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ORIGINAL CONTRIBUTION

Neuroprotective effect of cocoa flavonids on in vitro oxidative stress

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Abstract *Background* Cocoa is a rich source of flavonoids that, among other functions, can act as antioxidants. In living systems, the production of reactive oxygen species (ROS) activate an array of intracellular cascades, including mitogen-activated protein kinases (MAPK), that are closely associated with cell death or survival pathways. *Aim of the study* To ascertain the role of a cocoa extract and its main flavonoid, (-)-epicatechin, in an in vitro model of oxidative stress induced in a neuronal cell line. *Methods* We analyzed ROS production by fluorometry (dichlorofluorescein assay), and activation of MAPK pathways including extracellular signal-regulated kinases 1/2 (ERK 1/2), c-Jun N-terminal kinase (JNK), and p-38, by Western blot analysis. *Results* Cells incubated with cocoa extract or (-)-epicatechin, reduced ROS production in a dose-dependent manner, reaching 35% inhibition. pJNK and p38, involved in apoptosis, were down-modulated by cocoa extract and (-)-epicatechin with p38 inhibition reaching up to 70%. *Conclusions* Our results show that cocoa extract and (-)-epicatechin may exert a neuroprotective action by reducing ROS production and modulating MAPK activation.

Key words cocoa – c-JNK – MAPK – neuronal damage – oxidative stress

Abbreviations AD: Alzheimer's disease, ANOVA: Analysis of variance, Aβ: Amyloid-β protein, DA: diacetate, DCF: 2′,7′-dichlorofluorescein, DMSO: Dimethyl sulfoxide, ERK 1/2: Extracellular signal-regulated kinases 1/2, GSTPi: Glutathione-S-transferase enzyme-class Pi, H_2DCF: reduced DCF, JNK: c-Jun N-terminal kinase, LDH: Lactate dehydrogenase, MAPK: Mitogen-activated protein kinases, ORAC: Oxygen radical absorbance capacity, PMSF: Phenylmethylsulfonyl fluoride, ROS: Reactive oxygen species, RT: Room temperature, SOD: Superoxide dismutase, TBS: Tris buffered saline solution, TBST: TBS with 0.1% Tween 20

Introduction

Aerobic organisms are equipped with enzymatic and non-enzymatic antioxidant systems, in charge of controlling reactive oxygen species (ROS) levels, including free radicals. These mechanisms protect cells from oxidative stress continuously generated in the body as a consequence of normal metabolic processes, in both endogenous and neighboring cells and

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tissues [18]. Although ROS are well known to participate in physiological functions, their overproduction plays an important role in the development of many pathological processes, including neurodegenerative diseases [3, 35]. Some epidemiological studies have shown that high consumption of food containing polyphenols is associated with a lower incidence of Alzheimer's disease and dementia [5, 9]. Therefore, some neurodegenerative disorders characterized by an imbalance between oxidative and antioxidative species may be modulated by restoring antioxidant defenses through the intake of antioxidant-rich foods.

Polyphenols, abundant in cocoa, and therefore in chocolate, have been shown to be the main exogenous antioxidants present in human diet. Chocolate with a concentration of cocoa solids greater than 70% (i.e., dark chocolate) has a polyphenol content of approximately 5% in dried fat-free mass [41]. The cocoa polyphenols exist in various monomeric and polymeric states that differ depending on the source of the Cacao bean, the fermentation process, and the processing methods used during thermal treatment and free acid removal steps [6]. The predominant monomer is (-)-epicatechin, comprising approximately 35% of the total polyphenol content in the cocoa bean [41] with the remainder mainly composed of catechins and procyanidins [22, 25]. In addition, Cacao contains significant amounts of methylxanthines, especially theobromine [26].

Monomeric polyphenols and some dimeric procyanidins are readily adsorbed into the bloodstream, can be measured in serum a few hours after ingestion, and are associated with pharmacological effects [7, 17]. Notably the antioxidant activity of chocolate is exceedingly high on a percentage dry weight basis even when compared with traditionally healthy berries, beans and fruits. For instance, in comparison to the Acai berry (*Euterpe oleracea*), currently recognized as the most antioxidant rich natural foodstuff [34], dark chocolate and cacao powder have approximately 20 and 75% of the antioxidant activity per gram as measured in an oxygen radical absorbance capacity (ORAC) assay [20].

The antioxidant capacity of cocoa flavonoids and their main metabolites have been carefully characterized [21, 29, 42]. Flavonoids can act as antioxidants neutralizing directly free radicals, chelating metallic ions responsible for catalyzing lipid peroxidation, showing antiproliferative activity, and inducing antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase [22]. Intestinal and immune cellular models of oxidative stress have been used to demonstrate the protective effect of cocoa and its flavonoids [12, 28]. However, few evidences exist concerning the effect of these compounds on neuronal models both in vitro [15] and in vivo [10]. Moreover, it remains to be ascertained whether the cocoa prevention against oxidative damage is related to that neuroprotective effect.

The aim of the present study was to ascertain the antioxidative effect of a cocoa extract and its main flavonoid, (-)-epicatechin, on a human neuroblastoma cell line (SH-SY5Y) that is widely used for studying neuronal cell death induced by oxidative stress [2, 23]. We focused on the analysis of ROS production because of intracellular ROS levels are related with cell cycle progression. Moreover, we also determine the molecular mechanisms which may be influenced by ROS, specifically MAPK activation pathways including extracellular signal-regulated kinases 1 and 2 (ERK 1 and 2), which are involved in promoting cell survival by activating anti-apoptotic signaling pathways, and c-Jun N-terminal kinase (JNK) and p38 MAPK linked to neuronal cell death.

Materials and methods

Cocoa extract and (-)-epicatechin preparation

Natural Forastero cocoa powder from Malaysia was provided by Nutrexpa (Barcelona, Spain). Cocoa was subjected to an extraction of phenols following the method described by Andrés-Lacueva et al. [1] with some modifications. Briefly, 10 g of cocoa was mixed with 8 ml of deionized water at boiling point and vortexed for 1 min. Then, 32 ml of ethanol was added and the solution was shaken and vortexed for 3 min. The solution was centrifuged (15 min, 1,800*g*, 4°C) and the supernatant was concentrated under vacuum to a final volume of 4 ml, avoiding light exposure. To clean up the extract, the sample was applied to a Water Oasis HLB extraction cartridge and washed with water and 5% ethanol in water. The phenols were eluted with ethanol and the extract was evaporated under a stream of nitrogen, avoiding dryness, and reconstituted with dimethyl sulfoxide (DMSO). Total phenol content in the extract was determined according to the Folin-Ciocalteu method. The cocoa extract used in this study contained 20.4 mg/ml of total phenols expressed as catechin and stored at −80°C.

Stock solution of (-)-epicatechin (Sigma-Aldrich, St. Louis, MO, USA) was prepared in a minimum volume of DMSO and stored at −80°C. Just before cell treatment, cocoa extract and (-)-epicatechin solutions were diluted in fresh culture media to obtain the desired concentrations in cell suspensions.

Cell culture and study design

The human neuroblastoma S-SY5Y cell line (ATCC, Wiltshire, UK) was cultured in OptiMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% bovine calf serum, 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere until confluence and subcultured (1:10 split ratio) using trypsin (0.05% w/v) and EDTA (0.02%) both from Invitrogen.

To determine the effects of cocoa extract and (-)-epicatechin on ROS production, cells (2×10^4 cells/100 μl in serum-free media) were plated in 96 well-plates and allowed to attach overnight. Thereafter, cells were treated with cocoa extract (0.1–30 μg/ml) or (-)-epicatechin (0.3–29 μg/ml) for 30 min at 37°C. Compounds were added in low light and controls included cells treated with vehicle (DMSO), and ROS measured with DCF assay (described below).

To study the effects of cocoa extract and (-)-epicatechin on MAP kinase activation, a similar study design was applied. Cells (1×10^5 cells/500 μl in serum-free media) were plated in 24-well plates and incubated overnight. Cells were then exposed to cocoa extract (0.1–1 μg/ml), (-)-epicatechin (0.15–7.3 μg/ml) or DMSO (vehicle) for 30 min at 37°C. Cells were washed with serum-free media and stimulated with 1 mM of H_2O_2 plus 20 μM of $FeSO_4$ for 5 min at 37°C. Then, cells were washed once with PBS pH 7.4 to remove completely the media and were incubated with cell lysis buffer (Cell Signaling Technology, Danvers, MA USA) containing 1 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich) for 20 min on ice. Cell lysates were stored at −80°C until Western blot analysis.

Cytotoxicity assay

Cytotoxicity was determined using a LDH activity detection kit (Roche Diagnostics, Penzberg, Gerrmany). This assay is based on the colorimetric measurement of lactate dehydrogenase (LDH) released from the cytosol of damaged cells into the supernatant. Results are expressed as percentage of the maximum amount of releasable LDH, which was obtained by lysing cells with 1% Triton X-100.

ROS production by DCF assay

Intracellular ROS were quantified by using a fluorescent probe, reduced 2′,7′-dichlorofluorescein (H_2DCF) diacetate (DA) [40]. H_2DCF-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent H_2DCF which is oxidized to fluorescent DCF by ROS. Thus, DCF fluorescence intensity is proportional to intracellular ROS production.

Cells treated with cocoa extract or (-)-epicatechin were washed once with warm serum-free media and incubated with 10 μM of H_2DCF-DA in serum-free media (Molecular Probes, Carlsbad, CA, USA) for 30 min at 37°C. Then, cells were washed again and stimulated by adding 1 mM of H_2O_2 plus 20 μM of $FeSO_4$. Fluorescence was measured by fluorometry (excitation 538 nm, emission 485 nm). Results were expressed relative to values from untreated stimulated cells.

MAPK activation by western blot analysis

Protein samples were heated for 5 min at 95°C and separated by Sodium Dodecyl Sulphate/Polyacrylamide Gel Electrophoresis (10% acrylamide gel) using a Bio-Rad Mini-Protean III system (45 min, 170 V). Proteins were transferred to nitrocellulose membranes using Bio-Rad Trans Blot system (overnight, 30 V in 25 mM Tris, 192 mM glycine and 20% methanol). Following transfer, membranes were washed 1x with Tris buffered saline solution containing 0.1% of Tween 20 (TBST) and blocked with 10% nonfat milk powder dissolved in TBST for 1 h at room temperature (RT). Blots were then incubated with either rabbit anti-phospho-ERK1/2, rabbit anti-phospho-p38 or rabbit anti-phospho-SAPK/JNK (Cell Signaling Technology) in TBST for 2 h at RT. Thereafter, blots were washed 3x with TBST for 10 min, and incubated with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) in TBST for 1 h at RT. Following the removal of the secondary antibody, blots were washed 3x with TBST and 1x with Tris and were then developed with the Enhanced Chemiluminescence detection system (Amersham, Munich, Germany). Bands were analyzed using Bio-Profile software (LTF-Labortechnik, Wasserbutg, Germany). In order to normalize values, membranes were stripped and rehybridized with anti-mouse β-actin (Cell Signaling Technology).

Statistics

The software package SPSS 11.5 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Conventional one-way ANOVA was performed, considering total polyphenol concentration as an independent variable. When polyphenol concentration had a significant effect on the dependent variable, Bonferroni's

test was applied. Significant differences were accepted when $P < 0.05$.

Results

Cocoa and epicatechin effects on intracellular ROS and cytotoxicity

Intracellular ROS were analyzed using a DCF assay. Both cocoa extract and (-)-epicatechin reduced ROS production in a dose-dependent manner, up to 60% in non-stimulated S-SY5Y cells ($P < 0.05$) (Fig. 1). After 30 min of H_2O_2/Fe^{2+} stimulation, ROS production increased ~3-fold over baseline levels. Cocoa extract and (-)-epicatechin also diminished intracellular ROS in stimulated cells, reaching an inhibition of ~30% ($P < 0.05$) (Fig. 1).

In order to confirm that no cytotoxic effect was produced by polyphenolic compounds, intracellular lactate dehydrogenase (LDH) released to the media was determined (Fig. 1c). No increase in LDH activity was detected in supernatants from cells treated with cocoa extract or (-)-epicatechin for 30 min. H_2O_2/Fe^{2+} stimulation caused a 35 ± 2.9% of LDH leakage to the media ($P < 0.05$), without being modified by cocoa extract (0.1–30 μg/ml) or (-)-epicatechin (0.3–29 μg/ml) previous exposure.

Cocoa and epicatechin effects on MAP kinases activation

MAP kinase pathways are activated in response to cellular stress. In initial experiments SH-SY5Y cells showed the highest activation rate after 5 min of $H_2O_2/FeSO_4$ stimulation (data not shown), and this was the protocol used for the next experiments. Cocoa extract reduced, dose-dependently, both p38 (Fig. 2a, c) and JNK (46 and 54 kDa) (Fig. 2e) phosphorylation up to 75 and 85%, respectively, without affecting ERK activation levels (Fig. 3a). Epicatechin was also capable of diminishing p-p38 but only at higher concentrations than those found in cocoa extract (Fig. 2b, d). Moreover, (-)-epicatechin reduced p-JNK (46 kDa) (Fig. 2f) and diminished p-ERK1/2 achieving ~70% inhibition (Fig. 3b).

Discussion

The present work has studied the effects of cocoa extract and its mainly monomeric flavonoid, (-)-epi-

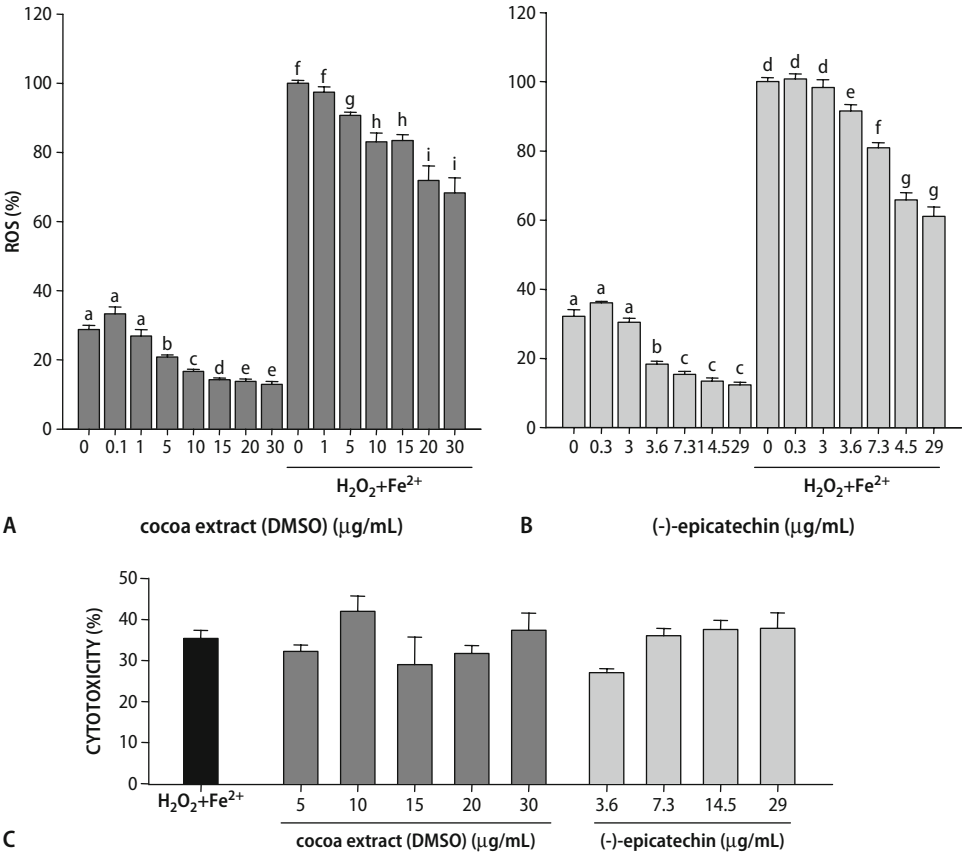

Fig. 1 Effect of cocoa extract (**a**) and (-)-epicatechin (**b**) on intracellular reactive oxygen species (ROS) production and LDH release (**c**) on SHSY5Y cells after stress oxidative induction. *Each bar* represents the mean of 10 values ±SEM from 3 individual experiments. Means within a graphic without a common letter differ, $P < 0.05$

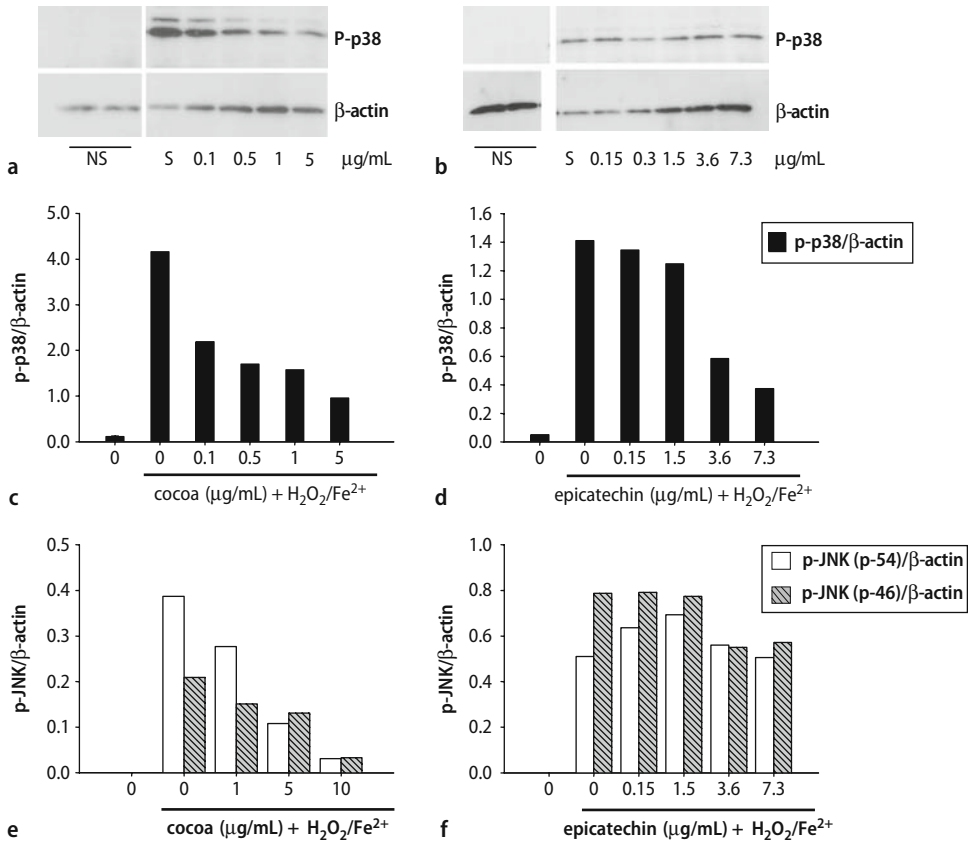

Fig. 2 Effect of cocoa extract and (-)-epicatechin on MAP kinase activation. Total protein western blots were probed with anti-phospho p38 (**a**, **b**). Densitometric quantification of p-p38 (**c**, **d**) and p-JNK (46 and 54 kDa) (**e**, **f**) from a representative experiment. Results are expressed relative to β-actin detected on the same blot, *S* stimulated, *NS* not stimulated

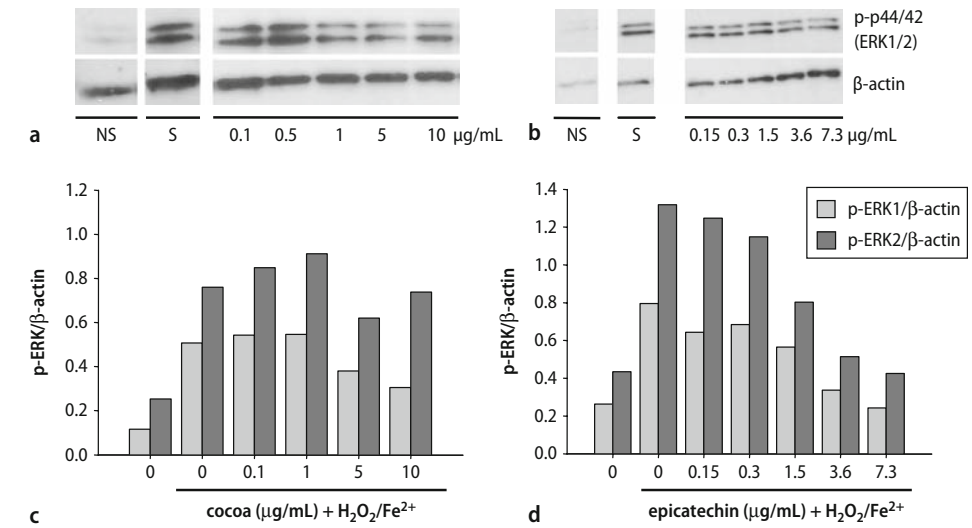

Fig. 3 Effect of cocoa extract and (-)-epicatechin on ERK1/2 activation. Representative western blots of p-ERK1/2 (**a**, **b**). Densitometric quantification of p-ERK1/2 (**c**, **d**) from a representative experiment. Results are expressed respect to those of β-actin

catechin, on cell viability, ROS production modulation and the activation of ERK1/2, JNK and p38 MAPKs in the human neuroblastoma SH-SY5Y cell line. Cocoa and epicatechin did not exert cytotoxic effects on the neuroblastoma cell line since the levels of intracellular LDH released to the media, a well established marker of cell injury were not modified after 30 min incubation [38]. The lack of cytotoxicity of cocoa compounds and (-)-epicatechin found here agrees with other previous in vitro studies using other cell lines [27, 28, 30]. The absence of cytotoxicity was not due to the short incubation time because longer exposure of cells to the cocoa extract or (-)-epicatechin have not shown decreased cell viability [13, 27, 28]. Unfortunately, these compounds did not show cell death protective effect in this model because

H_2O_2/Fe-induced cytotoxicity was neither reduced. However, others have demonstrated the benefits of the major flavonoids of cocoa, epicatechin and catechin, in preventing neuronal cell death induced by amyloid-β protein (Aβ) in PC12 cells in a dose-dependent manner [4, 15].

Elevated intracellular ROS have been linked to detrimental health in organisms [18], and therefore antioxidants have been considered a promising therapy for prevention and treatment of this overproduction. In this sense, our study showed that cocoa extract and (-)-epicatechin modulate antioxidant status by reducing ROS production in a dose-dependent manner. These results, agree with previous in vitro studies showing the inhibitory effect of cocoa and its main flavonoids on ROS production from activated immune cells [28], hepatoma cell lines [13, 14] or other polyphenols on neuronal cells [31]. In addition, in vivo studies have shown that continuous cocoa intake (10% for 3 weeks) enhances antioxidant capacity in tissues including liver, spleen and thymus by increasing the activity of antioxidant enzymes in young rats [29].

It is important to state that, in the present study, the maximum effect was similar in both cocoa extract and epicatechin conditions. Given that the precise mechanisms involved in flavonoid cell uptake remain to be well-established, it is difficult to know whether the inhibitory effect of cocoa is mainly due to the epicatechin present in the extract or other cocoa monomers which are also rapidly absorbed and may develop synergism among them [33], or whether metabolic processes are participating in this effect (i.e., procyanidin hydrolysis followed by cell uptake) [36]. In any case, although flavonoids and some of their metabolites are able to cross the blood-brain barrier [43], other compounds may be prevented from entering the brain and, since glial metabolism coexists in the same scenario as neuronal populations, the in vivo antioxidant effect of cocoa extract should be studied.

Enhanced production of ROS in the initial oxidative stress activates an array of intracellular signaling cascades that are closely associated with both cell death and survival pathways [16], such as the mitogen-activated protein kinases (MAPK). It is known that ERK1/2 activation promotes cell survival by activating anti-apoptotic signaling pathways, while JNK and p38 MAPK action is associated with neuronal cell death. Here, we have investigated the activation of these members of the MAPK pathway in H_2O_2-stimulated SH-SY5Y cells. In this model of neuronal oxidative stress, our results show that stimulation with H_2O_2 activates JNK phosphorylation, as was already described [32], but pretreatment with cocoa extract and epicatechin dose-dependently down-modulated pJNK levels. This inhibitory effect was higher in cocoa treated cells, likely due to the presence of additional compounds such as quercetin and theobromine. In this sense, quercetin treatment has been shown to suppress JNK activity and apoptosis [39] and theobromine-related molecule pentoxifylline showed antagonism of the JNK pathway [24]. Although the mechanism by which polyphenols are interfering in JNK activation induced by ROS remains to be studied, JNK inhibition may be associated with a decrease in apoptotic pathways and therefore with the suppression of some features involved in several neurodegenerative diseases [39]. It is also shown here that both cocoa and epicatechin induce a strong down-modulation of activated p38, triggered by oxidative stress, showing the cocoa extract the highest inhibition (up to 70%). Since p38 is known to promote apoptosis pathways, cocoa flavonoids may be considered therapeutic agents for neurodegenerative diseases.

On the other hand, our results showed that H_2O_2 stimulates increases in ERK1/2 pathways, as was already described in the same cell line [32]. The present study shows that cocoa extract added to SH-SY5Y neuroblastoma cells did not affect H_2O_2-induced p-ERK1/2 levels, whereas epicatechin reduced them up to ~70%. This decrease may provoke less survival signal, although there are authors who defend that the inhibition of the ERK1/2 pathway protects SH-SY5Y cells from cell death induced by H_2O_2 [32]. In fact, the role of ERK1/2 pathways in survival or death following oxidative stress is still controversial.

It should be taken into account that the intracellular signaling pathways involving MAPK are differentially activated against a variety of extracellular stimuli. In this sense, among them, ERK1/2 are mainly activated by mitogen and growth factors, whereas JNK and p38 respond to stress stimuli, such as ROS overproduction or inflammation. Moreover, ROS are also implicated in the activation of the inflammatory cascade and evidences of a neuroinflammatory mechanism contributing to the cascade of events that led to the progressive neuronal damage are observed in neurodegenerative disorders [35]. For this reason, down-modulation of both ROS overproduction and JNK and p38 MAPK activation by cocoa flavonoids might establish them as therapeutic anti-neuroinflammatory-agents, which may be useful in neurodegenerative diseases. However, this fact remains to be confirmed in other models more closely related to AD, Parkinson's disease or the aging-processes. In this sense, although a recent trial suggests no neuropsychological effects on healthy adults of a short term dark chocolate intake [8], several studies have demonstrated the positive effects of flavonoids and cocoa

on central nervous system following cell damage triggers [19]. Moreover, it is difficult to predict the in vivo concentrations that could produce beneficial neuroprotective effects after cocoa intake due to the few studies regarding bioavailability in the nervous system [11]. However, if high flavonoids concentrations were required, some enriched-cocoa formulations might be produced in the future, as recently described [37].

In summary, the present study has shown the potential neuroprotective action of cocoa flavonoids by reducing ROS production and modulating MAPK activation. Therefore, due to its worldwide consumption and the lack of toxicity in humans, cocoa may constitute a source of neuroprotectants with particular relevance to neurodegenerative diseases.

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