammatory actions of ethyl pyruvate remains to be elucidated, EP appears to have a role in protecting against lethal sepsis [10], hemorrhagic shock [8, 11] and ischemia/reperfusion-induced tissue injury [2, 7]. These results are based on the observed anti-inflammatory role of EP. 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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Fig. 1. Relative mRNA expressions and cytokine levels after ethyl pyruvate treatment of LPS-stimulated canine PMBCs. 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 $\pm$ SEM.

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