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Original article

Phytomodulatory proteins isolated from Calotropis procera latex promote glycemic control by improving hepatic mitochondrial function in HepG2 cells

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ABSTRACT

The medicinal uses of Calotropis procera are diverse, yet some of them are based on effects that still lack scientific support. Control of diabetes is one of them. Recently, latex proteins from *C. procera* latex (LP) have been shown to promote *in vivo* glycemic control by the inhibition of hepatic glucose production via AMP-activated protein kinase (AMPK). Glycemic control has been attributed to an isolated fraction of LP (CpPII), which is composed of cysteine peptidases (95%) and osmotin (5%) isoforms. Those proteins are extensively characterized in terms of chemistry, biochemistry and structural aspects. Furthermore, we evaluated some aspects of the mitochondrial function and cellular mechanisms involved in CpPII activity. The effect of CpPII on glycemic control was evaluated in fasting mice by glycemic curve and glucose and pyruvate tolerance tests. HepG2 cells was treated with CpPII, and cell viability, oxygen consumption, PPAR activity, production of lactate and reactive oxygen species, mitochondrial density and protein and gene expression were analyzed. CpPII reduced fasting glycemia, improved glucose tolerance and inhibited hepatic glucose production in control animals. Additionally, CpPII increased the consumption of ATP-linked oxygen and mitochondrial uncoupling, reduced lactate concentration, increased protein expression of mitochondrial complexes I, III and V, and activity of peroxisome-proliferator-responsive elements (PPRE), reduced the presence of reactive oxygen species (ROS) and increased mitochondrial density in HepG2 cells by activation of AMPK/PPAR. Our findings strongly support the medicinal use of the plant and suggest that CpPII is a potential therapy for prevention and/or treatment of type-2 diabetes. A common epitope sequence shared among the proteases and osmotin is possibly the responsible for the beneficial effects of CpPII.

Abbreviations: LP, Soluble latex proteins from *Calotropis procera*; CpPII, Major peptidase fraction treated with iodoacetamide; PPRE, PPAR response element; AMPK, AMP-activated kinase protein; PPAR, Peroxisome proliferator-activated receptor; UCP2, Mitochondrial uncoupling protein 2; HGP, Hepatic glucose production; ROS, Reactive oxygen species; DMEM, Dulbecco's minimal essential medium; DMSO, Dimethyl sulfoxide; FCCP, Oligomycin carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazine; OCR, Oxygen consumption rate; DHE, Dihydroethidium; OXPHOS, Oxidative phosphorylation; TBS-T, Tris buffered saline solution containing 0.1% Tween 20; AUC, Area under the curve; CTL, Control; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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1. Introduction

Calotropis procera is a plant found in tropical regions of the world. In many countries, it is regarded as having medicinal properties. In Brazil, there is no official documentation of the traditional use of plants, mainly used to prevent or treat human diseases. The Brazilian pharmacopeia is still in its infancy. However, the traditional use of medicinal plants by indigenous communities and people living in the poorest regions of the country is part of daily life. In Brazil, the latex from *Calotropis procera* (such as from other laticifer plants) is used in different forms and for different purposes (Silva, 2020). The latex is used in some communities in Northeast Brazil to treat hyperglycemia, but that is not consolidated because of the potential toxicity of the latex (Kaul and Sumeet, 2010). However, the ethnopharmacological use of the latex from *C. procera*, including treatment of diabetes, has been documented globally in studies stimulated by local traditional uses. Anti-hyperglycemic activity of the leaf extract (leaves of *C. procera* are full of latex) has been reported by Neto, 2013. Yadav, 2014 confirmed the traditional use of *C. procera* to treat diabetes, pain and inflammation for thousands of years in India.

The aqueous suspension of dried latex from *C. procera* has been shown to normalize blood glucose levels in humans, as well as in alloxan- and streptozotocin-induced diabetic rats (Kaul and Sumeet, 2010; Kumar and Padhy, 2011; Roy, 2005). However, little is known about the biochemistry and molecular mechanisms involved in the anti-hyperglycemic activity of compounds extracted from *C. procera* latex, nor about its different pharmacological activities, such as selective cytotoxicity (Viana, 2017) and anti-inflammatory activity (Kumar, 2015; Ramos, 2020). Therefore, more detailed studies about the proteins of the latex from *C. procera* are needed. Chitinases and proteases have been reported to be the most abundant proteins, as well as their different biochemical and structural aspects (Freitas, 2016; Ramos, 2013). Osmotin, a protein with biological effect similar to adiponectin, was identified as a minority protein present in *C. procera* latex (Freitas, 2015). Proteases and osmotin share a common antigenic epitope that permits cross-reaction of anti-osmotin antibodies with proteases (Freitas, 2015). These proteins are obtained from the latex in a common protein fraction, through ion-exchange chromatography (Freitas, 2020). To avoid the catalytic effect of proteases, which has been reported as harmful in some circumstances (Bezerra, 2017), the fraction is treated with iodoacetamide, which irreversibly inhibits proteolysis. This combined fraction encompassing catalytic-blocked proteases and osmotin is what we call CpPII in this study. The latex proteins from *Calotroips procera* and CpPII have been investigated in terms of toxicity and adverse effects. The most recent study, presented by Bezerra, 2017, reported safety aspects using these samples. However, the administration route must be considered. Many adverse effects caused by bacterial infection or side effects provoked by anti-metabolic drugs used to treat malignancies are eliminated in experimental animals when treated with latex proteins of *Calotropis procera* or CpPII (Oliveira, 2007; Oliveira, 2010; Alencar, 2017). Therefore, CpPII is currently a candidate for the attribution of the pharmacological activities associated with plants.

AMPK modulation has been shown to be an important pathway for mitochondrial function regulation (Madhavi, 2018). Mitochondria play a central role in energy metabolism by producing energy for the cells in form of adenosine triphosphate (ATP), and are also involved in the production and elimination of reactive oxygen species (ROS) (Mello et al., 2018). However, chronic hyperglycemia increases mitochondrial ROS production, and causes mitochondrial dysfunction, which contributes to insulin resistance (Gonzalez-Franquesa and Patti, 2017; Franko et al., 2015; Dey and Swaminathan, 2010). Thus, improvement of mitochondrial function also plays an essential role in glycemic control.

Since the latex from Calotropis procera is used in many communities around the globe to treat different human ailments, and the medicinal value of the plant has also been supported by scientific studies, the investigation of the molecular mechanisms underlying the reported potential is justified. Thus, the objective of this study was to evaluate the effect of CpPII, isolated from LP fractionation, on glycemic control of healthy mice and on mitochondrial function *in vitro*. While *in vivo* studies are important to investigate physiological effects of samples, the molecular aspects and the pathways involved, such as the signaling mechanisms, are more properly assessed in the cellular level by *in vitro* assays. Such assays have been used to study the hepatic effects of herbal medicines through use of HepG2 cells, also permitting evaluating mitochondrial response (Seo, 2017). This approach can provide further support of the medicinal usage of the plant and a better understanding of the active principles underlying the molecular mechanisms.

2. Materials and methods

2.1. Legal permission

The study of the latex from *Calotropis procera* reported in this manuscript was previously registered in the SiGen (Genetic Heritage and Associated Traditional Knowledge System) according to the current Brazilian legislation, under #A689147.

2.2. Animals

The experimental protocol was approved by the State University of Ceará Ethics Committee on Animal Research (#7839535/2016). Twenty-four male Swiss mice (20–30 g) were maintained in a room with controlled temperature (22 ± 2 °C) and humidity (50 ± 5%) and 12/12 h light–dark cycle, with *ad libitum* access to food and water. The animals were divided randomly into two groups: control group (n = 6) and CpPII fraction group (CpPII, n = 6).

2.3. Plant material

The latex of *Calotropis procera* (Aiton) Dryand (Apocynaceae) was obtained from plants from the city of Fortaleza (3°46′6″S/38°32′36″W). The latex was collected during the rainy season (march to june). A voucher specimen was identified by staff of the Prisco Bezerra Herbarium of Federal University of Ceará and registered under no 32663. The plant name was verified at http://www.theplantlist.org/1.1/browse/A/Apocynaceae/Calotropis/. *Calotropis procera* is commonly known as *ciúme* (“jealousy”) in the study region and most frequently cited as Sodom apple in the scientific literature.

2.4. CpPII fraction preparation

The fresh latex was collected in tubes (1:1, v/v distilled water) from the aerial parts of *Calotropis procera* in Fortaleza, and a voucher specimen was deposited in the Prisco Bezerra Herbarium of Federal University of Ceará. The latex of *C. procera* was obtained and processed by centrifugation (5000 × g for 10 min) to eliminate the precipitate enriched with rubber. After that, the supernatant was dialyzed by membranes with 8000 Da cutoff. The non-dialyzed fraction was lyophilized to obtain the laticifer proteins (CpLP), which were free from any toxicity or other undesired effects (Alencar, 2004). The CpLP fraction was separated by ion-exchange chromatography into three sub-fractions, CpPI, CpPII

and CpPIII, which were analyzed by polyacrylamide dodecyl sulfate electrophoresis gel, as previously described (Freitas, 2020; Ramos, 2009). After the extraction of these fractions, CpPII was treated with iodoacetamide (IAA) to produce CpPII-IAA and to inhibit the endogenous proteolytic activity reported by Ramos, 2013.

This fraction was composed of closely related isoforms of cysteine peptidases, estimated to represent 95% of the total proteins in CpPII, while the remaining 5% corresponded to osmotin. Those estimates were obtained by chromatography and electrophoresis analysis. Both proteins have previously been studied in terms of chemistry, biochemistry, enzymatic activity (formation) and structural aspects (Freitas, 2011; Ramos, 2013, 2015, 2020). Different aspects of their biological role in latex have been described recently (Ramos, 2019).

2.5. CpPII treatment and measurement of fasting glycemia in animals

All the treatments with CpPII *in vivo* were performed with 5 mg/kg of CpPII, a dose was chosen based on a previous study of glycemic control via AMPK in LP-treated animals (Oliveira, 2019), suspended in sterile saline solution, injected intravenously through the tail vein. The control group received only saline solution, administrated in the same volume. Fasting blood glucose levels were assessed by an Accu-Check® glucometer before treatment and 2, 3 and 4 h after administration of CpPII.

2.6. Intraperitoneal glucose and pyruvate tolerance testing

Intraperitoneal glucose (ipGTT) and pyruvate (ipPTT) tolerance tests were performed in 10 and 16 h-fasted animals. First, the animals were treated with CpPII, while the control group received only the same volume of a saline solution . Two hours after, the animals received glucose or pyruvate (2 g/kg, i.p.), then glycemia was measured after 30, 60, 90 and 120 min for ipGTT, and 15, 30, 60 and 120 min for ipPTT (Lee, 2015).

2.7. Cell culture and treatment

Hepatocellular carcinoma line cells, HepG2, were plated in low-glucose Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin and maintained at 37 °C with 5% CO_2. CpPII was diluted in DMEM until final concentration of 100 μg/mL. This concentration was chosen according to a previous *in vitro* study with soluble proteins from the latex, which showed immunomodulatory proprieties (Frankenfeld, 2014). Cells were incubated with CpPII or culture medium (control) for 3 h.

2.8. Cell viability assay

HepG2 cells were plated in 5×10^3 per well density in 96-well plates. After a 3 h pretreatment with CpPII (10, 25, 75 and 100 μg/mL), HepG2 cells were washed with PBS (twice) and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) for 1 h. After incubation, the medium was discarded and 100 μL of dimethyl sulfoxide (DMSO) was added for colorimetric assay reading. The absorbance of each well was measured at 540 nm using a spectrophotometer. The cell viability (%) was measured as viability percentage in relation to the control group.

2.9. Oxygen consumption

HepG2 cells were cultured in 6-well plates (6×10^5 cells). After the CpPII treatment, 5×10^5 cells in each chamber of the respirometer were resuspended in Krebs medium (1 mL) at 37 °C and supplemented (Oroborus Oxygraph-2 K). After that, respiration modulators were added as follows: oligomycin, carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazine (Cccp), and antimycin A, for oxygen consumption rate (OCR) monitoring. The cell parameters evaluated were ATP-linked respiration, and uncoupled, basal and spare capacity. The APT-linked respiration was calculated by the difference between OCR and basal respiration after oligomycin addition. The uncoupled capacity is the difference of OCR after addition of antimycin A and oligomycin. Finally, the spare capacity was calculated by the difference between maximum respiration (Cccp) and basal respiration.

2.10. PPRE luciferase reporter gene assay

PPRE luciferase reporter gene was performed in 96-well plates with transfected cells using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) with 0.02 μg of pRL-SV40 (E2231; Promega), 0.2 μg pf pPPRE X3-TK-luc (1015; Dr. Bruce Spiegelman; Addgene). The amount of plasmid DNA was maintained at 0.8 μg per well using the control plasmid pCDNA 3.1 (Addgene). After 24 h of transfection, cells were lysed with 100 mL of passive lysis buffer 1x (Promega) and luciferase activity was determined in 20 uL of lysate in white 96-well plates (Costar) using dual luciferase assay system (Promega), according to the manufacturer's instructions. Renilla luciferase activity (pRL-SV40) was used for normalization.

2.11. Measurement of lactate in the culture medium

HepG2 cells were cultured in 6-well plates (5×10^5 cells/ml) and, after CpPII-treatment, the cells were washed three times with PBS and then incubated with 2 mL of Krebs solution (25 mM glucose, pH 7.4) for 3 h. After that, Krebs solution (sample) was collected to measure lactate concentration, according to Bergmeyer (1974). Briefly, 20 μL sample was mixed with 148 μL of Tris-HCl hydrazine buffer (50 mM) and 2 μL of lactate dehydrogenase enzyme (11.4 ng/μL), then the first reading of fluorescence emission was performed at 360 excitation and 460 emission (E1). After the addition of 30 μL of NAD^+ (5 mM), the second fluorescence emission intensity was read (E2). The same assay was performed with a standard lactate curve (0–100 μm). Sample's lactate concentrtion was calculated by the difference between E1 and E2 and subsequent interpolation along the standard lactate curve.

2.12. Reactive oxygen species generation

HepG2 cells cultured in 96-well plates were treated with CpPII and then the wells were washed with PBS for the addition of the fluorescent probe DHE (dihydroethidium). The probe was diluted in PBS and glucose solution (5.5 Mm) for 20 min, and maintained in incubators with CO_2 (5%) at 37 °C. After the incubation, the probe was removed and cells were washed with PBS for addition to the test medium (glucose and PBS solution) in each well. The analysis was performed with excitation/emission of fluorescence at 518 nm/605 nm (DHE).

2.13. Mitochondrial density

To label the mitochondria by the fluorescence method, HepG2 cells were cultured and treated as described above. Thus, the cells were incubated for 30 min with MitoTracker Green probe at 30 nM (Invitrogen) and maintained in CO_2 incubators (5%) at 37 °C for the mitochondria visualization. After the incubation, the plate was read with a spectrophotometer with excitation of 579 nm and emission of 599 of nm. For nuclear labeling, 1 μg/mL of Hoersch solution was added to the medium (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 10 min at room temperature, and the fluo-

rescence was determined using excitation of 350 nm and emission of 461 nm. Mitochondrial density was defined as integrated MitoTracker Green fluorescence intensity (AU, arbitrary units).

2.14. Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was determined by monitoring fluorescence of tetramethyl rhodamine methyl ester (TMRM). HepG2 cells were treated with CpPII and incubated with 60 nM TMRM (Molecular Probes, Invitrogen, UK, T668) for 30 min at 37 ℃ in the dark. In the last 15 min, cells were added with 2 mM Cccp as positive control. Hoechst 33,342 (HO, 2 μg/mL) was used for nuclear staining. Cellular fluorescence was monitored by a Spectramax 190 microplate spectrophotometer. TMRM fluorescence intensity was quantified using relative fluorescence units (RFU): (RFU TMRM - RFU without probe)/(RFU HO – RFU without HO).

2.15. Confocal imaging

To determine MMP, images were analyzed accordingly to MitoTracker Red fluorescence intensity with an ImageXpress Micro Confocal High Content Imaging System. The MitoTracker signal was normalized to the Hoechst 33,342 signal. MMP was defined as integrated MitoTracker Red fluorescence intensity (AU, arbitrary units).

2.16. Western blotting

HepG2 cells were collected in Laemmli buffer and protein samples were transferred to membranes by electrotransfer from sodium dodecyl sulfate polyacrylamide gel (10%) using a Bio-Rad Trans-Blot system (Bio-Rad Laboratories, Inc., Tokyo, Japan). Membranes were blocked for 1 h at room temperature in 5% nonfat milk diluted in Tris buffered saline solution containing 0.1% Tween 20 (TBS-T). Then the membranes were washed in TBS-T and incubated with primary antibodies for OXPHOS, AMPK, p-AMPK and beta-actin (Sigma-Aldrich) overnight, under stirring at 4 °C. After that, the membranes were incubated with secondary antibodies for 2 h at room temperature. Finally, the detection of the blot was realized with a chemiluminescence kit (substrate ECL Western Clarity™, Bio-Rad, Hercules, CA, USA).

2.17. RNA extraction, cDNA preparation and real-time PCR

Total RNA was extracted from HepG2 cells with TRIzol™ reagent (Invitrogen). A high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA from the total RNA. A real-time PCR system (Applied Biosystems) was used to determine the extent of gene expression levels. The reaction mixture was composed of 6 μL of SYBR green PCR master mix (Applied Biosystems), forward primer (0.36 μL), reverse primer (0.36 μL), 2 μL of cDNA, and 3.28 μL of ultrapure water. Beta-actin was used as housekeeping gene. The sequences of the primers are shown in Table 1.

2.18. Statistical analysis

Results were expressed as mean ± SEM. Two-way repeated measure ANOVA followed by the Bonferroni test was used for comparison of glycemia evolution in each tolerance test. For other comparisons, the unpaired parametric *t*-test was used. Significance was set at $p < 0.05$. The analyses were performed using the statistical software GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1. CpPII promoted glycemic control in healthy mice

After 2 h, the animals treated with CpPII showed a significant reduction (23.3%) of fasting glycemia compared to control animals, and this reduction was maintained for 4 h after the treatment (Fig. 1A). Furthermore, the treatment with CpPII improved glucose tolerance of CpPII group compared to control group, as shown by the significant reduction of the area under the curve (AUC), during ipGTT, (Fig. 1B, insert). Moreover, CpPII showed reduction of hepatic gluconeogenesis by reducing glycemia at 60 and 120 min (55.7 and 64.8%, respectively) after pyruvate administration in control mice (Fig. 1C). The reduced AUC during ipPTT of the CpPII group compared to the control group proved this inhibition of hepatic glucose production (PHG) (Fig. 1D). We further investigated whether glycemic control caused by CpPII was related to the improvement of hepatic mitochondrial function.

3.2. CpPII increased liver cell viability, mitochondrial activity and mass

Considering the previous findings, we employed *in vitro* treatment of CpPII in HepG2 cells to determine the capacity of CpPII in improving mitochondrial function. First, the viability of the HepG2 cells treated with CpPII was analyzed by using the MTT assay. CpPII was not cytotoxic to HepG2 cells in the range of 10–100 μg/mL. Instead, the highest concentration of CpPII (100 μg/mL) significantly increased the cell viability in relation to the control group (Fig. 2A), so this concentration was chosen for all the *in vitro* experiments.

Fig. 2B summarizes the results of CpPII regarding respiratory activity on intact HepG2 cells by high-resolution oximetry. In the presence of CpPII, the basal oxygen consumption rate (OCR) increased significantly in comparison to control group. Addition of H-FoF1 ATP-synthase inhibitor oligomycin resulted, as expected, in a marked lowering of the respiration, which in the presence of CpPII was enhanced in HepG2 cells. To gain insight into the uncoupling mechanism of CpPII, we added the protonophoric uncoupler carbonylcyanide p-trifluoromethoxyphenylhydrazone (Cccp). In the presence of CpPII, OCR with Cccp resulted in an increase in comparison to control group. Addition of antimycin A, a known inhibitor of mETC complex III, to the cell suspension had a positive effect on the respiratory activity. Moreover, in the presence of CpPII, ATP-linked respiration and proton leak of the HepG2 cells increased significantly, but that did not affect the spare capacity compared to the control group (Fig. 2C, D and E).

Table 1
Sequence of primers.

Gene	Forward	Reverse
CS (citrate synthase)	GCTAAGGGTGGGGAAGAACC	GGAACAACCCGTCCTGAGTT
PDK4 (pyruvate dehydrogenase kinase 4)	GCAGTGGTCCAAGATGCCTT	GTTCAACTGTTGCCCGCATT
CPT-1 (carnitine palmitoyltransferase I)	CCAGACGAAGAACGTGGTCA	TGTGCTGGATGGTGTCTGTC
UCP2 (mitochondrial uncoupling protein 2)	AGTCCGGTTACAGATCCAAGG	GAGCATGGTAAGGGCACAGT
b-actin (beta-actin)	TCAGGTCATCACTATCGGCAATG	TTTCATGGATGCCACAGGATTC

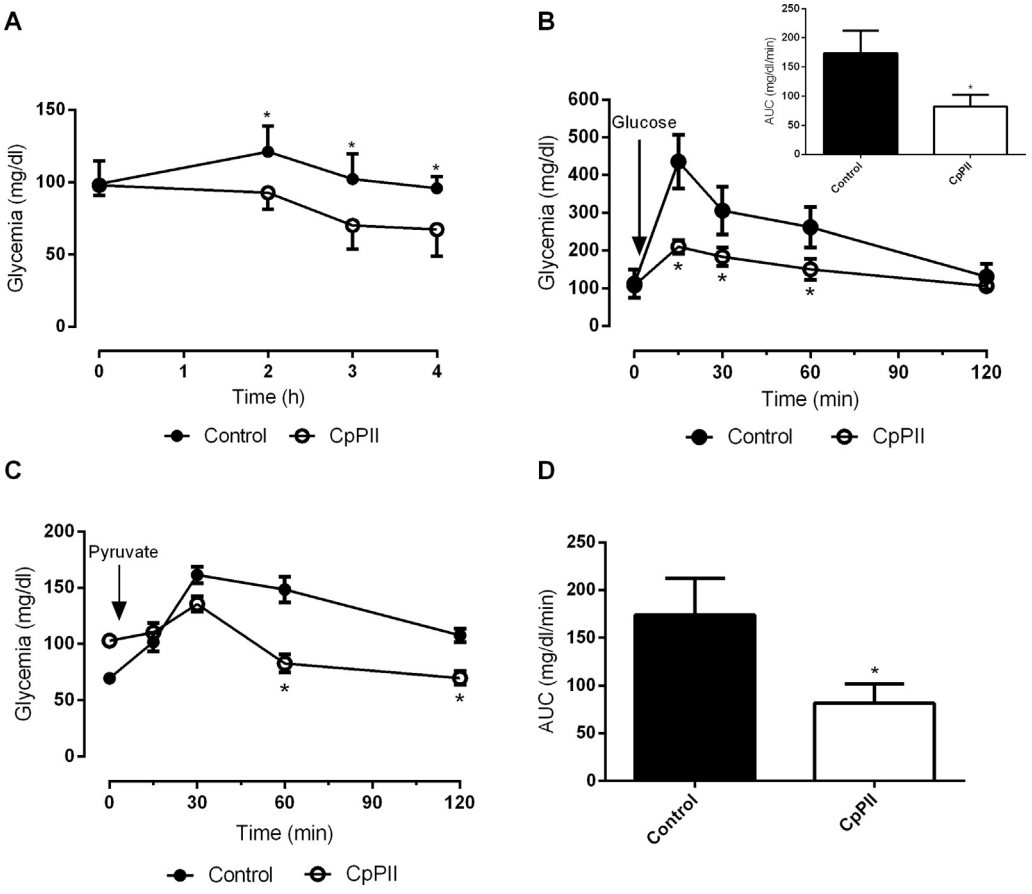

Fig. 1. Effect of the major peptidase fraction treated with iodoacetamide (CpPII) on glycemic control of healthy mice. The panel **A** shows fasted glycemia before, after and during for four hours to the treatment with CpPII (5 mg/kg, i.v.) or saline solution (0.1 mL / 100 g) (control group). The panels **B** and **C** show time-response curve of glycemia to the previously (2 h before) treatment with CpPII in fasted animals after the load administration of glucose (**B**), and its area under curve (insert) or pyruvate (**C**) and its area under the curve (**D**). Results are means ± SE (n = 6–8/group). *p < 0.05 vs. CTL. Data were analyzed by ANOVA followed by Bonferroni post-test and Student's *t*-test (for AUC).

Measurement of mitochondrial membrane potential (MMP) by the fluorescent probes tetramethylrhodamine methyl ester (TMRM) and MitoTracker Red was performed to assess other CpPII effects on the mitochondrial function. The treatment of HepG2 cells with CpPII stimulated a significant increase of MMP, indicating enhancement of mitochondrial activity (Fig. 2G, H and I). In addition, CpPII induced an increase of mitochondrial mass as detected by MitoTracker Green signal, a mitochondrial-selective fluorescent label (Fig. 2F).

3.3. CpPII increased oxidative metabolism and reduced ROS in liver cells

Having established that CpPII increased mitochondrial function, firstly we investigated whether CpPII could stimulate the oxidative metabolism by quantifying lactate concentrations. HepG2 cells treated with CpPII showed a significant reduction in lactate concentration (Fig. 3A) compared to the control. Besides that, we analyzed gene expression of metabolic oxidative enzymes, such as citrate synthase (CS), pyruvate dehydrogenase kinase 4 (PDK4) and carnitine palmitoyltransferase I (CPT-1) (Fig. 3B, C and D, respectively). CpPII induced a significant increase of CS and PDK4 gene expression in CpPII-treated HepG2 cells compared to the control group. However, CpPII did not have any effect on CPT-1 mRNA relative to controls. Moreover, CpPII induced a relevant increase (p = 0.06) in the UCP2 gene expression (Fig. 3E) and in the protein levels of mitochondrial complexes I, III and V (61.1%, 37.9% and 45.0% respectively), but there were no substantial differences between groups in complexes IV and II (Fig. 2F).

Then we tested if the effects of CpPII in increasing oxidative metabolism occurred by AMPK activation as well as by induction of PPAR. Fig. 3G shows a significant increase in AMPK phosphorylation in the HepG2 cells treated with CpPII compared to untreated cells. Besides that, CpPII significantly increased PPRE activity (71.5%) (Fig. 3H), suggesting that the improvement of mitochondrial function induced by CpPII occurred at least in part by the activation of AMPK/PPAR in HepG2 cells.

Finally, we tested if CpPII exerted liver mitochondrial function effects by activating AMPK and potentially suppressing free radicals. We observed that intracellular ROS level detected by DHE staining was strongly reduced by CpPII treatment in HepG2 cells (Fig. 3I).

4. Discussion

The folk and clinical medicinal uses of *Calotropis procera* have been reported extensively. The presence of laticifers in the plant (Ramos, 2019) suggested that latex constituents are involved in both toxic or medicinal effects. Our research group has demonstrated that secondary metabolites of latex from *Himatanthus drasticus* has activity similar to acarbose, a drug used in the treatment of diabetes (Morais, 2020). The first report suggesting the participation of the latex proteins in pharmacological benefits has described anti-inflammation in three different animal models

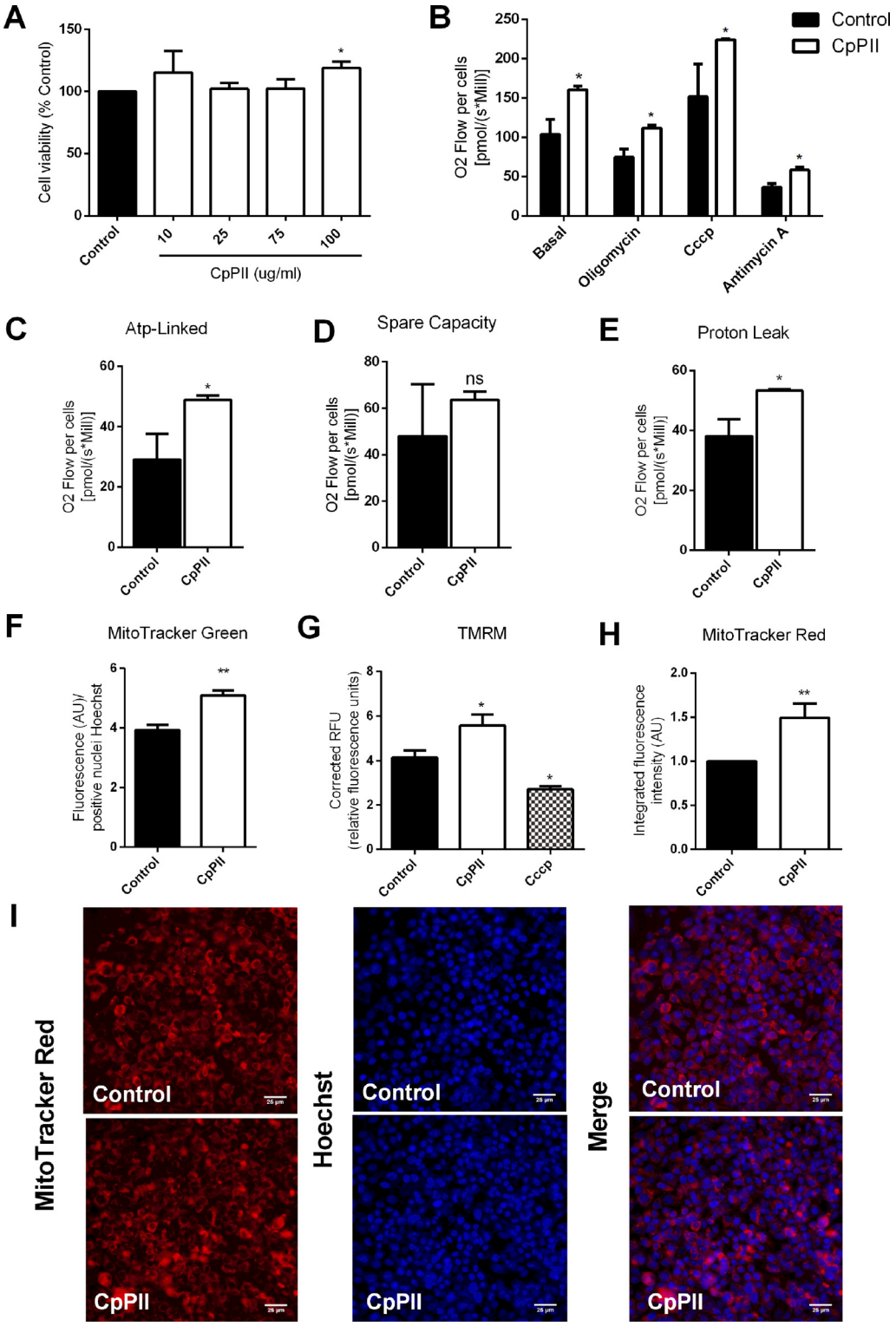

Fig. 2. Effect of the major peptidase fraction treated with iodoacetamide (CpPII) in mitochondrial function of HepG2 cells. Cell viability was performed in HepG2 cells after the treatment with CpPII on the range of 10–100 µg/mL by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (**A**). The panel **B** shows basal oxygen consumption rate and after oligomycin, oligomycin carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazine (Cccp) and antimycin A treatment. Panels C - H shows the ATP-linked, uncoupling respiration, spare capacity, measurement of mitochondrial density, mitochondrial membrane potential by tetramethyl rhodamine methyl ester (TMRM), and MitoTracker Red in HepG2 cells treated with CpPII, respectively. Panel I shows image analyses by MitoTracker Red. Results are means ± SE (n = 6–8/group). *p < 0.05 vs. CTL. Data were analyzed by Student's *t*-test.

(Alencar, 2004). Since then, the latex proteins have been implicated in modulation of different inflammatory processes of clinical relevance, such as severe bacterial infections (Nascimento, 2016) and colitis (Kumar et al., 2019), as well as in ameliorating side effects of antimetabolic drugs prescribed in cancer therapy (Alencar et al., 2017).

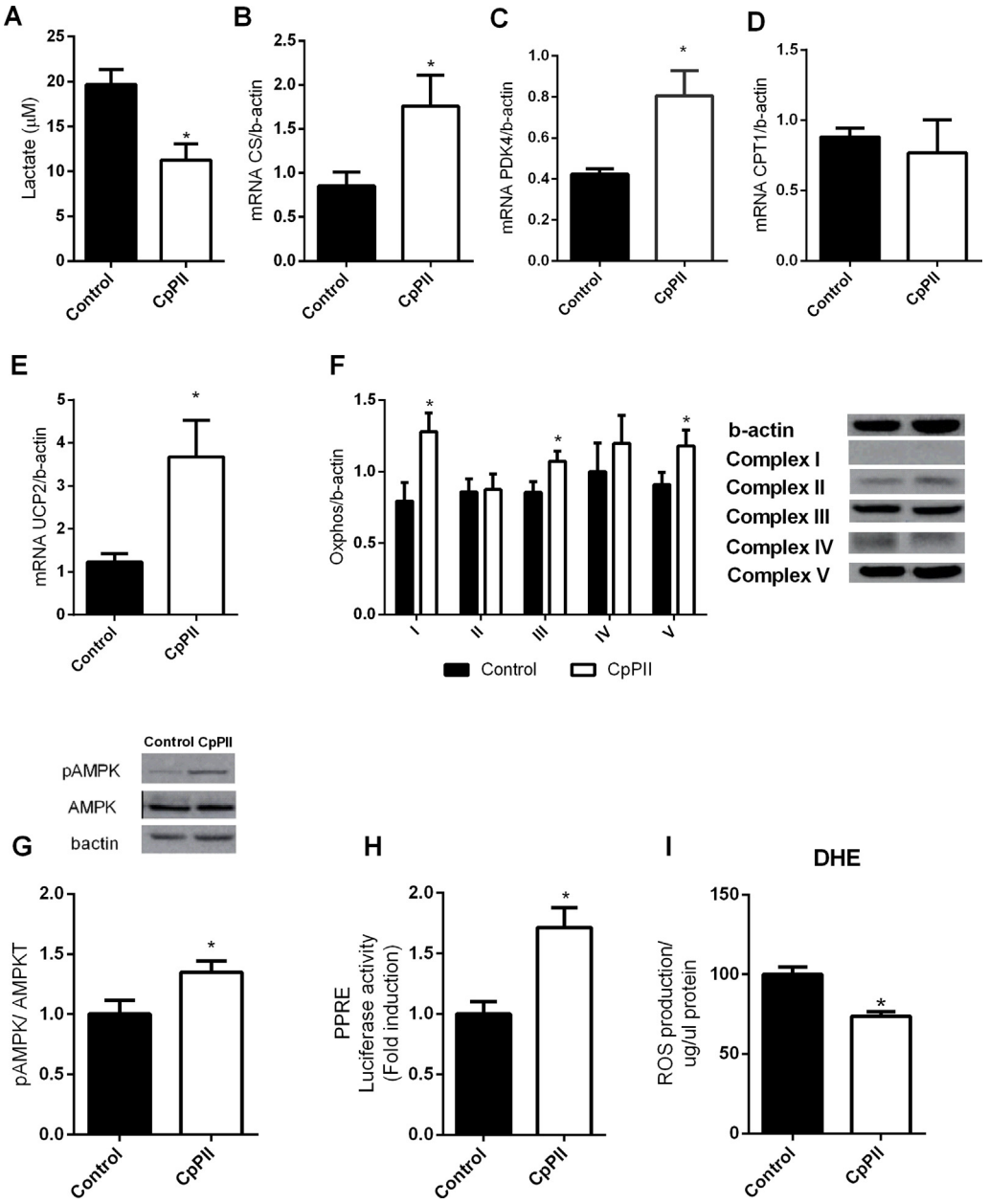

Fig. 3. Effect of the major peptidase fraction treated with iodoacetamide (CpPII) in oxidative metabolism and reactive oxygen species (ROS) generation HepG2 cells. The panel **A** shows concentration of lactate in HepG2 cells treated with CpPII. Panels B - E shows the genic expression of citrate synthase (CS), pyruvate dehydrogenase kinase 4 (PDK4), carnitine palmitoyltransferase I (CPT1) and peroxisome proliferator-activated receptor (UCP2) and panels F and G shows the protein expression of oxidative phosphorylation (OXPHOS) and phosphorylated AMP-activated kinase protein/ total AMP-activated kinase protein (p-AMPK/AMPK) in HepG2 cells treated with CpPII, respectively. The panel **H** shows peroxisome proliferator-activated receptor response element (PPRE) luciferase reporter gene assay and panel **I** shows oxygen species production by probe dihydroethidium (DHE) in HepG2 cells. Results are means ± SE (n = 6–8/group). *p < 0.05 vs. CTL. Data were analyzed by Student's *t*-test.

Among the latex proteins, proteases and osmotin are currently the leading candidates to promote physiological homeostasis in animals submitted to metabolic or physiological disturbances.

The benefits of *Calotropis procera* latex for treating diabetes have been widely claimed, but there is no consolidated information or scientific evidence supporting it. The recent study by Oliveira, 2019 provided important insights into this question. The study has showed that LP, the latex protein fraction of *C. procera*, promotes inhibition of hepatic glucose production and favors glycemic control via the AMPK pathway. Another study with *C. procera* latex provides protection against oxidative stress in 4-Nonylphenol-induced toxicity in catfish as animal model as surrogate for humans (Sayed, 2016). Latex also contains lignans which show stronger anti-inflammatory activity by the inhibitory potential against 5- and 15-lipoxygenase enzymes (Abdel-Mageed, 2016). These studies afforded scientific support for the use of the latex to control diabetes.

Latex is a highly complex substance, where soluble molecules and subcellular structures are present. In addition, latex accumulates rubber particles in suspension. Rubber particles easily precipitate in aqueous media. The soluble phase of latex contains a diversity of molecules that varies drastically among different species. Beneficial pharmacological properties and toxic effects are often found together in latex. Therefore, fractionation of latex for removing toxic compounds and chemically investingating particular latex fractions is important for a better understanding of latex

compounds' activity and potential for ensuring safer use in folk medicine regardless of the curative purpose as well.

In our study, the chemically well-defined fraction CpPII was assayed instead of LP. The data showed that CpPII promoted significant reduction of fasting glycemia, improved glucose tolerance and inhibited HGP in healthy mice. Furthermore, glycemia control promoted by CpPII can be associated with the improvement of hepatic mitochondrial function and the effects of CpPII appear to be mediated through activation of AMPK/PPAR.

The relation between hepatic gluconeogenesis and mitochondrial function in glycemic control was previously demonstrated (Lee, 2015; Lim et al., 2009). Such mitochondrial dysfunction causes insulin resistance, which induces elevation of gluconeogenesis enzymes levels, besides the extreme rise of hepatic gluconeogenesis (Lee, 2015; Lim et al., 2009; Blake, 2014). However, the activation of AMPK is associated with improvement of mitochondrial function and glucose metabolism (Takikawa, 2010). Some antidiabetic agents, such as metformin and pioglitazone, and natural products like berberine and resveratrol, have been shown to activate AMPK and stimulate mitochondrial oxidative phosphorylation (Bagul, 2018; Pawlyk, 2014).

In that respect, CpPII proved to be effective in glycemic control since it activated AMPK and promoted improvement of mitochondrial function by increasing oxygen consumption for ATP synthesis and respiratory complexes' components in HepG2 cells, favoring the improvement of oxidative metabolism and aerobic ATP synthesis. The efficacy of CpPII as an antihyperglycemic agent by inhibition of hepatic gluconeogenesis via AMPK activation might be comparable to metformin, which is the main first-line oral drug of choice in the management of type 2 diabetes (Chaudhury, 2017). However, CpPII interestingly does not promote mitochondrial impairment (Carvalho et al., 2008).

The same effect has been observed after the treatment with an isolated compound from roots of the plant *Salvia miltiorrhiza* Bunge in healthy rats, which resulted in the improvement of hepatic mitochondrial respiratory function via AMPK in HepG2 cells (Qiang, 2015). Another study using treatment with strawberry extract for 48 h raised the ATP-linked respiration in HepG2 cells via AMPK, demonstrating the role of AMPK in the improvement of mitochondrial function (Giampieri, 2017). Therefore, AMPK seems to play a critical role in the regulation of mitochondrial function.

Along with the marked improvement in mitochondrial function, shown by the improvement in OCR after CpPII treatment, we found a relevant increase in mitochondrial mass, indicated by the increase in citrate synthase gene expression, fluorescence microscopy of MitoTracker Red and biochemical markers of mitochondria, such as TMRM and MitoTracker Green.

Consistent with the increase in oxygen consumption, the improvement in mitochondrial oxidative metabolism by CpPII was demonstrated by the reduced lactate production and increased MMP, PDK4 and UCP2 mRNA expression by HepG2 cells. The decrease in lactate production suggests improvement of oxidative metabolism (Boenzi and Diodato, 2018). Similarly, a microbial fermentation byproduct of dietary fiber has been found to modulate energy metabolism and mitochondrial function, decreasing glycolysis, increasing β-oxidation, and enhancing the TCA cycle and oxidative phosphorylation by increasing MMP and oxidative enzymes in HepG2 cells (Xing, 2016). Although in this present study CpPII did not alter expression of CPT1 mRNA, a gene involved in fatty acid oxidation, it was recently demonstrated that upregulation of the PDK4 mRNA expression in the liver is a sensitive marker of increased mitochondrial fatty acid oxidation, by indicating an overall shift from glucose to fatty acids as the preferred oxidation fuel (Pettersen, 2019). Moreover, high UCP2 levels promote more efficient fatty acid oxidation, resulting in a beneficial effect on mitochondrial function (Kukat, 2014). Thus, CpPII can favor mitochondrial oxidative metabolism in HepG2 cells.

Mitochondrial respiration is an important source of ROS production and essential for the maintenance of redox balance (Nohl et al., 2003). Although the treatment with CpPII increased mitochondrial function, our results showed that CpPII reduced the ROS production in HepG2 cells. Similar to our study, Zhao, 2018 found a significant reduction of ROS content associated with the increase in gene and protein expression of mitochondrial complexes in HepG2 cells treated with a compound extracted from the pericarp of *Cyclocarya paliurus*, which has well-known antioxidant and hypoglycemic activity. Likewise, Tsai, 2016 reported that hepatocytes treated with an extract from *Garcinia mangostana* Linn significantly increased the rate of oxygen consumption and decreased ROS mitochondrial levels.

In addition, CpPII induced activation of AMPK, which may have enhanced the activity of PPAR. In the liver, PPAR and AMPK are well-known pathways correlated to improvement of the mitochondrial content and function (Wei, 2018). Further studies are required to better understand the interactions between AMPK and PPAR activity by CpPII.

5. Conclusions

In conclusion, CpPII increased hepatic mitochondrial function and reduced ROS production in HepG2 cells, an effect that may have contributed to inhibit the hepatic glucose production in the control animals, favoring glycemic control. Thus, this study provides new scientific evidence of the therapeutic use of *C. procera* latex in folk medicine against hyperglycemia and gives further insights in the molecular mechanisms underlying glycemic control and the latex compounds involved.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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