(RESEARCH ARTICLE)

Protective effects of Auranofin on the 6-hydroxydopamine model of Parkinson's disease in rats

Iana Bantim Felício Calou [1], Maria Janice Pereira Lopes [2], Melina Mottin [3], Andis Klegeris [4], Kelly Rose Tavares Neves [5], Gerly Anne de Castro Brito [5] and Glauce Socorro de Barros Viana [5, *]

[1] Federal University of Piaui, CSHNB, Picos, Brazil.
[2] Faculty of Medicine Estácio of Juazeiro do Norte, Ceará, Brazil.
[3] Institute of Biology, University of Brasilia, Brasilia, DF, Brazil.
[4] Department of Biology, University of British Columbia Okanagan Campus, Kelowna, BC, Canada.
[5] Department of Physiology and Pharmacology, Faculty of Medicine of the Federal University of Ceará, Ceará, Brazil.

Abstract

Auranofin (AU) presents anti-inflammatory effects and was shown to have a neuroprotective action. The objectives were to investigate the actions of AU in the 6-OHDA model of Parkinson's disease (PD). Methods: Male Wistar rats were distributed into sham-operated (SO, control), untreated 6-OHDA lesioned and 6-OHDA lesioned, and treated with AU (3 and 10 mg/kg, p.o. for 2 weeks) groups. Then, animals were euthanized, the striatum dissected and processed for measurements of dopamine (DA) and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), as well as immunohistochemical assays for inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and the Nuclear factor erythroid 2-related factor 2 (Nrf2). The influence of AU on the activation of the brain NrF2 was also pursued by molecular docking experiments. Results: While the 6-OHDA lesioned rats presented a decrease in the striatal DA and DOPAC concentrations, these effects were prevented after AU treatment. The 6-OHDA group showed increased expressions of iNOS and COX-2 and these effects were highly prevented by AU. While Nrf2 expression was significantly decreased in the 6-OHDA lesioned animals, values came back to those close to the SO group after AU treatment. Auranofin may bind to the Kelch domain of the Kelch-like ECH-associated Protein 1 (Keap1) and possibly inhibit Keap1/Nrf2 interaction. Significance: AU by decreasing iNOS and COX-2 expressions and by increasing the expression of Nrf2, emerge as a potential neuroprotective drug for the treatment of PD.

Keywords: Parkinson's disease; 6-OHDA; Patients; Oxidative stress; Neuroinflammation, Transcription factor; Auranofin

1. Introduction

Auranofin (AU), a gold-containing triethyl phosphine, was approved by the FDA (USA) for the treatment of rheumatoid arthritis in 1985. Since then, this drug has been investigated for potential therapeutic application in many other diseases including neurodegenerative disorders. The AU's main mechanism of action is through the inhibition of reduction/oxidation (redox) enzymes that are essential for maintaining intracellular levels of reactive oxygen species, ROS [1].

The pharmacological activity of AU is associated with its ability to induce the enzyme heme oxygenase-1 (HO-1). An earlier finding [2] indicates that auranofin leads to HO-1 upregulation by activating Keap1/Nrf2 signaling pathway (Kelch-like ECH-associated protein 1/Nrf2, Nuclear factor (erythroid-derived 2)-like 2). The Keap1/Nrf2 regulatory

* Corresponding author: Glauce Socorro de Barros Viana

pathway plays a central role in the protection of cells against oxidative, xenobiotic damage, and inflammation [3, 4]. It is used to monitor oxidative stress and is also closely associated with aging, by controlling the transcription of antioxidant enzymes [5].

Nrf2 and its endogenous inhibitor, Keap1, is a conserved intracellular defense mechanism to counteract oxidative stress [6]. In addition, Nrf2 activates the transcription of several cytoprotective genes, implicated in the protection of diseases, including cancers and neurodegenerative diseases [7, 8]. It is highly sensitive to reactive oxygen species (ROS) and reactive nitrogen species (RNS) and, in the cell nucleus, activates various antioxidant genes by binding to the antioxidant response elements [9].

Furthermore, AU has been shown to increase platelet ROS production and intracellular calcium concentration, and data show that thioredoxins (TrxRs) are an important part of platelet antioxidant defense [10]. TrxR inhibition effectively activates Nrf2 responses, indicating that Nrf2 is the primary mechanism by which TrxR inhibitors, including AU, increase HO-1 in some tissues such as lung epithelia [11]. Besides, TrxR is a potential target for anti-cancer drugs, and AU, a potent inhibitor of TrxR, can trigger mitochondrial-dependent apoptosis pathways [12].

AU inhibits the toxicity of stimulated primary human astrocytes and U-373 MG astrocytic cells towards human neuronal cells but did not inhibit the secretion of cytokines [13]. Was demonstrated that auranofin protected neuronal cells from the toxicity induced by hydrogen peroxide through the upregulation of heme-oxygenase (HOX)-1. These data point out a possible neuroprotective action of AU through its inhibition of astrocyte toxicity.

Furthermore, neurodegenerative diseases show an alteration of homeostatic responses related to the control of proteostasis and low-grade chronic oxidative, inflammatory, and metabolic stress. These are crucial events where the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) plays a defensive role [14]. The endogenous antioxidant response pathway is known to protect cells by increasing the expression of cytoprotective enzymes and is regulated by Nrf2. Regulating the expression of antioxidant genes, Nrf2 also exerts anti-inflammatory effects and modulates both mitochondrial function and biogenesis [15].

Protein dyshomeostasis is identified as the hallmark of many age-related neurodegenerative disorders, including PD, and the diseased brain shows the deposition of Lewy bodies composed of α-synuclein protein aggregates [16]. Thus, a defective proteostasis leading to the deposition of proteins, including α-synuclein in the biofluids, can be identified as potential biomarkers for early diagnosis of PD. Besides, degeneration of dopaminergic neurons, focal gliosis, up-regulations of nitric oxide synthase (iNOS) and cyclo-oxygenase-1- and -2 (COX-1 and COX-2) were also found in the brain of PD patients [17].

Nitric oxide (NO) is synthesized by iNOS (inducible nitric oxide synthase) and acts on key regulatory pathways, such as those associated with excitotoxicity processes. Activated glia has been shown to produce NO by triggering calcium mobilization. This culminates with the release of vesicular glutamate from astrocytes and microglia, resulting in neuronal death and contributing to age-associated susceptibility and neurodegeneration [18].

COX-2 (cyclo-oxygenase-2) is the rate-limiting enzyme in prostaglandin E_2 (PGE_2) synthesis and was shown to be up-regulated in brain dopaminergic neurons of both PD patients and MPTP-treated mice. Evidence indicates that COX-2 inhibition prevents the formation of the oxidant species, dopamine-quinone, which has been implicated in the pathogenesis of PD [19, 20]. COX-2 expression in the brain is associated with pro-inflammatory activities, which is crucial to PD pathogenesis [21], COX-2 promotes MPTP-induced neuronal cell death via the induction of autophagic mechanisms. Neuroinflammation is associated with autophagy and is probably one of the main pathological mechanisms in PD [22].

The aims of this paper were to investigate the action of AU in a PD model by exploring its activity on DA and DOPAC brain levels and brain inflammation-related enzymes as well. Besides, the neuroprotective action of AU as the result of its influence on the activation of Nrf2 was also pursued.

2. Material and methods

2.1. Drugs and reagents

Auranofin (AU) was supplied by Life Chemicals (Kyiv, Ukraine). Ketamine (5% Vetanarcol) and xylazine (2% Kensol) were purchased from König® (König, SA, Argentina). 6-Hydroxydopamine (6-OHDA) and the monoamine standards

used in the high-performance liquid chromatography (HPLC) were from Sigma-Aldrich (USA). All other reagents were of analytical grade.

2.2. Animals

Male Wistar rats (250 g) were housed at the Animal Facility of the Faculty of Medicine Estácio of Juazeiro do Norte (FMJ, Ceará, Brazil) on a 12 h/12 h light/dark cycle, at 23 °C. The animals had access to food and water *ad libitum*. All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals, USA, 2011.

2.3. 6-OHDA stereotaxic surgery and the 6-OHDA model of PD

The unilateral 6-OHDA injections were performed as described before [23]. Rats were randomly divided into four experimental groups with 4 animals per group (total of 16 animals). The 1st group was unlesioned and treated with the AU solvent (FO, controls); the 2nd group was 6-OHDA-lesioned and treated with the AU solvent (6-OHDA); the 3rd group was 6-OHDA-lesioned and treated with AU 3 mg/kg, i.p. daily for 2 weeks (6-OHDA 3); and the 4rth group was 6-OHDA-lesioned and treated with AU, 10 mg/kg, i.p., daily for two weeks (6-OHDA 10). To perform the surgery, the stereotaxic apparatus, model EEF 331 from Insight, Ribeirão Preto SP, Brazil was used. The animals were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.). Their heads were placed in a stereotaxic apparatus and held in place by ear bars and a nose cone. A midline incision was made on the top of the skull and the skin retracted. A sterile dental drill was used to drill a small hole above the injection site. A stainless steel needle connected to a Hamilton syringe was used to inject either 6-OHDA (dissolved in saline containing 0.2% ascorbic acid) or it's solvent into the right striatum at two points accordingly to the following coordinates: 1st point: AP, +0.5; LL, -2.5; DV, -5.0; 2nd point: AP, -0.5; LL, -3.7; DV: -6.5 from the bregma, according to the Atlas of Paxinos and Watson (2005). One injection of 1μl on each point (6 μg), corresponding to a total of 12 μg of 6-OHDA for both points, was performed at a rate of 0.5 μl/min. The injection needle was left in place for 5 min before it was slowly withdrawn. The wound was closed using standard sutures for the skin. AU administration started one h after the stereotaxic surgery and continued for the following 14 days once a day at either a 3 or 10 mg/kg dose. AU dissolved in 10% ethanol was given at 3 or 10 mg/kg by oral gavage at a volume of 1 ml/100 g animal weight in 10% ethanol. Control untreated animals received the same volume of 10% ethanol solution.

2.4. High-performance liquid chromatography (HPLC) analyses

Striatal concentrations of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by HPLC with an electrochemical detector (Shimadzu, Japan). Striatal tissues were extracted, and 10% (w/v) homogenates were prepared in 0.1 M $HClO_4$. Tissue samples were subsequently sonicated for 30 s and centrifuged at 15,000 g for 15 min at 4 °C. The supernatants were filtered (0.2 μm filter) and a 20 μl sample was injected into a C18 (5 μm, 250 × 4.6 mm) HPLC column (Shimadzu). The mobile phase included 0.163 M citric acid (pH 3.0), 0.02 mM ethylenediaminetetraacetic acid (EDTA), 0.69 mM sodium octane sulfonic acid, 4% v/v acetonitrile, and 1.7% v/v tetrahydrofuran. The concentrations of dopamine and DOPAC were determined by using the standards injected into the HPLC column on the day of measurements, and the results were expressed as ng/g tissue. The method followed that described before [23].

2.5. Immunohistochemistry

After quickly decapitation Coronal slices of striata from all tested groups, were used for immunohistochemical analyses as described before [23]. Striatal tissue slices were fixed in formalin for 24 h, followed by immersion in 70% alcohol and paraffin sectioning (5 μm). After slices were deparaffinized and rehydrated, the endogenous peroxidase was blocked by 3% H_2O_2 in phosphate-buffered saline (PBS). After an additional washing step, tissue slices were incubated overnight with one of the following primary rabbit polyclonal antibodies against tyrosine hydroxylase (TH, anti-tyrosine hydroxylase antibody, produced in rabbit) from Chemicon/Sigma (CA, USA), dopamine transporter (DAT, monoclonal antibody produced in rat IgG2a), cyclooxygenase (COX-2, unconjugated rabbit polyclonal antibody from human), inducible nitric oxide synthase (iNOS, monoclonal antibody produced in mice) (all from Santa Cruz Biotechnology, Dallas, TX, USA) and Nuclear factor erythroid 2-related factor 2 (Nrf2, affinity isolated antibody, produced in rabbit), from Sigma-Aldrich, MO, USA. Antibodies were diluted1:200 in 0.05 M Tris buffer, pH 7.2–7.6, containing 1% bovine serum albumin. The slices were washed two times with PBS, followed by the addition of a biotinylated secondary goat anti-rabbit antibody (1:100, DAKO/Agilent, Santa Clara, CA, USA) for 1 h. Then tissues were washed twice with PBS before streptavidin-peroxidase reagent (DAKO) was added for 40 min. Following another wash with 3,3′-diaminobenzidine (DAB) and 0.05% v/vH_2O_2 solution, prepared according to the manufacturer's instructions, was added to the tissue slices for 30 s. Finally, tissues were washed and mounted on glass slides. Digital images were acquired by Leica XPRO® optical microscope with a built-in camera under 40 x objective. Random sampling, in a blinded manner, was applied in the different animal treatment groups by analyzing five different fields of view from three tissue slices per animal. The Image J software (National Institute of Health, NIH) was used for densitometry analysis, and data

are expressed as area density in arbitrary units. Routine studies using the omission of primary antibodies established that no immunoreactivity was observed in these control sections.

2.6. Molecular docking calculations

Auranofin and THIQ, were docked into the human domain Kelch of Keap1 (PDB ID 6SP1) [24], using the Glide software [25] in extra precision mode. The protein structure at pH 7.4 was prepared by adding hydrogen atoms and minimizing the system energy.7.4 inserting protonation, according to Epik [26], and performing energy minimization. The protein grid coordinates (centered at x: -2.32 Å, y: -3.16 Å and z: 28.13 Å) were built based on the coordinates of the THIQ ligand co-crystallized in the structure used 6SP1 [24]. The PLIP server [27] was used to identify interactions between protein residues and the ligand. The VMD 1.9.3 program [28] was used for the visual inspection of the docking poses and to render the 3D molecular figures.

2.7. Statistical analyses

The results are presented as means plus standard error of the mean (SEM). One-way analysis of variance (ANOVA), followed by the Tukey post hoc test or two-tailed unpaired t-test for DA and DOPAC measurements, were used to assess the statistical significance of the effects observed. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Measurements of striatal dopamine (DA) and 3,4-Dihydroxyphenylacetic acid (DOPAC) by HPLC in rats subjected to the 6-OHDA model of PD (hemi- parkinsonian rats)

Parkinson's disease (PD) is associated with progressive dysfunction of the dopaminergic neurotransmission in the basal ganglia leading to neurodegeneration of dopaminergic neurons in the *substantia nigra pars compacta* (Snpc) and dopamine dyshomeostasis [29, 30]. Our results showed an 83% reduction of DA contents in the lesioned striatum as related to the SO control group. No significant change was observed in the 6-OHDA group treated with AU (3 mg/kg). However, a 31% decrease was observed in the 6-OHDA group after treatment with the higher dose of AU (10 mg/kg). [$F(3,10)=36.22$, $p<0.0001$]. Concerning DOPAC, the main neuronal metabolite of DA, contents a 56% decrease was noticed in the 6-OHDA-lesioned striatum compared with the SO group. Importantly, no significant differences were observed in the 6-OHDA groups after the treatments with both doses of AU. [$F(3,10)=11.08$, $p=0.0016$] (Fig. 1).
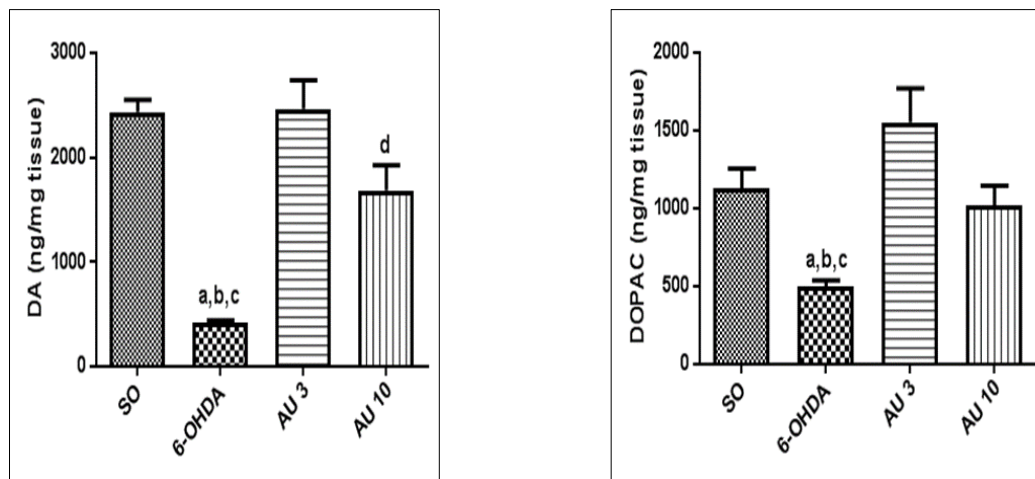

Figure 1 Effects of auronofin (AU) on the brain concentrations of dopamine (DA) and 3,4-Dihydroxyphenylacetic acid (DOPAC) in the striatum of hemiparkinsonian rats in groups of 4 animals per group. DA: a. vs. SO, p<0.0001; b. vs. 6-OHDA+AU3, p<0.0001; c. vs. 6-OHDA+AU10, p=0.0016; d. vs. SO, p=0.0420. DOPAC: a. vs. SO, p=0.0203; b. vs. 6-OHDA+AU3, p=0.0010; c. vs. 6-OHDA+AU10, p=0.0060 (Two-tailed unpaired t-test)

3.2. Immunohistochemistry analyses for the dopaminergic system markers, tyrosine hydroxylase (TH), and dopamine transporter (DAT) in the striatum of hemiparkinsonian rats.

Tyrosine hydroxylase (TH) is the enzyme catalyzing the formation of L-dihydroxyphenylalanine (L-DOPA), the rate-limiting step in the biosynthesis of DA. Thus, PD can be considered a TH-deficiency syndrome of the striatum [31]. We showed a 98% reduction in TH expression in the 6-OHDA-lesioned group compared with the SO group and, this change was prevented in the 6-OHDA groups after the treatments with both doses of AU [F(3,12)=320.2, p<0.0001] (Fig. 2). Lower changes were noticed in the 6-OHDA-lesioned group in DAT contents compared with the SO group (89% decrease), while around 23% reductions were seen in the 6-OHDA groups after AU treatments [F(3,12)=395.7, p<0.0001] (Fig. 3).
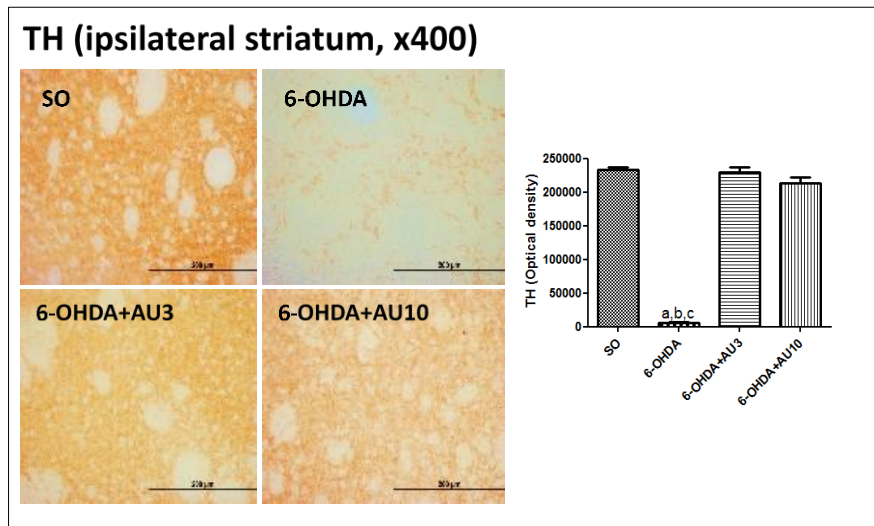

Figure 2 Representatives photomicrographs of the effects of auronofin (AU) on brain Tyrosine hydroxylase (TH) expressions in the striatum of hemiparkinsonian rats (scale= 200 μm). a. vs. SO, p<0.0001; b. vs. 6-OHDA+AU3, p<0.0001; c. vs. 6-OHDA+AU10, p<0.0001 (one-way ANOVA and the Tukey test for multiple comparisons)
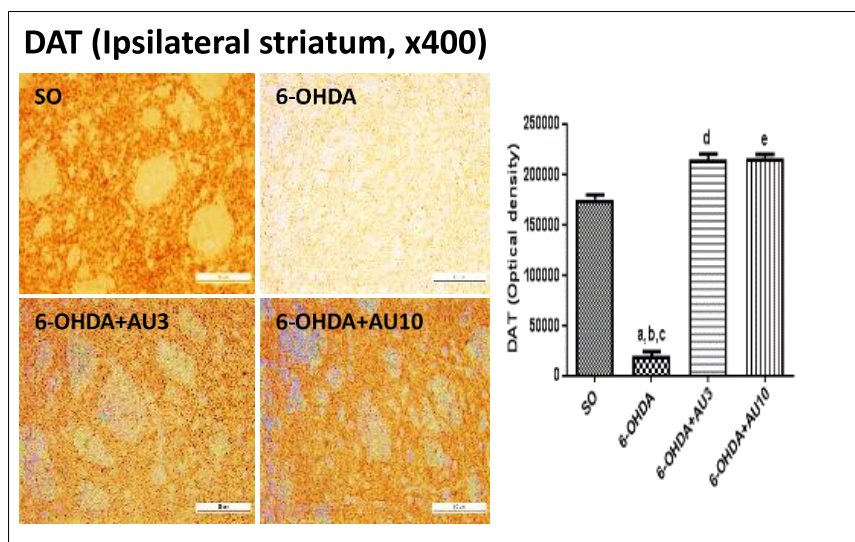

Figure 3 Representative photomicrographs of the effects of auronofin (AU) on brain dopamine transporter (DAT) expressions in the striatum of hemiparkinsonian rats (scale= 50 μm). a. vs. SO, p<0.0001; b. vs. 6-OHDA+AU3, p<0.0001; c. vs. 6-OHDA+AU10, p<0.0001; d. vs. SO, p<0.0003; e. vs. SO, p<0.0001 (one-way ANOVA and the Tukey test for multiple comparisons)

3.3. Immunohistochemistry analyses for the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the striatum of hemiparkinsonian rats.

The degeneration of dopaminergic neurons and focal gliosis are known to be pathological hallmarks of PD. Additionally, up-regulation of nitric oxide synthase- and cyclo-oxygenase-1- and -2-containing microglia were observed in the *substantia nigra* of PD patients' brains [16]. We observed drastic increases in the iNOS expression ($453*10^3$ times) in the 6-OHDA-lesioned striatum compared with the SO group. Much lower changes were noticed in the 6-OHDA groups after treatment with AU at the doses of 3 and 10 mg/kg ($86*10^3$ times and $51*10^3$ times, respectively. [$F_{(3,11)}=332.7$, $p<0.0001$] (Fig. 4). Concerning COX-2 changes, while an 18-times increase was observed in the 6-OHDA-lesioned group, the decreases were around 12- and 8-times after the treatments with AU (3 and 10 mg/kg, respectively) compared with the SO group [$F_{(3,16)}=37.86$, $p<0.0001$] (Fig. 5).
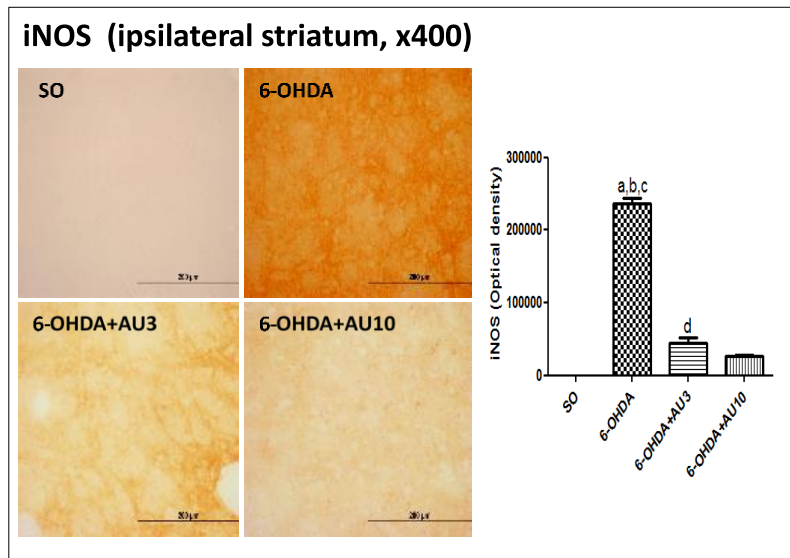

Figure 4 Representative photomicrographs of the effects of auronofin (AU) on brain inducible nitric oxide (iNOS) expressions in the striatum of hemiparkinsonian rats (scale= 200 µm). a. vs. SO, $p<0.0001$; b. vs. 6-OHDA+AU3, $p<0.0001$; c. vs. 6-OHDA+AU10, $p<0.0001$; d. vs. SO, $p=0.0011$ (one-way ANOVA and the Tukey test for multiple comparisons)
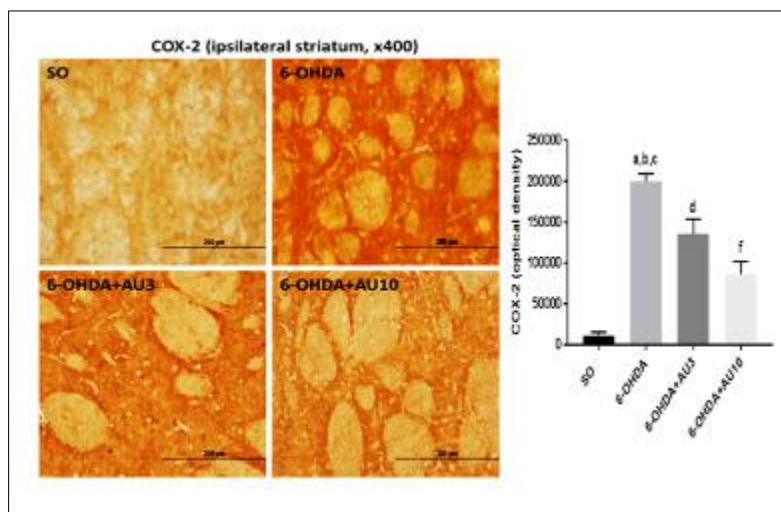

Figure 5 Representative photomicrographs of the effects of auronofin (AU) on brain cyclooxygenase-2 (COX-2) expressions in the striatum of hemiparkinsonian rats (scale= 200 µm). a. vs. SO, $p<0.0001$; b. vs. 6-OHDA+AU3, $p=0.0139$; c. vs. 6-OHDA+AU10, $p<0.0001$; d. vs. SO, $p<0.0001$ ((one-way ANOVA and the Tukey test for multiple comparisons)

3.4. Immunohistochemistry analysis for the Nuclear factor erythroid 2–related factor 2 (Nrf2) in the striatum of hemi parkinsonian rats

The Nuclear factor erythroid 2–related factor 2 (Nrf2) is an emerging regulator of cellular resistance to oxidants. Nrf2 controls the basal and induced expression of an array of antioxidant response element–dependent genes to regulate the physiological and pathophysiological outcomes of oxidant exposure [32]. In the present study, the 6-OHDA-lesioned striatum of hemiparkinsonian rats showed a 54% decrease in the Nrf2 expression compared with the control, SO group. On the other hand, only a 21% decrease in Nrf2 expression was noticed in the striatum of AU-treated rats with a dose of 10 mg/kg, suggesting a neuroprotective role for AU.
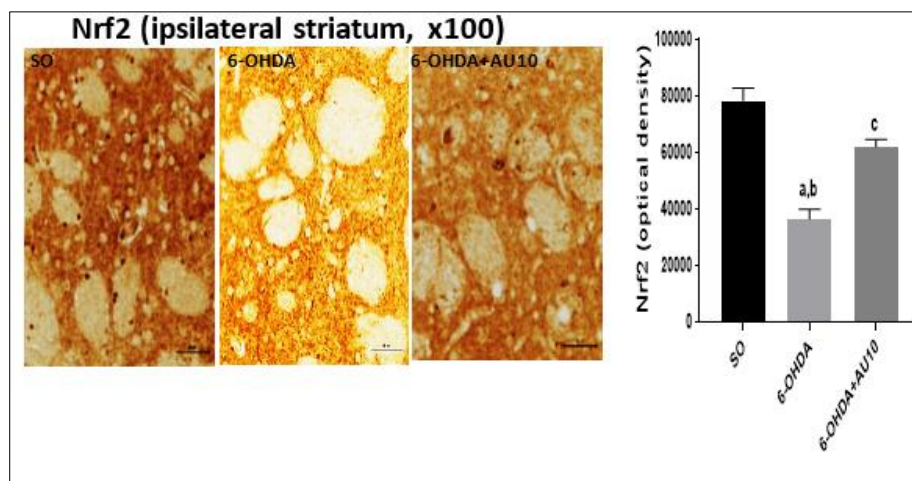

Figure 6 Representative photomicrographs of the effects of auronofin (AU) on brain Nuclear factor erythroid 2–related factor 2 (Nrf2) expressions in the striatum of hemiparkinsonian rats (scale= 50 μm). a. vs. SO, p<0.0001; b. vs. 6-OHDA+AU10, p=0.0011; c. vs. SO, p=0.0233

3.5. Molecular docking

To investigate the influence of auranofin in Keap1/Nrf2 interaction, its binding affinity, as well as its possible binding mode, we performed molecular docking calculations of auranofin against Keap1. Firstly, we performed a redocking calculation to validate the docking method and to verify if docking was able to recover a known complex's structure and interactions. Using the structure 6SP1, the docking of the Keap1 inhibitor, nonacidic tetrahydroisoquinoline (THIQ) compound, an inhibitor of Keap1/Nrf2 interaction (IC_{50} of 2.5 μM), at its original structure which presented a docking score of -9.98 Kcal·mol^{-1} was performed. The root means square deviation (RMSD) of THIQ docking pose in relation to its coordinates at crystal structure, and RMSD was 0.5 Å were calculated. RMSD calculation quantifies and compares the docking pose of the ligand with its co-crystallized pose. RMSD values below 2.0 Å indicate similar structures coordinates and, thus, docking reliability.

Auranofin docked at the Kelch domain of Keap1 and presented a good docking score of -8.10 Kcal·mol^{-1}, like the THIQ docking score. The docking pose of auranofin into the Kelch domain is shown in Fig. 7. Auranofin made H-bond with Ser602, salt bridge interactions with Arg415 and Arg483, and hydrophobic interactions with Tyr334 and Tyr525 residues. Several similar interactions were also observed in the crystal structure of Keap1-THIQ. The THIQ compound made H-bond with Ser602, Leu365, and Asn414, π-stacking interactions with Tyr572, salt bridges with Arg380 and Arg415, and hydrophobic interactions with Tyr334, Arg415, Ala565, and Tyr525. This suggests that auranofin may bind to the Kelch domain of Keap1 and possibly inhibit Keap1/Nrf2 interaction. By interrupting the interaction between Keap1 and Nrf2, auranofin could exert its neuroprotective activity.
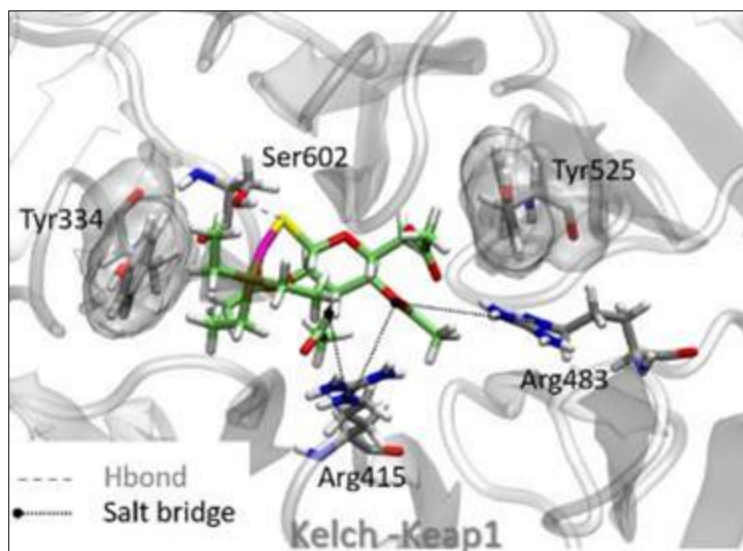
Figure 7 3D interactions of auranofin and Kelch domain of human Keap1. H-bonds, salt bridge, and hydrophobic interactions are shown as gray dotted lines, black dotted lines, and transparent surfaces, respectively. Sulfur, gold, oxygen, nitrogen, and hydrogen atoms are shown in yellow, pink, red, blue, and white, respectively. Carbon atoms of auranofin and protein residues are shown in green and gray, respectively

4. Discussion

Auronofin (AU) is a gold-based compound approved by the FDA (USA) for the treatment of rheumatoid arthritis. It has been investigated for its potential benefits in neurodegenerative diseases [1]. The main mechanism of action of auranofin is through the inhibition of reduction/oxidation enzymes that are essential for maintaining intracellular levels of reactive oxygen species (ROS), with consequent oxidative stress and apoptosis [1].

AU is a selective inhibitor of mammalian thioredoxin reductase (TrxR) playing a vital role in restoring cellular redox balance disrupted by reactive oxygen species (ROS) generation and oxidative damage [33]. TrxRs are the major cellular protein disulfide oxidoreductases critical for both redox homeostasis regulation of protein function and signaling. A growing number of transcription factors including NF-κB require thioredoxin reduction for DNA binding [34, 35, 36].

Both the cytosolic and mitochondrial forms of TrxR are known AU targets. Besides, AU was also reported to inhibit proteasome, the central component of the main cellular degradation pathway [37, 38]. Most importantly, the elimination of impaired proteins is essential for cell viability and its failure leads to a number of the neurodegenerative diseases that are prominent in the elderly population [39, 40, 41],

In the present work, it was investigated for the first time, the effects of the possible neuroprotective effect of AU on male rats subjected to the 6-OHDA model of PD treated daily for 14 days. This classical method involves the massive destruction of nigrostriatal dopaminergic neurons and is largely used to investigate motor and biochemical dysfunctions in PD [42, 43].

The model induced by 6-OHDA does not show all PD symptoms but reproduces the main cellular processes involved in PD, such as oxidative stress, neurodegeneration, neuroinflammation, and neuronal death by apoptosis [44]. When 6-OHDA is delivered into the striatum, as in the present study, induces slow, progressive, and partial damage to the nigrostriatal structure in a retrograde fashion over up to 3 weeks [45].

PD neurodegeneration leads to incapacitating motor abnormalities due to the loss of dopaminergic neurons in the *substantia nigra pars compacta, SNpc* [46]. Most importantly, one of the characteristic features of PD is that it primarily affects a restricted neuronal population, although other neurons are also affected, the dopaminergic neurons of *SNpc* are the most vulnerable, and the reason for this selective cell loss is still an open question [47].

Corroborating with the above literature data, we showed that the 6-OHDA-lesioned striatum group presents a great reduction in DA contents and this change was prevented in the 6-OHDA groups treated with both doses of AU (3 and 10 mg/kg). Interestingly, the effects observed with the lower dose were even better than those with the higher AU dose

indicating that the drug maximum response was already reached. The DA main metabolite, DOPAC showed results like that demonstrated with DA, indicating an ongoing neurodegenerative process.

The enzyme tyrosine hydroxylase (TH) catalyzes the formation of L-dihydroxyphenylalanine (L-DOPA), the rate-limiting step in DA biosynthesis thus, PD is a TH-deficiency striatum syndrome [31]. An earlier study [48], showed that injections of 6-OHDA into the medial forebrain bundle in rats destroy the dopaminergic neurons in the *SNpc*. According to these authors four weeks after the 6-OHDA injection, there was an 85% reduction in the number of TH-immunoreactive cells on the lesioned side, results like ours.

DAT (the dopamine transporter) is the most important mechanism for the re-uptake of extracellular dopamine into presynaptic terminals, regulating the amount of dopamine (DA) available for dopamine receptor stimulation after its release from the presynaptic terminal [49]. Previous pathological studies disclosed a severe depletion of DAT in the striatum of PD patients. Measurement of DAT was correlated with striatal dopamine levels in PD patients [50,51]. A reduction of DAT density is implicated in PD by neuroimaging where has been shown that dopamine turnover is elevated in early symptomatic PD [52] and pre-symptomatic individuals with monogenic mutations causal for parkinsonism.

We showed a great reduction in TH and DAT expressions in the striatum of 6-OHDA-lesioned rats. Most importantly, these changes were highly prevented in the 6-OHDA-lesioned groups after AU treatments with both doses. Interestingly, increases in DAT expression were demonstrated with a lower dose of 1 mg/kg (data not shown). The observation of increased expressions of TH and DAT suggests that AU presents neuroprotective effects in hemiparkinsonian rats.

Evidence [54], indicates the role of iNOS in the neurodegeneration of nigrostriatal dopaminergic neurons as observed in the 6-OHDA animal model of PD, the same as that one used by us. In addition, the brain expression of COX-2 was shown to be closely associated with pro-inflammatory activities and the subsequent generation of prostaglandins (PGs) are instrumental in neurodegenerative processes including in PD [18, 19].

Degeneration of dopaminergic neurons and focal gliosis are pathological hallmarks of PD and an up-regulation of iNOS and COX-1 and -2 in microglia have been found in parkinsonian [8]. An earlier study found that AU stimulated COX-1-dependent PGE2 production but inhibited COX-2-dependent PGE2 production in a concentration-dependent manner. The latter effect was found to be due to the inhibition of COX-2 protein induction [54]. Another study showed that AU inhibits both PGE2 production and NO production by inhibiting the upregulation of mRNA levels of COX-2 and NOS [55].

In the present study, a drastic increase in the iNOS expression in the striatum of 6-OHDA-lesioned rats compared with the 6-OHDA groups after AU treatments was observed. Concerning the COX-2 expression, although significant, the changes were lower and dose-dependent in the 6-OHDA-treated groups. These results corroborated with the above literature data, pointing out a neuroprotective effect of AU in this model of PD. AU has been shown to decrease the production of NO and the pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6 (tumor necrosis factor-alpha, interleukin-1 beta, interleukin-6) in macrophages besides inhibiting COX-2-dependent prostaglandin E2 (PGE2) production in a concentration-dependent manner [56], what corroborates with the results of the present study.

The nuclear factor erythroid 2-related factor 2 (Nrf2) is a well-known transcription factor for its function in controlling the basal and inducible expression of various antioxidants and detoxifying enzymes. Most neurodegenerative conditions such as Parkinson's disease are characterized by oxidative stress, misfolded protein aggregates, and chronic inflammation, the common targets of Nrf2 therapeutic strategies [57]. In addition to regulating the expression of antioxidant genes, Nrf2 has also been shown to exert anti-inflammatory effects and modulate both mitochondrial function and biogenesis [58].

In the present study, we showed that 6-OHDA decreases Nrf2 expression in the lesioned striatum of hemiparkinsonian rats. In addition, AU prevented this effect, and values of Nrf2 expression were close to those of the control group. Modulation of Nrf2 has been shown in several neurodegenerative disorders and its overexpression has become a potential therapeutic target for neurodegenerative disorders such as Parkinson's [59]. Evidence indicates that Nrf2-linked pathways are involved in protective mechanisms against oxidative stress by regulating antioxidant and phase II detoxifying genes. As such, Nrf2 deregulation has been linked to both aging and the pathogenesis of many human chronic diseases, including neurodegenerative diseases [60].

Nrf2 was also shown to play a pivotal role in controlling the expression of antioxidant genes that ultimately exert anti-inflammatory functions. Nrf2 is known to induce gene expression of antioxidant enzymes and proteasome subunits. Because both oxidative stress and protein aggregation have damaging effects on neurons, activation of the Nrf2

signaling should be beneficial against neurodegeneration and could counteract mitochondrial dysfunction in PD [61, 62, 63, 64]. Nrf2 is known to govern the gene expression of endogenous antioxidant synthesis and ROS-eliminating enzymes in response to various electrophilic compounds that inactivate the negative regulator Keap1. Additionally, evidence showed that mitochondrial ROS (mtROS) activate Nrf2 [65],

AU was shown to inhibit Nrf2 degradation by inducing the dissociation of the Nrf2 / Kelch-like ECH-associated protein 1 (Keap1) complex, which resulted in nuclear accumulation of Nrf2 [66]. Recent data demonstrated that AU-induced Nrf2 activation directly suppresses IL-1β synthesis independent of NF-κB and glutathione-mediated antioxidant mechanisms [67].

At the cellular level, oxidative stress, chronic neuroinflammation, mitochondrial dysfunction, and aberrant protein aggregation have been implicated in neurodegenerative diseases such as PD. The molecular docking experiments as shown in the present study, suggests that auranofin may bind to Kelch domain of Keap1 and possibly inhibit Keap1/Nrf2 interaction. Thus, activation of transcription factor Nrf2 may be a therapeutic strategy to counteract alterations as those demonstrated in the brain of PD patients [68].

5. Conclusion

The search for new treatments for PD is urgent given that the available ones do not cure or even slow down the progression of the neurodegenerative process. The oxidative stress is known to be involved in the initiation and progression of PD through the degeneration of dopaminergic neurons.

AU emerges as an option to be further studied considering that, in addition to its already proven anti-inflammatory activity, our study demonstrated its ability to reduce neuroinflammation, decreasing the striatal expression of iNOS and COX-2, as well as inhibiting an important pathway of signaling, via activation of Nrf2, which leads to increased oxidative stress and mitochondrial dysfunction, key players in the pathophysiology of PD as well as in the 6-OHDA-induced PD model.

In this way, AU was able to maintain normal levels of dopamine, as well as TH and DAT, demonstrating its neuroprotective role. and its possibility to be a new therapeutic strategy focused on nonconventional anti-inflammatory activity. Importantly, Nrf2 is essential for maintaining redox homeostasis by binding to the antioxidant response element of most genes coding for antioxidant enzymes. Nrf2 may provide a therapeutic option to mitigate oxidative stress-associated PD and AU as an Nrf2 activator could represent an important therapeutic strategy to be pursued for PD treatment .

 In addition, Nrf2 is closely related to redox metabolism, proteostasis and inflammation. Therefore, pharmacological activation of Nrf2 by AU as observed in the present work, is a promising therapeutic approach for neurodegenerative chronic diseases such as PD, which are underlined by mitochondria dysfunction, oxidative stress and neuroinflammation.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare they have no conflict of interests.

Statement of ethical approval

The project was approved by the Ethics Committee on Animal Experimentation of the Faculty of Medicine of the Federal University of Ceará (UFC).

Abbreviations

- AU- Auronofin
- TrxRs- Thioredoxin reductase
- ROS- Reactive oxygen species
- RNS- Reactive nitrogen species
- SNpc- Substantia nigra pars compacta
- PGE2-Prostaglandins E-2
- THIQ- tetrahydroisoquinoline (Keap 1 inhibitor)
- RMSD- Root means square deviation
- TNF-alpha- Tumor necrosis factor-alpha
- IL-1beta- Interleukin-1 beta
- IL-6- Interleukin 6
- HO-1- Heme oxygenase 1
- PD- Parkinson's disease
- 6-OHDA- 6-hydroxydopamine
- DA- dopamine
- DOPAC- 3,4-Dihydroxyphenylacetic acid
- TH- tyrosine hydroxylase
- DAT- Dopamine transporter
- iNOS- Inducible nitric oxide synthase
- COX-2- Cyclooxygenase-2
- Nrf2- Nuclear factor erythroid 2-related factor 2
- Keap1- Kelch like ECH associated protein 1

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