

Short Communication

Oleuropein, a natural extract from olive leaves, protects against renal ischemia-reperfusion injury via AMPK-independent regulation of autophagy and apoptosis

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Although recent studies have indicated the protective actions of oleuropein (OLE), its effect on autophagy under ischemia/reperfusion (I/R) conditions remains unclear. We aimed to assess whether OLE's action depends on the modulation of AMPK-mediated autophagy and apoptosis pathway in a rat model of renal I/R injury. OLE pretreatment activated autophagy markers through the phosphorylation of AMPK and its downstream target molecules and reduced apoptosis. However, Ara-A had no influence on these actions. Autophagy and apoptosis regulation by OLE were maintained despite AMPK inhibition. These findings suggest that OLE pretreatment reduces kidney injury following I/R, and this effect is related to the activation of autophagy and the inhibition of apoptosis. Importantly, the protective action of OLE is not dependent on the AMPK pathway.

Keywords: AMPK, Bax, Bcl-xL, Beclin-1, LC3 II, Oleuropein, p62

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Introduction

Renal ischemia-reperfusion injury (IRI) is the main cause of acute kidney injury, characterized by rapid and sudden kidney dysfunction leading to high morbidity and mortality rates. IRI occurs during many clinical settings, and remains the primary cause of graft dysfunction following kidney transplantation¹. Autophagy is an important regulator in the cell responses to IRI^{2,3}. Many regulators of autophagy have been described, including AMP-activated protein kinase (AMPK). In turn, activated AMPK promotes autophagy

by phosphorylation and activation of beclin-1, a crucial initiator of autophagosome formation⁴. Under stress, autophagy acts as a recycling system and helps remove damaged organelles and proteins¹. Furthermore, enhanced autophagy seems to play a key role in preventing cell apoptosis, so attenuating IRI⁵. Therefore, therapeutic agents targeting autophagy and its downstream apoptosis might provide a powerful strategy to reduce IRI. Recently, several studies investigated the efficiency of “natural” or alternative medicines in I/R conditions⁶. Polyphenols have attracted increased attention due to their low toxicity, easy accessibility, and multi-target potential⁷. Oleuropein (OLE), the main bioactive compound of olive leaves, has been shown to alleviate IRI by attenuating inflammation and apoptosis in ischemic brain⁸ and heart⁹. Despite these findings, the role of OLE on autophagy under I/R conditions remains unclear. In the present study, we attempted to investigate the potential of OLE to activate autophagy in a rat model of renal IRI. The AMPK-mediated autophagy pathway was investigated as a possible positive mechanism of OLE effects. Given the established role of AMPK in promoting both autophagy and apoptosis, we hypothesized that OLE confers its renoprotective effects, at least partially, through AMPK-dependent pathways.

Materials and Methods

Oleuropein preparation

Oleuropein (OLE) was extracted from olive leaves (*Olea europaea* L.) as previously described¹⁰. For oral administration to rats, OLE was dissolved in water.

Animals

The European guidelines regulating 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) were adhered to during experimentations. Male Wistar rats weighing between 180 g and 220 g were used and were kept in a 12:12 h light: dark cycle and in regulated temperature of 21±2°C. Food and water were freely available to rats. The animals (n = 6) were split into four experimental groups at random. Group Sham where rats were subjected to renal pedicle dissection without clamping. I/R group was similar to sham group, except that the renal pedicles were clamped

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for 60 minutes and then reperfused for 120 minutes. I/R + OLE group where 50 mg/kg of body weight of OLE was administrated to rats for three consecutive days before ischemia¹⁰. Finally, I/R + OLE + Ara-A group was similar to the I/R + OLE group, but rats were infused for 10 minutes with Ara-A at a rate of 100 μ g/kg per minute immediately before the onset of ischemia, with the infusion discontinued during both ischemia and reperfusion¹¹.

Surgery and experimental protocol

A well-established model of renal I/R injury was used¹². Briefly, after anaesthesia with 1.5% isoflurane at a rate of 0.8 L/min, abdominal incision was performed, and the renal pedicle was dissected and clamped. A heating pad maintained the body temperature at 37°C throughout the surgery. 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. 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 a commercially available kit (Beckman Coulter, Inc., USA).

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 using the Bradford method. Protein extracts (60 μ g) were then separated by SDS-PAGE electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were immunoblotted with antibodies directed against total AMPK, p-AMPK, p-PKA, LC3 I/II, p62, beclin-1, Bcl xl, Bax and β -actin (Cell Signaling, respectively). 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).

Statistical analysis

Data are expressed as mean \pm SEM (n = 6 for each group). Prior to statistical analysis, data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. They were 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 $P < 0.05$.

Results

Effect of OLE on AMPK phosphorylation

As indicated in Fig. 1a, phosphorylated AMPK (p-AMPK) levels were similar in Sham and I/R

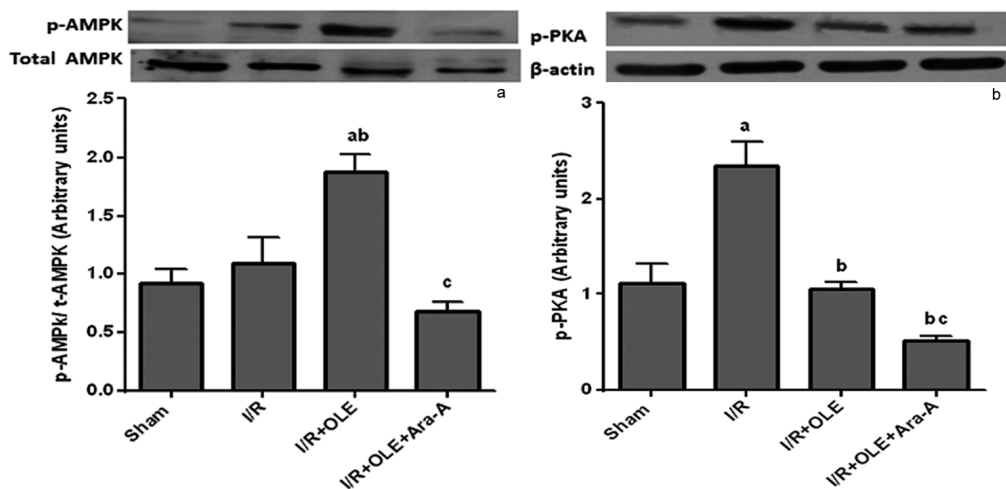


Fig. 1 — Effects of oleuropein (OLE) and AMPK inhibition on phosphorylated AMPK and PKA levels in renal tissues. Levels of phosphorylated a) AMPK and b) PKA in renal tissues. β -actin was used as a loading control. One representative blot of six independent experiments is shown at the top, whereas densitometric analysis is at the bottom. Data are expressed as mean \pm SEM. $ap < 0.05$ vs Sham; $bp < 0.05$ vs I/R; $cp < 0.05$ vs I/R+OLE.

groups. OLE increased p-AMPK protein levels when compared to the sham and I/R groups. As expected, Ara-A, an AMPK inhibitor, significantly reduced p-AMPK compared to the I/R+OLE group. Compared to the sham group, renal I/R greatly enhanced p-PKA (Fig. 1b). This activation was then significantly attenuated by OLE administration. After Ara-A administration, no significant changes were observed.

Effect of OLE pretreatment on renal function and injury

As indicated in Table 1, I/R induced a significant elevation in plasma creatinine concentration

compared to the sham group. OLE administration significantly prevented the creatinine decline. Next, we assessed cytolysis by measuring LDH activity. We found that I/R markedly increased LDH release as compared to the sham group, which was reversed by OLE treatment. Interestingly, the inhibition of AMPK suppressed the effects of OLE on renal function and injury parameters.

Effect of OLE on autophagy-related proteins

As shown in Fig. 2a and 2b, respectively, beclin-1 and LC3 II were significantly decreased while p62

Table 1 — Evaluation of creatinine concentration and lactate dehydrogenase activity in rats subjected to renal ischemia-reperfusion

	Sham	I/R	I/R+OLE	I/R+OLE+ara-A
Creatinine (μmol/L)	18±1	65±2 ^a	41±3 ^{ab}	56±5 ^{ac}
LDH (U/L)	699±79	2041±175 ^a	1140±130 ^b	1330±7 ^{ac}

Data are expressed as mean ± SEM (n = 6 for each group). ^a*p* <0.05 vs Sham; ^b*p* <0.05 vs I/R; ^c*p* <0.05 vs I/R+OLE

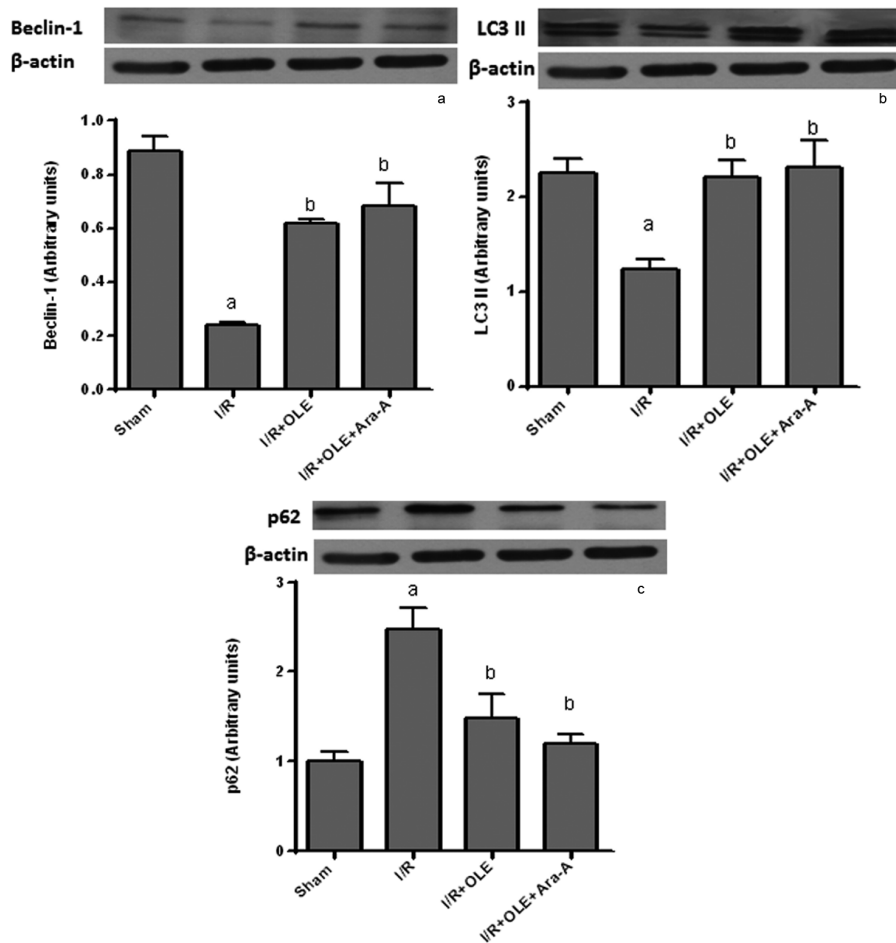


Fig. 2 — Effect of oleuropein (OLE) on autophagy markers in renal tissues after ischemia-reperfusion (I/R). Levels of a) beclin-1, b) LC3 II, and c) p62 in renal tissues. β-actin was used as a loading control. One representative blot of six independent experiments is shown at the top, whereas densitometric analysis is shown at the bottom. Data are expressed as mean ± SEM. *ap* <0.05 vs Sham; *bp* <0.05 vs I/R; *cp* <0.05 vs I/R+OLE.

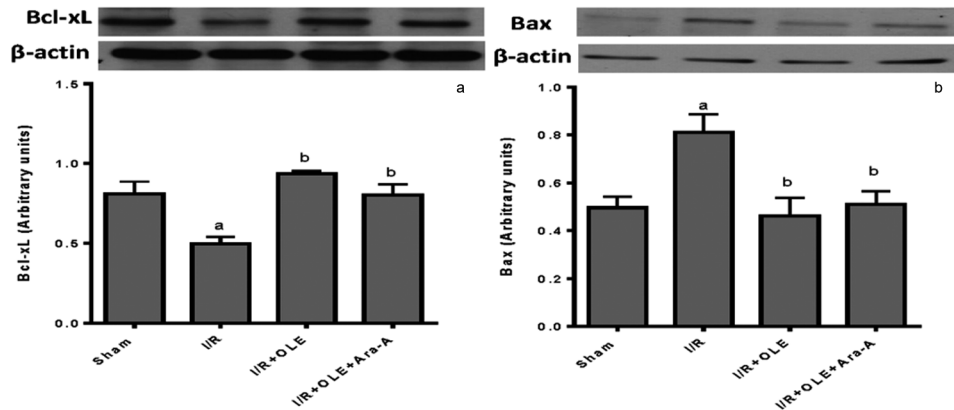


Fig. 3 — Effect of oleuropein (OLE) on apoptosis-related proteins in renal tissues following ischemia-reperfusion (I/R). Levels of a) Bcl xL, and b) Bax in renal tissues. β -actin was used as a loading control. One representative blot of six independent experiments is shown at the top, whereas densitometric analysis is shown at the bottom. Data are expressed as mean \pm SEM. *ap* <0.05 vs Sham; *bp* <0.05 vs I/R; *cp* <0.05 vs I/R+OLE.

levels (Fig. 2c) increased after I/R treatment compared with the sham group. Pretreatment with OLE significantly enhanced the LC3 II and beclin-1 levels and reduced p62 levels. After Ara-A administration, no significant changes were observed in any of these parameters.

Effect of OLE on apoptosis

As shown in Fig. 3, I/R induced the up-regulation of Bax expression and the downregulation of Bcl-xL expression. However, OLE treatment elevated the Bcl-xL levels and decreased Bax levels compared to the I/R group. AMPK inactivation did not affect any of these parameters.

Discussion

Polyphenols have been widely explored for their protective potential, often attributed to their antioxidative property^{13,14}. In the present study, we highlighted that OLE significantly reduced IRI in rat kidneys by improving autophagy and inhibiting apoptosis via an AMPK-independent 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. Our previous work proved that OLE could preserve kidney function parameters and membrane integrity. We further demonstrated the efficacy of OLE in counteracting renal IRI through its high antioxidant and anti-inflammatory capacity. We finally showed that OLE preserved mitochondrial integrity and reduced caspase 3-mediated apoptosis¹⁰.

Consistent with these observations, plasma creatinine concentration and LDH activity assessment revealed that OLE effectively preserved kidney

function parameters and maintained membrane integrity. This protective effect is likely attributed to the potent antioxidant capacity of OLE. These results align with our previous research, which also highlighted the effectiveness of OLE in mitigating renal IRI¹⁰. Importantly, these renoprotective actions of OLE on both renal function and injury markers were abolished after AMPK inhibition. This is in line with a study showing the direct antioxidant and cytoprotective effects of oleuropein via AMPK signaling¹⁵. Next, we investigated if the protective effect of OLE could be associated with autophagy and, if so, it could be related to the AMPK signaling pathway. Interestingly, our results demonstrated that oral administration of OLE significantly decreases the cAMP-dependent protein kinase A (PKA) phosphorylation, an AMPK inhibitor. Interestingly, this was accompanied by increased autophagic vesicles, confirmed by an increase of beclin-1 and LC3 II levels. Moreover, we noticed a significant decrease in phosphorylated p62, suggesting an enhanced autophagic flux. Accordingly, all these data suggest that OLE promotes autophagy. Our results are in agreement with other studies establishing a protective effect of OLE from neurodegeneration¹⁶ and Alzheimer's disease¹⁷ through the induction of autophagy. We further established that the protective effect of OLE is AMPK signaling pathway independent. Surprisingly, the expressions of beclin-1, LC3 II, and p62 were not dependent on AMPK phosphorylation. Our findings diverge from previous studies in other experimental models where OLE was shown to promote autophagy through AMPK activation¹⁶⁻¹⁸. However, numerous studies suggested

that oleuropein could engage alternative, AMPK-independent mechanisms to promote autophagy, such as Activating SIRT1¹⁹ or promoting TFEB nuclear translocation²⁰. Our results supported the idea that OLE stimulates autophagy through Beclin-dependent mechanisms, potentially regulated by SIRT1 and TFEB independently from AMPK pathways. Finally, we evaluated the impact of OLE administration on kidney apoptosis by determining Bcl-xl and Bax. In the present study, we found that OLE up-regulated Bcl-xl and down-regulated Bax expressions, thereby maintaining mitochondrial membrane stabilization and reducing the activation of the intrinsic apoptotic pathway^{8,9}. Consistent with our previous results, the inhibition of AMPK did not affect the protective role of OLE against mitochondria-mediated cell death. It is obvious that OLE regulates cell apoptosis through targeting numerous downstream pathways or by directly affecting mitochondrial function. This aligns with previous research indicating that oleuropein can modulate apoptosis through various AMPK-independent mechanisms^{19,21}.

In conclusion, our study determined that OLE has a beneficial effect on the kidneys under ischemic conditions. Its beneficial effects on renal function and membrane integrity are partially mediated by AMPK activation, as they were abolished by AMPK inhibition. However, OLE-induced autophagy and inhibited apoptosis are AMPK-independent. These findings highlighted the relevance of olive leaf-derived compounds as promising natural agents for plant-based interventions in renal injury.

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Disclosure statement

The authors declare there are no competing interests.

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