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Commentary

The biochemical basis for the anti-inflammatory and cytoprotective actions of ethyl pyruvate and related compounds

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ABSTRACT

Pyruvate is an important metabolic intermediate, and also is an effective scavenger of hydrogen peroxide and other reactive oxygen species (ROS). Pharmacological administration of pyruvate has been shown to improve organ function in animal models of oxidant-mediated cellular injury. However, pyruvate is relatively unstable in aqueous solutions, which could limit the therapeutic potential of this compound. Ethyl pyruvate (EP), a simple derivative of pyruvic acid, is also an ROS scavenger, but seems to exert pharmacological effects, such as suppression of inflammation, which are at least quantitatively different and in some instances are qualitatively distinct from those exerted by pyruvate anion. Treatment with EP has been shown to improve survival and/or ameliorate organ dysfunction in a wide variety of pre-clinical models of acute illnesses, such as severe sepsis, acute pancreatitis and stroke. Using other animal models, some studies have demonstrated that more prolonged treatment with EP can ameliorate inflammatory bowel disease or slow the rate of growth of malignant tumors. In a clinical trial of patients undergoing cardiac surgery, treatment with EP was shown to be safe, but it failed to improve outcome. The true therapeutic potential of EP and related compounds remains to be elucidated. In this review, some of the biochemical mechanisms, which might be responsible for the pharmacological effects of EP, are discussed.

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1. Pyruvate

Pyruvate (CH_3COCOO^-), the anionic form of a simple alpha-keto acid, plays a key role in intermediary metabolism as a product of glycolysis and the starting substrate for the tricarboxylic acid (TCA) cycle. In the reaction catalyzed by the enzyme, lactate dehydrogenase, pyruvate is reduced by nicotinamide adenine dinucleotide (NADH) to form lactate and the oxidized form of the co-factor (NAD^+). Under aerobic conditions, pyruvate enters into mitochondria, where it undergoes oxidative decarboxylation in a reaction catalyzed by the enzyme complex, pyruvate dehydrogenase, to form acetyl coenzyme A and carbon dioxide.

Although it is an important metabolic intermediate, pyruvate is also an important endogenous scavenger of certain reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2). Pyruvate scavenges H_2O_2 by virtue of a nonenzymatic oxidative decarboxylation reaction, which was first described by Holleman in 1904 [1].

H_2O_2 is a highly reactive compound, which is capable of oxidizing and thereby damaging numerous cellular constituents, including lipids, proteins, and nucleic acids. H_2O_2, which is generated by the partial reduction of molecular oxygen, is produced in cells directly or after secondary reactions as a result of the leakage of electrons from the electron transport chain in mitochondria or as a consequence of a variety of other enzymatic reactions, including those catalyzed by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase. Scavenging of H_2O_2 by endogenously generated pyruvate is probably a key cellular defense against oxidative stress, particularly in proliferating cells [2,3].

In addition to H_2O_2, other important ROS in biological systems include superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^\bullet$), and peroxynitrite ($ONOO^-$) [4]. Although $O_2^{\bullet -}$ is only moderately reactive, it can under go a one-electron reduction to form H_2O_2 or react with nitric oxide (NO) to form the potent oxidizing and nitrosating agent, $ONOO^-$ [5]. Additionally, in the presence of certain transition metal cations, $O_2^{\bullet -}$ and H_2O_2 (or hypochlorous acid, another biologically important oxidant) can interact, yielding the extremely reactive free radical, $OH^\bullet$ [6]. Evidence has been presented to support the view that pyruvate is not only capable of scavenging H_2O_2, but also $OH^\bullet$ [7] and peroxynitrite [8].

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In view of its ability to scavenge ROS, pyruvate has been studied extensively as a cytoprotective agent. In 1985, Andrae et al. demonstrated that pyruvate and some other related longer chain alpha-ketoacids are capable of protecting cultured cells against the lethal effects of H_2O_2 [9] Subsequently, Salahudeen et al. showed that therapeutic administration of pyruvate protected rodents against H_2O_2-induced renal injury [10]. Since ROS are implicated in the pathogenesis of organ damage due to shock or ischemia/reperfusion (I/R) injury [11], it is not surprising that many laboratories have investigated the therapeutic potential of pyruvate in these conditions. Indeed, the number of publications, which have reported results from work along these lines, is too extensive to provide a comprehensive list here. Some especially notable papers are those showing that pharmacological doses of pyruvate ameliorated tissue injury and/or improved survival in animal models of hemorrhagic shock [12–14] as well as myocardial [15,16], intestinal [17] or hepatic [18] I/R injury.

Despite pyruvate's efficacy as a scavenger of ROS, its use as a therapeutic agent might be limited due to its poor stability in solution. In aqueous solutions, pyruvate spontaneously undergoes an aldol-type condensation reaction to yield parapyruvate (4-hydroxy-4-methyl-2-ketoglutaric acid) [19,20]. This compound, in turn, can undergo spontaneous cyclization and dehydration to form a lactone, which exists in aqueous solutions as ketone and enol tautomers [19]. Parapyruvate has the potential to be a metabolic poison, since it inhibits the TCA cycle by blocking the activity of the key enzyme, 2-ketoglutaric acid dehydrogenase [19,20]. It is not known whether inhibition of mitochondrial function by parapyruvate (and related compounds) is anything more than an *in vitro* phenomenon. At a minimum, however, the possibility of parapyruvate-induced mitochondrial dysfunction should be a cause for concern with regard to developing pyruvate as therapeutic agent.

2. Ethyl pyruvate

In an effort to develop a more stable aqueous form of pyruvate, Sims et al. studied the biological effects of a closely related compound, ethyl pyruvate (EP), using an intestinal I/R model in rats [21]. Whereas pyruvate is the anionic form of a monocarboxylic acid, EP is the ester formed by the condensation of pyruvic acid with the 2-carbon alcohol, ethanol. Sims et al., believing that EP would be more stable in aqueous solution than pyruvate, subjected rats to 60 min of intestinal ischemia followed by 60 min of reperfusion, and randomized the animals to receive either Ringer's lactate solution (lactate concentration = 28 mM), a 28 mM solution of sodium pyruvate dissolved in a Ringer's-like solution of sodium chloride, potassium chloride and calcium chloride, or a 28 mM solution of EP in the same Ringer's-type formulation. Although intestinal mucosal injury was ameliorated by both sodium pyruvate and EP, an equimolar dose of the ester was significantly more protective than pyruvate anion.

The positive results with EP obtained by Sims et al. were predated by results from two earlier studies, which were carried out by Varma and colleagues. In the first of these studies, both EP and pyruvate protected rodent lens tissue from oxidative damage in culture, but EP was more effective in this regard than the parent carboxylate anion [22]. In the second of these studies, the same laboratory showed that topical application of a 5% solution of EP delayed lens cataract formation in young rats fed a high galactose diet [23].

Although it was postulated that EP would be more stable than pyruvate in an aqueous solvent, subsequent (unpublished) observations suggest that EP, when it is dissolved in water, gradually undergoes spontaneous hydrolysis to form pyruvic acid and ethanol and also gradually undergoes other reactions, including hydration (to form the *gem* diol, ethyl-2,2-dihydroxypropanoate) as well as the ethyl ester analogue of parapyruvate. Accordingly, in many subsequent (pre-clinical and clinical) studies, EP solutions have been prepared just prior to administration to experimental animals or human subjects.

3. EP: a pluripotent pharmacological agent

Since the publication of the paper by Sims et al. in 2001 [21], EP has been the subject of more than 150 peer-reviewed papers. As summarized in Table 1, the pharmacological effects of EP are quite diverse, and include: down-regulation of the secretion of pro-inflammatory cytokines; enhanced anti-tumor immunity; amelioration of redox-mediated damage to cells and tissues; inhibition of apoptosis (under some circumstances) or promotion of apoptosis (under other circumstances); and support of cellular ATP synthesis. These pharmacological actions of EP are discussed in greater detail in the remainder of this review.

4. Anti-inflammatory effects of EP

Results from studies published over the past 8 years provide robust support for the notion that EP is an effective anti-inflammatory agent. Preliminary evidence for the anti-inflammatory effects of EP came from studies carried out by Yang et al. [24] and Venkataraman et al. [25]. Using a murine model of hemorrhagic shock, Yang et al. demonstrated that resuscitation with a solution containing EP down-regulated activation of the pro-inflammatory transcription factor, NF-κB, as well as the expression of several pro-inflammatory proteins, such as TNF, IL-6,

Table 1
Summary of the pharmacological effects of ethyl pyruvate.

Pharmacological effect	Proposed mechanism(s)
Inhibition of redox-mediated cellular damage	Nonenzymatic scavenging of H_2O_2 Scavenging of other reactive intermediates (e.g., $ONOO^-$)
Inhibition of inflammation	Decreased NF-κB-dependent signaling due to decreased intracellular GSH concentration Decreased NF-κB-dependent signaling due to covalent modification of p65 Decreased JAK/STAT-dependent signaling due to scavenging of ROS Inhibition of the enzyme, glyoxalase-1, leading to increased intracellular levels of methylglyoxal
Cytoprotection	Augmented ATP production Scavenging of ROS
Inhibition of apoptosis	Scavenging of ROS Inhibition of up-regulated expression of pro-apoptotic proteins
Inhibition of cancer cell growth	Improved anti-neoplastic immunity due to down-regulated expression of the enzyme, indoleamine 2,3-dioxygenase Promotion of cancer cell apoptosis

cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS), in liver and intestinal mucosa. Using a rat model of shock induced by injection of *Escherichia coli* lipopolysaccharide (LPS), Venkataraman and co-workers showed that resuscitation with a solution containing EP decreased circulating levels of IL-6 and nitrite/nitrate (products of nitric oxide metabolism) and increased circulating levels of the counter-regulatory cytokine, IL-10.

These findings were substantially extended by Ulloa et al. [26], who made a number of key observations. First, these authors showed that LPS-induced secretion of the pro-inflammatory "alarm-phase" cytokine, tumor necrosis factor (TNF), was inhibited in a concentration-dependent fashion when RAW 264.7 murine macrophage-like cells were incubated with graded concentrations of EP. Second, they demonstrated that treatment with EP improved survival in mice challenged with a lethal dose of LPS or rendered septic by cecal ligation and perforation (CLP). Importantly, the therapeutic benefit of EP was evident in the CLP model of polymicrobial sepsis even though the compound was injected 24 h after the onset of infection. Third, these authors showed that EP inhibited secretion of the pro-inflammatory DNA-binding protein, high mobility group box 1 (HMGB1), from LPS-stimulated RAW 264.7 cells and decreased circulating levels of HMGB1 in septic mice. Finally, Ulloa et al. reported that EP inhibited activation of two key pro-inflammatory signaling pathways, namely those involving NF-κB and p38 mitogen-activated protein kinase (MAPK), when RAW 264.7 cells are incubated with LPS.

Numerous other studies have confirmed and extended these early findings. For example, Johansson et al. showed that EP inhibited the adhesion of human neutrophils to LPS-, TNF- or IL-1β-stimulated human umbilical vein endothelial cells (HUVECs) [27]. Treatment with EP also down-regulated the expression of key adhesion molecules, such as ICAM-1, E-selectin, and VCAM-1, on the surface of immunostimulated HUVECs. In addition, EP inhibited the secretion of IL-8 and G-CSF by HUVECs stimulated with LPS or IL-1β. More recently, Dong et al. [28] and Cai et al. [29] showed that resuscitation with an EP-containing colloid solution inhibited TNF production in pigs or rats subjected to hemorrhagic shock.

Further evidence for the anti-inflammatory properties of EP is provided by the results from a series of experiments carried out by Davé and co-workers, who sought to determine whether treatment with EP could ameliorate the severity of colitis in IL-10$^{-/-}$ mice, a murine model of inflammatory bowel disease. In these studies, 10-week-old IL-10$^{-/-}$ mice with established colitis were randomized to receive either EP (40 mg/kg in 500 μL of Ringer's lactate solution) or a similar volume of vehicle by intraperitoneal injection every other day for 2 weeks. Treatment with EP significantly ameliorated the severity of colitis as evidenced by improved histology scores and decreased colonic wall thickening. Of note, fecal HMGB1 levels were increased in IL-10$^{-/-}$ mice as compared to wild-type controls. Treatment with EP significantly decreased the content of HMGB1 in fecal material from IL-10$^{-/-}$ mice.

In addition to the reports by Ulloa et al. [26] and Davé et al. [30] already cited, many other studies have shown that EP effectively inhibits secretion of the pro-inflammatory DNA-binding protein, HMGB1. For illustrative purposes, three such studies will be mentioned here. In a study of experimental severe acute pancreatitis (SAP) induced by the retrograde injection of artificial bile into the pancreatic duct of rats, circulating levels of HMGB1 were significantly higher in animals with SAP than in controls at 24 and 48 h after induction of the disease [31]. Delayed treatment with EP, starting 12 h after the injection of artificial bile, significantly decreased serum HMGB1 levels in rats with SAP. In a study of pulmonary fibrosis induced in C57Bl/6 mice by the intratracheal administration of bleomycin, levels of HMGB1 in broncho-alveolar lavage fluid (BALF) were significantly greater at 14 days after bleomycin instillation than after instillation of vehicle [32]. Treatment with intraperitoneal EP (40 mg/kg per day from days 3 through 13 after bleomycin instillation) significantly decreased the concentration of HMGB1 in BALF on day 14 after bleomycin challenge. In a study of rats subjected to a 30% full-thickness cutaneous scald injury, serum was collected and regulatory T cells (Tregs) were isolated from the spleens of burned and sham-burned animals [33]. Circulating HMGB1 levels were elevated on days 1, 3, 5 and 7 after the burn trauma. Furthermore, in comparison to Tregs from sham-burned animals, Tregs from burned rats expressed higher levels of CTLA-4 and Foxp3 and produced more IL-10, an immunosuppressive cytokine, in tissue culture. All of these changes were prevented when the burned animals were treated with EP at 6, 12, 24, 36 and 48 h after the burn injury.

5. Mechanisms responsible for the anti-inflammatory effects of EP

Although it is firmly established that EP is an effective anti-inflammatory agent, the biochemical mechanisms responsible for this effect remain to be established with confidence. A number of possibilities have been suggested. These potential mechanisms are not mutually exclusive, and it seems plausible that the basis for the anti-inflammatory properties of EP is multifactorial. Four hypotheses to account for the anti-inflammatory effects of EP will be reviewed here.

The first hypothesis is based on the recognition that all of the members of the NF-κB family of proteins share a characteristic motif, consisting of one cysteine and three arginine residues in a RxxRxRxxC pattern in the DNA-binding region [34,35]. The sulfhydryl group of this critical cysteine residue (Cys^{62} in the NF-κB protein, Rel A or p65) is essential for DNA-binding and transcription-activating activity. Because of the three positively charged arginine residues nearby, this cysteine residue is very susceptible to oxidation (e.g., mixed disulfide formation) [36]. 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, which 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 [35,37–40].

Song et al. investigated the hypothesis that EP modifies NF-κB-dependent pro-inflammatory signaling by modifying intracellular reduced GSH levels [41]. In their studies, stimulation of RAW 264.7 murine macrophage-like cells with LPS significantly increased intracellular levels of GSH relative to those observed in unstimulated cells, but this effect was prevented by co-incubation of the cells with EP. Incubation of the cells with graded concentrations of EP inhibited LPS-induced DNA binding by NF-κB, but, if the cells were co-incubated with the cell-permeable GSH analogue, glutathione ethyl ester, EP-mediated inhibition of LPS-induced NF-κB activation was prevented. These data support the notion that EP may inhibit signaling by NF-κB, in part, by changing intracellular redox balance in a way that favors oxidation (mixed disulfide formation) at a key cysteine residue in one of subunits of the pro-inflammatory transcription factor.

In order to understand the evidence supporting the second hypothesis, it is necessary to briefly review our current understanding of the canonical pathway for NF-κB activation. In resting cells, the homo- or heterodimeric forms of NF-κB exist in the cytoplasm in an inactive form due to binding by a third inhibitory protein, called IκB [42,43]. Five IκB-like proteins have been identified, IκBα, IκBβ, IκBε, IκBζ and Bcl-3 [42,44]. Upon stimulation of the cell by a pro-inflammatory trigger – for

example, cytokines like TNF or IL-1β or the bacterial product, LPS – IκB is phosphorylated, which targets the molecule for ubiquitination and subsequent proteasomal degradation. Phosphorylation of IκB is mediated by an enzyme complex called IκB kinase [45]. Phosphorylation, release and degradation of IκB permits translocation of the transcriptionally competent form of NF-κB into the nucleus where it can bind to *cis*-acting elements in the promoter regions of various NF-κB-responsive genes.

In studies using LPS-stimulated RAW 264.7 cells as a model system, Han et al. showed that EP inhibited NF-κB-mediated signaling in a concentration-dependent fashion [46]. Under control conditions, stimulation of RAW 264.7 cells resulted in rapid disappearance of IκBα and IκBβ, as expected. When the cells were co-incubated with EP, LPS-triggered disappearance of IκBα and IκBβ was not affected. These observations suggest that EP-mediated inhibition of NF-κB-mediated signaling occurs downstream of the proximal steps in the canonical NF-κB activation pathway. In this regard, EP seems to be similar to some other NF-κB inhibitors, which interfere with DNA-binding by the transcription factor as a result of oxidation or alkylation of key sulfhydryl residues in the protein [47–50]. Although Han et al. did not obtain direct evidence that EP is capable of covalently modifying the transcriptionally active form of NF-κB, these investigators showed that EP inhibited DNA binding by wild-type p65 homodimers, which were over-expressed in transiently transfected human embryonic kidney 293 cells. However, EP failed to inhibit the DNA-binding activity of homodimers of an over-expressed mutant form of a p65 with substitution of serine for cysteine at position 38 in the protein. Taken together, these results support the hypothesis that EP inhibits DNA-binding by covalently modifying the NF-κB subunit, p65, at Cys^{38}.

Whereas the first and second hypotheses propose that EP modulates pro-inflammatory signaling by promoting the oxidation of at least one key sulfhydryl residue in an NF-κB subunit, the third hypothesis is based on a seemingly opposite concept. Specifically, the third hypothesis proposes that EP acts as an anti-oxidant, and, on this basis, inhibits pro-inflammatory signaling. This hypothesis is supported by data, which verify that EP is an anti-oxidant and H_2O_2 scavenger. For example, Tawadrous et al. showed that treatment with EP ameliorated hepatic malondialdehyde formation, a marker for oxidant-induced lipid peroxidation, in rats subjected to hemorrhagic shock and resuscitation [51]. Using various *in vivo* animal models of oxidative stress, other investigators have confirmed that treatment with EP ameliorates or abrogates injury-induced lipid peroxidation [52,53]. In another important study, Fedeli et al. used a luminal chemiluminescence assay to provide direct evidence that EP is an effective H_2O_2 and $O_2^{\bullet -}$ scavenger [54]. Indeed, in these studies, EP was shown to be more potent than pyruvate anion as an H_2O_2 scavenger and more effective than pyruvate anion as an $O_2^{\bullet -}$ scavenger. Wang et al. also provided direct evidence that EP is an effective H_2O_2 scavenger [55]. Chen et al. showed that EP is not an effective $OH^{\bullet}$ scavenger, but prevents the formation of $OH^{\bullet}$ from $ONOO^-$ [56].

Intracellular signaling, which is mediated by various Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins, is important in the activation of many immune and inflammatory responses [57]. Prompted by the recognition that ROS generated by NADPH oxidase can initiate activation of the JAK/STAT signaling pathways, Kim et al. carried out an extensive series of *in vitro* and *in vivo* studies, which were designed to test the hypothesis that the anti-inflammatory effects of EP are due to scavenging of ROS, leading to inhibition of JAK/STAT signaling [58]. Using BV2 murine microglia-like cells, these authors showed that incubation with graded concentrations of EP inhibited LPS-induced expression of several JAK/STAT-responsive genes, including iNOS, COX-2, IL-1β, IL-6 and TNF, in a dose-dependent fashion. Using the same reductionist model system, Kim and co-workers also demonstrated that EP inhibited LPS-induced tyrosine phosphorylation of the STAT proteins, STAT1 and STAT3, as well as phosphorylation of the upstream signaling protein, JAK2. Additionally, these investigators showed that EP as well two other extensively studied ROS scavengers, namely N-acetylcysteine (NAC) and ascorbic acid (vitamin C), inhibited ROS-dependent fluorescence in LPS-stimulated BV2 cells loaded with ROS-responsive probe, 2′,7′-dichlorodihydrofluorescein diacetate. Finally, Kim et al. reported that EP, NAC and ascorbic acid all inhibited LPS-induced STAT activation as well as iNOS and COX-2 expression, although EP was at least 3-fold more potent than the other two ROS scavengers. Collectively, these findings support the contention that at least some of the anti-inflammatory effects of EP are related to its ability to scavenge H_2O_2 and/or other ROS.

The fourth and perhaps most novel hypothesis to explain the anti-inflammatory effects of EP was proposed by Hollenbach et al., who proposed a mechanism involving the enzyme, glyoxalase (Glo)-1 [59]. Localized to the cytosol of cells, Glo-1 is responsible for detoxification of the glycolytic byproduct, methylglyoxal (MGO) as well as other highly reactive α-oxoaldehydes. Using heparinized human whole blood as an assay system, Hollenbach and co-workers showed that addition of graded concentrations of exogenous MGO down-regulated LPS-induced production of several cytokines, including TNF, IL-6 and IL-1β. In view of this observation, one would predict that an agent, which was capable of increasing intracellular levels of MGO, would similarly down-regulate LPS-triggered production of these cytokines. Compounds, which are capable of inhibiting the activity of Glo-1, would be expected to increase intracellular MGO levels. It is noteworthy, therefore, that p-bromobenzylglutathione cyclopentyl diester (BGCD), a pro-drug form of a known Glo-1 inhibitor, potently inhibited LPS-induced TNF, IL-6 and IL-1β, in anticoagulated human whole blood. As expected, graded concentrations of EP similarly inhibited secretion of these cytokines in LPS-stimulated human whole blood cultures. Remarkably, however, EP inhibited Glo-1 activity in a cell-free system, an observation, which suggests that inhibition of Glo-1, leading to intracellular accumulation of MGO (or related electrophiles), might account for the anti-inflammatory effects of EP.

Although most studies of the immunomodulatory effects of EP have focused on the compound's anti-inflammatory effects, recently published findings suggest that EP also can *enhance* T cell mediated anti-neoplastic immunity, at least in experimental models of cancer. Muller et al. showed that treatment with EP significantly inhibited tumor outgrowth in syngeneic mice challenged with $Bin1^{-/-}$ MR KEC cells or B16-F10 melanoma cells [60]. This effect of EP was associated with decreased expression of indoleamine 2,3-dioxygenase (IDO), an NF-κB-responsive inducible enzyme. Increased expression of IDO has been associated with a less favorable prognosis in various forms of human cancer, whereas, in various animal models of cancer, systemic blockade of IDO activity with small-molecule inhibitors suppressed the outgrowth of tumors. In the studies by Muller et al., EP failed to affect tumor outgrowth in congenitally athymic, T cell-deficient mice, and also failed to affect tumor outgrowth in IDO knock-out mice. Collectively, these data support the view that EP can enhance T cell-dependent immunity against malignant cells by inhibiting NF-κB-mediated upregulation of the enzyme, IDO.

6. Comparison of the pharmacological effects of pyruvate anion with those of EP or other pyruvate esters

Sims et al. evaluated EP because the ester might be more stable in aqueous solution than the parent carboxylate anion [21]. However, it is now clear that the pharmacological actions of certain

pyruvate esters, such as EP and methyl pyruvate (MP), are at least quantitatively different (and in some cases may be qualitatively distinct) from those produced by pyruvate anion. This notion was suggested by the original work carried by Sims et al., wherein the preservation of normal ileal mucosal histology was much more impressive when rats subjected to mesenteric I/R were treated with EP rather than an molar equivalent dose of pyruvate [21]. Similarly, in another early study, which compared the pharmacological effects of pyruvate and EP, the ester was more effective than the anion for ameliorating oxidant-induced damage to the lens [22]. The greater efficacy of EP as compared to pyruvate was attributed to greater penetration by the ester through the lens [23].

In certain disease models, such as rodents or pigs subjected to hemorrhagic shock, treatment with either pyruvate [13,14,61,62] or EP [24,28,29,51,63,64] improved survival and/or ameliorated organ damage. In these studies of hemorrhagic shock, the total dose of sodium pyruvate administered has ranged from about 18 mmol/kg [14] to as much as 120 mmol/kg [13]. In contrast, the total dose of EP administered has ranged from about 1.2 mmol/kg [24] to 2.5 mmol/kg [64]. These data suggest that in models of hemorrhagic shock, the effects of pyruvate and EP are qualitatively similar, but EP is about 10-fold to 100-fold more potent.

The notion that pyruvate and EP exert similar pharmacological effects albeit at different doses tends to be supported by a very recent study, which was a head-to-head comparison of sodium pyruvate and EP in a rat model of hemorrhagic shock [12]. Sodium pyruvate was administered as a hypertonic solution and the total dose delivered was 10 mmol/kg. EP was administered as a nearly isotonic solution in a Ringer's-type formulation, and the total dose delivered was approximately 0.9 mmol/kg (i.e., about 10-fold less than the sodium pyruvate dose). Treatment with either sodium pyruvate or EP ameliorated biochemical evidence of hepatocellular damage, and both compounds down-regulated the expression of various pro-inflammatory proteins in the liver. In these studies, hypertonic sodium pyruvate solution was somewhat more efficacious than isotonic EP solution, but interpretation of the comparative efficacy of the two compounds is confounded by the large difference in administered dose.

Recently, Zeng et al. assessed the degree of neuronal protection afforded when neonatal rat brain slices are subjected to an oxidant stress (incubation with 2 mM H_2O_2 for 1 h) and then "rescued" with 20 mM pyruvate or 20 mM EP in artificial cerebrospinal fluid [65]. The primary read-out was the ratio of intracellular adenosine diphosphate (ADP) concentration divided by intracellular adenosine triphosphate (ATP) concentration as assessed by ^{31}P nuclear magnetic resonance spectroscopy. By this measure, treatment with pyruvate actually was worse than no treatment at all, whereas treatment with EP was protective. EP provided almost complete protection against H_2O_2-induced apoptosis, whereas pyruvate at the same concentration provided no protection at all.

In many studies, particularly those focusing on anti-inflammatory effects, the pharmacological actions of EP have been shown to be qualitatively different from those of pyruvate anion. For example, Sappington et al. showed that incubating cultured Caco-2 transformed human intestinal epithelial cells with a cocktail of pro-inflammatory cytokines increased the permeability of monolayers to fluorescein-labeled dextran, but this effect was blocked in concentration-dependent fashion by EP [66]. Similar concentrations of pyruvate had no effect on cytokine-induced epithelial hyperpermeability in this reductionist model system.

In another relevant study by Johansson et al., which was cited already above, EP (at a final concentration of 10 mM) markedly inhibited the adhesion of human neutrophils to LPS-, TNF- or IL-1β-stimulated HUVECs and significantly down-regulated the expression of key adhesion molecules, such as ICAM-1, E-selectin, and VCAM-1, on the surface of immunostimulated HUVECs [27]. In these experiments, 10 mM sodium pyruvate (i.e., the same final concentration) had no effect on any of the measured parameters. The investigators recognized that hydrolysis of EP yields one molecule of ethanol for every molecule of pyruvate formed. Accordingly, they also evaluated the effects of simultaneously adding equimolar concentrations of sodium pyruvate and ethanol, and observed no effects of the combination of agents on cytokine-induced adhesion of neutrophils or expression of adhesion molecules by HUVECs.

In a follow-up study from the same group, 10 mM EP significantly inhibited the adhesion of neutrophils to A549 human transformed pulmonary epithelial cells activated by exposure to IL-1β or TNF [67]. Incubation of cytokine-activated A549 cells with 10 mM sodium pyruvate failed to block adhesion of neutrophils; in other words, the pharmacological effects of EP and sodium pyruvate were qualitatively different. Similarly, 10 mM EP significantly blocked the expression of adhesion molecules, such as ICAM-1, on cytokine-activated A549 cells, whereas the same concentration of sodium pyruvate was without effect. An equimolar mixture of sodium pyruvate and ethanol, the compounds produced when EP undergoes hydrolysis, also failed to block adhesion molecule expression on A549 cells stimulated with IL-1β or TNF.

Similar and closely related results were obtained in a very recent study by Mizutani et al. [68]. These authors evaluated the effects of 5 mM EP, 5 mM pyruvate, 5 mM ethanol or a mixture of 5 mM pyruvate and 5 mM ethanol on TNF-induced NF-κB activation in cultured A549 human alveolar epithelial cells [68]. Whereas EP significantly inhibited activation of the NF-κB signaling pathway (as assessed via several different read-outs), this effect was not observed with equimolar concentrations of pyruvate, ethanol or pyruvate plus ethanol.

In another pyruvate *versus* EP study, Kim et al. reported that 5, 10 or 20 mM EP protected primary rat microglial cultures from loss of viability induced by incubation with LPS or H_2O_2 [69]. In contrast, pyruvate, when tested at the same concentrations, failed to exert a protective effect on microglial cells exposed to LPS. When microglial cells were incubated with 200 μM H_2O_2, 5 mM pyruvate and 5 mM EP were similarly and significantly protective. However, higher concentrations of EP were more protective, whereas the protective benefit of pyruvate was lost when concentrations greater than 5 mM were evaluated. EP dose-dependently inhibited nitric oxide production by LPS-stimulated microglial cells, but equivalent concentrations of pyruvate were without effect.

Kim et al. also compared the pharmacological effects of EP and pyruvate *in vivo* in a model of transient cerebral I/R induced by transient occlusion of the middle cerebral artery (MCA) in rats for 1 h [69]. In one experiment, sodium pyruvate (250, 500 or 1000 mg/kg) was administered by intraperitoneal injection 30 min after the induction of cerebral ischemia. Infarct volumes were assessed 2 days after reperfusion. Interestingly, the dose–response relationship for pyruvate was U-shaped; whereas, the 500 mg/kg dose significantly reduced infarct volume, neither the 250 mg/kg dose nor the 1000 mg/kg dose decreased infarct volume. If administration of sodium pyruvate (500 mg/kg) was delayed until 4 or 12 h after reperfusion, there was no effect on infarct volume. However, delayed administration of a much lower dose of EP (40 mg/kg) at 4 or 12 h after reperfusion still provided significant neuroprotection against focal cerebral ischemia.

Another recent study evaluated the pharmacological effects of equimolar doses of the sodium salts of three different α-keto carboxylic acids or EP in rats subjected to 50 min of multivisceral ischemia (induced by cross-clamping of the proximal abdominal aorta) followed by 60 min of reperfusion [70]. The test compounds were sodium pyruvate, sodium benzoylformate, sodium 4-hydroxyphenylpyruvate, and EP, and the total dose of each compound

was 0.86 mmol/kg administered over 20 min by intravenous infusion starting 2 min before the start of reperfusion. Controls were subjected to multivisceral I/R, but received only an equivalent volume of Ringer's lactate solution (RLS). Although all four compounds significantly ameliorated hepatic lipid peroxidation to a similar extent, only EP and sodium benzoylformate significantly decreased circulating TNF levels (relative to RLS-treated controls). Similarly, only EP and sodium benzoylformate significantly ameliorated the development of intestinal mucosal hyperpermeability. Benzoylformate, which is not an ester but rather the anionic form of an α-carboxylic acid, is considerably more lipophilic than pyruvate. EP, being an ester, is also much more lipophilic than pyruvate. Thus, as will be discussed further below, the pharmacological differences between pyruvate and pyruvate esters, such as EP or MP, likely relates to differences in their tendencies to permeate membranes rather than the presence or absence of the ester linkage *per se*.

Pharmacological differences between pyruvate anion and pyruvate esters have been noted previously in a study of the medicinal chemistry of hypoglycemic agents and the biology of insulin release from pancreatic β cells. Siggins et al. reported that some α-keto acid esters of 2-chloroethanol possess hypoglycemic activity in glucose-challenged fasted rats [71]. Whereas pyruvic acid was pharmacologically inactive, 2-chloroethyl pyruvate effectively decreased blood glucose concentration, being more potent than the well-studied oral hypoglycemic agent, tolbutamide. Along related lines, Mertz et al. reported that insulin secretion by cultured mouse pancreatic islet cells was stimulated by adding MP but not sodium pyruvate to the culture medium [72]. These investigators further showed that MP, but not sodium pyruvate, caused closure of ATP-sensitive potassium channels, triggered a sustained rise in intracellular calcium ion concentration, and increased insulin secretion more effectively than glucose. Zawalich and Zawalich subsequently showed that MP, but not sodium pyruvate, is a potent insulin secretagogue in freshly isolated rat pancreatic islet cells [73]. The authors of this study speculated that esterification renders pyruvate more "membrane-permeable" and thereby allows higher levels of the compound to accumulate in mitochondria. This notion is supported by data reported by Malaisse et al., who showed that MP caused less lactate production than sodium pyruvate in pancreatic islets, a finding that is consistent with decreased cytoplasmic metabolism and increased mitochondrial metabolism by the ester [74]. Furthermore, Rocheleau et al. reported that the addition of MP (instead of sodium pyruvate) caused a larger and more sustained increase in the cellular content of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenenine dinucleotide phosphate (NADPH) in islet cells [75]. The NADH/NADPH response also occurred faster when islet cells were incubated with MP instead of glucose or sodium pyruvate. These data are consistent with the view that the pyruvate ester stimulates mitochondrial production of both NADPH and NADH. Thus, the differential permeability hypothesis seems likely to be right, at least with respect to the effect of pyruvate esters on insulin secretion. EP probably penetrates through biological membranes more readily than pyruvate because it is more lipophilic. As already noted previously, another more lipophilic derivative of pyruvic acid, benzoylformate, is the conjugate anion of a carboxylic acid (i.e., benzoylformate is not an ester), but nevertheless demonstrated pharmacological properties similar to those of EP in a rodent model of I/R injury [70]. Support for the notion that EP readily permeates biological membranes is provided by a recent publication authored by Hegde et al., who showed that topically applied EP penetrated the cornea of the eye, leading to accumulation of pyruvate or EP (the assay method was not capable of distinguishing between the two compounds) in the aqueous humor [76].

7. Modulation of apoptosis by EP

Depending upon the model system under study, EP has been shown to decrease or increase the incidence of apoptosis. For example, in studies using a rodent model of hepatic I/R injury, treatment with EP down-regulated hepatocellular apoptosis as assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining [77]. Similarly, in a study, which utilized PC12 rat pheochromocytoma cells a reductionist *in vitro* model system, EP (0.1–10 mM) inhibited cell death and DNA fragmentation (an indicator of apoptosis) induced by adding 1 mM dopamine to the cultures [55]. EP also inhibited dopamine-induced upregulation of the expression of two key pro-apoptotic proteins, p53 and Bax, and prevented activation of the apoptosis effector protein, caspase 3, in dopamine-challenged cells. As already noted, EP (but not pyruvate) ameliorated H_2O_2-induced apoptosis in neonatal rat brain slices [65].

Two other recent studies of I/R-induced injury also showed that treatment with EP can decrease the incidence of apoptosis in susceptible cell populations. Both of these studies employed models of transient spinal cord ischemia followed by reperfusion, although one study utilized mice [78] and the other utilized rabbits [79]. In the rabbit study, treatment with EP (40 mg/kg intravenously) as long as 6 h after reperfusion significantly improved hind-limb motor function (evaluated at 72 h after reperfusion) and significantly decreased the number of apoptotic motor neurons (as assessed by TUNEL staining) in spinal cord sections obtained at 72 h after reperfusion. In the murine study, treatment with EP (75 mg/kg) at 1 and 6 h after reperfusion significantly improved functional outcome and decreased apoptosis in spinal cord tissue sections evaluated (with annexin V staining) at 24 h after reperfusion.

In an extensive series of experiments, Epperly et al. showed that the survival of mice was significantly improved when EP (70 mg/kg per injection) was administered intraperitoneally before and after (daily for 5 days) whole body exposure to a lethal dose (9.75 Gy) of ionizing radiation [80]. In order to better understand the basis for this *in vivo* observation, these investigators carried out additional studies, using cultured 32Dcl3 mouse hematopoietic progenitor cells. When these cells were exposed to ionizing radiation (10 Gy), their clonigenic potential was impaired, mitochondrial cytochrome *C* was released into the cytoplasm, and caspase 3 was activated. All of these markers of increased cellular apoptosis were prevented when EP (10 mM) was present in the medium.

From the preceding, it is apparent that EP is capable of preventing apoptosis in a variety of *in vivo* and *in vitro* model systems. Interestingly, however, in some other circumstances, EP actually is capable of promoting apoptosis. For example, when cultured A549 cells were subjected to glucose deprivation (GD), cellular viability was compromised and necrosis was the mechanism for death [81]. Addition of 2 mM EP to the culture medium switched the mechanism of cell death induced by GD from necrosis to apoptosis. In addition, GD-induced release of the pro-inflammatory DNA-binding protein, HMGB1, was blocked by the presence of 2 mM EP in the culture medium. This latter finding is consistent with previously published data, showing that HMGB1 is released by necrotic but not apoptotic cells [82].

In another recent study, Liang et al. reported that treatment with EP (80 mg/kg intraperitoneally administered once per day for 9 days) significantly inhibited the growth of liver tumors in mice injected into the portal vein with MC38 syngeneic murine colorectal cancer cells [83]. TUNEL staining showed that tumor cell apoptosis was increased in mice treated with vehicle as compared to mice treated with vehicle. In a companion series of *in vitro* studies, 40 mM EP promoted apoptosis of cultured MC38 cells as evidenced by decreased cell viability, increased percentage of TUNEL-positive cells and increased cleavage of poly-ADP-ribosyl-polymerase.

8. Metabolic effects

As already noted, pyruvate is a key metabolic intermediate, which functions as the penultimate product of anaerobic glycolysis and the starting substrate for the TCA cycle.

Methyl pyruvate, which, like EP, is an aliphatic ester of pyruvic acid, can substitute for pyruvate as substrate for certain enzymes, such as lactate dehydrogenase and glutamate-alanine transaminase [84]. Thus, it is conceivable that EP can directly influence intracellular metabolism, even without hydrolysis and liberation of the natural endogenous substrate, pyruvate anion. In any event, whether acting itself as a metabolic substrate or as cell-permeable source of pyruvate, it is clear from *in vitro* [65] and *in vivo* [85] studies that EP can enhance ATP biosynthesis, particularly by cells subjected to redox stress.

9. Other pyruvate-like compounds

As should be apparent from this review, the pharmacological effects of pyruvic acid and its simple aliphatic esters, MP and EP, have been investigated extensively. Although other chemical relatives of pyruvic acid have been investigated less extensively, results from a few noteworthy studies have been reported. For example, Stanley et al. synthesized dipyruvyl acetyl glycerol (DPAG) and studied its pharmacological effects in a porcine model of myocardial I/R injury [86]. DPAG is an ester of the simple sugar alcohol, glycerol, which contains two pyruvyl, $CH_3C(=O)C(=O)$–, residues and one acetyl, $CH_3C(=O)$–, residue per molecule. When administered to pigs during 2 h of myocardial reperfusion, DPAG (8 mg/kg per min) reduced myocardial infarct size.

In another relevant study, Varma and Hegde showed that α-ketoglutarate, the anionic form of an alpha-keto dicarboxylic acid, inhibited oxidative stress and cataract formation in rats challenged with sodium selenite [87]. Similarly, Desagher et al. reported that several alpha-keto carboxylates, including alpha-ketoglutarate and oxaloacetate, ameliorated cell death when primary cultures of rat striatal neurons were exposed to H_2O_2 [88]. More recently, Sappington et al. showed that diethyl oxalproprionate, an esterified alpha-keto dicarboxylic acid, improved survival and ameliorated gut mucosal barrier dysfunction in mice challenged with a lethal dose of LPS [89].

Pyruvic acid can exist either as a ketone (i.e., 2-oxoproprionic acid) or as an enol (i.e., 2-hydroxyacrylic acid). In aqueous solutions of pyruvic acid, the enol tautomer is present in only trace amounts [90]. Data are lacking, regarding the relative proportions the keto *versus* enol tautomers of EP (or MP) in aqueous solutions. Nevertheless, *ab initio* calculations suggest that pyruvate can react with ROS only via oxidative carboxylation to form acetate and carbon dioxide, whereas EP can react with ROS both via oxidative carboxylation and via formation of hydroxylated adducts at the 3-carbon [91]. Accordingly, it is conceivable that the enol tautomer of EP contributes, at least to some extent, to some of the pharmacological properties of the compound. In this regard, it is noteworthy that two compounds, 2-acetamidoacrylate and its methyl ester, which recapitulation the enol structure of pyruvate (or EP), have been shown to have anti-inflammatory and anti-neoplastic properties both *in vitro* and *in vivo* [60,89,92]. Methyl 2-acetamidoacrylate is at least 100-fold more potent than EP with regard to suppressing LPS-induced nitric oxide production by cultured RAW 264.7 macrophage-like cells [89].

10. Summary

More than 150 papers, which report findings from studies of EP in animal models of human disease, have appeared in the literature. Because of space limitations, it has been impossible to exhaustively review this experience here. Briefly, however, acute administration of EP has been shown to improve survival and/or ameliorate organ damage or dysfunction in animal models hemorrhagic shock [28,29,51,63,64,93], endotoxic shock [25,26], bacterial peritonitis [26,94,95], ethanol-induced acute liver injury [96], liver injury due to extrahepatic biliary occlusion [97], necrotizing pancreatitis [98–100], cardiac I/R injury [85], hepatic I/R injury [77], stroke [101,102], whole body radiation-induced injury [80,103], cutaneous burn injury [53], zymosan-induced multiple organ system dysfunction syndrome [104], and spinal cord I/R injury [78,79]. More chronic administration of EP on a once daily or once every other day basis for 10–14 days has been shown to decrease the number of liver tumors in a murine model metastatic cancer [83], slow tumor growth in mice [60], and ameliorate colitis in IL-$10^{-/-}$ mice [30]. Collectively, these reports as well as others not cited here, support the view that EP is a remarkably effective drug for a wide range of therapeutic indications (at least in animal models).

Prompted by some of the pre-clinical data cited here, Bennett-Guerrero et al. carried out a randomized, prospective clinical trial of EP in selected high-risk patients, who underwent cardiopulmonary bypass and cardiac surgical procedures [105]. Although EP was shown to be quite safe in this study, there was no evidence of therapeutic benefit. There are myriad potential reasons for the negative results in this clinical trial (e.g., the timing or dose of EP was wrong; the wrong patient population was selected to study the therapeutic effects of EP; EP is effective in animals but not humans). It remains to be determined whether the disappointing results in this trial will relegate EP for all time to the category of "promising therapeutic agents, which failed to show benefit in human patients." However, even if EP is not pursued further as a therapeutic agent in clinical medicine, future efforts to understand the pharmacology of this compound, particularly with regard to its applications as a radioprotectant and a therapeutic for cancer, seem warranted, as the insights gained with regard to mechanism(s) of action may lead to the development of even more promising compounds.

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