Evidence that Glutathione Depletion is a Mechanism Responsible for the Anti-inflammatory Effects of Ethyl Pyruvate in Cultured LPS-stimulated RAW 264.7 Cells

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Ethyl pyruvate inhibits lipid peroxidation and depletes GSH

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ABBREVIATIONS: EMSA, electrophoretic mobility shift assay; EP, ethyl pyruvate; FBS, fetal bovine serum; GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); GSH-Et, glutathione ethyl ester; HMGB1, high mobility group B; LPS, lipopolysaccharide; MDA, malondialdehyde; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; RT-PCR, reverse transcriptase polymerase chain reaction; TBARS, thiobarbituric acid reactive substances;
ABSTRACT
Ethyl pyruvate (EP), an effective scavenger of reactive oxygen species, is also an anti-
inflammatory agent in a variety of in vivo and in vitro model systems. In order to gain a better understanding of the molecular basis for the anti-inflammatory effects of EP, we compared the pharmacological properties of EP and N-acetyl-L-cysteine (NAC), a well-studied scavenger of reactive oxygen species and a precursor for the endogenous anti-oxidant, glutathione (GSH). The studies were performed using RAW 264.7 murine macrophage-like cells that were stimulated with lipopolysaccharide (LPS). Although EP and NAC both inhibited LPS-induced nitric oxide and IL-6 secretion, the former compound was considerably more potent than the latter. EP markedly inhibited iNOS, IL-6 and IL-10 mRNA induction, whereas the effects of NAC were minimal. EP inhibited LPS-induced NF-κB DNA binding to a much greater extent than did NAC. Both compounds inhibited LPS-induced lipid peroxidation, but the two compounds had qualitatively different effects on cellular levels of GSH. Whereas NAC increased GSH levels, EP had the opposite effect. The anti-inflammatory effects of EP were partially reversed when RAW 264.7 cells were treated with a cell permeable GSH analogue, glutathione ethyl ester. These data support the view that the anti-inflammatory effects of EP are mediated, at least in part, by its ability to deplete cellular GSH stores. Moreover, the findings presented here suggest that an unusual combination of biochemical effects—inhibition of lipid peroxidation and GSH depletion—might account for the anti-inflammatory effects of EP.
INTRODUCTION

Ethyl pyruvate (EP), a simple aliphatic ester derived from pyruvic acid, has been shown to be an effective anti-inflammatory agent in a variety of *in vivo* and *in vitro* model systems (Fink, 2003; Riedemann et al., 2003). For example, when mice with hemorrhagic shock are resuscitated with a balanced salt solution containing EP instead of a control solution without EP, activation of the proinflammatory transcription factor, NF-κB, is inhibited and the expression of several proinflammatory genes is down-regulated (Yang et al., 2002). Similarly, EP increases survival time and reduces circulating levels of IL-6 and nitrite (NO₂⁻), a marker of nitric oxide (NO) production in rats injected with a lethal dose of lipopolysaccharide (LPS) (Venkataraman et al., 2002). EP also inhibits activation of NF-κB in Caco-2 human enterocyte-like cells stimulated with a mixture of TNF-α, IL-1β and IFN-γ (Sappington et al., 2003) and RAW 264.7 murine macrophage-like cells stimulated with lipopolysaccharide (LPS) (Ulloa et al., 2002). Pretreating mice with EP prior to administration of a lethal dose of LPS inhibits secretion of key proinflammatory cytokines, such as TNF-α and high mobility group B1 (HMGB1), and improves long-term survival (Ulloa et al., 2002). Remarkably, treating mice with EP 12 or 24 h after the onset of severe infection ameliorates the development of renal dysfunction (Miyaji et al., 2003), improves survival (Ulloa et al., 2002), and decreases circulating concentrations of both TNF-α (Miyaji et al., 2003) and HMGB1 (Ulloa et al., 2002). EP also blocks the release of HMGB1 from LPS-stimulated RAW 264.7 cells (Ulloa et al., 2003; Ulloa et al., 2002).

The biochemical mechanisms responsible for the salutary anti-inflammatory effects of EP remain to be elucidated. One possibility is suggested by the close chemical similarity between EP and pyruvate, a compound that is known to be an effective scavenger of reactive oxygen species (ROS) (Adickes and Andresen, 1943; Bunton, 1949; Melzer and Schmidt, 1988; Dobsak et al., 1999). An oxidizing environment, particularly in the cytosol, has been implicated in the activation of pro-inflammatory signaling molecules, such as NF-κB (Rahman et al., 2001; Livolsi et al., 2001; Schoonbrodt et al., 2000; Schreck et al., 1992; Oka et al., 2000) and p38 mitogen activated protein kinase (Hashimoto et al., 2001). Accordingly, EP, by scavenging ROS, might inhibit activation of pro-inflammatory pathways induced by various stimuli. This notion is supported by our laboratory’s observation that EP ameliorates lipid peroxidation in rats subjected to hemorrhagic shock and resuscitation (Tawadrous et al., 2002).

Another potential mechanism to explain the anti-inflammatory effects of EP, however, derives from the recognition that all of the members of the NF-κB family of proteins share a
characteristic motif with one cysteine and three arginine residues in the DNA-binding region (Dröge, 2002). The sulfhydryl group of this critical cysteine residue (Cys\(^{62}\)) is essential for DNA-binding activity. Because of the three positively charged arginine residues nearby, the cysteine residue is very susceptible to oxidation (e.g., mixed disulfide formation) (Galter et al., 1994). Hence, depletion of the important intracellular anti-oxidant, glutathione (GSH), which shifts the intracellular redox milieu toward a more oxidized state, might interfere with binding to DNA by the activated NF-κB complex through this mechanism. This idea is supported by results from several studies that document that LPS- or TNF-α-induced inflammation and/or NF-κB activation are down-regulated by prior administration of pharmacological agents that promote depletion of cellular GSH stores (Kefer et al., 2001; Wei et al., 1999; Jones et al., 1999; Nathens et al., 1996; Nathens et al., 1998). Since pyruvate is known to react with cysteine to form an unstable thiazolidine adduct (Dröge et al., 1994), it is conceivable that EP might function as a GSH depleting agent and exert anti-inflammatory effects on this basis.

In the present study, we sought to obtain data that might help to elucidate the mechanism(s) responsible for the anti-inflammatory effects of EP. Our approach was to use LPS-stimulated RAW 264.7 cells as a reductionist in vitro model system, and compare the effects of EP with those of N-acetyl-L-cysteine (NAC), a well-studied antioxidant, on the expression of inflammatory mediators, the formation of thiobarbituric acid reactive substances (TBARS) as an index of lipid peroxidation, and changes in cellular GSH levels. Although both EP and NAC inhibited LPS-induced TBARS formation and cytokine and NO release, these two compounds had qualitatively different effects on cellular concentrations of GSH. Whereas NAC increased cellular GSH levels as expected (Haddad, 2002b; Dobashi et al., 2001), EP had the opposite effect. Furthermore, the anti-inflammatory effects of EP were partially reversed if the cells were treated with glutathione ethyl ester (GSH-Et), an established approach for increasing cellular levels of GSH (Buchmuller-Rouiller et al., 1995). Collectively, these findings support the view that the anti-inflammatory effects of EP are mediated, at least in part, by a mechanism that involves depletion of intracellular GSH.
METHODS

Cell culture and reagents. All reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise indicated. RAW 264.7 mouse macrophage-like cells were purchased from the American Type Cell Collection (ATCC, Manassa, VA) and grown in DMEM medium (BioWhittaker; Walkersville, MD) supplemented with 10% defined fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified incubator under an atmosphere containing 5% CO2 95% air. For stimulation, the cells (1 × 10^6/well) were transferred to 6, 12, or 24-well polystyrene culture plates (Falcon, Franklin Lakes, NJ) in DMEM plus 10% FBS. After overnight incubation, the culture medium was removed and the cells were incubated in DMEM containing 5% FBS with or without graded concentrations of EP or NAC in the presence or absence of 10 ng/ml Escherichia coli LPS (serotype O111:B4) for the time periods indicated. Cell viability was assessed by trypan blue exclusion and lactate dehydrogenase assay (Promega, Madison, WI). Under the conditions employed in this study, neither EP nor NAC appeared to have any effect on the viability of RAW 264.7 cells.

Measurement of nitrite concentration. As an indicator of (NO) synthesis, nitrite (NO_2^-) concentrations in conditioned media (containing phenol red) were determined by mixing 100 µl of Griess reagent [0.1% N-1-naphylenediamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid] with the same volume of culture supernatant and incubating for 10 min in darkness at room temperature. Samples were analyzed in duplicate. Absorbance was measured at 540 nm using an MRX microplate reader (Dynex Technologies, Chantilly, VA). A freshly prepared solution of sodium nitrite was used to generate a standard curve.

Cytokine assays. The concentrations of IL-6 and IL-10 in culture supernatants were determined using ELISA kits (Pharmingen, San Diego, CA) according to the instructions provided by the manufacturer. Concentrations of IL-6 or IL-10 were calculated by comparison with standard curves.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from RAW 264.7 cells with RNA-Bee™ reagent (TEL-TEST, Friendswood, TX) according to the manufacturer’s instructions. The amount of RNA was determined spectrophotometrically. Two
micrograms of RNA from each sample was reverse transcribed to cDNA by using 200 U/µl Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was allowed to proceed for 10 min at 25 °C followed by 60 min at 42 °C. The reaction was terminated by heating to 95 °C for 5 min.

The PCR reaction was performed in a total volume of 50 µl containing Taq polymerase buffer (Invitrogen), deoxynucleotide mixture (0.2 mM each), MgCl₂ (1.5 mM), Taq polymerase (2.5 U) (Invitrogen), oligonucleotide primers (0.5 µM each), and 2.5 µl of the RT product. The specific primers for TNF-α, IL-6, IL-10 and iNOS are indicated in Table 1. Amplification was performed for 33-35 cycles. Each cycle consisted of denaturation for 1 min at 95°C, annealing for 1 min at the appropriate primer-specific temperature (Table 1), and extension for 1.5 min at 72 °C. An additional incubation at 72 °C for 10 min was executed after the last cycle. To document equal loading of RNA, primers for 18S ribosomal RNA were amplified under the same conditions (Table 1). Ten µL of PCR products were analyzed on 1.5% agarose gels containing 0.2 µg/ml ethidium bromide. Gels were scanned in a NucleoVision imaging workstation (NucleoTech, San Mateo, CA), and quantified using GelExpert™ release 3.5. Results are reported as relative band densities, using 18S RNA as a standard for normalization.

Assessment of NF-κB DNA binding. Nuclear extracts for electrophoretic mobility shift assay (EMSA) were prepared by incubating 1 × 10⁶ RAW 264.7 cells on ice for 15 min with 1 ml of cell lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM Na₂EDTA, 0.5 mM PMSF, 2 µg/ml aprotinin, and 2 µg/ml leupeptin). After adding 62.5 µl of 10% NP-40, the mixture was centrifuged at 5,000 g at 4 °C for 10 min. The crude nuclear pellet was suspended in 200 µl of nuclear lysis buffer (50 mM HEPES [pH 7.9], 50 mM KCl, 300 mM NaCl, 0.1 mM Na₂EDTA, 1 mM DTT, 0.1 mM PMSF, 2 µg/mL aprotinin, 2 µg/mL leupeptin, and 10% glycerol) and incubated on ice for 30 min. The suspension was centrifuged at 16,000 g at 4 °C for 30 min. The supernatant (nuclear proteins) was collected and stored at -80 °C until assayed. Nuclear protein concentration was determined using the Bio-Rad protein assay kit with BSA as a standard (Bio-Rad Laboratories, Hercules, CA).

EMSA to assess NF-κB DNA binding was performed using an appropriate consensus oligonucleotide probe. The sequence of the double-stranded NF-κB oligonucleotide was as
follows: sense: 5'-AGT TGA GGG GAC TTT CCC AGG C-3'; antisense: 3'-TCA ACT CCC CTG AAA GGG TCC -5' (Promega, Madison, WI). The oligonucleotides were end-labeled with [γ-32P]ATP (Du Pont-New England Nuclear, Boston, MA) using T4 polynucleotide kinase (Promega). Nuclear proteins (5 µg) were incubated with γ-32P-labeled NF-κB probe at room temperature for 30 min in a binding buffer that consisted of 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 250 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 20% glycerol, and 0.25 mg/ml poly(dI-dC), the total volume of binding reaction mixture being 20 µl. The mixture was subjected to electrophoresis on a 4% nondenaturing polyacrylamide gel in 0.5× TBE buffer. After electrophoresis, the gels were vacuum-dried and exposed to Kodak X-Omat AR film (Rochester, NY) between two intensifying screens at –80 °C. Gels were scanned in a NucleoVision imaging workstation (NucleoTech, San Mateo, CA), and quantified using GelExpert™ release 3.5.

Estimation of lipid peroxidation. Cells were washed with cold phosphate-buffered saline twice and then lysed with 500 µl of 2% SDS. TBARS were determined using the method of Jentzsch et al. (Jentzsch et al., 1996). Briefly, 200 µl of cell lysate or malondialdehyde (MDA) standard, which was prepared by hydrolysis of 1,1,3,3-tetramethoxypropane, were mixed with 25 µl butylhydroxytoluene in ethanol (0.03 mM) and 200 µl orthophosphoric acid (0.2 mM) in a 2 ml Eppendorf tube and vortexed for 10 s. Twenty-five µl of 2-thiobarbituric acid reagent (800 mg of TBA dissolved in 50 ml 0.1 mM NaOH) was added and the tube was vortexed again. The reaction mixture was then incubated at 90 °C for 45 min in a water bath. The tubes were put on ice to stop the reaction. After cooling to room temperature, TBARS were extracted once with 500 µl n-butanol (ICN Biomedicals, Aurora, Ohio). To facilitate phase separation, 50 µl saturated NaCl solution was added and the test tubes were centrifuged at 12,000 rpm for 1 min in an Eppendorf centrifuge. Two hundred µl of the upper butanol phase were placed into a flat-bottom 96-well multititer plate. Absorption was read at 530 and 570 nm for correcting baseline absorption in a MRX Revelation microplate reader. MDA equivalents were calculated using the difference in absorption at the two wavelengths and quantification was carried out with the aid of appropriate calibration curves.

Determinations of cellular levels of reduced and oxidized glutathione (GSH and GSSG, respectively). Cells were washed twice with cold phosphate-buffered saline and scraped in 100
µl of 4.3% sulfosalicylic acid and 392.5 µl of 0.1 M phosphate buffer. The mixture was centrifuged and the supernatants neutralized with 7.5 µl 5 M KOH per 500 µl total volume. The GSH recycling method was performed as described previously (Novelli, 1997). GSH standards were prepared in the background buffer and treated in the same way as the samples. Fifty µl samples of cell lysates or standards were transferred into the wells of microtiter plates. After addition of 100 µl of reaction mixture [0.1 M sodium phosphate buffer (pH 7.5) containing 2.5 mM EDTA, 0.15 mM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.2 mM NADPH, and 1.0 U/ml GSH reductase], absorbance at 405 nm was read at 30 sec intervals over 5 min using a MRX Revelation microplate reader. To measure GSSG concentration, 100 µl of the above cell lysate was incubated with 100 µl of 35 mM N-ethylmaldimide (NEM) for 0.5 h at room temperature to sequester all free (reduced) GSH. Remaining excess NEM was sequestered by adding 100 µl of 35 mM L-cysteine. Fifty µl of the cell lysates or standards were assayed as described above. The concentrations of total GSH and GSSG were calculated on the basis of a calibration curve obtained with authentic GSH as standards. Protein concentration was determined using the Bio-Rad protein assay kit with BSA as a standard. Results are expressed as nmol/µg protein.

**Statistical analysis.** All results are expressed as means ± SD. In general, the statistical significance of differences among groups was assessed using one-way analysis of variance followed by the Student-Newman-Keul’s test for multiple comparisons. Summary statistics are presented for RT-PCR and EMSA data, but these results were not subjected to statistical analyses since the method employed was only semi-quantitative and the samples sizes (N=3 per condition) were small (Yang et al., 2002). SigmaStat statistical software (Jandel Scientific, San Rafael, CA) was used for data processing, and results were considered statistically significant when the P value was < 0.05.
RESULTS

EP and NAC inhibit LPS-induced NO, IL-6, and IL-10 production by RAW 264.7 cells. Murine macrophage-like RAW 264.7 cells were treated with LPS (10 ng/ml) in the presence or absence of various concentrations of either NAC or EP for 24 h. At the end of this period, concentrations of NO\textsubscript{2}−, IL-6 and IL-10 were measured in the conditioned media. Both EP and NAC inhibited NO, IL-6 and IL-10 release in a dose-dependent manner (Figures 1-3). The pharmacological effects of EP and NAC on NO\textsubscript{2}− concentrations in conditioned media were quite similar (Figure 1). Although qualitatively similar, the effects of EP and NAC on LPS-induced IL-6 production were quantitatively different. Whereas co-incubation of LPS-stimulated RAW 264.7 cells with 20 mM EP inhibited IL-6 secretion by >90%, co-incubation with a similar concentration of NAC decreased IL-6 production by only about 30% (Figure 2). Quantitative differences between the concentration-dependent pharmacological effects EP and NAC were even more striking when LPS-induced IL-10 production was assessed (Figure 3). Whereas 5 mM NAC almost completely inhibited IL-10 secretion, a similar concentration of EP inhibited the release of IL-10 by only about 30% relative to LPS-stimulated control cells incubated in the absence of the compound.

EP and NAC have distinct effects on LPS-induced iNOS, IL-6, IL-10 mRNA expression. RAW 264.7 cells were incubated in the presence or absence of LPS (10 ng/ml). Graded concentrations of either EP or NAC were added to some of the cultures. RNA was isolated after 4 h of incubation. As expected, treatment of RAW 264.7 cells with LPS increased expression of iNOS, IL-6 and IL-10 transcripts (Figure 4 and Table 2). Even at the highest concentration studied (20 mM), NAC had only minimal effects on steady-state levels of iNOS, IL-6 and IL-10 mRNA in LPS-stimulated RAW 264.7 cells. In contrast, EP, particularly at higher concentrations (10 or 20 mM), markedly inhibited expression of all three of these genes.

EP and NAC inhibit LPS-induced NF-κB DNA binding. EP has been shown to inhibit activation of NF-κB in RAW 264.7 cells (Ulloa et al., 2002). In contrast, prior studies indicate that NAC fails to inhibit LPS-induced activation of NF-κB in RAW 264.7 cells (Wadsworth and Koop, 1999) although NAC has been shown to inhibit activation of NF-κB in this cell type.
caused by other stimuli, such as ionizing radiation (Pajonk et al., 2002) and pro-oxidant dicarbonyls (Fan et al., 2003). In order to compare the effects of the two compounds on LPS-induced NF-κB DNA binding under identical assay conditions, RAW 264.7 cells were incubated for 1 h with or without LPS in the presence or absence of 20 mM EP or 20 mM NAC. When nuclear extracts were prepared from cells incubated under control conditions (i.e., in the absence of LPS), it was apparent that detectable NF-κB DNA binding was present even in unstimulated cells (Figure 5). Both EP and NAC inhibited DNA binding by NF-κB in unstimulated cells, but EP was clearly more active than NAC in this regard. Although basal NF-κB activation was evident in unstimulated cells, when RAW 264.7 cells were stimulated with LPS for 1 h, binding of NF-κB to DNA was clearly increased. Treatment with 20 mM NAC moderately decreased NF-κB DNA binding in LPS stimulated cells, but 20 mM EP dramatically decreased NF-κB DNA binding in LPS stimulated cells such that the band intensity was similar to that observed when extracts were prepared from control cells incubated with EP.

*Both EP and NAC inhibit LPS-induced lipid peroxidation.* We used TBARS formation as an indicator of lipid peroxidation. Both EP (Tawadrous et al., 2002) and NAC (Davreux et al., 1997; Cuzzocrea et al., 2000) have been shown to inhibit lipid peroxidation under various conditions that are associated with redox stress. In order to compare the effects of the two compounds on lipid peroxidation in endotoxin-stimulated macrophages, RAW 264.7 cells were incubated for 24 h under control conditions or with LPS in the absence or presence of graded concentrations of either EP or NAC. Both EP and NAC significantly inhibited LPS-induced lipid peroxidation in a concentration-dependent fashion (Figure 6). At the highest concentration studied, EP was clearly more effective than NAC at preventing the formation of TBARS. Because TBARS formation was virtually undetectable when cells were incubated with 20 mM EP, we considered the possibility that residual EP might interfere with the spectrophotometric assay system employed. Accordingly, additional experiments were carried out to measure TBARS concentrations in solutions containing TBARS standards in the presence or absence of graded concentrations of EP (0-20 mM). These studies documented that EP does not interfere with the TBARS assay (data not shown).
EP and NAC exert differential effects on intracellular GSH concentrations in LPS-stimulated RAW 264.7 cells. Results from several prior studies indicate that LPS-induced NF-κB activation, iNOS expression and pro-inflammatory cytokine production are inhibited by agents that deplete intracellular levels of GSH (Wang et al., 1999; Buchmuller-Rouiller et al., 1995; Wei et al., 1999; Kang et al., 1999; Kefer et al., 2001). We hypothesized that the anti-inflammatory effects of EP might occur as a result of this mechanism. Accordingly, we measured GSH and GSSG levels in RAW 264.7 cells incubated for 24 h with or without LPS in the absence or presence of graded concentrations of EP or NAC. Stimulation of RAW 264.7 cells with LPS significantly increased intracellular levels of GSH relative to those observed in unstimulated cells (Figure 7). Whereas co-incubation of the LPS-stimulated cells with NAC significantly increased GSH levels, co-incubation of the cells with EP had just the opposite effect.

Treatment with GSH-Et partially reverses the anti-inflammatory effects of EP. GSH-Et is a lipophilic cell-permeable compound that has been used previously to increase cellular levels of GSH (Buchmuller-Rouiller et al., 1995; Celli et al., 1998). We reasoned that incubating EP-treated RAW 264.7 cells with GSH-Et might reverse the effects of EP on LPS-induced NF-κB activation and mediator release, if EP exerts its anti-inflammatory actions by depleting cellular levels of GSH. In order to test this hypothesis, we pre-incubated RAW 264.7 cells growing in multiwell plates with either 20 mM EP or medium for 60 min (T=-75 to –15 min). After the cells were washed with cold PBS three times, cultures were subjected to a second 15 min pre-incubation period (T=-15 to 0 min). During this period, the cells were incubated with either fresh medium or medium containing 6 mM GSH-Et. Finally, the cells were stimulated for either 60 min or 24 h with LPS-containing or LPS-free medium to assess NF-κB activation and mediator release.

As expected, incubating RAW 264.7 cells with LPS for 60 min increased DNA binding by NF-κB (Condition 3 versus Condition 1 in Figure 8). Pre-treating the cells with 20 mM EP for 60 min decreased LPS-induced NF-κB DNA binding (Condition 4 versus Condition 3). However, incubating the cells with 6 mM GSH-Et for 15 min after exposing them to EP partially restored LPS-induced NF-κB DNA binding (Condition 6 versus Condition 4). Interestingly,
incubating cells with GSH-Et clearly increased NF-κB DNA binding even in the absence of LPS stimulation (Condition 2 versus Condition 1).

Table 3 shows the effects of EP with or without subsequent treatment with GSH-Et on the release of NO and IL-6 from RAW 264.7 cells exposed for 24 h to LPS. As expected, EP markedly decreased IL-6 and NO$_2^-$ concentrations in LPS-stimulated RAW 264.7 cell supernatants (Condition 4 versus Condition 3). If the EP-treated cells were washed and then exposed to GSH-Et for 15 min, the concentrations of IL-6 and (to a greater extent) NO$_2^-$ in conditioned media were significantly greater than when the cells were exposed to EP alone (Condition 6 versus Condition 4). Treatment of cells with GSH-Et in the absence of LPS stimulation slightly (albeit significantly) increased the basal secretion of IL-6, although this treatment had no discernable effect on the basal secretion of NO (Condition 2 versus Condition 1).

Intracellular GSH and GSSG levels were measured in the cells pre-incubated with medium, EP and/or GSH-Et prior to being incubated for 24 h in the absence or presence of LPS (Table 4). Incubation of cells with GSH-Et (in the absence of either EP or LPS) significantly increased intracellular GSH concentration (Condition 1 versus Condition 2). Consistent with the results from a slightly different experiment depicted in Figure 7, incubating naïve RAW 264.7 cells with LPS for 24 h significantly increased the intracellular concentration of GSH (Condition 1 versus Condition 3 in Table 4). Also consistent with the results from the earlier experiment, pre-incubating cells with 20 mM EP for 60 min prior to exposing them to LPS for 24 h significantly decreased GSH concentration (Condition 4 versus Condition 3). Compared to cells incubated with LPS without prior exposure to either EP or GSH-Et, cells that were pre-incubated with EP and then GSH-Et before adding LPS had a significantly lower concentration of GSH (Condition 6 versus Condition 3). However, compared to cells that were pre-incubated with EP only, cells that were pre-incubated with EP and then GSH-Et before adding LPS had a significantly higher concentration of GSH (Condition 6 versus Condition 4). This last result supports the view that GSH-Et partially reversed EP-mediated GSH depletion.
DISCUSSION

EP is a simple aliphatic ester derived from pyruvic acid that has been shown to be an effective anti-inflammatory agent in a variety of in vivo and in vitro model systems (Fink, 2003). It is well-known that pyruvate is an ROS scavenger (Adickes and Andresen, 1943; Bunton, 1949; Melzer and Schmidt, 1988; Dobsak et al., 1999) and data are available suggesting that EP is as well (Varma et al., 1998; Tawadrous et al., 2002). NAC, a derivative of the amino acid, L-cysteine, is both an ROS scavenger itself (Aruoma et al., 1989) and a precursor for the synthesis of the important endogenous anti-oxidant, GSH (Fernandez et al., 1999). Since redox-sensitive signaling pathways are thought to regulate the expression of various pro- and anti-inflammatory mediators involved in the innate immune response (Haddad, 2002a; Garg and Aggarwal, 2002; Janssen-Heininger et al., 2000), we sought to compare the pharmacological effects of similar concentrations of EP and the widely employed reagent, NAC.

Our studies showed that these two anti-oxidants have quantitatively distinct effects on LPS-stimulated RAW 264.7 murine macrophage-like cells. Although both EP and NAC inhibited DNA binding by the pro-inflammatory transcription factor, NF-κB, in LPS-stimulated RAW 264.7 cells, EP was considerably more effective in this regard than NAC. In comparison to NAC, EP also was a more potent inhibitor of iNOS, IL-6 and IL-10 mRNA expression. Whereas 20 mM NAC decreased the mean IL-6 concentration in conditioned media from LPS-stimulated RAW 264.7 cells by about 30%, the same concentration of EP decreased the mean concentration of this cytokine by more than 90%. NAC, even at a relatively low concentration (5 mM), almost completely abrogated the release of the anti-inflammatory cytokine, IL-10, from RAW 264.7 cells incubated for 24 h with LPS. In contrast, 5 mM EP decreased LPS-stimulated IL-10 release by only about 20%. With regard to IL-6 release, the results presented here are in agreement with data from in vivo studies wherein it was shown that treatment with EP significantly decreased circulating IL-6 levels in endotoxemic rats (Venkataraman et al., 2002). However, in contrast to the in vitro data presented here, treatment with EP significantly increased plasma IL-10 levels in rats challenged with LPS. Nevertheless, to the extent that the IL-6 /IL-10 ratio is an indicator of inflammatory “tone” (Taniguchi et al., 1999), the in vitro data presented here and the in vivo results reported previously are consistent in showing that EP is an anti-inflammatory agent.
When steady-state mRNA levels for iNOS, IL-6 and IL-10 were assessed using RT-PCR, differences between the effects of EP and NAC were even more apparent. For example, 20 mM EP reduced iNOS and IL-6 mRNA expression to almost undetectable levels in LPS-stimulated RAW 264.7 cells, whereas the same concentration of NAC had little or no effect on expression of these transcripts under similar conditions. Interestingly, EP also suppressed IL-10 mRNA expression in LPS-stimulated RAW 264.7 cells to a greater extent than NAC. However, NAC suppressed IL-10 secretion to a much greater degree than EP. While our data insufficient to provide a mechanism for the discordance between the pharmacological effects of EP and NAC on IL-10 mRNA expression and IL-10 secretion, discordance between changes in IL-10 transcript levels and changes in IL-10 secretion has been noted before (Spuck et al., 2003; Ledeboer et al., 2002). In this context, it is important to recognize that our measurements of IL-10 concentration in samples of conditioned media assessed the cumulative secretion of the cytokine over a 24 h period of incubation, whereas our semi-quantitative estimates of IL-10 transcript levels represented a “snap-shot” at a single point in time (i.e., 4 h after stimulation of cells with LPS).

Results from several studies support the view that an oxidizing milieu promotes activation of the important pro-inflammatory transcription factor, NF-κB, in monocytes or macrophages stimulated with LPS or various cytokines. For example, Legrand-Poels et al. reported that LPS induces ROS formation in cultured human U937 promonocytes (Legrand-Poels et al., 1997). Moreover, these authors showed that relatively high concentrations of NAC (10-30 mM) inhibit LPS-induced NF-κB activation in these cells (Legrand-Poels et al., 1997). More recently, Sanlioglu et al. showed that LPS-mediated stimulation of RAW 264.7 murine macrophage-like cells promotes ROS formation and NF-κB activation via a process that is dependent on activation of Rac1, a GTP-binding protein that is thought to be involved in the generation of ROS via the enzyme, NADPH oxidase (Sanlioglu et al., 2001). These authors also showed that 25 mM NAC inhibits LPS-induced NF-κB activation and TNF synthesis by LPS-stimulated RAW 264.7 cells (Sanlioglu et al., 2001). In studies employing very high concentrations of LPS (10 µg/ml), Chen et al. reported that 20 mM NAC inhibits endotoxin-induced NF-κB activation in RAW 264.7 cells (Chen et al., 1995). Our data are generally consistent with these observations, since we also observed evidence of redox stress (TBARS...
formation) in LPS-stimulated RAW 264.7 cells and showed partial inhibition of LPS-induced NF-κB activation when these cells were incubated with 20 mM NAC.

Conflicting findings, however, have been reported by other investigators. For example, Chandel et al. showed that LPS fails to induce ROS formation in J774.1 murine macrophage-like cells (Chandel et al., 2000). Furthermore, these authors found that co-incubating the cells with NAC (500 µM) fails to abolish NF-κB DNA binding activity and increases TNF mRNA expression in response to stimulation with LPS (Chandel et al., 2000). In another study, Parmentier et al. showed that 5 mM NAC increases both LPS-induced NF-κB activation and IL-1β release by a THP-1 human myelomonocytic cells differentiated into macrophages (Parmentier et al., 2000). Similarly, Wadsworth and Koop reported that 30 mM NAC fails to inhibit LPS-induced NF-κB DNA binding in RAW 264.7 cells stimulated by LPS (Wadsworth and Koop, 1999).

Like NAC, EP ameliorated LPS-induced lipid peroxidation in RAW 264.7 cells, a finding that supports the view that EP is capable of scavenging ROS. Since EP and NAC were similarly effective with respect to amelioration of LPS-induced TBARS formation but exhibited differential pharmacological effects with respect to inhibition of LPS-induced NF-κB activation and mediator secretion, we investigated the effects of these two agents on cellular levels of the reduced and oxidized forms of glutathione (GSH and GSSG, respectively). Consistent with previously reported findings from other laboratories (Parmentier et al., 2000; Neuschwander-Tetri et al., 1996), we showed that treatment with NAC increased intracellular levels of GSH but had only a minimal effect on the levels of GSSG. The effect of EP was qualitatively different. In a dose-dependent fashion, incubating cells with EP significant decreased cellular levels of GSH and (and to a much lesser degree) GSSG. Whereas NAC increased the GSH/GSSG ratio in LPS-stimulated RAW 264.7 cells, EP had the opposite effect.

The effect of EP on cellular GSH levels might offer a clue to its mechanism of action as an anti-inflammatory agent. The results from several prior studies document that LPS- or TNF-α-induced inflammation and/or NF-κB activation is down-regulated by prior exposure of cells or animals to pharmacological agents that promote depletion of cellular GSH stores (Wei et al., 1999; Jones et al., 1999; Nathens et al., 1996; Nathens et al., 1998; Kefer et al., 2001). The basis for this pharmacology is thought to be enhanced formation of mixed disulfides involving critical
cysteine residues in the proteins making up NF-κB when the GSH/GSSG ratio is relatively low (Galter et al., 1994).

If the anti-inflammatory effects of EP are mediated by GSH depletion, then using a pharmacological approach to increase intracellular GSH levels after exposing cells to EP should reverse its effects on NF-κB activation and mediator release. When we tested this idea experimentally by using the cell permeable GSH analogue, GSH-Et, our findings were consistent with the hypothesis that the inflammatory effects of EP are partially mediated via GSH depletion. LPS-induced NF-κB DNA binding, as assessed by EMSA, was greater in cells treated with both EP and GSH-Et than in cells treated with EP alone. Similarly, nitrite and IL-6 concentrations were slightly, but significantly, higher in supernatants from LPS-stimulated cells that were previously treated with both EP and GSH-Et than in supernatants from LPS-stimulated cells previously treated with only EP.

It is important to note that treatment with 6 mM GSH-Et provided only partial restoration of LPS responsiveness in cells previously treated with EP. The failure of GSH-Et to completely reverse the effects of EP could reflect that the anti-inflammatory effects of the latter compound are only partially dependent on GSH depletion. Alternatively, incubation with 6 mM GSH-Et might have been inadequate to restore intracellular GSH levels or reverse the formation of mixed disulfides involving key cysteine residues in NF-κB subunits. This latter notion is supported by the data depicted in Table 3, which indicate that GSH levels were substantially lower in LPS-stimulated cells incubated with 20 mM EP and 6 mM GSH-Et than they were in LPS-stimulated cells incubated in the absence of either EP or GSH-Et (Condition 6 versus Condition 3).

Incubation of RAW 264.7 cells with GSH-Et in the absence of either LPS or EP increased the basal level of NF-κB DNA binding (Figure 8) and the secretion of IL-6 (Table 2). These subtle but consistent effects of incubation with GSH-Et might be explained by either of two plausible mechanisms. First, it is possible that the GSH-Et preparation we used was contaminated with traces of LPS. In other words, small of amounts of contaminating LPS might have been responsible for the increases in NF-κB DNA binding and IL-6 secretion that were observed when cells were incubated with GSH-Et. Alternatively, because incubating RAW 264.7 cells with GSH-Et (in the absence of LPS or EP) significantly increased intracellular levels of GSH (Condition 2 versus Condition 1 in Table 4), it is possible that the subtle pro-inflammatory effects of GSH-Et were caused by the same mechanism that has been offered here
to explain the anti-inflammatory effects of EP. Thus, according to our proposed model, agents, such as EP or diethyl maleimide (Wei et al., 1999; Jones et al., 1999; Nathens et al., 1996; Nathens et al., 1998; Kefer et al., 2001), that decrease intracellular GSH concentration are anti-inflammatory, whereas an agent, GSH-Et, that increases cellular GSH concentration is pro-inflammatory.

It is also conceivable that GSH-Et per se was biologically active in our assays, and did not function merely as a precursor for the formation of GSH. This idea is supported by previously published data, showing that GSH-Et-mediated protection against the damaging effects of ultraviolet radiation correlates more closely the dose of this thiol compound than to intracellular GSH levels (Steenvoorden et al., 1998).

We observed that LPS increased cellular concentrations of GSH in RAW 264.7 cells. Since some studies have reported that LPS induces the formation of ROS in macrophages, we were somewhat surprised by this observation. However, this same finding has been reported from studies examining the effects of LPS-stimulation on GSH levels in primary cultures of murine peritoneal macrophages (Sato et al., 2001; Sato et al., 1995). The mechanism underlying this phenomenon apparently is related to LPS-mediated induced expression of a cysteine transporter (Sato et al., 2001; Sato et al, 1995).

In summary, we showed that both EP and NAC inhibited the formation of TBARS, a marker of lipid peroxidation, following stimulation of RAW 264.7 cells with LPS. EP, however, was considerably more potent than NAC as an inhibitor of LPS-induced NF-κB activation and NO and IL-6 secretion. Whereas NAC increased cellular levels of GSH, EP had the opposite effect. We are unaware of any other compounds that have been shown to both inhibit lipid peroxidation and deplete cellular GSH levels. This unusual combination of biochemical actions may explain the remarkable anti-inflammatory effects of EP.
ACKNOWLEDGEMENTS

This research was supported by grants from the Defense Advanced Research Projects Agency (N65236-00-1-5434) and the National Institutes of Health (GM 068481).
REFERENCES

Adickes F and Andresen G (1943) Zur kenntnis der reihe der normalen aliphatischen \( \beta \)-oxysauren und der \( \alpha \)-ketosauren. *Ann Chem* **50**:41-57.


FIGURE LEGENDS

Figure 1. Effects of EP and NAC on LPS-induced accumulation of NO₂⁻ in conditioned supernatants from RAW 264.7 cells. Stimulated cells (solid bars) were incubated for 24 h with 10 ng/ml LPS in the presence or absence of graded concentrations of EP or NAC. Unstimulated cells (open bars) were incubated under the same conditions but in the absence of LPS. Depicted are representative data from an experiment that was repeated three times with similar results. Results are expressed as means ± SD (n=3 per condition). * indicates P < 0.05 versus cells treated with LPS alone.

Figure 2. Effects of EP and NAC on LPS-induced accumulation of IL-6 in conditioned supernatants from RAW 264.7 cells. Stimulated cells (solid bars) were incubated for 24 h with 10 ng/ml LPS in the presence or absence of graded concentrations of EP or NAC. Unstimulated cells (open bars) were incubated under the same conditions but in the absence of LPS. Depicted are representative data from an experiment that was repeated three times with similar results. Results are expressed as means ± SD (n=3 per condition). * indicates P < 0.05 versus cells treated with LPS alone.

Figure 3. Effects of EP and NAC on LPS-induced accumulation of IL-10 in conditioned supernatants from RAW 264.7 cells. Stimulated cells (solid bars) were incubated for 24 h with 10 ng/ml LPS in the presence or absence of graded concentrations of EP or NAC. Unstimulated cells (open bars) were incubated under the same conditions but in the absence of LPS. Depicted are representative data from an experiment that was repeated three times with similar results. Results are expressed as means ± SD (n=3 per condition). * indicates P < 0.05 versus cells treated with LPS alone.

Figure 4. Effects of EP and NAC on LPS-induced iNOS, IL-6 and IL-10 mRNA expression in RAW 264.7 cells. The cells were incubated for 4 h in the presence or absence of 10 ng/ml LPS in the presence or absence of EP (5-20 mM) or NAC (5-20 mM). RNA was extracted and expression of iNOS, IL-6 and IL-10 assessed using semi-quantitative RT-PCR. Results depicted are typical of those obtained in experiments that were repeated three times.
Figure 5. Effects of EP and NAC on LPS-induced NF-κB activation in RAW 264.7 cells. The cells were incubated for 1 h in the presence or absence of 10 ng/ml LPS in the presence or absence of EP (20 mM) or NAC (20 mM). Nuclear extracts were prepared and EMSA performed. The results shown in Panel A are representative of three separate experiments. Band densities (arbitrary units) for the gel depicted in Panel A were as follows: Lane 1, 80; Lane 2, 16; Lane 3, 40; Lane 4, 312; Lane 5, 10; Lane 6, 69. Panel B shows the results of a typical supershift assay wherein nuclear extracts were pre-incubated with 2 µL (2 µg/µL) of anti-p50, anti-p65 or anti-c-Rel antibodies (Santa Cruz Biotechnology; Santa Cruz, CA) for 1 h prior to the addition of radiolabeled probe.

Figure 6. Effects of EP and NAC on LPS-induced lipid peroxidation in RAW 264.7 cells. The cells were incubated for 1 h in the presence or absence of 10 ng/ml LPS in the presence or absence of graded concentrations of EP or NAC. Lipid peroxidation was assayed as described in the text. Results, expressed as nM malondialdehyde (MDA) per ml of cell lysate, are means ± SD (n=3 per condition). * indicates P < 0.05 versus cells incubated with LPS in the absence of EP or NAC (hatched bar); † indicates P < 0.05 versus cells incubated with LPS and the same concentration of NAC; ‡ indicates P <0.05 versus control cells (C) incubated in the absence of LPS (open bar).

Figure 7. Effects of EP and NAC on intracellular GSH and GSSG levels in RAW 264.7 cells. The cells were incubated for 24 h in the presence or absence of 10 ng/ml LPS in the presence or absence of graded concentrations of EP or NAC. Intracellular GSH and GSSG levels were assayed as described in the text. Results, expressed as nM malondialdehyde (MDA) per ml of cell lysate, are means ± SD (n=3 per condition). * indicates P < 0.05 versus cells incubated with LPS in the absence of EP or NAC (hatched bar); † indicates P < 0.05 versus cells incubated with LPS and the same concentration of NAC; ‡ indicates P <0.05 versus control cells (C) incubated in the absence of LPS (open bar).

Figure 8. Effects of EP pretreatment followed by GSH-Et pretreatment on LPS-induced NF-κB activation in RAW 264.7 cells. The cells were incubated for 1 h in the presence or absence of 10
ng/ml LPS after pretreatments as indicated in the figure and the text. Nuclear extracts were prepared and EMSA performed. The gel shown is representative of similar results obtained in three experiments. Band densities (arbitrary units) for the depicted gel were as follows: Condition 1, 14; Condition 2, 42; Condition 3, 224; Condition 4, 39; Condition 5, 291; Condition 6, 63.
### TABLE 1. Primers, annealing temperatures and cycle numbers for semi-quantitative PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence: sense (s)/antisense (as)</th>
<th>Fragment size (bp)</th>
<th>Annealing (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
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<td>iNOS</td>
<td>a: CACCACAAGGCCACATCGGATT</td>
<td>426</td>
<td>60</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>as: CCGACCTGATGTTGCCATTGTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
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<td>62</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>as: ATGCTTAGGCATAACGCACACTAGGT</td>
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<td></td>
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</tr>
<tr>
<td>IL-10</td>
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<td>237</td>
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<td>30</td>
</tr>
<tr>
<td></td>
<td>as: CTATGCAGTTGATGAAGATGTCAAAA</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>18S</td>
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<td>58</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>as: CGCCCGCTCCCCAAGATCCACTAC</td>
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TABLE 2. Effects of EP and NAC on LPS-induced iNOS, IL-6 and IL-10 mRNA expression in RAW 264.7 cells.

<table>
<thead>
<tr>
<th>LPS</th>
<th>EP (mM)</th>
<th>NAC (mM)</th>
<th>iNOS</th>
<th>IL-6</th>
<th>IL-10</th>
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<tbody>
<tr>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0.23±0.02</td>
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<td>5</td>
<td>0</td>
<td>0.38±0.02</td>
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</tr>
<tr>
<td>-</td>
<td>10</td>
<td>0</td>
<td>0.33±0.01</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>-</td>
<td>20</td>
<td>0</td>
<td>0.11±0.01</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
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<td>-</td>
<td>0</td>
<td>5</td>
<td>0.30±0.01</td>
<td>Undetectable</td>
<td>Undetectable</td>
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<tr>
<td>-</td>
<td>0</td>
<td>10</td>
<td>0.26±0.02</td>
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<tr>
<td>-</td>
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<td>20</td>
<td>0.23±0.01</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
<td>1.11±0.02</td>
<td>0.64±0.01</td>
<td>1.49±0.01</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>0</td>
<td>0.71±0.01</td>
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<td>1.33±0.05</td>
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<tr>
<td>+</td>
<td>10</td>
<td>0</td>
<td>0.56±0.01</td>
<td>0.39±0.01</td>
<td>0.72±0.03</td>
</tr>
<tr>
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<td>0</td>
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<td>0.13±0.01</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>5</td>
<td>1.05±0.01</td>
<td>0.67±0.02</td>
<td>1.32±0.03</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>10</td>
<td>0.97±0.04</td>
<td>0.67±0.07</td>
<td>1.25±0.07</td>
</tr>
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<td>+</td>
<td>0</td>
<td>20</td>
<td>0.97±0.05</td>
<td>0.69±0.08</td>
<td>0.99±0.05</td>
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</tbody>
</table>

Results are expressed as mean±SD relative band densities normalized to 18S RNA band densities.
TABLE 3. Effect of EP and GSH-Et on the release of NO and IL-6 by LPS-stimulated RAW 264.7 cells.

<table>
<thead>
<tr>
<th>Condition</th>
<th>1st pre-incubation (T=-75 to -15 min)</th>
<th>2nd pre-incubation (T=-15 to 0 min)</th>
<th>LPS (T=0 to 24 h)</th>
<th>Nitrite (µM)</th>
<th>IL-6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium</td>
<td>Medium</td>
<td>-</td>
<td>8.4±0.8*</td>
<td>1±1*</td>
</tr>
<tr>
<td>2</td>
<td>Medium</td>
<td>6 mM GSH-Et</td>
<td>-</td>
<td>8.1±0.1</td>
<td>35±5‡</td>
</tr>
<tr>
<td>3</td>
<td>Medium</td>
<td>Medium</td>
<td>+</td>
<td>83.4±4.6</td>
<td>5145±348</td>
</tr>
<tr>
<td>4</td>
<td>20 mM EP</td>
<td>Medium</td>
<td>+</td>
<td>25.9±1.1*</td>
<td>298±19*</td>
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<tr>
<td>5</td>
<td>Medium</td>
<td>6 mM GSH-Et</td>
<td>+</td>
<td>84.2±3.4</td>
<td>5325±314</td>
</tr>
<tr>
<td>6</td>
<td>20 mM EP</td>
<td>6 mM GSH-Et</td>
<td>+</td>
<td>34.6±1.1†*</td>
<td>343±11†*</td>
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</tbody>
</table>

RAW 264.7 cells growing in multiwell plates were incubated with 10 ng/ml *E. coli* LPS for 24 h. Results are means±SD (n=6 for all conditions except Condition 2; n=3 for Condition 2). * indicates p<0.05 versus Condition 3; † indicates p<0.05 versus Condition 4. ‡ indicates p<0.05 for the contrast, Condition 1 versus Condition 2.
<table>
<thead>
<tr>
<th>Condition</th>
<th>1st pre-incubation (T=-75 to -15 min)</th>
<th>2nd pre-incubation (T=-15 to 0 min)</th>
<th>LPS (T=0 to 24 h)</th>
<th>GSH (mmole/µg)</th>
<th>GSSG (mmole/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium</td>
<td>Medium</td>
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<td>3.3±0.3*</td>
<td>1.1±0.1</td>
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<tr>
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<td>Medium</td>
<td>6 mM GSH-Et</td>
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<td>4.2±0.2‡</td>
<td>1.2±0.1</td>
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<td>3</td>
<td>Medium</td>
<td>Medium</td>
<td>+</td>
<td>5.1±0.2</td>
<td>1.3±0.1</td>
</tr>
<tr>
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<tr>
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<td>5.4±0.8</td>
<td>1.4±0.3</td>
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<tr>
<td>6</td>
<td>20 mM EP</td>
<td>6 mM GSH-Et</td>
<td>+</td>
<td>3.0±0.1*†</td>
<td>1.0±0.1</td>
</tr>
</tbody>
</table>

RAW 264.7 cells growing in multiwell plates were incubated with 10 ng/ml *E. coli* LPS for 24 h. Results are means±SD (n=3 per condition). * indicates p<0.05 versus Condition 3; † indicates p<0.05 versus Condition 4. ‡ indicates p<0.05 for the contrast, Condition 1 versus Condition 2.
Figure 1

Graph showing the concentration of [NO₂⁻] (µM) in response to different concentrations of EP and NAC.

- **EP (mM):**
  - 0 mM (control) vs. 5 mM, 10 mM, and 20 mM.
  - The concentration of [NO₂⁻] increases with EP concentration.
  - Asterisks (*) indicate statistically significant differences.

- **NAC (mM):**
  - 0 mM (control) vs. 5 mM, 10 mM, and 20 mM.
  - The concentration of [NO₂⁻] decreases with NAC concentration.
  - Asterisks (*) indicate statistically significant differences.

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Figure 2

[IL-6] (pg/ml) vs. EP (mM)

[IL-6] (pg/ml) vs. NAC (mM)

* indicates statistically significant difference.
Figure 3

[IL-10] (pg/ml) vs EP (mM)

[IL-10] (pg/ml) vs NAC (mM)

* indicates significant difference from control.
Figure 4

<table>
<thead>
<tr>
<th></th>
<th>iNOS</th>
<th>IL-6</th>
<th>IL-10</th>
<th>18S</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EP</td>
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<td>10</td>
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<tr>
<td>NAC</td>
<td>-</td>
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</table>

The figure shows gel electrophoresis results for iNOS, IL-6, IL-10, and 18S with LPS, EP, and NAC treatments.
Figure 5

(A) NF-κB

<table>
<thead>
<tr>
<th></th>
<th>LPS</th>
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<th>NAC</th>
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<tbody>
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<tr>
<td>P50</td>
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<td>-</td>
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</tr>
<tr>
<td>P65</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>c-Rel</td>
<td>+</td>
<td>+</td>
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</table>

(B) NF-κB

CTRL
P50
P65
+c-Rel
Figure 6

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Figure 7

Concentration of EP or NAC (in mM) added to LPS

GSH (nmole/µg)

C  LPS  5  10  20

EP  NAC

Concentration of EP or NAC (in mM) added to LPS

GSSG (nmole/µg)

C  LPS  5  10  20

EP  NAC

*† *† *†

Figure 7
Figure 8

<table>
<thead>
<tr>
<th>Condition</th>
<th>1st pre-incubation (T=-75 to -15 min)</th>
<th>2nd pre-incubation (T=-15 to 0 min)</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
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</tr>
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<td>Medium</td>
<td>Medium</td>
<td>+</td>
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<td>4</td>
<td>20 mM EP</td>
<td>Medium</td>
<td>+</td>
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<tr>
<td>5</td>
<td>Medium</td>
<td>6 mM GSH-Et</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>20 mM EP</td>
<td>6 mM GSH-Et</td>
<td>+</td>
</tr>
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</table>