Involvement of AMP-activated protein kinase in the protective effect of melatonin against renal ischemia reperfusion injury

Kaouther HADJ AYED TKA1, Asma MAHFOUDH BOUSSAID1, Kaouther KESSABI2, Rym KAMMOUN3, Imed MESSAOUDI3, Sonia GHOUL MAZGAR3, Joan ROSELLO CATAFAU4, Hassen BEN ABDENNEBI1,*

1Unit of Molecular Biology and Anthropology Applied to Development and Health (UR12ES11), Faculty of Pharmacy, University of Monastir, Monastir, Tunisia
2Laboratory of Genetics, Bio-Diversity and Valorization of Bio-Resources (LR11ES41), Institute of Biotechnology, University of Monastir, Monastir, Tunisia
3Laboratory of Histology and Embryology, Faculty of Dental Medicine, University of Monastir, Monastir, Tunisia
4Unit of Experimental Hepatic Ischemia-Reperfusion, Institute of Biomedical Investigations, Higher Council of Scientific Investigations, Barcelona, Spain

Abstract: We investigated the implication of AMP-activated protein kinase (AMPK) in melatonin-induced protection in a renal ischemia/reperfusion (I/R) model. Animals were divided into four groups: rats of the sham group were not subjected to renal I/R. Rats in the I/R group received the vehicle 30 min before renal ischemia for 1 h followed by reperfusion for 6 h. Rats in the Mel+I/R group received melatonin (10 mg/kg) 30 min before the onset of I/R. Rats in the Mel+I/R+araA group received araA (AMPK inhibitor, 100 µg/kg per min for 10 min) just before melatonin administration. The results showed that melatonin treatment induced a significant improvement in renal function, morphology, and antioxidant status, as well as Akt pathway activation. It also decreased cytolysis and endoplasmic reticulum stress. Inhibition of AMPK by araA administration completely abolished the effects of melatonin in regard to the above parameters except for Akt and some of its downstream target molecules. These results demonstrate that the effects of melatonin on renal I/R are, for the most part, linked to AMPK activation, with the exception of the Akt pathway, which seems to be independent of AMPK.

Key words: Ischemia/reperfusion, kidney, melatonin, AMP-activated protein kinase, oxidative stress, Akt

1. Introduction
Acute kidney injury remains an important health concern due to the high morbidity and mortality rates. It is most frequently caused by ischemia/reperfusion (I/R) syndrome (Kumagai et al., 2009). Ischemia starves cells of oxygen and nutrients, resulting in depletion of ATP, accumulation of waste products, and production of reactive oxygen species (ROS). Restitution of blood flow with reperfusion sustains renal injury through further generation of ROS (Bidmon et al., 2012). ROS are capable of reacting with proteins, lipids, and nucleic acids leading to protein denaturation, lipid peroxidation, and DNA damage (Bidmon et al., 2012; Wang et al., 2012a). Many antioxidants have been the focus of studies for developing strategies for use in I/R therapy (Mahfoudh Boussaid et al., 2007; Wang et al., 2012a; Aydogan et al., 2013). Along with others, we have shown that the antioxidant molecule, melatonin, has a significant protective action against renal damage that occurs during I/R injury (Aktoz et al., 2007; Ersoz et al., 2009; Hadj Ayed Tka et al., 2015). Effectively, there is a wide range of intracellular pathways that intervene in melatonin protection, but a cogent description of how they relate to each other is lacking.

A growing body of evidence has proven that AMP-activated protein kinase (AMPK) is a key modulator of cell survival under conditions that increase intracellular AMP, such as hypoxia (Hardie and Carling, 1997). It is a master metabolic regulator that orchestrates the regulation of cellular energy homeostasis (Sid al., 2013). Moreover, AMPK was shown to play a crucial role in defending various organs including the liver (Peralta et al., 2001), the kidney (Lempiäinen et al., 2012), and the heart (Morrison et al., 2011) against I/R injury. On the basis of this, it would be interesting to investigate its possible involvement in melatonin-induced protection during I/R. Therefore, the aim of this study was to explore whether and to what extent AMPK mediates this protection.
2. Materials and methods

2.1. Animals and drugs

All animal care and experimental protocols complied with the European Union Regulations (Directive 86/609 EEC) for animal experiments. Adult male Wistar rats (200–250 g) were housed under standard conditions (12 h light/dark cycle) and were permitted ad libitum access to water and food. Melatonin (Sigma, St Louis, MO, USA) was dissolved in ethanol and further diluted in saline (0.9% NaCl) to produce a final alcohol concentration of 1% and was administered at a dose of 10 mg/kg via intraperitoneal (i.p.) route (Ersoz et al., 2009; Sehajpal et al., 2014). Adenine 9-β-D-arabinofuranoside (araA; Sigma), an inhibitor of AMPK, was dissolved in saline (0.9% NaCl) and was infused at a dose of 100 µg/kg per min intravenously (i.v.) for 10 min (Zaouali et al., 2013).

2.2. Experimental groups

Rats were randomly assigned to one of the following experimental groups, each of them consisting of six animals:

- Sham group: animals in this group were sham operated, and both renal pedicles were excised but were not subjected to I/R.

- I/R group: animals received the vehicle (ethanol + NaCl 0.9%, i.p.) 30 min before they were subjected to 60 min of renal ischemia followed by reperfusion for 6 h (Ersoz et al., 2009).

- Mel+I/R group: animals were injected with melatonin (10 mg/kg) 30 min before the onset of I/R.

- Mel+I/R+araA group: same treatment as in the previous group, but animals were treated with araA (100 µg/kg per min) just before melatonin injection.

2.3. Surgery and experimental design

Anesthesia was achieved with an injection of ketamine hydrochloride (50 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and the body temperature was maintained at 36–38 °C by placing the rats on a thermal blanket. Animals were submitted to a midline laparotomy, and ischemia was induced by bilateral renal pedicle clamping for 60 min (with the exception of the sham group) with smooth vascular clamps, as previously described (Mahfoudh Boussaid et al., 2012). After the clamps were released, the incisions were closed by running suture, and rats were allowed to recover and kept warm. Five hours later, animals were again submitted to anesthesia and catheters were inserted into: i. the bladder for urine collection during the last 30 min of reperfusion; ii. the right carotid artery to control the mean arterial pressure (Pression Monitor BP-1; Pression Instruments, Sarasota, FL, USA) and collect blood samples; and iii. the left jugular vein for mannitol (10%) and heparin (50 U/mL) infusion (Minipuls 3 peristaltic pump, Gilson, France). At the end of the reperfusion period (6 h), rats were euthanized and plasma, urine, and tissue samples were stored at −80 °C until analyzed.

2.4. Renal function

As previously described by Mahfoudh Boussaid et al. (2012), renal function was assessed in terms of creatinine clearance calculated using the following formula:

\[ \text{Creatinine clearance (µL/min per g kidney weight) = (creat u \times V)/creat p} \]

where

- \( creat u \) = creatinine concentration in urine (µmol/L),
- \( V \) = urine flow (µL/min/g kidney weight), and
- \( creat p \) = creatinine concentration in plasma (µmol/L).

Creatinine concentrations in plasma and urine were measured using a standard diagnostic kit (BioMérieux, France).

2.5. Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity in plasma was measured to evaluate cytolysis using a commercial assay kit, according to manufacturer’s instructions (Spinreact, Spain).

2.6. Lipid peroxidation

Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) in renal tissue with the thiobarbiturate reaction (Ben Mosbah et al., 2006).

2.7. Histological analysis

Renal biopsies were fixed in 10% formaldehyde solution and then dehydrated and processed in paraffin according to routine protocols. Renal specimens were then sectioned (8 µm) and stained with standard hematoxylin-eosin (H&E). A total of 10 sections per kidney were observed by light microscopy at a magnification of 40–400 by an experienced pathologist who had no information about the treatment groups. The extent of renal necrosis was semiquantitatively evaluated and scored from 0 to 4, as reported by Jablonski et al. (1983). The scoring system is as follows: 0: normal; 1: necrosis of individual cells; 2: necrosis of all cells in adjacent proximal convoluted tubule (PCT), with survival of surrounding tubules; 3: necrosis confined to distal third PCT with bands of necrosis extending across inner cortex; 4: necrosis of all three segments of PCT.

2.8. Superoxide dismutase activity

The total (Cu-Zn and Mn) superoxide dismutase (SOD) activity in renal tissue was determined according to the method of Marklund and Marklund (1974). It is based on the inhibition of pyrogallol oxidation. One unit of SOD was defined as the enzyme amount causing 50% inhibition of pyrogallol oxidation. The SOD activity was measured at 420 nm and expressed as U/mg protein.

2.9. Catalase activity

The renal activity of catalase was measured using the method described by Claiborne (1985). It consists of
following the decomposition rate of hydrogen peroxide spectrophotometrically at 240 nm. Results were expressed as µmol H₂O₂ decomposed/mg protein per min.

2.10. Glutathione peroxidase activity
Glutathione peroxidase (GPx) activity in the kidney was quantified by measuring the decrease in absorbance at 412 nm of glutathione (GSH) in the presence of H₂O₂, as reported by Flohe and Gunzler (1984). The activity was expressed as µmol oxidized GSH/min per mg protein.

2.11. Sulphydryl content
The sulphydryl content was estimated by measuring protein-bound sulphydryls (PSH) and GSH concentrations in the renal tissue. PSH concentration was determined by subtracting the nonprotein sulphydryl (NPSH) content from the total sulphydryl content (TSH) (Sedlak and Lindsay, 1968). TSH and NPSH contents were measured at 412 nm after the reaction with 5’-dithiobis 2-nitrobenzoic acid (DTNB). Total GSH concentration in the kidney was quantified at 412 nm by DTNB reaction, according to the method described by Tietze (1969).

2.12. Western blotting
The kidney samples were homogenized as described previously (Mahfoudh Boussaid et al., 2012). Tissue lysates were then resolved by SDS-PAGE electrophoresis, and proteins were transferred onto polyvinylidene fluoride membranes. Blots were probed with antibodies against the following proteins: total and p-AMPK, total and p-Akt, total and phosphorylated glycogen synthase kinase-3 beta (p-GSK3β), heat shock protein 70 (HSP70), p-P70S6K (Cell Signaling Technology Inc., Beverly, MA, USA), total and phosphorylated RNA-activated protein kinase (PKR)-like ER kinase (p-PERK), activating transcription factor 4 (ATF4), CCAAT/enhancer binding protein homologous protein (CHOP), tumor necrosis factor receptor-associated factor 2 (TRAF2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), heme oxygenase 1 (HO-1), and b-actin (Sigma, St Louis, MO, USA) and subsequently probed with horseradish-peroxidase–conjugated polyclonal antibodies. The bands were visualized using an enhanced chemiluminescence kit (Bio Rad Laboratories, Hercules, CA, USA). The values were obtained by densitometric scanning and the Quantity One software program (Bio Rad Laboratories).

2.13. Statistical analysis
Data are expressed as mean ± SEM values. Differences between groups were examined for statistical significance using one-way ANOVA followed by Newman–Keuls multiple comparisons (Graph Pad Prism software, version 4 for Windows). The level of statistical significance was set at P < 0.05.

3. Results
Immunoblot analysis of Figure 1 indicates that p-AMPK levels were similar in both sham and I/R groups. AMPK phosphorylation was significantly enhanced by melatonin treatment compared to I/R group (P < 0.05). As expected, the use of araA, an AMPK inhibitor, dramatically decreased the levels of p-AMPK in comparison to all other groups (P < 0.05).

As illustrated in the Table, animals that underwent renal I/R exhibited a significant increase in LDH and MDA levels, while their creatinine clearance and PSH content decreased significantly in comparison to values obtained from sham-operated rats (P < 0.05). Melatonin treatment induced a significant reduction in LDH and MDA and markedly raised the creatinine clearance and PSH concentration as compared to the I/R group (P < 0.05). The inhibition of AMPK suppressed the effects of melatonin in all of these parameters.

Histological evaluation of renal injury is consistent with these biochemical parameters. In fact, kidneys of the sham group did not show any histopathological changes (Figure 2A), whereas features of renal damage and necrosis including a consistent loss of nuclei, cell swelling, tubular dilatation, and hemorrhage were markedly observed in those belonging to the I/R group (Figure 2B). Although a lower degree of injury was observed after melatonin treatment (Figure 2C), the association of melatonin with araA negated this improvement, and renal tissues demonstrated severe tubular damage (Figure 2D).

These results were represented graphically in terms of necrosis grading scale among the different groups (Figure 2E).

![Figure 1](image_url)

**Figure 1.** Western blot showing protein levels of phosphorylated and total AMP-activated protein kinase in renal tissues after in vivo ischemia/reperfusion. Representative blot is shown at the top, and densitometric analysis is shown at the bottom. Results are expressed as mean ± SEM (n = 6 for each group). * P < 0.05 vs. sham; # P < 0.05 vs. I/R; § P < 0.05 vs. Mel+I/R.
**Table.** Evaluation of creatinine clearance, lactate dehydrogenase activity, protein-bound sulfhydryls, and malondialdehyde concentrations among the different experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>I/R</th>
<th>Mel+I/R</th>
<th>Mel+I/R+araA</th>
</tr>
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<tbody>
<tr>
<td>Creatinine clearance (μL/min/g)</td>
<td>613 ± 47</td>
<td>10 ± 3*</td>
<td>115 ± 3**</td>
<td>11 ± 2*§</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>700 ± 81</td>
<td>3838 ± 386*</td>
<td>1512 ± 412*</td>
<td>8486 ± 918**§</td>
</tr>
<tr>
<td>PSH (µg/mg protein)</td>
<td>15 ± 1.5</td>
<td>6 ± 0.8*</td>
<td>17 ± 1.5*</td>
<td>9 ± 0.6§</td>
</tr>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>0.122 ± 0.02</td>
<td>0.6 ± 0.07*</td>
<td>0.28 ± 0.04*</td>
<td>0.5 ± 0.02§</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (n = 6 for each group). * P < 0.05 vs. sham; # P < 0.05 vs. I/R; § P < 0.05 vs. Mel+I/R.

**Figure 2.** Representative histological photographs of kidney tissues from sham (A); I/R (B); Mel+I/R (C); and Mel+I/R+araA (D) groups, (H&E) × 200. Semiquantitative assessment of renal necrosis among the different experimental groups using the Jablonski score (E). H: hemorrhage, L: nuclei loss, and D: tubular dilatation. Results are expressed as mean ± SEM (n = 6 for each group). * P < 0.05 vs. sham. # P < 0.05 vs. I/R; § P < 0.05 vs. Mel+I/R.
We then examined the possible involvement of melatonin in stimulating other antioxidant systems and whether this stimulation could be mediated by changes in AMPK phosphorylation. I/R resulted in a significant reduction in HO-1 level (Figure 3A) and GSH concentration (Figure 3B) along with diminution of SOD (Figure 3C), catalase (Figure 3D), and GPx (Figure 3E) activities in the kidney in comparison to sham-operated animals (P < 0.05). Rats treated with melatonin had significantly higher antioxidant enzyme activities and HO-1 and GSH content in their kidneys than nontreated animals (P < 0.05). In contrast, the administration of AMPK inhibitor completely abolished the melatonin-mediated antioxidant action; thus, catalase, SOD, and GPx activities and HO-1 and GSH amounts returned to I/R levels.

As a next step, we investigated the relationship between melatonin, Akt, and AMPK pathways. Western blot analyses show that p-Akt (Figure 4A), HSP70 (Figure 4B), and p-P70S6K (Figure 4C) levels were markedly reduced after I/R when compared to the sham group (P < 0.05). However, there were no significant changes between the I/R and sham groups regarding GSK-3β phosphorylation (Figure 4D). Intraperitoneal injection of melatonin produced a significant rise in p-Akt, HSP70, p-P70S6K, and p-GSK-3β amounts in comparison to their relative I/R groups. After araA administration, no significant changes were observed in any of these parameters.

Finally, we focused on the role of AMPK in the melatonin-mediated effect on endoplasmic reticulum (ER) stress parameters. When compared to the sham-operated group, renal I/R greatly enhanced p-PERK (Figure 5A), ATF4 (Figure 5B), CHOP (Figure 5C), and TRAF2 levels (Figure 5D) (P < 0.05). This activation was then significantly attenuated by melatonin administration compared to the I/R groups (P < 0.05). Combined pretreatment of the rats with araA and melatonin significantly inhibited the effects of melatonin alone; thus, p-PERK, ATF4, CHOP, and TRAF2 levels rose noticeably (P < 0.05).

Figure 3. Western blot showing protein levels of heme oxygenase 1 and β-actin. Representative blot is shown at the top, and densitometric analysis is shown at the bottom (A). Glutathione concentration (B), superoxide dismutase activity (C), catalase activity (D), and glutathione peroxidase activity (E) in renal tissues after in vivo ischemia/reperfusion. Results are expressed as mean ± SEM (n = 6 for each group). * P < 0.05 vs. sham; # P < 0.05 vs. I/R; § P < 0.05 vs. Mel+I/R.
4. Discussion

In this study, we shed some light on the molecular mechanisms whereby melatonin modulates renal I/R injury. These mechanisms appear to be dependent on AMPK activation. The examination of AMPK phosphorylation levels showed that melatonin treatment greatly enhanced AMPK activation. Given the key role of AMPK in modulating several signaling pathways, we hypothesized that melatonin exerts its I/R-related protection through its ability to activate AMPK. As a result, we examined the effects of melatonin on some intracellular axes with and without the use of an AMPK inhibitor.

The current study showed that exogenous melatonin improved the renal function and cell integrity of ischemic kidneys. Indeed, this effect was completely abolished by the use of araA. In another context, it is well known that oxidative damage to proteins and lipids is reflected by a decrease in PSH level (Dubey et al., 1996) and an increase in the level of MDA (Aktoz et al., 2007). Herein, melatonin preconditioning was found to preserve the PSH pool and decrease MDA formation. Again, this effect was notably suppressed subsequent to AMPK inhibition, suggesting its involvement in the melatonin-related protective mechanism regarding protein and lipid oxidation, renal function, and cellular deterioration.

Oxidative damage to proteins and lipids might result from alterations in antioxidant defense capacities. Our study provides evidence that renal I/R causes a significant increase in oxidative status, measured as a lower protein level of HO-1 as well as lower catalase, SOD, and GPx activities and GSH content. As expected, pre-ischemic melatonin treatment effectively enhanced these antioxidant systems. The blockage of AMPK activation using araA completely suppressed the antioxidant action of melatonin. This implies that the induction of antioxidants produced by melatonin involves activation of the AMPK signaling system. Interestingly, it has been proven that p-AMPK induces expression of genes involved in antioxidant defense, such as SOD, catalase, and HO-1 (Wang et al., 2012b). This allows it to control the redox balance and mitochondrial function and suppress the cell death induced by oxidative stress, in addition to its traditional role as an energy sensor (Kim et al., 2009).
With the aim of investigating other pathways that melatonin could activate and discovering whether this activation requires AMPK, the phosphorylation levels of Akt and its downstream targets HSP70, P70S6K, and GSK-3β were examined. Of marked interest was the observation that melatonin treatment provoked significant enhancement in p-Akt, HSP70, p-P70S6K, and p-GSK-3β levels after renal I/R. This is important since Akt has been shown to promote cell survival signals, in part, through GSK-3β and P70S6K phosphorylation (Mahfoudh Boussaid et al., 2012; Park et al., 2014) as well as HSP70 enhancement (Yeh et al., 2010). Notably, AMPK inhibition had no significant effect on the melatonin-induced activation of Akt, as our data showed. The examination of HSP70, p-P70S6K, and p-GSK-3β levels further supported the hypothesis that AMPK was not involved in the melatonin-mediated activation of Akt. In light of these findings, we suggest that there is no interaction between the AMPK and Akt/HSP70/P70S6K/GSK-3β signaling cascades.

The last step of this investigation consists of evaluating the role of AMPK in the melatonin-mediated effect on ER stress. We observed that the blockage of AMPK activation restrained the melatonin-induced decrease in p-PERK, ATF4, CHOP, and TRAF2. This confirms a close relationship between AMPK and ER stress modulation. Consistent with the present study, Zaouali et al. (2013) reported the relevance of AMPK inhibition on an ER-stress rise in hepatic I/R.

In conclusion, the present study elucidated some of the molecular mechanisms whereby melatonin mediates renoprotection during I/R. The signaling cascades involved in this protection appear to be dependent on AMPK. This may be particularly true for antioxidant capacities and ER stress modulation, while the effect of melatonin on the Akt pathway seems to be independent of AMPK.

**Acknowledgment**

This work was supported by the Tunisian Ministry of Higher Education and Scientific Research.
References


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