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Original article

AMPK activation mediates the antioxidant and anti-inflammatory effects of oleuropein in renal ischemia-reperfusion injury

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Abstract

In the present study, we investigated the protective effect of OLE in a renal ischemia/reperfusion (I/R) rat model. We assessed whether OLE action depends on the modulation of AMPK-mediated oxidative stress and inflammation pathways. Rats were subjected to 60-min of renal ischemia followed by 120-min of reperfusion. OLE (50 mg/kg, per os) was administered for three consecutive days before I/R. Ara-A, an AMPK inhibitor, was infused at a rate of 100 µg/kg per min for 10 min before the onset of ischemia. Our results showed that OLE treatment improved significantly renal function, cell integrity and antioxidant status. These effects were completely blunted after Ara-A administration. Rats' pretreatment with OLE reduced NF-κB pathway through the phosphorylation of AMPK and its downstream target molecule COX 2, an effect that was abolished following AMPK inhibition. In conclusion, this study reveals that OLE pretreatment attenuates kidney injury after I/R and this beneficial effect is related to the activation of AMPK pathway. These results emphasize oleuropein therapeutic potential for limiting oxidative and inflammatory damages associated with renal I/R.

Keywords: Oleuropein, renal ischemia, oxidative stress, Inflammation, AMPK.

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1. Introduction

Renal ischemia-reperfusion injury (IRI) is the leading cause of acute kidney injury marked by an abrupt decline in kidney function with high morbidity and mortality rates [1,2]. Complex mechanisms are involved in the pathogenesis of IRI, including among others increased production of reactive oxygen species (ROS) and inflammatory responses [3].

AMPK, a serine/threonine kinase, is a key sensor of intracellular energy homeostasis and participates in the regulation of the production and the consumption of energy [4]. Ischemic stress causes a dramatic drop in ATP levels and activates AMPK, which activates cytoprotective pathways and redirects cellular metabolism toward energy conservation [5]. Numerous research demonstrated that AMPK significantly reduces oxidative stress by boosting antioxidant defense mechanisms.

Simultaneously, AMPK downregulates pro-inflammatory cytokines and inhibits NF-κB pathway activation [6]. AMPK is a potential therapeutic target for ischemia-related diseases through reducing tissue damage and promoting cellular survival.

Oleuropein (OLE), the main bioactive compound of olive leaves, has been reported to exhibit several pharmacological activities including antioxidant, anti-inflammatory and anti-apoptotic effects [7]. In fact, OLE has been shown to alleviate IRI and reduce the secondary injury associated to cardiac [8] and cerebral [9] ischemia. We recently reported that OLE is effective in protecting kidney against IRI [10]. We demonstrated that it decreases oxidative stress as well as inflammation. Despite the growing evidence supporting the protective actions of OLE, its ability to modulate oxidative stress and inflammation through AMPK activation during renal I/R has not yet been clarified.

In the present study, we attempted to clarify the role of OLE in a rat model of renal IRI. The AMPK-mediated pathway was investigated as a possible positive mechanism of OLE effects.

2. Materials and methods

Extraction and purification procedures of oleuropein from olive leaves

OLE extraction from olive leaves and purification were previously described [10]. The purity and identity of OLE were verified by high-performance liquid chromatography (HPLC) analysis, which showed a single major peak corresponding to oleuropein, with a purity greater than 95%. The retention time and UV spectrum were consistent with those reported for standard oleuropein. These analyses confirmed the suitability of the purified compound for in vivo experiments.OLE was dissolved in water for oral administration to rats.

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Animals

All animal procedures were performed according to the European guidelines governing animal care (Directive 2010/63/EU) and the ethic committee on the research in life sciences and health of the High Institute of Biotechnology of Monastir, Tunisia (cer-svs10/2020). We used male Wistar rats weighing 180–220 g. Rats were housed in a 12:12 h light:dark cycle with controlled temperature (21±2°C). Animals had free access to water and food.

Animals were randomly divided into four experimental groups (n = 6):

- Group 1 (Sham): rats were subjected to dissection of renal pedicle without their clamping.
- Group 2 (I/R group): Same as sham group but renal pedicles were clamped for 60 min and then reperfused for 120 min.
- Group 3 (I/R+OLE): Same as I/R group, but animals were treated with OLE orally at a dose of 50 mg/Kg of body weight for three consecutive days before ischemia [10]. The dose of OLE and the pretreatment period of three consecutive days were selected based on our previous studies and other reports demonstrating significant antioxidant and anti-inflammatory effects without toxicity in ischemia–reperfusion models. This pretreatment regimen was chosen to ensure adequate bioavailability and activation of protective signaling pathways prior to ischemic insult.
- Group 4 (I/R+OLE+Ara-A): Same as I/R+OLE group, but before the onset of ischemia the rats were infused for 10 min with Ara-A at a rate of 100 µg/kg per min [11].

Surgery and experimental protocol

A well-established model of renal I/R injury was used. Briefly, after anesthesia with 1.5 % isoflurane at a rate of 0.8 L/min, abdominal incision was performed and the renal pedicle was dissected and clamped. After the clamps were removed and at the end of reperfusion (120 min), plasma and tissue samples were collected and stored at -80 °C until analysis.

The short reperfusion period reflects acute injury mechanisms. Longer reperfusion times would be required to assess sustained renal dysfunction and recovery. Sham animals were subjected to the same protocol without renal pedicle clamping.

Renal function

Creatinine concentration in plasma was measured at 520 nm using a standard kit, in accordance with the manufacturer's instructions (Beckman Coulter, Inc., USA).

Determination of lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was quantified at 340 nm using commercially available kit (Beckman Coulter, Inc., USA).

Determination of C-reactive protein concentration

To evaluate inflammation, C-reactive protein (CRP) concentration in plasma was measured at 600 nm using a commercially available kit (Beckman Coulter Inc., USA).

Determination of antioxidant enzymes activities

The activity of catalase was determined as described by Clairbone at 240 nm (Claiborne, 1985). Superoxide dismutase (SOD) activity was estimated according to Marklund and Marklund at 420 nm [12]. The enzymatic activity of glutathione peroxidase (GPx) was assessed, spectrophotometrically at 240 nm by the method of Floche and Gunzler [13].

Determination of oxidative stress parameters

The concentrations of glutathione (GSH), malondialdehyde (MDA) and sulfhydryl proteins (PSH) in kidney homogenates were measured. GSH was measured according to the method of Tietze [14]. MDA was quantified using the thiobarbituric acid at 530nm as previously described [15]. The determination of PSH level was assessed according to the method of Sedlack and Lindsay [16].

Western Blot analysis

Renal samples were homogenized in a buffer containing 50 mM Tris, 320 mM sucrose, protease/phosphatase inhibitor cocktail and 0.5% CHAPS (all from Sigma-Aldrich, Madrid, Spain). Homogenates were centrifuged at 13000 rpm for 10 min at 4 °C, and cleared supernatants were used for immunoblotting.

Protein concentrations were determined according to the Bradford method. Protein extracts (60 μg) were then separated by SDS-PAGE electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were immunoblotted with antibodies directed against total AMPK, p-AMPK and β-actin (Cell Signaling), COX 2 (Cayman), p-IκB (Abcam).

Membranes were then incubated with peroxidase-linked secondary antibodies (1:5000) for 60 min at room temperature. Immunoreactive bands were detected using the Pierce Detection System (Rockford, IL).

Densitometric analysis of Western blot bands was performed using ImageJ software. Protein expression levels were normalized to β-actin and expressed as relative fold change compared with the Sham group. All signals were quantified within the linear range of detection, and identical exposure settings were used for all samples.

Statistical analysis

Data are expressed as mean ± SEM (n = 6 for each group). Prior to statistical analysis, data distribution was assessed for normality using the Shapiro–Wilk test. They were then, compared statistically using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test (Graph Pad Prism software 6.01). Data are considered statistically significant at $P < 0.05$.

3. Results

Effect of OLE on AMPK phosphorylation

As indicated in Fig. 1, phosphorylated AMPK (p-AMPK) levels were similar in sham and I/R groups. OLE increased p-AMPK protein levels when compared to sham and I/R groups. As expected, the use of Ara-A, an AMPK inhibitor, significantly reduced p-AMPK in comparison to I/R+OLE group (p = 0.0023).

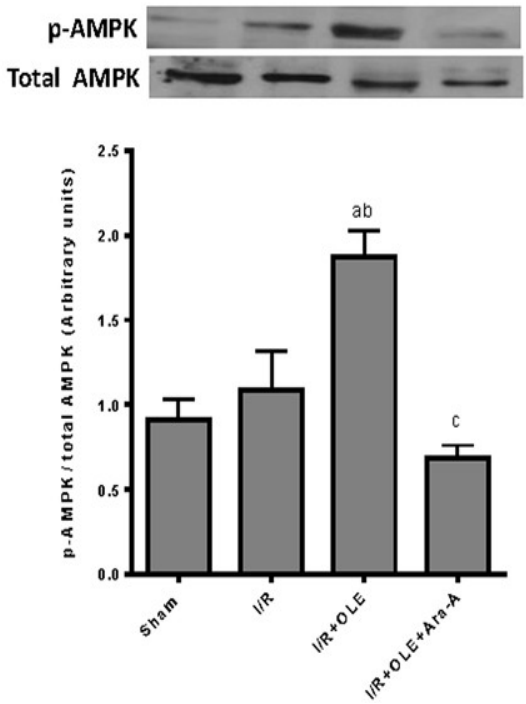

Fig. 1. Levels of phosphorylated AMPK in renal tissues. Representative Western blots of phosphorylated AMPK (p-AMPK) and total AMPK are shown (upper panels). Densitometric analysis of p-AMPK was normalized to total AMPK and expressed as fold change relative to the Sham group (lower panel). Data are presented as mean ± SEM (n = 6 per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Different letters indicate statistically significant differences ($p < 0.05$): a vs Sham, b vs I/R, c vs I/R + OLE.

Effect of OLE pretreatment on renal function and injury

A widely known biochemical indicator of renal function is the measurement of creatinine concentration in plasma. As indicated in Fig. 2A, I/R induced a significant elevation in plasma creatinine concentration in comparison to sham group. OLE administration significantly prevented the creatinine decline in I/R group ($p < 0{,}0001$). Next, we assessed cytolysis by measuring LDH activity (Fig. 2B). Our results showed that I/R markedly increased LDH release as compared to sham group, which was reversed by OLE pretreatment ($p < 0{,}0001$). Interestingly, the inhibition of AMPK suppressed the effects of OLE on both renal function and injury parameters.

Effect of OLE on antioxidant enzymes activities and oxidative stress

We next examined the effects of OLE pretreatment on the preservation of the antioxidant enzymes activities (Fig. 3) and on the extent of the oxidative stress (Fig. 4) in renal tissues.

We observed that the renal activities of SOD, Catalase, and GPx and the level of GSH decreased significantly in I/R operated rats as compared to sham group ($p = 0{,}0065$, $p = 0{,}0001$, $p = 0{,}0053$ and $p = 0{,}019$ respectively). Our data also showed that I/R significantly increased MDA concentration ($p = < 0{,}0001$) and decreased PSH levels ($p = 0{,}0047$) compared to the sham group. OLE administration effectively reversed these changes, whereas co-treatment with Ara-A completely abolished the protective effects of OLE.

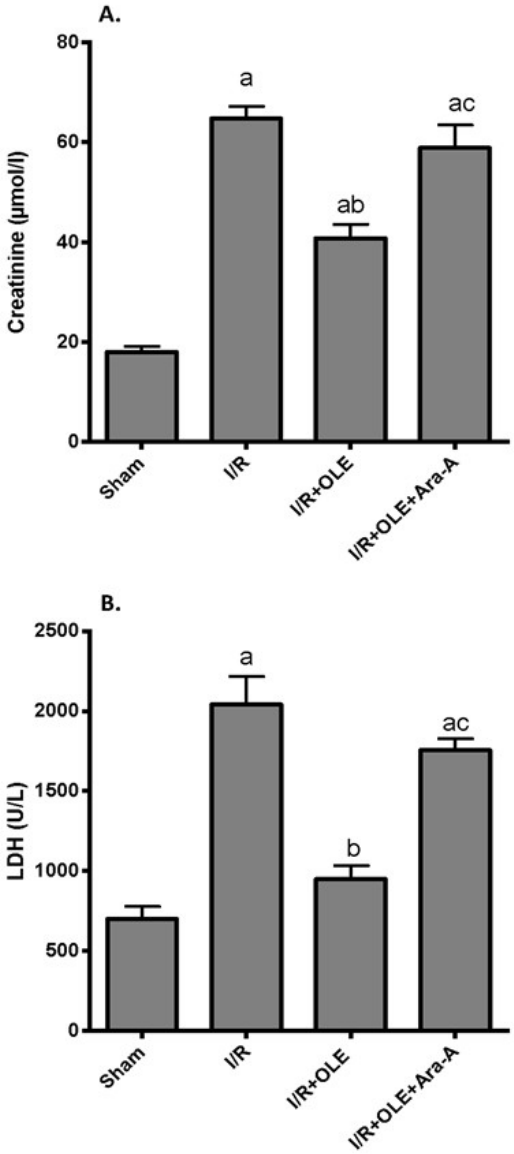

Fig. 2. Evaluation of renal function and cellular injury. Plasma creatinine concentration (A) and lactate dehydrogenase (LDH) activity (B) were measured as indicators of renal dysfunction and cytolysis, respectively. Data are expressed as mean ± SEM (n = 6 per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Different letters indicate statistically significant differences ($p < 0.05$): a vs Sham group; b vs I/R group; c vs I/R + OLE group.

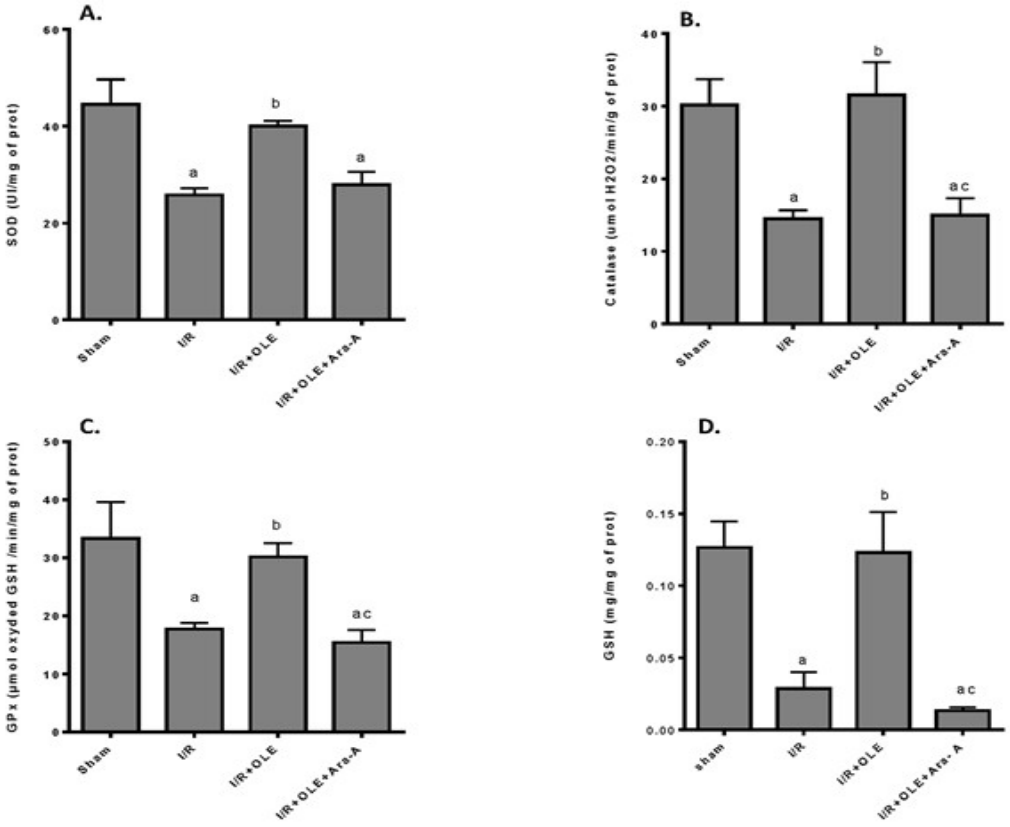

Fig. 3. Effect of oleuropein on antioxidant defense systems in renal tissues. Activities of superoxide dismutase (SOD) (A), catalase (B), and glutathione peroxidase (GPx) (C), as well as reduced glutathione (GSH) concentration (D), were assessed in kidney homogenates. Data are expressed as mean ± SEM (n = 6 per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Different letters indicate statistically significant differences ($p < 0.05$): a vs Sham group; b vs I/R group; c vs I/R + OLE group.

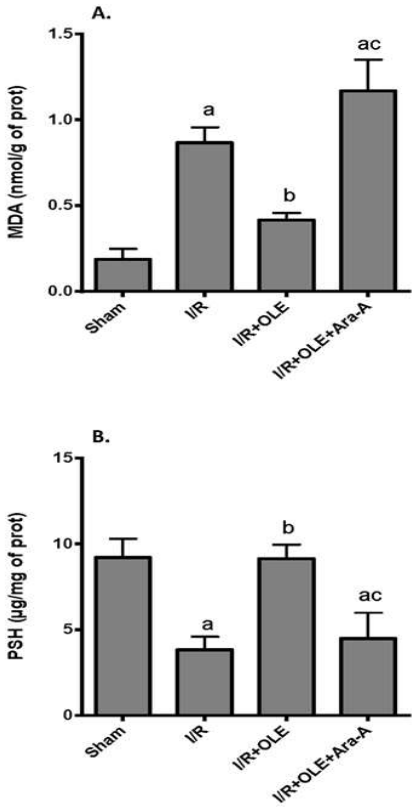

Fig. 4. Effect of oleuropein on oxidative stress markers in renal tissues. Concentrations of malondialdehyde (MDA) (A) and sulfhydryl protein groups (PSH) (B) were measured as indices of lipid peroxidation and protein oxidation, respectively. Data are expressed as mean ± SEM (n = 6 per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Different letters indicate statistically significant differences ($p < 0.05$): a vs Sham group; b vs I/R group; c vs I/R + OLE group.

Effect of OLE pretreatment on renal inflammatory parameters

We then determined the impact of OLE on the expression of the inflammatory markers p-IκB, COX 2 and CRP (Fig. 5). We observed that I/R increased their levels compared with sham group. OLE diminished significantly the expression of these markers ($p = 0.0078$, $p < 0.0001$, and $p < 0.0001$, respectively). In contrast, the administration of AMPK inhibitor suppressed the anti-inflammatory action of OLE regarding all these parameters.

4. Discussion

Polyphenols have been widely studied for their protective properties, often mediated by their antioxidant potential [7]. For instance, numerous investigations have focused on OLE because of its multiple beneficial effects in several conditions including I/R [8,9]. In the present study, we demonstrated that OLE significantly attenuated I/R injury in rat kidneys by preserving antioxidant capacities and by inhibiting inflammation through an AMPK-dependent pathway.

I/R is characterized by a rapid induction of a cascade of events promoting cellular damage and leading to cell death and organ dysfunction [17]. Our measurements of creatinine concentration and LDH activity in plasma showed that OLE was able to preserve kidney function and membrane integrity likely through its high antioxidant capacity.

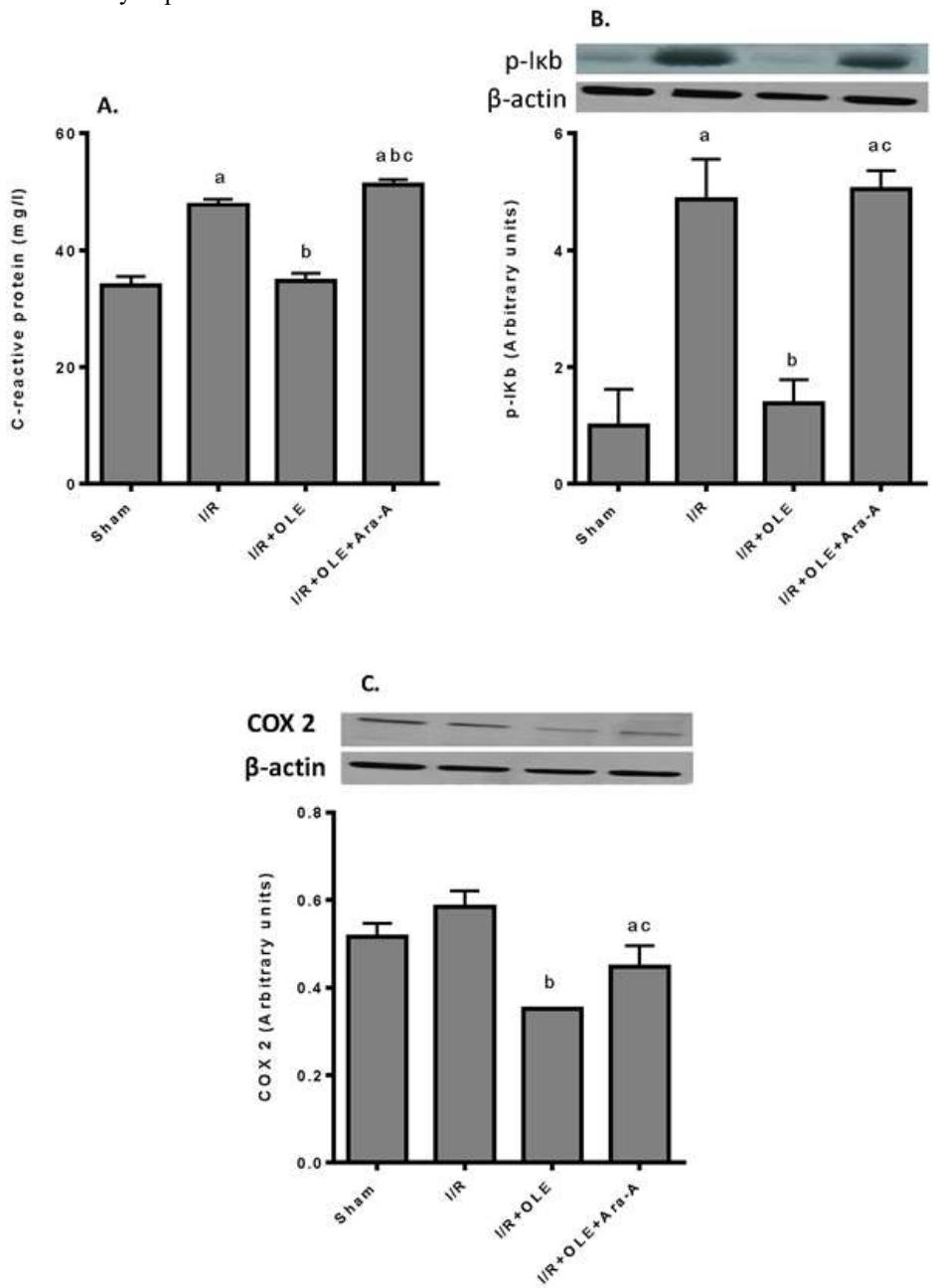

Fig. 5. Effect of oleuropein on inflammatory markers in plasma and renal tissues. Plasma C-reactive protein (CRP) concentration (A) and renal protein expression levels of phosphorylated IκB (p-IκB) (B) and cyclooxygenase-2 (COX 2) (C) were evaluated. Representative Western blots are shown in the upper panels, and corresponding densitometric analyses normalized to β-actin are shown in the lower panels. Data are expressed as mean ± SEM (n = 6 per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Different letters indicate statistically significant differences ($p < 0.05$): a vs Sham group; b vs I/R group; c vs I/R + OLE group.

These results are consistent with those of our previous work which indicated the efficacy of OLE in counteracting renal I/R injury [10].

Oxidative stress plays a critical role in the pathophysiology of I/R-induced kidney injury. I/R generates ROS and causes the depletion of antioxidant defenses [18]. Our results showed that OLE possesses antioxidant properties. This is in line with previous studies showing that OLE could act as a free radical scavenger, by decreasing ROS levels [19,20]. In parallel, OLE pretreatment enhanced the activity of the antioxidant enzymes, which is consistent with several studies highlighting its protective properties [10,21].

Numerous data have indicated that oxidative stress is closely associated with inflammation [22]. ROS can activate NF-κB, which then dissociates from IκB and translocates to the nucleus to regulate the production of inflammatory mediators such as COX 2 [23]. In the current study, we observed NF-κB activation and elevated COX 2 expression in response to renal ischemia. Additionally, CRP levels

rapidly elevated in various inflammatory states and are correlated with impaired kidney function [24,25]. In fact, a previous report indicated that CRP exacerbates kidney IRI by down-regulating autophagy and activating apoptosis [26]. Our results demonstrated that OLE reduced NF-κB activation, COX 2 expression and CRP concentration after renal I/R, in agreement with previous findings [10,23].

Dysfunction of AMPK signaling pathway contributes significantly to I/R-induced oxidative stress and inflammation [2,27]. It is widely demonstrated that AMPK controls the enzymes and the signaling pathways that determine redox balance including SOD, catalase, and GPx. Indeed, Activation of AMPK limits ROS formation and lipid peroxidation, further mitigating oxidative damage [27].

Moreover, AMPK signaling inhibits the inflammatory response by inhibiting NF-κB signaling through stabilization of IκB, resulting in reduced transcription of pro-inflammatory mediators such as COX 2 [28]. The current study showed that pharmacological inhibition of AMPK abolished the ability of oleuropein to enhance antioxidant defenses, as evidenced by reduced SOD, CAT, GPx, GSH, and PSH levels, and to decrease MDA accumulation. Furthermore, AMPK inhibition reversed the suppressive effects of oleuropein on IκB degradation and COX-2 expression. Collectively, these results suggest that the antioxidant and anti-inflammatory effects of oleuropein in renal I/R are mediated directly through AMPK activation. Nevertheless, future studies using genetic approaches or more selective AMPK modulators will be necessary to further confirm the causal involvement of AMPK.

5. Conclusion

In conclusion, we demonstrated that OLE has a protective effect on kidney under ischemic conditions. We also highlighted that OLE could reduce oxidative stress and inflammation through AMPK activation after ischemia in rat kidney. To our knowledge, this study provides new evidence supporting the involvement of AMPK activation in the protective effects of OLE against renal ischemia–reperfusion injury.

Acknowledgements

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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