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Prophylactic effect of ethyl pyruvate on renal ischemia/reperfusion injury mediated through oxidative stress

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Abstract

Purpose As *oxidative stress* (*OXS*) has been shown to play a primary role in renal ischemia/reperfusion injury (RIRI), we investigated whether *antioxidant* such as ethyl pyruvate (EPy) might effectively prevent RIRI. Possible prophylactic effects of EPy and mannitol (Mann), one of perioperative agents often used, were tested against harmful OXS in vitro.

Methods Hydrogen peroxide (H_2O_2) was used to exert OXS on the renal proximal tubular MDCK cells. Severity of OXS and protective effects of EPy and Mann were assessed by lipid peroxidation assay and cell viability test, respectively. The cytotoxic mechanism of H_2O_2 was explored by examining the status of glycolysis, metabolic signaling pathways, cell cycle, and induction of apoptosis.

Results Although H_2O_2 (500 μM) increased OXS by ~3.5 times of controls, EPy (1 mM) fully reduced it to the basal level. Cell viability declined to merely 10% by H_2O_2 was regained to > 90% with EPy. Hexokinase activity and ATP level also declined significantly by H_2O_2, but they sustained 80–90% with EPy. Additionally, H_2O_2 led to the modulations of metabolic signaling regulators, a G_1 cell cycle arrest, and induction of apoptosis, which were yet prevented with EPy. Unlike EPy, Mann had virtually little effects.

Conclusions OXS can indeed lead to the significant cell viability reduction through its adverse cellular effects, ultimately resulting in RIRI. However, EPy appears to prevent these effects and protect MDCK cells, while Mann does not. Thus, EPy could be a more effective prophylactic renoprotective agent (than Mann) against oxidative renal cell injury including RIRI.

Keywords Ethyl pyruvate · Oxidative stress · Antioxidant · Renal cell injury

Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion, hydroxyl radicals, etc. [1], are capable of causing cellular injury or dysfunction in renal cells as well as many other cells. In fact, accumulating data suggest that various renal cell injuries, caused by renal ischemia/reperfusion, nephrotoxic agents, or even by extracorporeal shock wave lithotripsy (ESWL), could be primarily attributed to *oxidative stress* (generation of ROS) [2, 3]. In particular, renal ischemia/reperfusion injury (RIRI) would result in the significant postoperative morbidity and mortality, often accompanied by acute renal failure (ARF) [4]. To protect the kidneys from such RIRI during surgery and in the immediate postoperative period, the perioperative renoprotective interventions using various pharmaceutical agents have been routinely employed [5]. Mannitol (Mann) is one of those agents being clinically used, but its usefulness or actual effectiveness has been debated ever since. Particularly, when the Mann infusion rate exceeds the rate of urinary excretion (for the uncertain reason) [6] that could cause osmotic nephrosis leading to ARF. Due to this puzzling Mann-induced ARF and the poor efficacy of other agents, a safer and more effective agent for prevention of RIRI needs to be actively explored.

If oxidative stress is indeed the primary cause of RIRI (and other renal cell injuries), it is possible that certain *antioxidants* might be able to significantly reduce the incidence of RIRI. In fact, antioxidants have been reported to have

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beneficial or protective effects on cellular injury/damage associated with oxidative stress [7]. Those include vitamins (vitamins C/E), uric acid, Mann, reduced glutathione (GSH), and Mann has been routinely used as a perioperative intervention but its efficacy is elusive. Meanwhile, pyruvate is another antioxidant and ROS scavenger [8], having been considered as a therapeutic agent for various pathologic conditions induced by oxidative stress. For example, pyruvate, or rather, *sodium pyruvate* (NaPy) has been shown to prevent/minimize myocardial, intestinal, or hepatic ischemia/reperfusion injury (due to oxidative stress) in animal models [9]. However, the clinical utility of NaPy is limited by its instability in aqueous solution. Once NaPy is dissolved in solution, it is rapidly converted to parapyruvate, which is an inhibitor of a critical step in the TCA cycle (conversion of α-ketoglutarate to succinyl coenzyme A) [8, 9]. Aqueous NaPy also undergoes hydration to form pyruvate hydrate [9]. Neither parapyruvate nor pyruvate hydrate acts as antioxidants capable of scavenging free radicals.

To overcome such drawbacks, *ethyl pyruvate* (EPy), a simple aliphatic ester derived from pyruvate, has been introduced [9, 10]. EPy is found to be safer, more stable, and effective than NaPy. EPy has the prophylactic or beneficial effects on organ systems; e.g., it reduces or alleviates ischemia/reperfusion injury (mesenteric, hepatic, and myocardial) and hemorrhagic or endotoxic shock in animals [11–13]. More beneficial effects of EPy have been also documented. Nevertheless, the exact mechanism underlying those beneficial or therapeutic effects of EPy with antioxidant activity has not been fully understood and more studies are certainly required.

Accordingly, for prevention of RIRI induced by oxidative stress, we investigated whether EPy and/or Mann might have protective effects on acute renal cell injury induced by hydrogen peroxide (H_2O_2) capable of exerting oxidative stress. To have an insight into the prophylactic mechanism of EPy or Mann (against H_2O_2), we also explored antioxidant activity of these agents, the status of glycolysis and cell cycle, and induction of apoptosis. More details are described, and the interesting findings are also discussed herein.

Methods

Cell culture

The renal tubular epithelial MDCK cells (American Type Culture Collection, Manassas, VA) were employed as our in vitro experimental model. They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 ∝g/ml). For experiments, MDCK cells were seeded at an initial cell density of 2×10^5 cells/ml in 6-well plates or T-75 flasks for 24 h and treated with specified concentrations of H_2O_2, EPy, Mann, or their combinations for another 24 h. Cell viability was then determined by MTT assay as described below.

MTT assay (cell viability test)

Cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay following the vendor's protocol (Sigma-Aldrich, St. Louis, MO). Briefly, MTT reagent (1 mg/ml) was added to each well in the 6-well plate, followed by 3-h incubation at 37 °C. After removing MTT reagent, dimethyl sulfoxide (DMSO) was added to each well and absorbance of formazan solution (purple) was read on a microplate reader. Cell viability was then expressed by the percent (%) of viable cells relative to the control reading (100%). The same experiment was repeated separately three times.

Lipid peroxidation (LPO) assay

The severity of oxidative stress was assessed by LPO assay that measures the formation of malondialdehyde (MDA), an end product from peroxidation of polyunsaturated fatty acids [14]. The amount of MDA formed will indicate severity of oxidative stress: *the more MDA formed, the greater oxidative stress*. The detailed procedures were described in the vendor's protocol (Abcam, Cambridge, MA). Briefly, the reaction was initiated by mixing cell lysates with thiobarbituric acid (TBA) solution and incubated in a boiling water bath (~ 100 °C) for 1 h. Samples were then read at A_{532} on a microplate reader. The amount of MDA formed was calculated from the MDA standards and expressed by fold-increase relative to that of control (1). The same experiment was repeated three times.

Hexokinase (HK) assay

HK activity was determined by the HK Colorimetric Assay Kit (BioVision, Milpitas, CA), following the manufacturer's protocol. Cell lysates (20 μg per sample) and NADH standards were prepared in the 96-well plate, and the reaction was started by the addition of reaction mix (containing substrate). Immediately the plate was placed in a microplate reader and the absorbance changes with time were monitored at 450 nm for 20 min with 5-min intervals. All readings were calculated and normalized, and then, HK activity was expressed by the % of sample activity relative to the controls (100%). The same experiment was repeated three times.

Determination of cellular ATP level

The cellular ATP level was determined using the ATP Colorimetric Assay Kit (BioVision) following the vender's protocol. Cells were first lysed in ATP assay buffer, and cell lysates were deproteinized with $HClO_4$ and neutralized with KOH. The ATP standards and samples (50 µl per sample) were prepared in the 96-well plate, and the reaction was initiated by the addition of reaction mixture. The plate was then incubated at room temperature for 30 min in the dark. After incubation, all ATP standards and samples were read at 570 nm on a microplate reader and ATP content was calculated by applying the sample readings to the ATP standards. The ATP level was then expressed by the % of sample readings relative to the controls (100%). The same experiment was repeated three times.

Cell cycle analysis

A BD FACscan flow cytometer (Becton–Dickinson, Franklin Lakes, NJ), equipped with a double discrimination module, was employed for cell cycle analysis. Cells (~ 1×10^6 cells) were resuspended in propidium iodide solution and incubated for 1 h at room temperature. Approximately 10,000 nuclei from each sample were analyzed on a flow cytometer, and CellFit software was used to quantify cell cycle compartments to estimate the % of cells distributed in the different cell cycle phases. The same experiment was repeated three times.

Western blot analysis

Briefly, aliquots (10 µg each) of cell lysates/extracts obtained from MDCK cells were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (blot). The blot was first incubated with the primary antibodies against three metabolic regulators, AMP-activated protein kinase (p-AMPK), serine/threonine protein kinase B (p-Akt), and mammalian target of rapamycin (p-mTOR) (Santa Cruz Biotechnology, Santa Cruz, CA), or against two apoptotic regulators, bcl-2 and Bax (Santa Cruz Biotechnology) for 90 min, followed by 30-min incubation with the appropriate secondary antibody conjugates. The specific immunoreactive protein bands were then detected by chemiluminescence following the manufacturer's protocol (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The same experiment was repeated at least three times.

Statistical analysis

All data were presented as mean ± SD (standard deviation), and statistical differences between groups were assessed with either one-way ANOVA or the unpaired Student's *t* test. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Effect of H_2O_2 on cell viability and protective effects of EPy and/or Mann

We first examined how H_2O_2 would affect *cell viability* in MDCK cells because cell viability indicates the percent (%) of viable cells following H_2O_2 treatment. After cells were seeded in the 6-well plate for 24 h, they were treated with varying concentrations (0–500 µM) of H_2O_2 for another 24 h and subjected to MTT assay. Cell viability was significantly ($P < 0.03$) reduced to ~ 50% with 300 µM H_2O_2 and further went down to ~ 10% ($P < 0.01$) with 500 µM (Fig. 1). Thus, these results show that H_2O_2 would significantly reduce cell viability in MDCK cells. As H_2O_2 (500 µM) appears to be effective and suitable, this concentration was then used in the rest of our study.

We next examined if EPy and/or Mann might prevent the cell viability reduction induced by H_2O_2 (500 µM). Following 24-h cell seeding, cells were treated with H_2O_2 (500 µM) alone or in combination with either EPy (1 mM) or Mann (10 mM) for 24 h and cell viability was determined. The results showed that EPy maintained > 90% cell

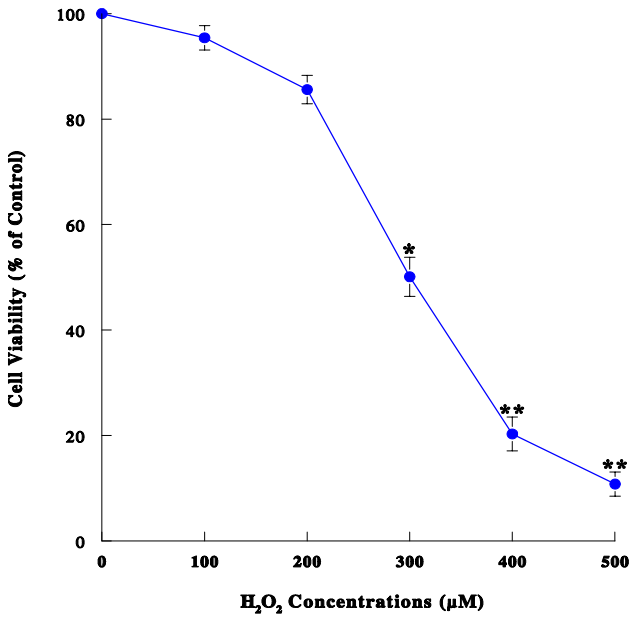

Fig. 1 Dose-dependent effects of H_2O_2 on MDCK cell viability. After cells were treated with varying concentrations of H_2O_2 (0–500 µM) for 24 h, cell viability was determined by MTT assay and expressed by the percent (%) relative to controls (100%). All data are mean ± SD (standard deviation) from three independent experiments (*$P < 0.03$; **$P < 0.01$)

viability, which was once reduced to ~10% ($P < 0.01$) by H_2O_2 treatment (Fig. 2). In contrast, Mann had little effects on H_2O_2-induced cell viability reduction (Fig. 2). Thus, EPy is capable of maintaining high (~90%) cell viability following H_2O_2 exposure, whereas Mann (with 10 times higher concentration of EPy) yet has little or no effects.

Reduction of oxidative stress with EPy

To verify if oxidative stress is responsible for H_2O_2-induced cell viability reduction, we directly assessed how much oxidative stress (*severity* of oxidative stress) was actually exerted by H_2O_2 and diminished with EPy. Cells were exposed to H_2O_2 (500 μM) alone or in combination with either Epy (1 mM) or Mann (10 mM) for 6 h and subjected to LPO assay. The severity of oxidative stress is determined by the amount of MDA formed—*the more MDA formed, the greater oxidative stress*. Compared to the basal MDA level (1) of controls, H_2O_2 led to a ~3.1-fold increase ($P < 0.01$) in the MDA level (Fig. 3). However, such a MDA increase was almost completely prevented with EPy, maintaining the MDA level virtually the same as the control level (Fig. 3). Mann yet had no effect on such oxidative stress. Thus, EPy, not Mann, may have antioxidant activity capable of effectively reducing oxidative stress and protecting the cells from it (i.e., renoprotection). It should be noted that Mann was omitted from the rest of our study because it seems to have little effects on H_2O_2-mediated cellular alterations.

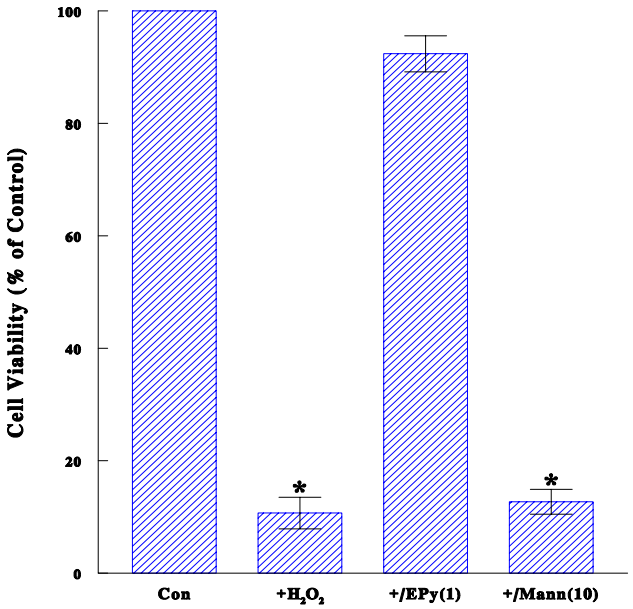

Fig. 2 Protective effects of EPy or Mann against H_2O_2. Cells were treated with H_2O_2 (500 μM) alone or in combination with either EPy (1 mM) or Mann (10 mM) for 24 h and cell viability was determined by MTT assay. Cell viability was then expressed by % relative to controls (100%). The data are mean ± SD from three separate experiments (*$P < 0.01$ compared with control)

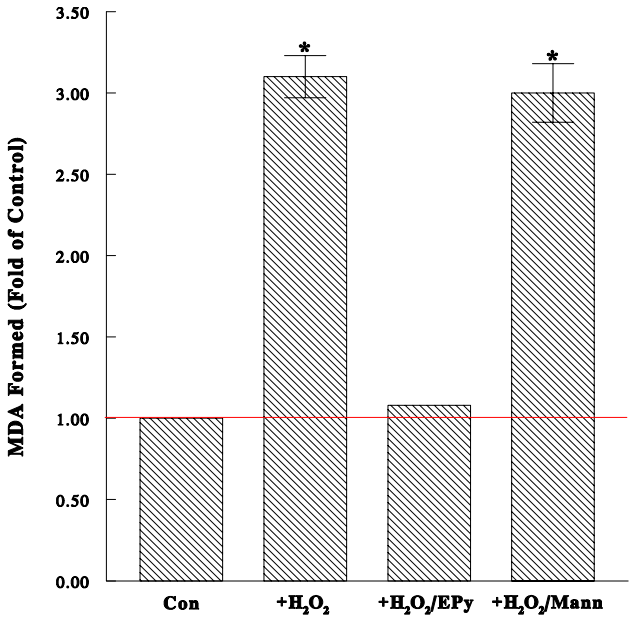

Fig. 3 Antioxidant activity of EPy. Cells were exposed to H_2O_2 (500 μM) alone or in combination with either Epy (1 mM) or Mann (10 mM) for 6 h and subjected to LPO assay. The amounts of MDA formed were measured and expressed by fold-increase relative to controls (1). All data are mean ± SD from three separate experiments (*$P < 0.01$ compared with controls)

Inhibitory effect of H_2O_2 versus prophylactic effect of EPy on glycolysis

To explore the mechanism through which H_2O_2 reduces cell viability, we focused on *glycolysis* because energy (ATP) metabolism is the most essential and crucial event required for cellular activity, survival, and proliferation [15, 16]. The status of two key glycolytic parameters, hexokinase (HK) [17] activity and cellular ATP synthesis, was examined. Cells were exposed to H_2O_2 (500 μM) alone or in the presence of EPy (1 mM) for 24 h and assayed for HK and ATP accordingly. As shown in Fig. 4, compared to controls (100%), H_2O_2 exposure declined the HK and ATP levels to ~45% and ~30%, respectively, indicating the inhibition of glycolysis. However, EPy significantly ($P < 0.05$) increased such H_2O_2-reduced HK and ATP levels to ~90% and ~80%, respectively (Fig. 4). Thus, H_2O_2 may inhibit glycolysis (leading to the growth cessation or cell death) but EPy could effectively *reverse* such an inhibition by sustaining the high levels of HK and ATP.

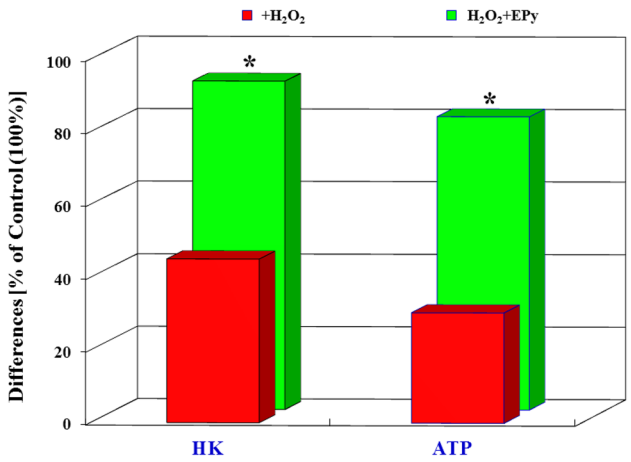

Fig. 4 Effects of EPy on glycolytic parameters inhibited by H_2O_2. Cells exposed to H_2O_2 (500 μM) alone or in the presence of EPy (1 mM) for 24 h were assayed for HK activity and ATP level. The results were normalized and expressed by % relative to the respective controls (100%). All data are mean from three independent experiments (*$P < 0.05$ compared with respective H_2O_2-treated cells)

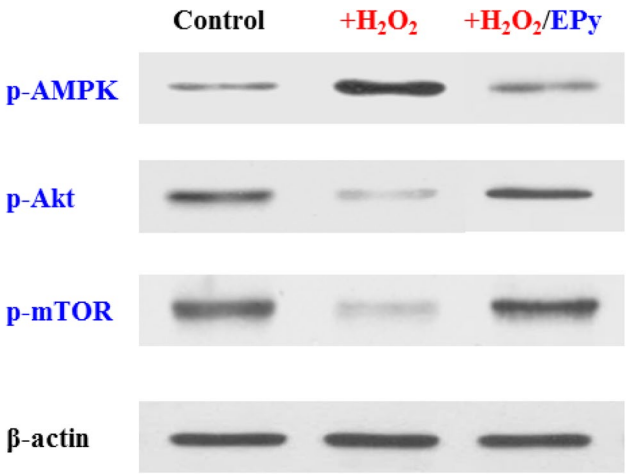

Fig. 5 Effects of EPy on metabolic regulators modulated by H_2O_2. Cells exposed to H_2O_2 (500 μM) alone or in the presence of EPy (1 mM) for 24 h were analyzed for three key metabolic regulators using Western blots. Autoradiographs of p-AMPK, p-Akt, and p-mTOR expressed in control, H_2O_2-treated or H_2O_2/EPy-treated cells are shown for comparison. Beta-actin is also shown as a loading control

Effects of H_2O_2-induced glycolysis inhibition on metabolic signaling pathways

ATP (energy) reduction/depletion, due to the glycolysis inhibition, has been shown to affect metabolic signaling pathways, which play a pivotal role in cell proliferation and survival [16, 18]. We then analyzed three key metabolic regulators, AMPK [19], Akt [20], or mTOR [21], following 24-h exposure of cells to H_2O_2 alone or its combination with EPy. Western blot analysis revealed that the expression of AMPK was enhanced or phosphorylated (activated), whereas those of Akt and mTOR were reduced or dephosphorylated (inactivated) by H_2O_2 (Fig. 5). However, no such alterations in these regulators were seen in the presence of EPy (Fig. 5). Therefore, activation of AMPK concomitant with inactivation of Akt/mTOR by H_2O_2 suggests the cells undergoing cell death, although EPy appears to prevent such a death process.

Interruption of cell cycle by H_2O_2 but inhibition with EPy

In addition, the glycolysis inhibition has been also shown to interfere with cell cycle [22], this possibility was tested next. Cells were exposed to H_2O_2 or EPy alone or their combination (H_2O_2/EPy) for 24 h and subjected to cell cycle analysis. The results showed that H_2O_2 led to a ~41% *increase* ($P < 0.05$) in G_1-phase cell number concomitant with a ~47% *decrease* ($P < 0.05$) in S-phase cell number, compared to those in controls (Fig. 6). This cell accumulation in the G_1 phase is known as a G_1 cell cycle arrest [23], which would subsequently lead to the cell growth/viability reduction or cell death. However, EPy sustained nearly the same cell distribution pattern as the controls under oxidative stress (Fig. 6), indicating inhibition of cell cycle arrest but promotion of cell growth. Thus, although H_2O_2 may specifically block the G_1–S-phase progression, EPy could lift such a blockade, allowing the cells to continue growing.

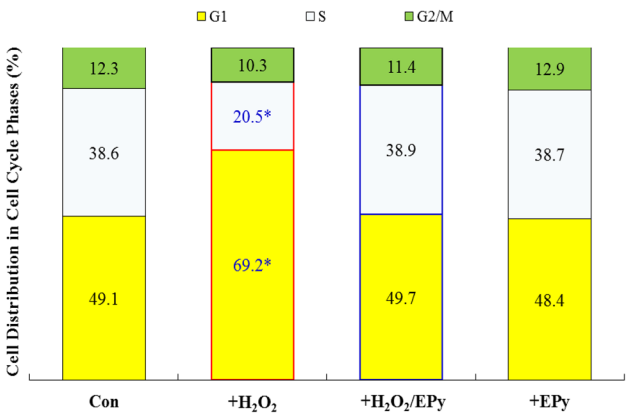

Fig. 6 Preventive effect of EPy on cell cycle arrest. Cells were exposed to H_2O_2 (500 μM) or EPy (1 mM) alone or the combination of H_2O_2 and EPy for 24 h and subjected to cell cycle analysis. The data are representatives of three independent experiments (*$P < 0.05$ compared with controls)

Induction of apoptosis by H_2O_2 but inhibition with EPy

Lastly, it was important to address whether H_2O_2-induced *cell death* might be primarily linked to *apoptosis* (programmed cell death). After cells were exposed to H_2O_2 alone or in the presence of EPy for 24 h, they were analyzed for two key apoptosis regulators, bcl-2 and Bax [24], using Western blots. Analysis revealed that bcl-2 expression was reduced or down-regulated, while Bax was enhanced or up-regulated by H_2O_2 exposure (Fig. 7). However, no such changes were seen with EPy as both bcl-2 and Bax looked quite similar to those in controls (Fig. 7). Since bcl-2 is anti-apoptotic while Bax is pro-apoptotic [24], the observed modulations of these regulators rather indicate induction of apoptosis. Thus, H_2O_2 appears to more likely induce apoptosis, accounting for the significant cell viability reduction, which can be yet inhibited with EPy.

Discussion

It has been well documented that oxidative stress would exert the adverse or cytotoxic effects on a variety of cells including renal cells [2, 3]. Particularly, renal cell injury is very serious and can lead to various renal diseases and disorders. For instance, RIRI is common during renal surgery and appears to result from oxidative stress [4], implying its potential prevention with *antioxidants*. In fact, Mann has been routinely utilized to protect the kidneys from RIRI, but its efficacy is vague and disputable. Hence, a more effective antioxidant must be found.

We were interested in ethyl pyruvate (EPy), which was an antioxidant with the prophylactic or therapeutic effects on inflammation and oxidative cell injury [9–13]. To address whether EPy could be a viable agent for prevention of RIRI, its antioxidant activity was examined on MDCK cells under oxidative stress exerted by H_2O_2. Mann was also tested for comparison.

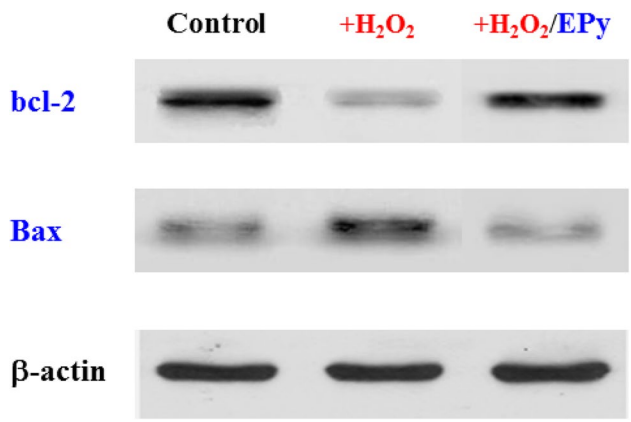

Fig. 7 Inhibition of apoptosis with EPy. Cells treated with H_2O_2 (500 μM) alone or in the presence of EPy (1 mM) for 24 h were analyzed for bcl-2 and Bax using Western blots. Expressions of bcl-2 and Bax on autoradiogram are shown, and β-actin is used as the protein loading control

We examined whether EPy or Mann would prevent the cell viability reduction induced by H_2O_2. Although H_2O_2 (500 μM) drastically reduced cell viability to ~ 10% (i.e., a ~ 90% reduction), EPy (≥1 mM) maintained ~ 90% cell viability under H_2O_2 attack whereas no such effect was seen with Mann. H_2O_2 was indeed found to exert a ~ 3.1-fold greater oxidative stress (than controls) but EPy almost completely reduced such oxidative stress, keeping it almost at the basal level (as controls). Mann had no effect. Thus, these results suggest that EPy is capable of protecting renal cells from oxidative stress through its antioxidant activity.

Nevertheless, unlike our study (focusing on antioxidant activity), many previous studies of EPy have focused on its "anti-inflammatory" activity mostly in animals and some cell cultures. For instance, one study was performed to demonstrate the renoprotective effect of EPy against RIRI under hyperglycemia in the rats [25]. They specifically examined the status of several pro-inflammatory parameters, such as high-mobility group box 1 (HMGB1), nuclear factor-kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), etc. [10, 12, 26], to corroborate the notion that RIRI is substantially mediated through *inflammation* on the rat kidneys. As a result, compared to RIRI under normoglycemia, it was *severer* under hyperglycemia in terms of tubular damage, blood urea nitrogen and creatinine (renal function), pro-inflammatory parameters, and apoptosis. However, the status of RIRIs in both cases was significantly ameliorated with EPy, implying the potent renoprotective effect of EPy with *anti-inflammatory* activity.

Another study describes the protective effect of EPy against cisplatin-induced nephrotoxicity in the rats [27]. It has been shown that cisplatin was one of typical nephrotoxic agents, inducing renal injury and dysfunction [28]. EPy was capable of protecting the rat kidneys from nephrotoxicity of cisplatin, presumably through its anti-inflammatory and antioxidant activities. In fact, the severity of oxidative stress by cisplatin against the rat kidneys was examined and confirmed. Histopathological examination of kidney specimens also indicated the cisplatin-induced inflammatory modifications such as inflammatory cell infiltration, tubular necrosis/dilation. However, EPy significantly diminished oxidative stress and made renal injury/damage less remarkable. Nonetheless, the protective effect of EPy on "RIRI" (*nephrotoxin-induced renal injury* instead) has not been yet addressed in this study.

Compared to these two in vivo studies, our in vitro study thus far describes that RIRI appears to be primarily induced

by *oxidative stress*, which can be significantly reduced/diminished with EPy in MDCK cells.

To better understand *how* H_2O_2-exerted oxidative stress would result in a cell viability reduction and EPy might prevent it, we examined the possible effects of H_2O_2 on glycolysis and cell cycle. Both HK activity and ATP synthesis have significantly declined by H_2O_2 exposure, indicating the shutdown (inhibition) of glycolysis and ultimate cell death. However, EPy sustained the high (> 80%) HK and ATP levels during H_2O_2 attack, suggesting that EPy may significantly prevent or reverse the glycolysis inhibition (induced by H_2O_2).

In addition, we found that the three metabolic signaling regulators, AMPK, Akt, and mTOR [19–21], were *modulated* by H_2O_2 due to possible ATP depletion (Fig. 5). AMPK was *phosphorylated* (activated), while Akt was *dephosphorylated* (inactivated) by H_2O_2. It has been shown that both AMPK and Akt should be *phosphorylated* to become active and functional [19, 20]. In fact, AMPK activation is known to be cytotoxic to various cancer cells and promote apoptosis [29], whereas Akt activation would inhibit apoptosis to promote cell survival [20, 30]. Hence, as AMPK will work oppositely to Akt [19], activation or inactivation of these two parameters would govern an anabolic or catabolic process. Moreover, *activation* of AMPK and *inactivation* of Akt is known to *inactivate* the master orchestrator of cell proliferation known as mTOR, which is a protein kinase working with Akt to promote cell proliferation [21]. This is indeed consistent with our finding that mTOR (along with Akt) was *dephosphorylated* or *inactivated* by H_2O_2 (Fig. 4). Thus, AMPK activation concomitant with Akt/mTOR inactivation induced by H_2O_2 herein would ultimately lead to cell death, feasibly accounting for the cell viability reduction. Nevertheless, no such modulations of these regulators have been seen with EPy, suggesting that the cells remain intact or uninjured (from H_2O_2 attack).

Further study showed that the glycolysis inhibition by H_2O_2 certainly induced a G_1 cell cycle arrest, leading to the growth cessation and cell death [23]. EPy yet effectively inhibited a G_1 arrest, promoting cell proliferation instead. This also supports the notion that glycolysis and cell cycle are closely interlinked as reported [22]. We eventually found that cell death induced by H_2O_2 was primarily associated with apoptosis, seemingly accounting for the resulting cell viability reduction. However, such induction of apoptosis could be entirely blocked with EPy.

After all these studies, it is important to address the potential clinical relevance of EPy. Initially, EPy was widely used as a food additive and then its safety has been confirmed in a Phase I (toxicology) clinical trial [8]. Especially, this Phase I approval is significant because EPy could be used as a perioperative renoprotective agent against IRI during a variety of surgeries (e.g., a Phase II clinical trial of EPy in cardiac surgery with cardiopulmonary bypass [31]) being performed. We also demonstrated that EPy was indeed significantly superior to Mann and a potent antioxidant for prevention of RIRI. Moreover, those past studies of EPy included multivisceral *ischemia and reperfusion injury* (IRI), hepatic IRI, cerebral ischemic injury, other inflammatory organ injuries involving pancreas, lung, kidney, heart, etc., hemorrhage, systemic inflammation, endotoxic/septic shock, inflammatory arthritis, pro-inflammatory gene expression in monocytes, etc. [10–13, 32–37], although all studies were carried out using animals or cell cultures. EPy was found to act as an anti-inflammatory agent to prevent or alleviate those adverse conditions by modulating key pro-inflammatory parameters (HMGB1, NF-κB, TNF, IL-6, etc.). Although a few studies of antioxidant activity of EPy against oxidative stress (such as our study) have been performed, accumulating data suggest that EPy has both anti-inflammatory and antioxidant activities [9–13], capable of ameliorating and reducing detrimental inflammation and oxidative stress, thereby preventing various critical illnesses. These EPy studies could be then applied to the clinical settings, and such adverse conditions might be alleviated, prevented, or treated with EPy. Taken together, EPy appears to be a promising agent with broad clinical implications, particularly in the prevention and/or amelioration of renal injuries including RIRI. Further evaluation of EPy is warranted.

Last of all, for confirmation of the safety and actual efficacy of EPy, our next study should be performed using the rats (in vivo), focusing on the role of oxidative stress, inhibition of glycolysis, induction of apoptosis, and renal function in the rat kidneys under RIR induced by the classic procedures involving a 40–45-min clamping of renal arteries (ischemia) followed by a 24-h reperfusion [25]. Such study may provide us with valuable information toward a clinical trial of EPy and is currently underway in our laboratory.

Conclusions

Oxidative stress may play a critical role in RIRI as evidenced by this study. H_2O_2 can exert severe oxidative stress, resulting in a profound cell viability reduction, the glycolysis inhibition, the modulations of metabolic signaling pathways, a cell cycle arrest, and ultimate apoptosis. However, EPy is capable of protecting renal cells from H_2O_2 attack and diminishing a variety of adverse effects. Mann yet has no such beneficial effects. Therefore, it is conceivable that EPy should be considered as a safer, more effective perioperative renoprotective agent against RIRI as well as a variety of acute clinical conditions commonly encountered in the medical practice.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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