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ORIGINAL PAPER

Protective Effects of Ursolic Acid Against Cytotoxicity Induced by Corticosterone: Role of Protein Kinases

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Abstract

Neuronal hippocampal death can be induced by exacerbated levels of cortisol, a condition usually observed in patients with Major depressive disorder (MDD). Previous in vitro and in vivo studies showed that ursolic acid (UA) elicits antidepressant and neuroprotective properties. However, the protective effects of UA against glucocorticoid-induced cytotoxicity have never been addressed. Using an in vitro model of hippocampal cellular death induced by elevated levels of corticosterone, we investigated if UA prevents corticosterone-induced cytotoxicity in HT22 mouse hippocampal derived cells. Concentrations lower than 25 µM UA did not alter cell viability. Co-incubation with UA for 48 h was able to protect HT22 cells from the reduction on cell viability and from the increase in apoptotic cells induced by corticosterone. Inhibition of protein kinase A (PKA), protein kinase C (PKC) and, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), but not phosphoinositide 3-kinase(PI3K), by using the pharmacological the inhibitors: H-89, chelerythrine, KN-62, and LY294002, respectively totally abolished the cytoprotective effects of UA. Finally, UA abrogated the reduction in phospho-extracellular signal–regulated kinases 1 and 2 (ERK1/2) but not in phospho-c-Jun kinases induced by corticosterone. These results indicate that the protective effect of UA against the cytotoxicity induced by corticosterone in HT22 cells may involve PKA, PKC, CaMKII, and ERK1/2 activation. The cytoprotective potential of UA against corticosterone-induced cytotoxicity and its ability to modulate intracellular signaling pathways involved in cell proliferation and survival suggest that UA may be a relevant strategy to manage stress-related disorders such as MDD.

Keywords Corticosterone · Cytotoxicity · HT22 cells · Neuroprotection · Protein kinase · Ursolic acid

Introduction

Major depressive disorder (MDD) is a highly prevalent psychiatric disorder responsible for a great burden on society [1], resulting in impaired social functioning [2] and elevated costs to the health-care system and economy [3]. A strong association between stress and MDD has been reported since nearly 80% of cases of this disorder are preceded by chronic stressful life events [4]. This relationship can be explained by the neuro-hormonal hypothesis of depression [5], which postulates that depressive symptoms may occur as a consequence of an altered stress response. Therefore, an augment in the activity of the hypothalamic–pituitary–adrenal axis and a reduction in their negative feedback mechanism leads to high systemic levels of glucocorticoids [6–8]. In the central nervous system (CNS), excessive levels of glucocorticoids may cause atrophy and neuronal death in the hippocampus [9, 10] of rodents and non-human primates [11–16], and human patients [17–19]. Based on these observations, neuronal cells exposed to glucocorticoids such as corticosterone have been a valid in vitro model of stress because they mimic neurochemical and morphological features observed in the hippocampus of depressed patients [20–24]. In this regard, the HT22 mouse hippocampal neuronal cell line is highly sensitive to glucocorticoids [25] and

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has been widely used to understand neurotoxic processes in the hippocampus as well as to establish novel neuroprotective agents that could counteract the cytotoxicity of corticosterone [20–24].

Currently, there is an increasing interest in pentacyclic triterpenes such as ursolic acid (UA) due to their wide range of pharmacological activities [26]. UA constitutes a phytochemical and bioactive compound commonly found in medicinal herbs, like ginseng (*Panax ginseng*), calendula (*Callendula officinalis*), rosemary (*Rosmarinus officinalis*), as well as fruits such as apple (*Malus domestica*), pear (*Pyrus pyrifolia*), and plum (*Prunus domestica*) [27]. Several in vivo and in vitro studies have demonstrated that UA has anti-inflammatory, hepatoprotective, antioxidant, chemopreventive, and neuroprotective properties [26, 28]. These effects combined with its low toxicity makes UA a promising alternative pharmacological resource for the management of MDD. Our group showed that UA elicits antidepressant-like effects in the tail suspension test and forced swim test [29–31], two predictive behavioral tests used to verify antidepressant-like effects of compounds [32–34]. The antidepressant-like effect of UA in mice depends on protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CAMK-II), and mitogen-activated protein kinase (MAPK) modulation [30]. Evidence from in vitro studies suggests that UA may exert anti-stress effects by antagonizing the corticotropin-releasing hormone receptor 1, reducing cortisol release, and by inhibiting the activity of 11β-hydroxysteroid dehydrogenase type 1 enzyme, which catalyzes the conversion of inactive cortisone to active cortisol [35, 36]. However, the antidepressant-like effect of UA has not been evaluated in an in vitro model of stress induced by corticosterone. Therefore, the aim of this study was to investigate the ability of UA to counteract the corticosterone-induced cytotoxicity, as well as, the role of protein kinases in this effect.

Materials and Methods

Drugs

BisBenzimide H 33342 trihydrochloride (Hoechst33342), corticosterone, chelerythrine, RU-486, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium high glucose (DMEM), piperazine ethane sulfonic acid, H89, propidium iodide (PI), KN62, sodium dodecyl sulfate (SDS), and UA were obtained from Sigma-Aldrich (São Paulo, Brazil). LY294002 was purchased from Tocris (Biogen Científicas, Madrid, Spain). Penicillin, pyruvate, heat-inactivated fetal bovine serum (FBS), streptomycin, and trypsin 0.05%, and ethylenediamine tetraacetic acid (EDTA) were purchased from Invitrogen (São Paulo, Brazil). L-glutamine and sodium bicarbonate obtained from Vetec (Duque de Caxias, Brazil). Corticosterone, chelerythrine, RU-486, H89, KN62, LY294002 and UA were dissolved in a final concentration of 0.1% DMSO.

Culture of HT22 Cells

Immortalized mouse hippocampal HT22 cells were a gift from Dr. Pamela Maher (Salk Institute, La Jolla, CA, U.S.A.). HT22 cells were cultured in DMEM high glucose (pH 7.4), supplemented with 10% heat-inactivated FBS, maintained and sub-cultured at 37 °C in a humidified atmosphere with 5% CO_2 as previously described [21]. Cells were treated with drugs before confluence in DMEM with 10% FBS. Cells were used at passages below 13. All experiments were conducted in triplicate using different cell batches.

Cell Viability Assay

MTT, a soluble tetrazolium salt that is converted into an insoluble purple formazan by viable cells (mitochondrial dehydrogenases activity of living cells), can be measured by a quantitative colorimetric assay. At the end of the treatment period, the medium was removed and MTT reagent (at a final concentration of 0.5 mg/ml, dissolved in Hank's Balanced Salt Solution, pH 7.4) was added to each well and incubated for 2 h at 37 °C. Then, the insoluble formazan was dissolved with DMSO and the optical density (OD) was estimated spectrophotometrically at 540 nm. Control cells treated with medium (DMEM + 10% FBS) were considered as 100% viable cells. Cellular viability is proportional to absorbance [37].

Detection of Cell Death Using Propidium Iodide (PI) and Hoechst

The double-staining PI/Hoechst is widely used to evaluate cell membrane integrity since damage to plasma membranes allows PI uptake [38]. Right after the treatments, PI (1 µg/ml) and Hoechst 33342 (5 µg/ml) were added to the medium of each well and incubated for 20 min at 37 °C, as previously described [39]. Then, HT22 cells were washed with PBS 100 mM and observed in an inverted fluorescent microscope Axiovert 40 CFL (Carl Zeiss). Analyses were conducted taking random photographs of 3 wells for each treatment using an Axiocam MRc coupled to the microscope [39]. Cells positive for PI^+ and $Hoechst^+$ were analyzed as particles using Image J v1.50b (NIH) "watershed plugin" (minimal size of 0.001 pixels) as previously described [39]. PI^+ cells were expressed as a percentage of total cells ($Hoechst^+$). Each experimental condition was conducted in triplicate and 5 independent experiments were performed.

Flow Cytometry

The number of viable, early apoptotic, and late apoptotic cells was evaluated by flow cytometry using Annexin V-phycoerythrin (PE)/7-aminoactinomycin D (7-AAD) double staining kit (BD Bioscience, Madrid, Spain) as previously reported [21], and in accordance with the manufacturer's instructions. The cells were analyzed using a FC 500 MPL Cytometer (Beckman Coulter, Madrid, Spain). At least 10,000 events per well were represented in the dot plots. The percentage of viable (annexin$^-$/7AAD$^-$), early apoptotic (annexin$^+$/7AAD$^-$), and late apoptotic (annexin$^+$/7AAD$^+$) cells was measured. In addition, the percentage of total apoptosis was calculated based on all the annexin$^+$/7AAD$^-$ (early apoptotic) plus the annexin$^+$/7AAD$^+$ (late apoptotic), and it was represented as bar graphs. The experimental conditions were conducted in triplicates and four independent experiments were performed.

Western Blot

After the incubation was completed, the medium was removed, wells were washed with 100 mM PBS and mechanically dissociated in 200 µl SDS-stopping solution (containing 4% SDS, 2 mM EDTA, 50 mM Tris) with the aid of a cell scraper. Immediately, the samples were placed in a dry bath at 100 °C for 5 min. Thereafter, samples were vortexed and centrifuged for 10 min at 13,000 rpm. Finally, the supernatant was collected and was added sample dilution solution (40% glycerol, 100 mM Tris, bromophenol blue, pH 6.8) in the ratio 25:100 (v/v) and β-mercaptoethanol (final concentration 8%). The protein content of the samples was quantified by the method described by Peterson [40], using bovine serum albumin (BSA) as standard. The electrophoresis was performed by applying 40 µg of protein per condition in a 10% SDS-PAGE minigel, and then, transferred to nitrocellulose membranes [21]. After blocking with 2% BSA, the membranes were incubated overnight (at 4 °C) with the following primary antibodies: anti-JNK and anti-phosphorylated JNK, anti-ERK1/2 and anti-phosphorylated ERK1/2 (See Table 1). Moreover, the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody or (HRP)-conjugated anti-mouse IgG antibody (See Table 1). Immunoreactivity was detected in a ChemiDoc© Bio-Rad photodocumentation system. All blocking and incubation steps were followed by three times (5 min) washing with TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5). All membranes were incubated with mouse anti-β-actin (1:2000) antibody to verify that equal amounts of protein for each sample were loaded on the gel. Measurement of the optic density (OD) of the protein bands was performed using ImageLab software. The phosphorylation level was determined as a ratio of the OD of the phosphorylated band/the OD of the total band. The immunocontent of ERK1/2 and JNK was determined as a ratio of the optic density (OD) of each protein band/the OD of the β-actin band. Data are expressed as a percentage of the control group.

Statistical Analysis

Data are represented as mean + S.E.M. Comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test when appropriate. $P < 0.05$ was considered significant.

Results

Corticosterone Induces Time- and Concentration-Dependent Cytotoxicity

To analyze the effect of corticosterone on cell viability, HT22 cells were treated with increasing concentrations of corticosterone (5, 50, 100, 500 or 1000 µM) or vehicle (0.1% DMSO), and afterward, the cell viability was evaluated by MTT assay. Incubation of HT22 cells with 50 or 100 µM reduced to almost 40% their viability, while higher concentrations of corticosterone (500 and 1000 µM) reduced approximately 54% of cell viability when compared to vehicle (Fig. 1a). In a parallel experiment, we investigated the time-course of corticosterone-induced cytotoxicity. Thus, HT22 cells were incubated for

Table 1 Description of antibodies used in this study

Primary antibody	Type	Source	Company	Code	Dilution
Anti p-JNK1/2	Polyclonal	Rabbit	Cell signaling	#9251S	1/1000
Anti t-JNK1/2	Polyclonal	Rabbit	Sigma	J4500	1/1000
Anti p-ERK1/2	Monoclonal	Mouse	Sigma	M8159	1/5000
Anti t-ERK1/2	Monoclonal	Rabbit	Sigma	M5670	1/20,000
Anti β-actin	Monoclonal	Mouse	Santa Cruz	sc-4778	1/2000
Anti-rabbit IgG	Policlonal	Rabbit	Millipore	AP132P	1/2500
Anti-mouse IgG	Policlonal	Mouse	Millipore	AP308P	1/2500

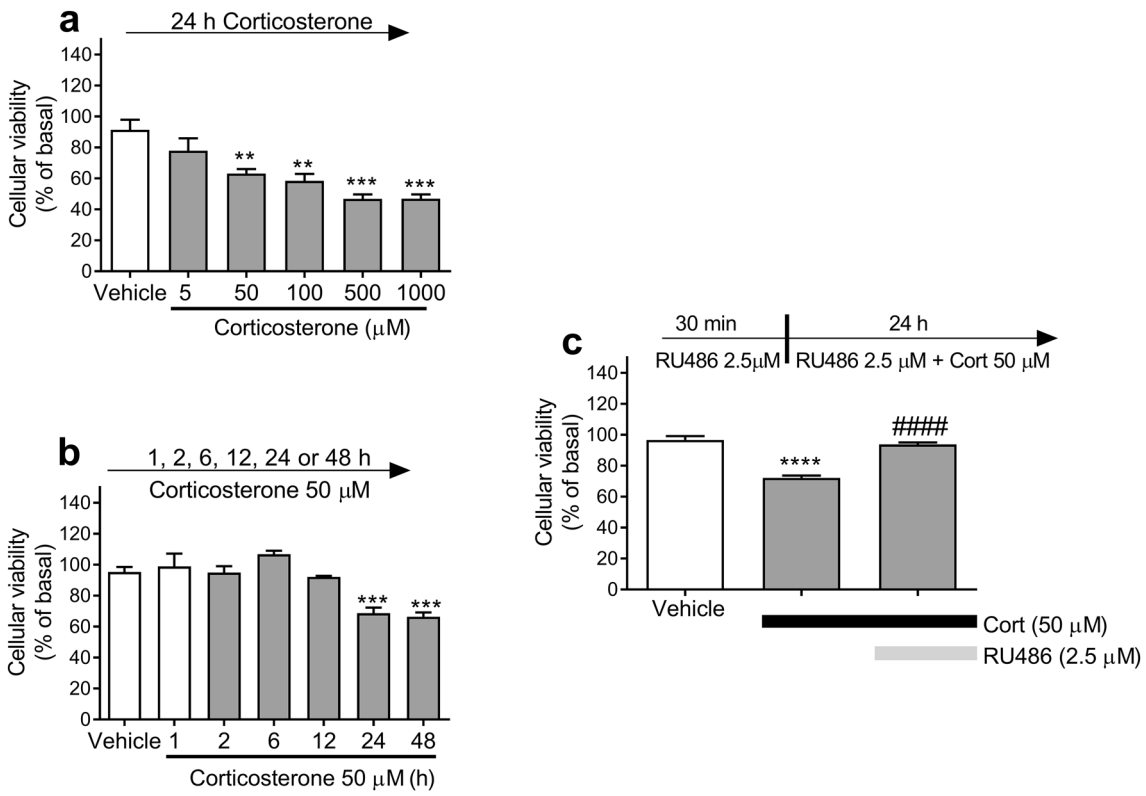


Fig. 1 Corticosterone induces toxicity in HT22 cells. **a** HT22 cells were treated with vehicle (0.1% DMSO) or corticosterone (5, 50, 100, 500 or 1000 μM) (n = 3). **b** HT22 cells were treated with vehicle (0.1% DMSO) or corticosterone (50 μM) in a time-response curve (n = 4–5). **c** Incubation of HT22 cells with RU486 (2.5 μM) or vehicle (0.1% DMSO) followed by incubation with corticosterone (50 μM) for an additional 24 h-period prevented the cytotoxic effect of corticosterone (n = 3–6). At the end of treatments, cell viability was measured by MTT assay. Results are expressed considering the basal condition as 100% of cell viability (mean + S.E.M.). $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$, as compared to the vehicle group. $^{\#\#\#\#}P < 0.0001$, as compared to the corticosterone-group (one-way ANOVA followed by Newman–Keuls post hoc test). *Cort* corticosterone

1, 2, 6, 12, 24 or 48 h with the lower cytotoxic concentration of corticosterone (50 μM) or vehicle (0.1% DMSO) and, at the end of the incubation period, MTT was analyzed. Incubation for 24 or 48 h with corticosterone reduced cell viability to almost 40% (Fig. 1b). In addition, the statistical analysis showed that 24 h of corticosterone incubation corresponds to the minimal amount of time needed to induce cytotoxicity in HT22 cells (Fig. 1b).

Considering that HT22 cells can express glucocorticoid receptors (GR), we examined if GR activation is involved in the corticosterone-induced cytotoxicity observed in our experiments. To this end, HT22 cells were pre-incubated with RU486 (2.5 μM), a GR antagonist or vehicle (0.1% DMSO) for 30 min, and co-incubated with RU486 and corticosterone 50 μM or vehicle (0.1% DMSO) for 24 h. The statistical analysis revealed that pre-treating HT22 cells with RU486 significantly prevented the cytotoxic effects elicited by corticosterone (Fig. 1c), confirming the participation of glucocorticoid receptors in its toxic effect.

Time and Dose–Response of HT22 Cells Followed by UA Treatment

To test the effect of UA on cell viability, HT22 cells were treated for 24 h with increasing concentrations of UA (5, 15, 25 or 50 μM) or vehicle (0.1% DMSO). In this experiment, 5, 15 or 25 μM of UA did not alter cell viability. However, incubation with 50 μM UA reduced by almost 60% the viability of HT22 cells (Fig. 2a). We also measured changes in cell viability after incubation with 5 or 15 μM at different time points (24, 48 or 72 h). The statistical analysis revealed that UA did not significantly affect cell viability at the different time points analyzed (Fig. 2b).

UA Decreases Corticosterone-Induced Cell Toxicity

Once the non-toxic concentrations of UA were established, we investigated whether they would be able to prevent the cytotoxicity induced by corticosterone. The incubation for 24 h with UA (5 or 15 μM) prior to the co-incubation

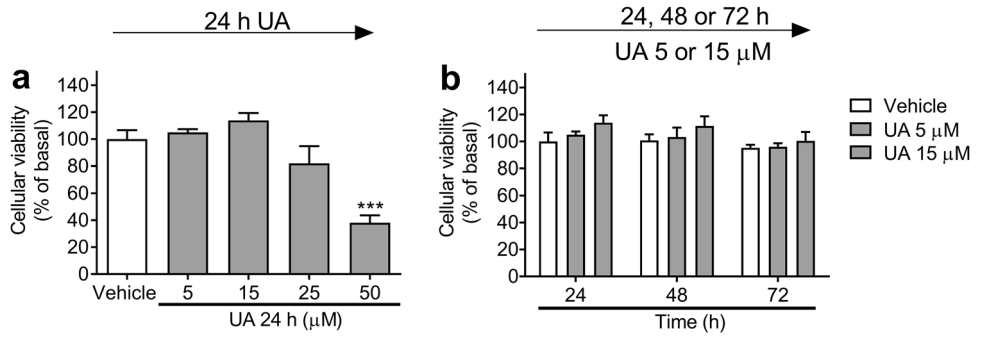
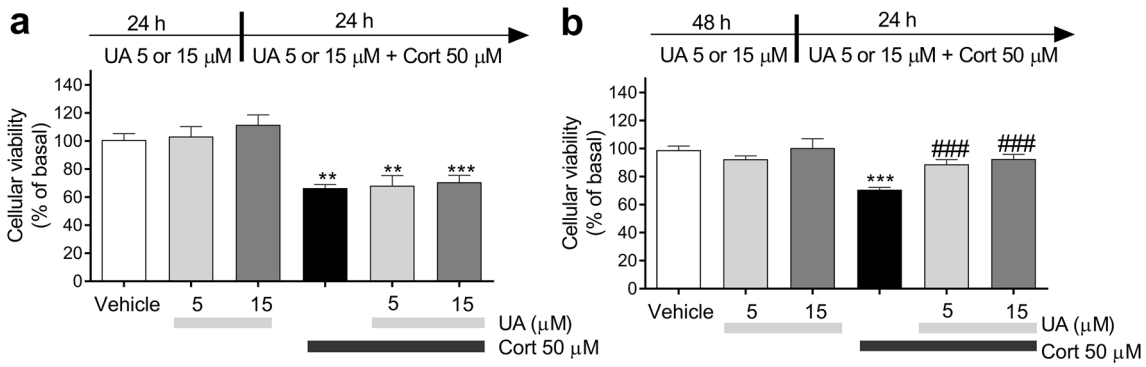


Fig. 2 Effect of UA on HT22 cells. Dose–response curve of UA (5, 15, 25 or 50 μM) or vehicle (0.1% DMSO) on viability of HT22 cells (n = 4). **a** HT22 cells were incubated with UA (5, 15, 25 or 50 μM) or vehicle in a time-response curve (n = 4–6). **b** The results are expressed as baseline percentage (mean + S.E.M. One-way ANOVA followed by Newman–Keuls post hoc test). $^{***}P < 0.001$, as compared to vehicle



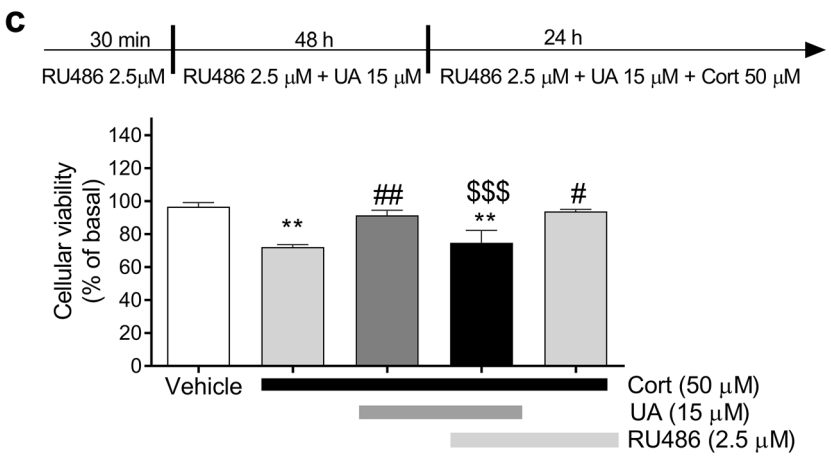


Fig. 3 UA produces cytoprotective effects against corticosterone in HT22 cells. The incubation for 24 h with UA (5 or 15 μM) prior to the incubation with corticosterone for a further 24 h did not alter the cytotoxic effect of corticosterone in HT22 cells (n = 6). **a** The incubation of HT22 cells for 48 h with UA (5 or 15 μM) prior to the incubation with corticosterone for a further 24 h prevented the cytotoxic effect of corticosterone on HT22 cells (n = 6). **b** Co-incubating HT22 cells with RU486 (2.5 μM) prevented the cytoprotective effect exerted by UA (15 μM). **c** The results are expressed as baseline percentage (mean + S.E.M. One-way ANOVA followed by Newman-Keuls post hoc test). $^{**}P < 0.01$ and $^{***}P < 0.001$, as compared to vehicle, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, as compared to corticosterone; $^{\$\$\$}P < 0.001$, as compared to UA + corticosterone group. Cort: corticosterone

with 50 µM corticosterone for another 24 h, was not able to prevent the cytotoxic effect produced by corticosterone (Fig. 3a). We also tested whether a longer pre-incubation time with UA (48 h) would be able to prevent corticosterone-induced cytotoxicity. In this second protocol, 48 h incubation with 5 or 15 µM UA, prior to co-incubation with corticosterone, prevented corticosterone-induced cytotoxicity (Fig. 3b). Another independent experiment showed that incubation with RU486 (2.5 µM) prevented the cytoprotective effect of UA (15 µM) against corticosterone-induced cytotoxic effect (Fig. 3c). None of the UA concentrations per se altered the viability of HT22 cells.

UA Prevents Corticosterone-Induced Cell Membrane Permeability

To further investigate the cytoprotective effect of UA against corticosterone, we evaluated membrane permeability to PI after different treatment conditions (Fig. 4a). First, HT22 cells were pre-incubated with 15 µM UA for 48 h before the

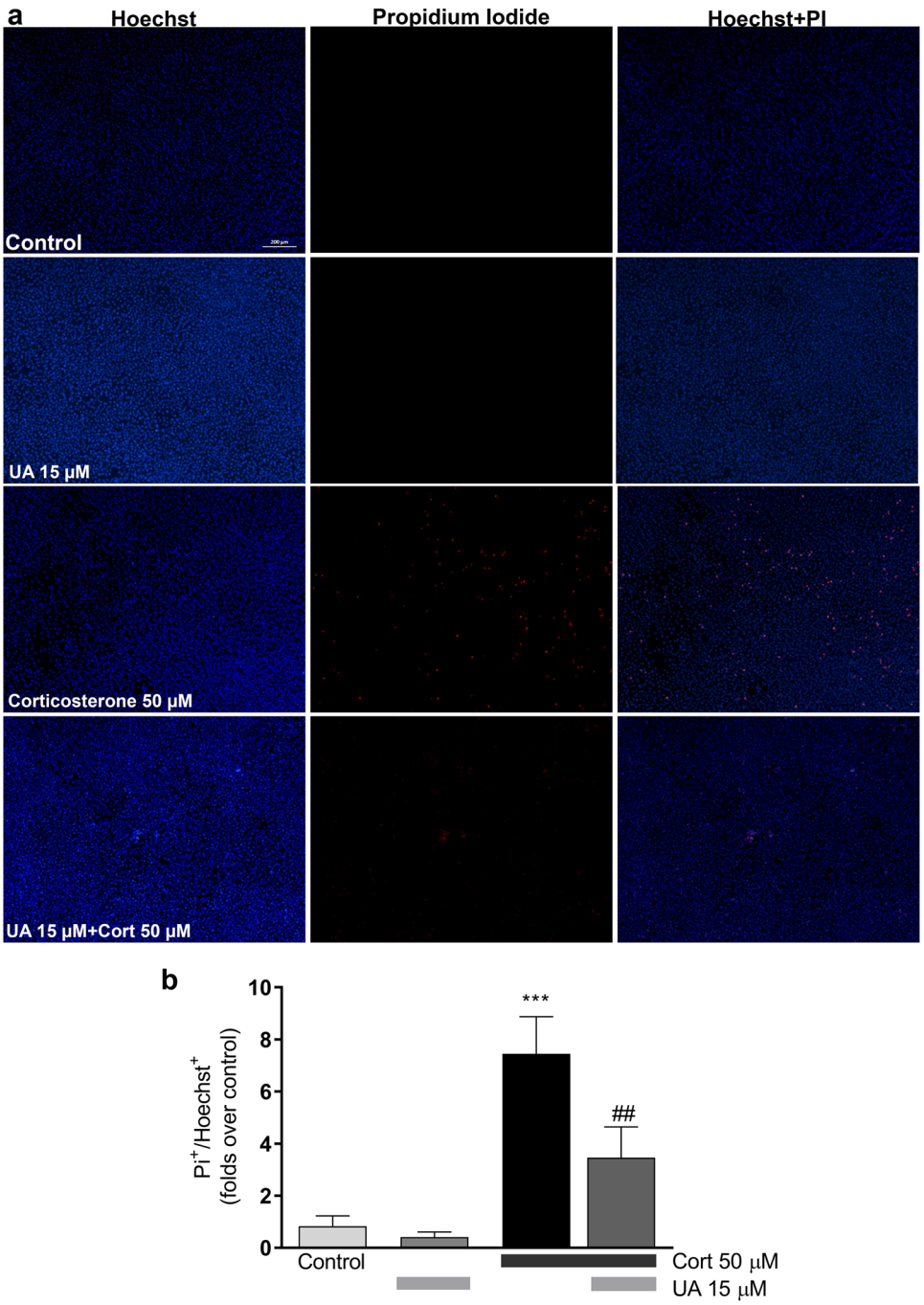


Fig. 4 Cytoprotective effect of UA against corticosterone-induced cell permeability. **a** Representative fluorescence microphotograph obtained after Hoechst and PI double staining in control, UA, corticosterone, and UA followed by corticosterone-treated groups. The treatment of cells with UA for 48 h before corticosterone treatment significantly prevented the corticosterone-induced increase in the percentage of PI^+ cells. Scale bar: 200 µm. **b** The percentage of $PI^+/Hoechst^+$ cells was measured from 5 independent experiments using ImageJ particles analysis plugin and compared to the percentage of control-treated group. All data are expressed as mean + SEM. One-way ANOVA analysis was used for comparison among multiple groups followed by Newman Keuls post hoc test. $^{***}P < 0.001$, as compared to control group; $^{\#\#}P < 0.01$, as compared to corticosterone (Cort) treatment group

co-incubation with 50 µM corticosterone for an additional 24 h-period. This concentration was selected because UA was able to prevent the reduction in the cell viability induced by corticosterone assessed by the MTT assay. The statistical analysis using one-way ANOVA revealed that corticosterone increased by almost eight (8) times the number of PI^+ cells (Fig. 4b). Interestingly, UA treatment was able to attenuate the corticosterone-induced increase in PI^+ cells.

UA Prevents Corticosterone-Induced Apoptosis

In the next experiment, we evaluated the number of viable, early apoptotic and late apoptotic cells by flow cytometry. HT22 cells were treated with 50 µM corticosterone (24 h), 15 µM UA (72 h) or 15 µM UA for 48 h plus a co-incubation with 50 µM corticosterone for 24 h. We observed that corticosterone caused a two-fold increase in the percentage of apoptosis as compared to the control condition, a conclusion based on the percentage of all annexin$^+$/7AAD$^-$ (early apoptosis) plus the annexin$^+$/7AAD$^+$ (late apoptosis) (Fig. 5a). In addition, either UA alone or in combination with corticosterone (after a pre-incubation with UA for 48 h), showed a similar number of apoptotic cells compared to the control condition (Fig. 5b). This result indicates that UA is able to counteract the increased number of apoptotic cells induced by corticosterone treatment.

Role of Protein Kinases in the Cytoprotective Effect of UA Against Corticosterone-Induced Cytotoxicity

To test the role of different protein kinases in the cytoprotective effect of UA, cells were pre-treated with pharmacological inhibitors LY294002 (3 µM), KN62 (10 µM), H89 (2 µM) or chelerythrine (0.1 µM) or vehicle (0.1% DMSO) 30 min before the addition of 15 µM UA and maintained during the entire experiment (Fig. 6a). When HT22 cells were treated with LY294002, a PI3K inhibitor, we observed that incubation with this inhibitor did not prevent the cytoprotective effect of UA against corticosterone (Fig. 6b). Next, using KN62 (10 µM) we evaluated the influence of CAMKII on the neuroprotective effect of UA against corticosterone-induced cytotoxic effect. One-way ANOVA analysis revealed that pre-treatment ofHT22 cells with KN62 prevented UA cytoprotective effect (Fig. 6c). Using H89 (2 µM) we investigated the involvement of PKA in the neuroprotective effect of UA against corticosterone-induced cytotoxicity. We observed that this inhibitor also prevented the neuroprotective effects of UA in this model (Fig. 6d). Finally, we investigated whether PKC inhibition with chelerythrine (0.1 µM) would prevent the neuroprotective effect of UA against corticosterone-induced cytotoxicity. In fact, treatment with this PKC inhibitor also prevented the

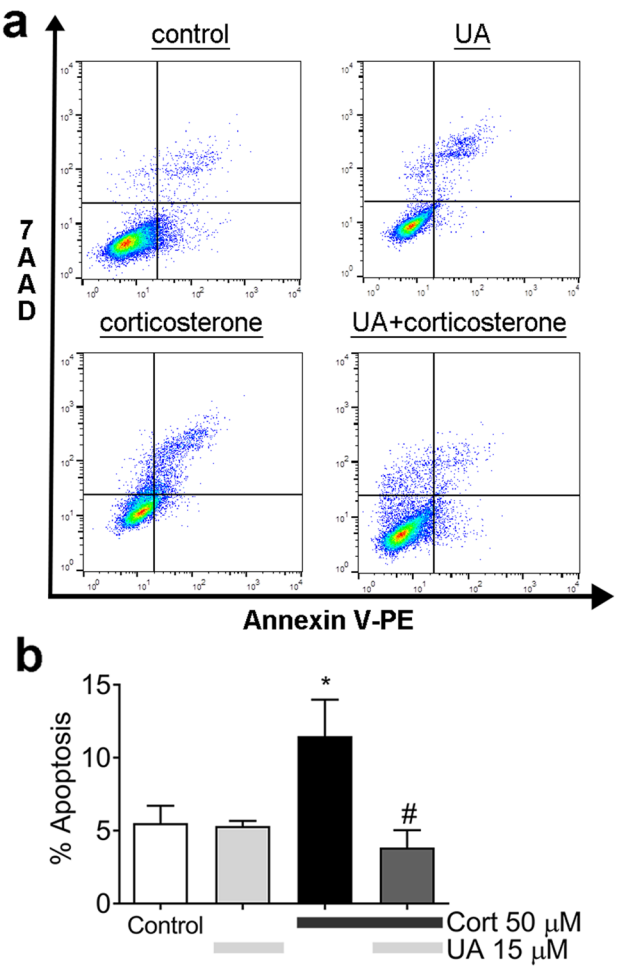


Fig. 5 Cytoprotective effect of UA against corticosterone-induced apoptosis**. a** Representative plot diagrams of cells incubated with control, UA, corticosterone, and UA followed by a co-incubation with corticosterone. Cells were collected, stained with annexin V-PE and 7AAD, and analyzed by flow cytometry. The treatment of cells with UA for 48 h before corticosterone treatment significantly prevented the corticosterone-induced increase in the number of apoptotic cells. **b** Quantification of 7AAD and annexin FACS data. Averaged apoptotic population (annexin$^+$/7AAD$^-$and annexin$^+$/7AAD$^+$) for each group is represented. All data are expressed as mean + SEM. One-way ANOVA analysis was used for comparison among multiple groups followed by Newman Keuls post hoc test. $^*P < 0.05$, as compared to control group, and $^{\#}P < 0.05$, as compared to corticosterone (Cort)-treatment group

neuroprotective effects of UA against corticosterone-induced cytotoxicity in HT22 cells (Fig. 6e).

Next, we investigated if corticosterone would alter the ratio between the phosphorylated and total forms of ERK1/2 and JNK (protocol in Fig. 7a). OD analysis of immunoblots incubated with a specific antibody for anti-p-ERK1/2 revealed that the treatment for 3 h with 50 µM corticosterone reduced approximately 80% the phosphorylation levels of ERK1 and 2 (Fig. 7c, d, respectively). However, 48 h pre-incubation with 15 µM of UA prevented this reduction

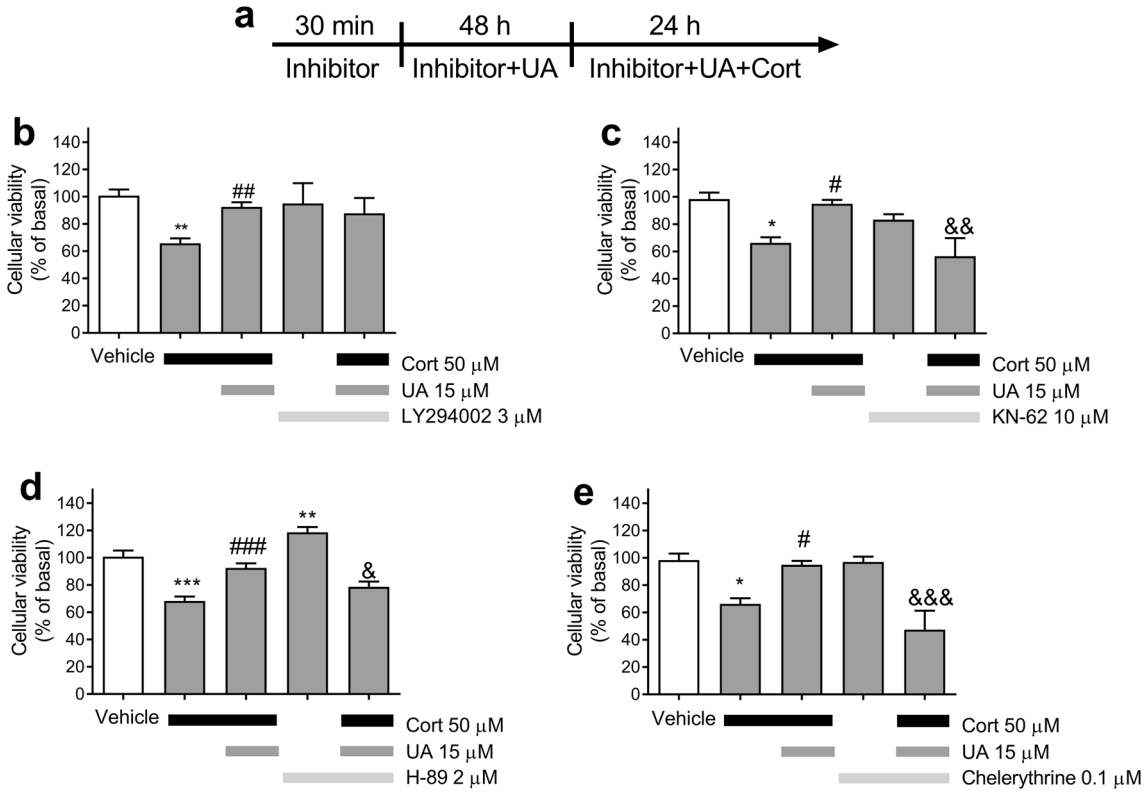


Fig. 6 The cytoprotective effect of UA against corticosterone-induced cytotoxicity was prevented by H-89, KN-62 and chelerythrine, but not LY294002. **a** Experimental protocol. HT22 cells were incubated for 30 min prior to the treatment with UA (15 μM) with the following inhibitors: LY294002 (PI3K inhibitor, n = 3–6, **b**), KN-62 (CaMKII inhibitor, n = 5, **c**), H-89 (PKA inhibitor, n = 4–6, **d**) or chelerythrine (PKC inhibitor, n = 4–5, **e**). Inhibitors were present throughout the treatment. The treatment of cells with KN-62, H-89 or chelerythrine significantly abolished the protective effect of UA against the cytotoxic effect induced by corticosterone. The results are expressed as baseline percentage (mean + S.E.M). Results analyzed by one-way ANOVA followed by Newman-Keuls post hoc test). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, as compared to vehicle. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, as compared to corticosterone group. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, and $^{\&\&\&}P < 0.001$, as compared to treatment with UA and corticosterone. Cort: corticosterone

(Fig. 7c, d, respectively). In addition, HT22 cells treated for 3 h with 50 μM of corticosterone also presented a reduction of approximately 40% in the ratio of phosphorylated forms of JNK (Fig. 7g). However, pre-incubation for 48 h with 15 μM UA, prior to the co-incubation with corticosterone 50 μM, was not able to prevent the reduction in JNK phosphorylation levels (Fig. 7g). None of the treatments altered the immunocontents of the total forms of ERK or JNK (Fig. 7e, h, respectively).

Discussion

In the present study, we demonstrate, for the first time, that UA exhibits cytoprotective effects against corticosterone-induced cytotoxicity in HT22 cells. In this model, incubation of HT22 cells for 48 h with low concentrations of UA (5 or 15 μM) was able to prevent the reduction in cell viability, the increase of PI positive cells, and the increased number of apoptotic cells induced by corticosterone. Previous reports have shown the neuroprotective effects of triterpenoid compounds such as CDDO-MA, piperine, asian acid and tormentic acid against neurotoxic insults, including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, 3-nitropropionic acid and Aβ peptides [41–44]. Therefore, our findings suggest that the plant-derived triterpenoid family appears to share neuroprotective properties.

Behl et al. demonstrated that HT22 cells are sensitive to glucocorticoid stimulation, which makes them an excellent model for studying glucocorticoid-induced cell death [25]. Our results reinforce these and other findings by authors who used this same model in HT22 cells [20–24]. As previously reported, our results confirmed that the cytotoxic effects of corticosterone can be abolished by RU486 [21, 25]. This steroid antagonizes corticosterone competitively by binding to GR [45]. Therefore, these data indicate that the corticosterone-induced cell death is likely mediated through GR activation.

One of the main findings of our study relies on the fact that only 48 h, but not 24 h, of UA incubation, produced a

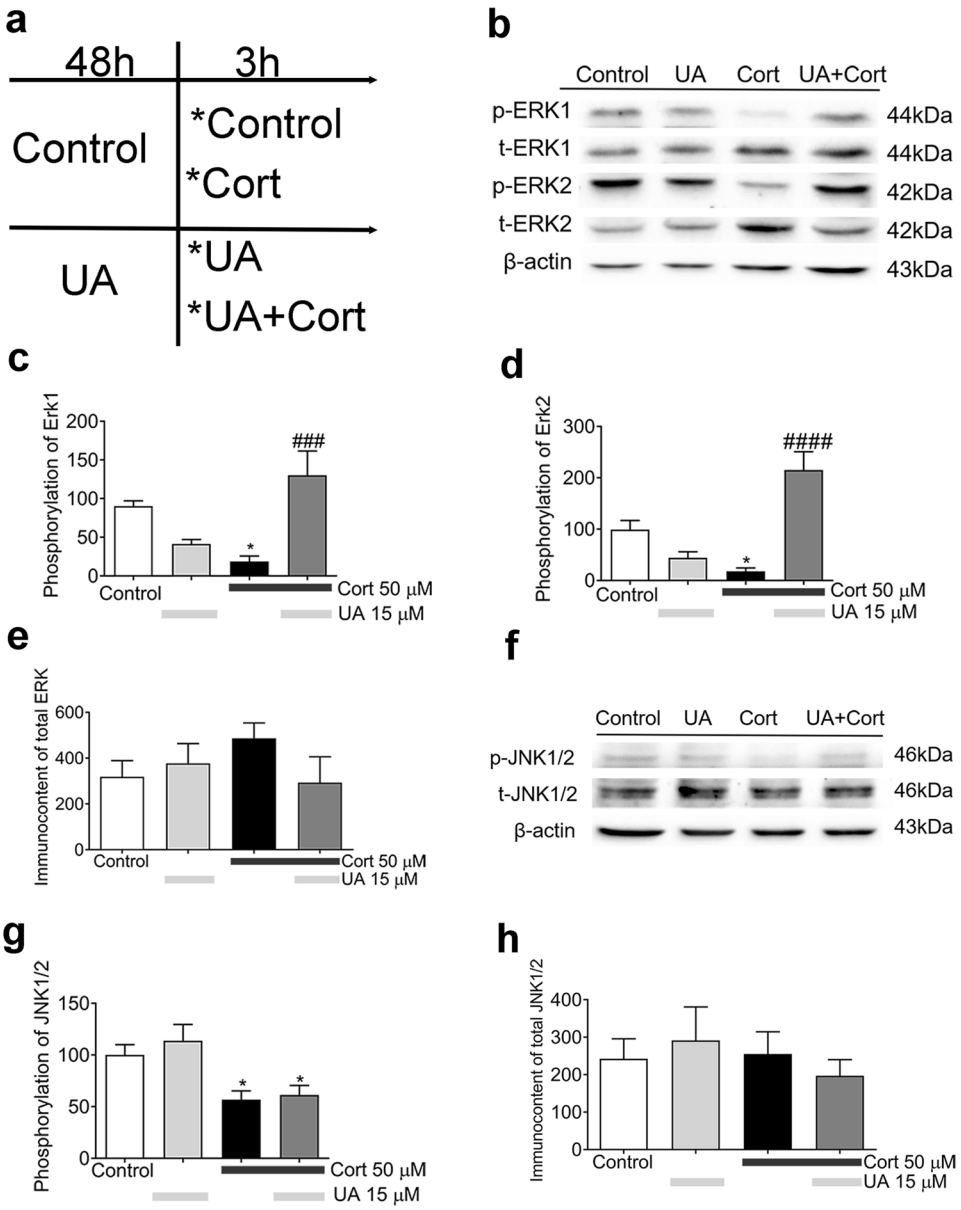


Fig. 7 Evaluation of MAPK proteins phosphorylation levels of HT22 cells exposed to corticosterone and/or UA. **a** Experimental protocol. **b** Representative immunoblots for the phosphorylated and total forms of ERK. The column histogram represents the semi-quantification by optical densitometry of the OD ratio between the phosphorylated form and the OD of the total form of ERK1 (p44 kDa; **c**), ERK2 (p42 kDa; **d**) and the OD ratio between the total form of ERK1 and 2 and the OD of the β-actin (**e**). **f** Representative immunoblots for the phosphorylated and total forms of JNK. The column histogram represents the semi-quantification by optical densitometry of the OD ratio between the phosphorylated form and the OD of the total form of JNK (p46 kDa; **g**) and the OD ratio between the total form of JNK (p46 kDa; **h**) and the OD of the β-actin. Each column represents the percentage relative to the control of 6 independent experiments (mean + S.E.M., one-way ANOVA followed by Newman–Keuls post hoc test). $^{*}P < 0.05$, as compared to control group, $^{###}P < 0.001$, and $^{####}P < 0.0001$, as compared to the corticosterone-treated group. Cort: corticosterone, p: phosphorylated, t: total

cytoprotective effect against corticosterone-induced toxicity. Previous studies have also shown that UA is able to protect PC12 cells against Aβ-induced cytotoxicity at the same concentration range, and with the same incubation time observed in our study [46, 47]. A longer incubation time suggests that the neuroprotective effect of UA is possibly dependent on the modulation of intracellular signaling pathways and other late-onset targets such as the transcription of genes related to survival and production of neurotrophic factors. Confirming the findings obtained in the MTT assay, we observed that UA was able to prevent the increase in the number of cells labeled with PI and the percentage of apoptotic cells induced by corticosterone. Shih et al. demonstrated that UA protects neurons from primary hippocampal cultures of rats against kainate (KA)-induced necrosis at the same concentration range tested in our study (10 and 15 μM) [48]. However, they found that the neuroprotective effect of UA against KA-induced excitotoxicity was produced after a shorter (10 min) time of UA incubation, while we observed cytoprotective effects only after a 48 h incubation. These differences might be related to the cell type or the specific pharmacological nature of the toxic stimuli.

Importantly, we observed that the cytoprotective effect of UA was abolished by RU486, which may indicate an interaction between UA and GR function. In this regard, it has been suggested that UA is able to act as a ligand of nuclear receptors for glucocorticoids since it has a chemical structure similar to members of the estradiol family [49–51]. In fact, merotain (also known as URA, a liposome-embedded UA) competes with RU486 for binding to GR and regulates

the expression of extracellular matrix metalloproteinases dependent on GR activation [49]. In addition, URA has the ability to bind GR and stimulate collagen production or suppress keratin and ceramide gene expression in keratinocytes [51]. Based on these studies, we may speculate that the cytoprotective effect of UA against corticosterone-induced cytotoxicity could be mediated by the modulation of GR function; however, further studies are required to confirm this hypothesis.

Noteworthy, it has been shown that, in response to corticosterone, there is a modulation in the phosphorylation state and in the activity of protein kinases [52]; especially those participating in cell survival signaling cascades [53, 54]. Among these, we highlight PI3K, PKA, CaMKII, and PKC-dependent signaling pathways. In our study we observed that the exposure to a concentration of LY294002 that does not alter the viability of HT22 cells did not prevent the cytoprotective effect of UA against the corticosterone-induced cytotoxicity. This result suggests that the PI3K signaling pathway is not involved in the cytoprotective effect of UA. In contrast, exposure of HT22 cells to H-89, KN-62 or chelerythrine prevented the cytoprotective effect of UA against corticosterone-induced cytotoxicity. Therefore, these data indicate that the activation of PKA, PKC and CaMKII may be involved in the cytoprotective mechanism of UA against the corticosterone-induced cytotoxicity. Accordingly, a previous study from our group showed that the activation of PKA, PKC, and CaMKII, but not PI3 K, is required for the antidepressant-like effects of UA in mice [30].

MAPK dependent signaling pathways are involved in the response of cells to stress and can activate transcription factors and promote proliferation [55] and inflammatory responses [56]. Conversely, the signaling pathways dependent on ERK activation may stimulate the production of neurotrophic factors and promote neuroprotection in HT22 cells [57]. An interesting finding in our study was that the reduction of ERK1/2 phosphorylation induced by corticosterone was significantly prevented by pre-incubating HT22 cells with UA. This finding suggests that activation of ERK1/2 can mediate the cytoprotective effects of UA against corticosterone-induced cytotoxicity [58]. Accordingly, it has been suggested that ERK activation may protect HT22 cells against cell death induced by glucose deprivation by activating cAMP response element-binding protein (CREB), which in turn, stimulates the brain-derived neurotrophic factor (BDNF) expression [57]. In addition, other antidepressant drugs induce ERK1/2 phosphorylation, such as fluoxetine in astrocytes [59], and imipramine, which increases ERK1/2 phosphorylation in the hippocampus of mice submitted to a chronic restraint stress protocol [60]. In fact, the cytoprotective effect of apelin-13 against corticosterone-induced cytotoxicity has been shown to be mediated by the activation of ERK-dependent signaling pathways in HT22 cells [61]. Moreover, it was reported that GR activation may trigger ERK1/2 and JNK dephosphorylation by a mechanism dependent on MKP-1 (MAPK phosphatase-1) activation [62]. Our study did not address if this mechanism might be operating in HT22 cells. Noteworthy, UA despite counteracting corticosterone-induced ERK1/2 dephosphorylation was not able to prevent the reduction in JNK phosphorylation in response to corticosterone. Therefore, future studies will be necessary to underlie the mechanism involved in the modulation of MAPKs by UA.

An intriguing result of our study was the observation of a dual effect of UA in HT22 cells. Low concentrations (5 or 15 µM) of UA did not alter the viability of these cells and also acted as a cytoprotective agent. In contrast, a higher UA concentration (50 µM) reduced cell viability. A similar profile has also been reported in rodent leukemic cells, in which UA presented antioxidant effects up to 10 µM and cytotoxic effects at concentrations over 20 µM [63]. In glioblastoma multiforme DBTRG-05MG cells, 15 µM UA did not alter cellular viability; however, concentrations over 20 µM caused a reduction in the viability, increased reactive oxygen species production and induced necrotic cell death [64]. Accordingly, contrasting experimental results regarding the effects of UA have been reported, i.e., this compound may be antioxidant, anti-inflammatory and protective or prooxidant and cytotoxic, depending on the tissue, cell type and experimental conditions [65, 66]. In this regard, some authors suggest that UA, as well as other triterpenoid compounds, may act biphasically depending on the concentration and experimental conditions [65, 66].

In conclusion, considering that neuronal death can occur through several mechanisms [67], that share many intracellular signaling pathways [67]; the discovery and study of compounds with an antidepressant activity that produce neuroprotection through different mechanisms are of particular interest [68]. Regarding this issue, it has been proposed that multiple target drugs are often more efficient molecules than single-target drugs [69]. In this study, UA was able to activate different intracellular signaling pathways associated with neuroprotection against neuronal death, which makes this compound a promising therapeutic strategy for the treatment of diseases associated with neurodegenerative processes and stress-related disorders.

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Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflict of interest.

References

1. Aschbacher K, Epel E, Wolkowitz OM, Prather AA, Puterman E, Dhabhar FS (2012) Maintenance of a positive outlook during acute stress protects against pro-inflammatory reactivity and future depressive symptoms. Brain Behav Immun 26(2):346–352
2. Cassano P, Fava M (2002) Depression and public health: an overview. J Psychosom Res 53(4):849–857
3. Hunter RM, Nazareth I, Morris S, King M (2013) Modelling the cost-effectiveness of preventing major depression in general practice patients. Psychol Med 44(7):1381–1390
4. Brown GW, Harris T (1978) Social origins of depression: a reply. Psychol Med 8(4):577–588
5. Gold PW, Goodwin FK, Chrousos GP (1988) Clinical and biochemical manifestations of depression. Relation to the neurobiology of stress (1). N Engl J Med 319(6):348–353
6. Sapolsky RM, Uno H, Rebert CS, Finch CE (1990) Hippocampal damage associated with prolonged glucocorticoid exposure in primates. J Neurosci 10(9):2897–2902
7. Uno H, Tarara R, Else JG, Suleman MA, Sapolsky RM (1989) Hippocampal damage associated with prolonged and fatal stress in primates. J Neurosci 9(5):1705–1711
8. Sapolsky RM, Krey LC, McEwen BS (1985) Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. J Neurosci 5(5):1222–1227
9. Greisen MH, Altar CA, Bolwig TG, Whitehead R, Wörtwein G (2005) Increased adult hippocampal brain-derived neurotrophic factor and normal levels of neurogenesis in maternal separation rats. J Neurosci Res 79(6):772–778
10. Smith MA, Makino S, Kvetnansky R, Post RM (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J Neurosci 15(3Pt1):1768–1777
11. Czéh B, Michaelis T, Watanabe T, Frahm J, de Biurrun G, van Kampen M, Bartolomucci A, Fuchs E (2001) Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. Proc Natl Acad Sci USA 98(22):12796–12801
12. Lucassen PJ, Vollmann-Honsdorf GK, Gleisberg M, Czéh B, De Kloet ER, Fuchs E (2001) Chronic psychosocial stress differentially affects apoptosis in hippocampal subregions and cortex of the adult tree shrew. Eur J Neurosci 14(1):161–166
13. Krishnan V, Nestler EJ (2011) Animal models of depression: molecular perspectives. Curr Top Behav Neurosci 7:121–147
14. Uno H, Eisele S, Sakai A, Shelton S, Baker E, DeJesus O, Holden J (1994) Neurotoxicity of glucocorticoids in the primate brain. Horm Behav 28(4):336–348
15. Lee HJ, Kim JW, Yim SV, Kim MJ, Kim SA, Kim YJ, Kim CJ, Chung JH (2001) Fluoxetine enhances cell proliferation and prevents apoptosis in dentate gyrus of maternally separated rats. Mol Psychiatry 6(6):610, 725–618
16. Moretti M, Colla A, de Oliveira Balen G, dos Santos DB, Budni J, de Freitas AE, Farina M, Rodrigues ALS (2012) Ascorbic acid treatment, similarly to fluoxetine, reverses depressive-like behavior and brain oxidative damage induced by chronic unpredictable stress. J Psychiatr Res 46(3):331–340
17. Vythilingam M, Heim C, Newport J, Miller AH, Anderson E, Bronen R, Brummer M, Staib L, Vermetten E, Charney DS, Nemeroff CB, Bremner JD (2002) Childhood trauma associated with smaller hippocampal volume in women with major depression. Am J Psychiatry 159(12):2072–2080
18. Gilbertson MW, Shenton ME, Ciszewski A, Kasai K, Lasko NB, Orr SP, Pitman RK (2002) Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma. Nat Neurosci 5(11):1242–1247
19. Bremner JD, Randall P, Scott TM, Bronen RA, Seibyl JP, Southwick SM, Delaney RC, McCarthy G, Charney DS, Innis RB (1995) MRI-based measurement of hippocampal volume in patients with combat-related posttraumatic stress disorder. Am J Psychiatry 152(7):973–981
20. Behl C, Lezoualc'h F, Trapp T, Widmann M, Skutella T, Holsboer F (1997) Glucocorticoids enhance oxidative stress-induced cell death in hippocampal neurons in vitro. Endocrinology 138(1):101–106
21. Freitas AE, Egea J, Buendía I, Navarro E, Rada P, Cuadrado A, Rodrigues ALS, López MG (2015) Agmatine induces Nrf2 and protects against corticosterone effects in hippocampal neuronal cell line. Mol Neurobiol 51(3):1504–1519
22. Xu Y, Zhang C, Wang R, Govindarajan SS, Barish PA, Vernon MM, Fu C, Acharya AP, Chen L, Boykin E, Yu J, Pan J, O'Donnell JM, Ogle WO (2011) Corticosterone induced morphological changes of hippocampal and amygdaloid cell lines are dependent on 5-HT7 receptor related signal pathway. Neuroscience 182:71–81
23. Xu Y, Pan J, Chen L, Zhang C, Sun J, Li J, Nguyen L, Nair N, Zhang H, O'Donnell JM (2013) Phosphodiesterase-2 inhibitor reverses corticosterone-induced neurotoxicity and related behavioural changes via cGMP/PKG dependent pathway. Int J Neuropsychopharmacol 16(4):835–847
24. Zheng Y, Huang J, Tao L, Shen Z, Li H, Mo F, Wang X, Wang S, Shen H (2015) Corticosterone increases intracellular Zn(2+) release in hippocampal HT-22 cells. Neurosci Lett 588:172–177
25. Behl C, Trapp T, Skutella T, Holsboer F (1997) Protection against oxidative stress-induced neuronal cell death–a novel role for RU486. Eur J Neurosci 9(5):912–920
26. Ramos-Hryb AB, Pazini FL, Kaster MP, Rodrigues ALS (2017) Therapeutic potential of ursolic acid to manage neurodegenerative and psychiatric diseases. CNS Drugs 31(12):1029–1041
27. Ramos-Hryb AB, Cunha MP, Kaster MP, Rodrigues ALS (2017) Chapter 6—natural polyphenols and terpenoids for depression treatment: current status. In: Atta-ur-Rahman (ed) Studies in natural products chemistry. Elsevier, Amsterdam, pp 181–221
28. López-Hortas L, Pérez-Larrán P, González-Muñoz MJ, Falqué E, Domínguez H (2018) Recent developments on the extraction and application of ursolic acid. A review. Food Res Int 103:130–149
29. Machado DG, Neis VB, Balen GO, Colla A, Cunha MP, Dalmarco JB, Pizzolatti MG, Prediger RD, Rodrigues AL (2012) Antidepressant-like effect of ursolic acid isolated from *Rosmarinus officinalis L.* in mice: evidence for the involvement of the dopaminergic system. Pharmacol Biochem Behav 103(2):204–211
30. Ramos-Hryb AB, Cunha MP, Pazini FL, Lieberknecht V, Prediger RDS, Kaster MP, Rodrigues ALS (2017) Ursolic acid affords antidepressant-like effects in mice through the activation of PKA, PKC, CAMK-II and MEK1/2. Pharmacol Rep 69(6):1240–1246
31. Colla AR, Oliveira A, Pazini FL, Rosa JM, Manosso LM, Cunha MP, Rodrigues ALS (2014) Serotonergic and noradrenergic

systems are implicated in the antidepressant-like effect of ursolic acid in mice. Pharmacol Biochem Behav 124:108–116
32. Colla AR, Rosa JM, Cunha MP, Rodrigues ALS (2015) Anxiolytic-like effects of ursolic acid in mice. Eur J Pharmacol 758:171–176
33. Taviano MF, Miceli N, Monforte MT, Tzakou O, Galati EM (2007) Ursolic acid plays a role in *Nepeta sibthorpii Bentham* CNS depressing effects. Phytother Res 21(4):382–385
34. Jeon SJ, Park HJ, Gao Q, Pena IJ, Park SJ, Lee HE, Woo H, Kim HJ, Cheong JH, Hong E, Ryu JH (2015) Ursolic acid enhances pentobarbital-induced sleeping behaviors via GABAergic neurotransmission in mice. Eur J Pharmacol 762:443–448
35. Richard EJ, Illuri R, Bethapudi B, Anandhakumar S, Bhaskar A, Chinampudur Velusami C, Mundkinajeddu D, Agarwal A (2016) Anti-stress activity of *Ocimum sanctum*: possible effects on hypothalamic-pituitary-adrenal axis. Phytother Res 30(5):805–814
36. Rollinger JM, Kratschmar DV, Schuster D, Pfisterer PH, Gumy C, Aubry EM, Brandstötter S, Stuppner H, Wolber G, Odermatt A (2010) 11beta-Hydroxysteroid dehydrogenase 1 inhibiting constituents from *Eriobotrya japonica* revealed by bioactivity-guided isolation and computational approaches. Bioorg Med Chem 18(4):1507–1515
37. Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 89(2):271–277
38. Riccardi C, Nicoletti I (2006) Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 1(3):1458–1461
39. Liu Q, Qiu J, Liang M, Golinski J, van Leyen K, Jung JE, You Z, Lo EH, Degterev A, Whalen MJ (2014) Akt and mTOR mediate programmed necrosis in neurons. Cell Death Dis 5:e1084
40. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83(2):346–356
41. Mao QQ, Huang Z, Ip SP, Xian YF, Che CT (2012) Protective effects of piperine against corticosterone-induced neurotoxicity in PC12 cells. Cell Mol Neurobiol 32(4):531–537
42. Xu MF, Xiong YY, Liu JK, Qian JJ, Zhu L, Gao J (2012) Asiatic acid, a pentacyclic triterpene in *Centella asiatica*, attenuates glutamate-induced cognitive deficits in mice and apoptosis in SH-SY5Y cells. Acta Pharmacol Sin 33(5):578–587
43. Yang L, Calingasan NY, Thomas B, Chaturvedi RK, Kiaei M, Wille EJ, Liby KT, Williams C, Royce D, Risingsong R, Musiek ES, Morrow JD, Sporn M, Beal MF (2009) Neuroprotective effects of the triterpenoid, CDDO methyl amide, a potent inducer of Nrf2-mediated transcription. PLoS ONE 4(6):e5757
44. Zhao Q, Ye J, Wei N, Fong C, Dong X (2016) Protection against MPP(+)-induced neurotoxicity in SH-SY5Y cells by tormentic acid via the activation of PI3-K/Akt/GSK3β pathway. Neurochem Int 97:117–123
45. Gallagher P, Young AH (2006) Mifepristone (RU-486) treatment for depression and psychosis: a review of the therapeutic implications. Neuropsychiatr Dis Treat 2(1):33–42
46. Heo HJ, Cho HY, Hong B, Kim HK, Heo TR, Kim EK, Kim SK, Kim CJ, Shin DH (2002) Ursolic acid of *Origanum majorana L.* reduces Abeta-induced oxidative injury. Mol Cells 13(1):5–11
47. Hong SY, Jeong WS, Jun M (2012) Protective effects of the key compounds isolated from *Corni fructus* against β-amyloid-induced neurotoxicity in PC12 cells. Molecules 17(9):10831–10845
48. Shih YH, Chein YC, Wang JY, Fu YS (2004) Ursolic acid protects hippocampal neurons against kainate-induced excitotoxicity in rats. Neurosci Lett 362(2):136–140
49. Cha HJ, Park MT, Chung HY, Kim ND, Sato H, Seiki M, Kim KW (1998) Ursolic acid-induced down-regulation of MMP-9 gene is mediated through the nuclear translocation of glucocorticoid receptor in HT1080 human fibrosarcoma cells. Oncogene 16(6):771–778
50. Mortiboys H, Aasly J, Bandmann O (2013) Ursocholanic acid rescues mitochondrial function in common forms of familial Parkinson's disease. Brain 136(Pt10):3038–3050
51. Yarosh DB, Both D, Brown D (2000) Liposomal ursolic acid (merotaine) increases ceramides and collagen in human skin. Horm Res 54(5–6):318–321
52. Maiyar AC, Phu PT, Huang AJ, Firestone GL (1997) Repression of glucocorticoid receptor transactivation and DNA binding of a glucocorticoid response element within the serum/glucocorticoid-inducible protein kinase (sgk) gene promoter by the p53 tumor suppressor protein. Mol Endocrinol 11(3):312–329
53. Lang F, Stournaras C (2013) Serum and glucocorticoid inducible kinase, metabolic syndrome, inflammation, and tumor growth. Hormones 12(2):160–171
54. David S, Kalb RG (2005) Serum/glucocorticoid-inducible kinase can phosphorylate the cyclic AMP response element binding protein, CREB. FEBS Lett 579(6):1534–1538
55. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286(5443):1358–1362
56. Kumar S, Boehm J, Lee JC (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat Rev Drug Discov 2(9):717–726
57. Rössler OG, Giehl KM, Thiel G (2004) Neuroprotection of immortalized hippocampal neurones by brain-derived neurotrophic factor and Raf-1 protein kinase: role of extracellular signal-regulated protein kinase and phosphatidylinositol 3-kinase. J Neurochem 88(5):1240–1252
58. Pace TW, Hu F, Miller AH (2007) Cytokine-effects on glucocorticoid receptor function: relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. Brain Behav Immun 21(1):9–19
59. Mercier G, Lennon AM, Renouf B, Dessouroux A, Ramaugé M, Courtin F, Pierre M (2004) MAP kinase activation by fluoxetine and its relation to gene expression in cultured rat astrocytes. J Mol Neurosci 24(2):207–216
60. Leem YH, Yoon SS, Kim YH, Jo SA (2014) Disrupted MEK/ERK signaling in the medial orbital cortex and dorsal endopiriform nuclei of the prefrontal cortex in a chronic restraint stress mouse model of depression. Neurosci Lett 580:163–168
61. Zou Y, Wang B, Fu W, Zhou S, Nie Y, Tian S (2016) Apelin-13 protects PC12 cells from corticosterone-induced apoptosis through PI3K and ERKs activation. Neurochem Res 41(7):1635–1644
62. Wu W, Pew T, Zou M, Pang D, Conzen SD (2005) Glucocorticoid receptor-induced MAPK phosphatase-1 (MPK-1) expression inhibits paclitaxel-associated MAPK activation and contributes to breast cancer cell survival. J Biol Chem 280(6):4117–4724
63. Ovesná Z, Kozics K, Slamenová D (2006) Protective effects of ursolic acid and oleanolic acid in leukemic cells. Mutat Res 600(1–2):131–137
64. Lu CC, Huang BR, Liao PJ, Yen GC (2014) Ursolic acid triggers nonprogrammed death (necrosis) in human glioblastoma multiforme DBTRG-05MG cells through MPT pore opening and ATP decline. Mol Nutr Food Res 58(11):2146–2156
65. Liby KT, Yore MM, Sporn MB (2007) Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. Nat Rev Cancer 7(5):357–369
66. Tannock LR (2011) Ursolic acid effect on atherosclerosis: apples and apples, or apples and oranges? Atherosclerosis 219(2):397–398
67. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N (2013) Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta 1833(12):3448–3459

68. Yakovlev AG, Faden AI (2004) Mechanisms of neural cell death: implications for development of neuroprotective treatment strategies. NeuroRx 1(1):5–16
69. Csermely P, Agoston V, Pongor S (2005) The efficiency of multi-target drugs: the network approach might help drug design. Trends Pharmacol Sci 26(4):178–182