Plant Foods for Human Nutrition (2020) 75:161–168
https://doi.org/10.1007/s11130-020-00807-1

ORIGINAL PAPER

Cocoa Flavanols Protect Human Endothelial Cells from Oxidative Stress

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Published online: 18 March 2020

Abstract

Oxidative stress may cause functional disorders of vascular endothelia which can lead to endothelial apoptosis and thus alter the function and structure of the vascular tissues. Plant antioxidants protect the endothelium against oxidative stress and then become an effective option to treat vascular diseases. Cocoa flavanols have been proven to protect against oxidative stress in cell culture and animal models. In addition, epidemiological and interventional studies strongly suggest that cocoa consumption has numerous beneficial effects on cardiovascular health. The objective of this study was to test the chemo-protective effect of realistic concentrations of a cocoa phenolic extract and its main monomeric flavanol epicatechin on cultured human endothelial cells submitted to an oxidative challenge. Both products efficiently restrained stress-induced reactive oxygen species and biomarkers of oxidative stress such as carbonyl groups and malondialdehyde, and recovered depleted glutathione, antioxidant defences and cell viability. Our results demonstrate for the first time that a polyphenolic extract from cocoa and its main flavonoid protect human endothelial cells against an oxidative insult by modulating oxygen radical generation and antioxidant enzyme and non-enzyme defences.

Keywords epicatechin · catechins · procyanidins · EA.hy926 cells · chocolate flavanols · endothelial dysfunction

Abbreviations

CPE	cocoa polyphenolic extract
CVD	cardiovascular disease
DCF	2′,7′-dichlorofluorescein
DCFH	2′,7′-dichlorohydrofluorescein
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNPH	dinitrophenylhydrazone
EC	epicatechin
FBS	foetal bovine serum
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
LDL	low density lipoprotein
MDA	malondialdehyde
NADPH	nicotine adenine dinucleotide phosphate reduced salt
NO	nitric oxide
OPT	*o*-phthaldialdehyde
ROS	reactive oxygen species
t-BOOH	*tert*-butylhydroperoxide
TEP	1,1,3,3-tetraethoxypropane

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11130-020-00807-1) contains supplementary material, which is available to authorized users.

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Introduction

Oxidative stress seems to be the common underlying mechanism for the development of endothelial dysfunction; when reactive oxygen species (ROS) production is not balanced by antioxidant defense systems, the generated oxidative stress may cause

functional disorders of vascular endothelia which can lead to endothelial apoptosis and thus alter the function and structure of the vascular tissues [1]. Therefore, prevention of oxidative stress is one of the main objectives nowadays of cardiovascular research and consequently, considerable efforts have been made in the last years for the identification of natural antioxidants and dietary products containing these bioactive compounds, which may provide valuable strategies to prevent oxidative stress and the development of cardiovascular disease (CVD).

Polyphenols are some of the most abundant phytochemicals in plant foods and increasing evidence from cohort studies indicate that the intake of some of these compounds may help to reduce the development of CVDs and CVDs mortality risk [2]. Polyphenols encompass several families of compounds, the most represented in plant foods being phenolic acids and flavonoids [3]. A major group of flavonoids is constituted by flavanols that are abundant in green tea, red wine, cocoa and various fruits such as apples [4]. Among them, cocoa beans are one of the richest known sources of flavonoids; indeed, cocoa is the food that has the highest flavonoid content on a per-weight basis [4, 5]. The main constituents are flavanols, present as monomeric (−)-epicatechin (EC) and (+)-catechin, together with their oligomers, the so-called proanthocyanidins, responsible for cocoa bitterness. Epidemiological and interventional studies strongly suggest that cocoa consumption, as well as vegetables and fruit intake, has numerous beneficial effects on cardiovascular health [6] including an improvement in vascular function [2, 7]. Various potential mechanisms, including the increased bioavailability of NO [8] and the anti-inflammatory and antioxidant effect [9], are supposed to be responsible for the protective properties of cocoa. However, scarce research is available on the intrinsic mechanisms involved in such effects at cellular level. Therefore, the objective of this study was to test the chemoprotective effect of a cocoa phenolic extract (CPE) and its main monomeric flavanol EC on endothelial cells submitted to an oxidative challenge. To this purpose, a human endothelial cell line, EA.hy926, was used as a cell culture model of endothelium and treatment with a strong pro-oxidant, *tert*-butylhydroperoxide (t-BOOH), was used to reproduce a cell culture condition of oxidative stress in order to study the possible protective mechanisms through which cocoa flavanols protect endothelial function.

Materials and Methods

Reagents and Materials

Tert-butylhydroperoxide (t-BOOH), (−)-epicatechin (>95% of purity), glutathione reductase (GR), reduced (GSH) and oxidized glutathione, dichlorofluorescin (DCFH), *o*-phthaldialdehyde (OPT), nicotine adenine dinucleotide phosphate reduced salt (NADPH), 2,4-dinitrophenylhydrazine (DNPH), H_2O_2, 1,1,3,3-tetraethoxypropane (TEP), gentamicin, penicillin G and streptomycin were purchased from Sigma Chemical Co. (Madrid, Spain). Acetonitrile, methanol of HPLC grade, dimethyl sulfoxide (DMSO) were acquired from Panreac (Barcelona, Spain). Bradford reagent was from BioRad Laboratories S.A. DMEM culture media and foetal bovine serum (FBS) were from Cultek (Madrid, Spain). All other reagents were of analytical quality.

Sample Preparation

Natural Forastero cocoa powder (Idilia Foods, Barcelona, Spain) was used to prepare the CPE. Extraction of soluble polyphenols and characterization of the different components by HPLC has been detailed elsewhere [10]. Briefly, the polyphenolic profile of CPE showed that monomeric EC and catechin were the major flavanols in the extract, together with appreciable amounts of procyanidins B1 and B2. Additionally, theobromine was present in high amounts while only traces of caffeine were detected in the extract (supplementary data)

Cell Culture and Treatment

EA.hy926, a human hybrid cell line, was a kind gift from Profs. Patricio Aller and Carmelo Bernabeu, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain. The cell line was cultured and passaged in Biowhittaker DMEM media supplemented with 10% fetal bovine serum. Cells were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37 °C and grown in DMEM medium supplemented with 10% FBS and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin [11]. Different concentrations of CPE (2.5, 5, 10 and 20 μg/mL) and EC (2.5, 5, 10 and 20 μM), dissolved in serum-free culture medium, were added to the cell plates for 18 h to study a direct/basal effect of the cocoa extract and its main phenolic compound. In order to evaluate the protective effect of the CPE and EC against an oxidative insult, two different approaches were carried out, co-treatment and pre-treatment. In the co-treatment assay EA.hy926 cells were simultaneously treated for 18 h with 100 μM t-BOOH plus any of the four different CPE or EC concentrations, whereas in the pre-treatment assay cells were first treated with tested doses of CPE or EC for 18 h, then washed and submitted to a new media containing 200 μM t-BOOH for 4 h.

Determination of Cell Redox Status, Biomarkers of Oxidative Damage and Cell Viability

Cellular ROS were quantified by the DCFH assay using microplate reader and the assay has been described elsewhere [10, 11]. GSH was quantitated by the fluorometric assay

described in Browne & Armstrong [12] with some modifications. Assay of GPx activity is based on the oxidation of GSH by GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH by GR as described by Martín et al. [10]. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione [10]. Malondialdehyde (MDA) was analyzed by high-performance liquid chromatography (HPLC) as its DNPH derivative [10], measured in an Agilent 1100 Series HPLC-DAD. MDA values are expressed as nmol of MDA/mg protein. Protein oxidation of cells was measured as carbonyl groups content according to a published method [11]. Analyses of GSH, GPx, GR, MDA and carbonyl groups were performed on cell lysates. Protein was measured by the Bradford reagent. Cell viability was determined by using the crystal violet assay [11].

Statistics

Statistical analysis of data was as follows: prior to analysis the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous. The level of significance was $p < 0.05$. A SPSS version 23.0 program has been used.

Results and Discussion

Biological activities of cocoa flavanols include scavenging of active oxygen species, prevention of LDL oxidation, anti-inflammatory activity, inhibition of tumor cell growth, regulation of apoptotic and survival/proliferation pathways, anti-diabetic effects and cardiovascular benefits [1–3, 10, 13]. All these properties make cocoa polyphenolic fraction an interesting candidate for vascular chemo-protection and, in the present study, the effects of the complex mix of flavanols in CPE have been tested in endothelial cells and the results compared to those with an individual major flavanol EC. The results of the present study demonstrate for the first time that a polyphenolic extract from cocoa powder and its main monomeric flavanol, EC, have the capacity to protect human endothelial cells against an oxidative insult by modulating oxygen radical generation and enzyme and non-enzyme antioxidant defenses.

CPE used in this study has been confirmed as a realistic representative of cocoa-derived products [10, 14, 15]. Some authors have reported concentrations up to 35 μM of EC in rat serum 1 h after oral administration of EC and 0.2–0.4 μM EC have been detected after consumption of 50 g of chocolate [10]; actual EC concentrations in tested doses of CPE ranged from 33 nM in the dose of 2.5 μg CPE/mL to 0.265 μM in that of 20 μg CPE/mL. Cultured EA.hy926 cells have been recently proved a reliable *in vitro* model of endothelial tissue [8, 11, 16] and their viability was not altered by treatment with concentrations up to 20 μg/mL CPE and 20 μM EC for 18 h. When cells were submitted to 100 μM t-BOOH for 18 h or 200 μM t-BOOH for 4 h a dramatic increase in ROS production was observed, but this increase was partially or completely avoided by a co-treatment or pre-treatment with any of the CPE and EC (Fig. 1). Due to their phenolic structure, flavanols have a remarkable oxidant-scavenging capacity related to the hydrogen donating ability and the stability of the phenoxyl radicals formed [3]. In this study, high levels of ROS generated during the stress period are being more efficiently quenched in cells co-treated or pre-treated with CPE or EC resulting in a reduced cell oxidative damage. A similar attenuation of ROS has been reported for grape stilbene resveratrol in endothelial cells [17] and analogous cocoa phenolic extracts in hepatic [10] and pancreatic [13] cells.

As the main non-enzymatic antioxidant defense within the cell, it is accepted that GSH depletion reflects a milieu of intracellular oxidation, whereas an increase in GSH concentration places the cell in a more favorable position against a potential oxidative insult [10–12, 16]. When cells were submitted to t-BOOH a significant depletion of GSH levels was observed (Fig. 2c, d), and this alteration was successfully prevented by most of CPE and EC treatments (Fig. 2), only the highest CPE concentration did not recover the depleted GSH; a fact that has been explained by conjugation of monomeric flavanols to the free sulfhydryl group of reduced glutathione [10]. This protective effect on the cellular antioxidant stores, also observed in endothelial cells treated with plant extracts [11, 16], is essential since maintaining GSH concentration above a critical threshold while facing a stressful situation represents an enormous advantage for cell survival.

Activation of GPx and GR is an critical mechanism of the cell antioxidant defense to face oxidative insults by quenching ROS over-production; however, a rapid return of the antioxidant enzyme activities to basal values once the oxidative challenge has been overcome will reestablish favorable conditions for the cell to cope with a new oxidative insult [10, 11, 16]. Cells submitted to t-BOOH showed a substantial increase of GPx activity as antioxidant response to the oxidative insult, and a full recovery from this increased activity was achieved when cells were co-treated with 5–10 μg/mL of CPE or 5–10 μM EC (Fig. 3c,d). In the pre-treatment assay, all tested doses of CPE and EC evoked a partial but significant recovery of the stress-enhanced GPx activity (Fig. 3e,f). A remarkable increase in the activity of GR was observed after treatment with t-BOOH (Fig. 4c-f), but its activity dose-dependently decreased when cells were co-treated with 2.5–20 μg/mL of CPE (Fig. 4c) and a full recovery

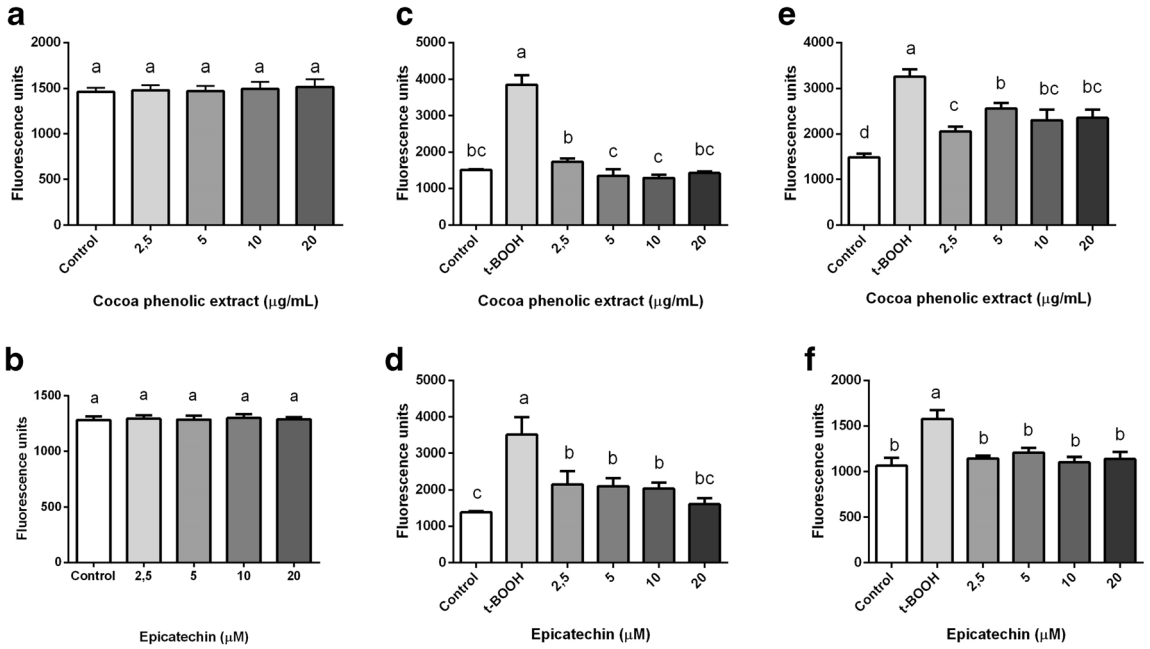

Fig. 1 Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on reactive oxygen species production by EA.hy926 cells. (**a**, **b**) direct effect of CPE and EC; (**c**, **d**) effect of co-treatment of 100 μM t-BOOH plus noted CPE or EC concentrations for 18 h; (**e**, **f**) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 μM t-BOOH. Results are means ± SD (n=3, 4 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data

of the GR activity was observed with a co-treatment with EC (Fig. 4d). A pre-treatment of cells with CPE or EC was able to significantly reduce the stress-enhanced GR activity to values that were similar to those of control cells. This phenomenon is

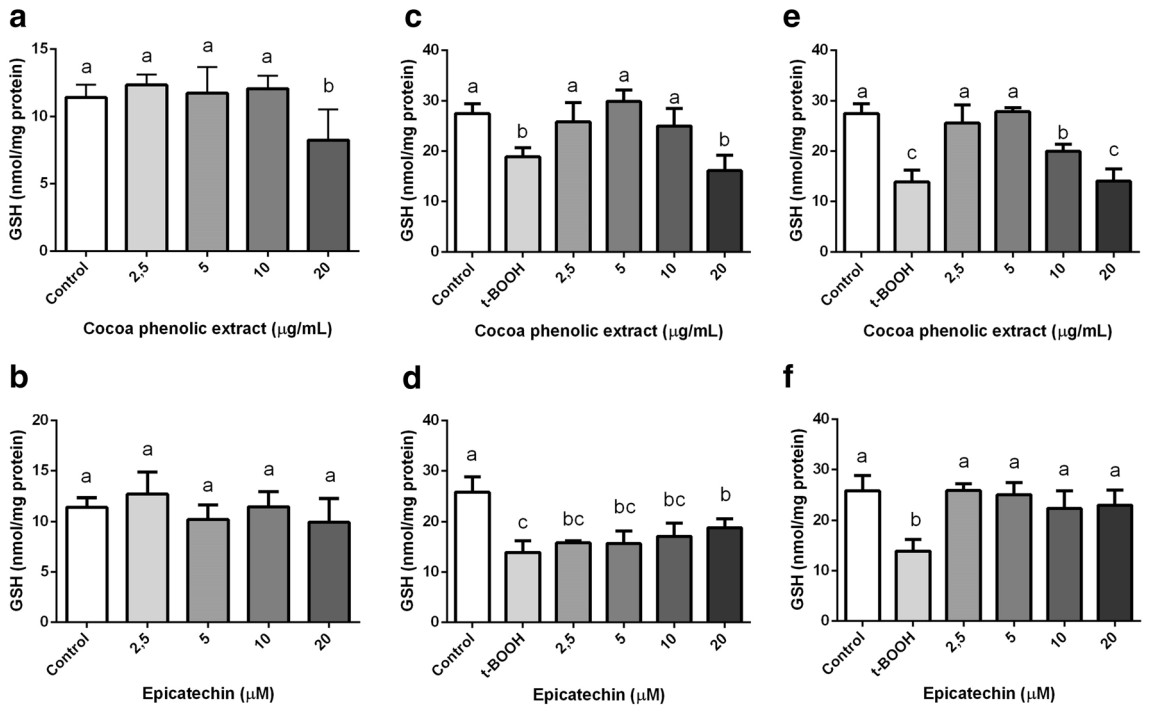

Fig. 2 Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on glutathione concentration in EA.hy926 cells. (**a**, **b**) direct effect of CPE and EC; (**c**, **d**) effect of co-treatment of 100 μM t-BOOH plus noted CPE or EC concentrations for 18 h; (**e**, **f**) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 μM t-BOOH. Results are means ± SD (n=3, 4 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data

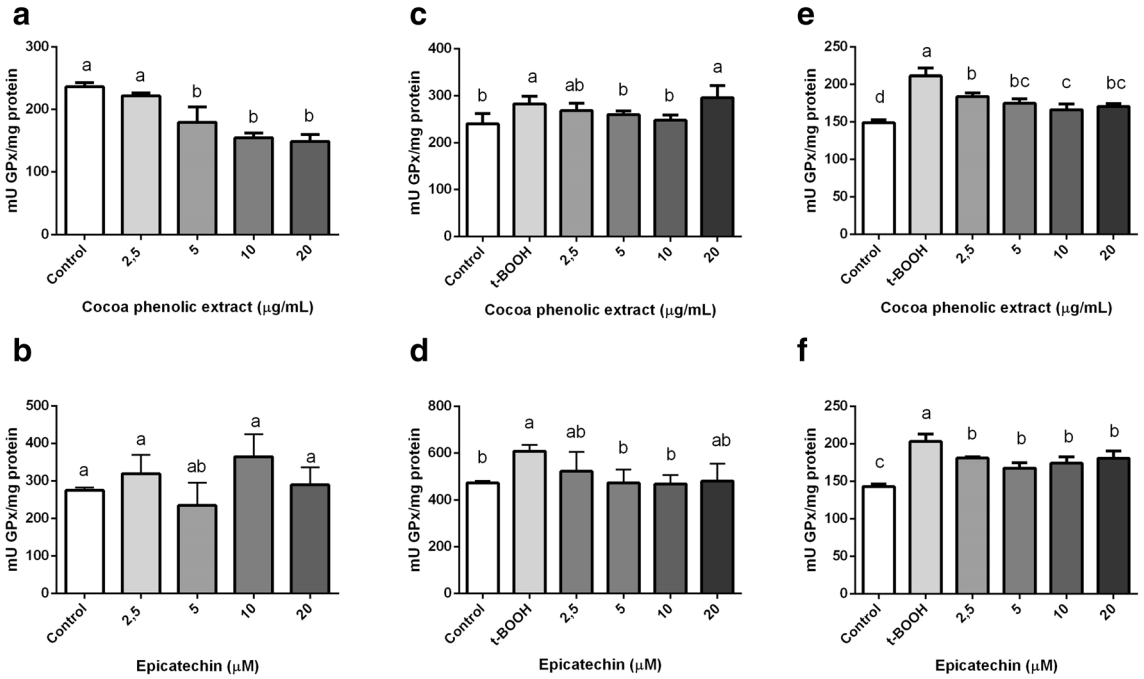

Fig. 3 Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on glutathione peroxidase activity in EA.hy926 cells. (**a**, **b**) direct effect of CPE and EC; (**c**, **d**) effect of co-treatment of 100 μM t-BOOH plus noted CPE or EC concentrations for 18 h; (**e**, **f**) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 μM t-BOOH. Results are means ± SD (n=3 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data

consistent with previous results with EC and CPE in other cell types [10, 13, 15]. Hence, while cells submitted to oxidative stress are still fighting to overcome the insult, those treated with the antioxidants have more efficiently controlled the stressful situation and returned to a balanced redox status consequently reducing cell damage.

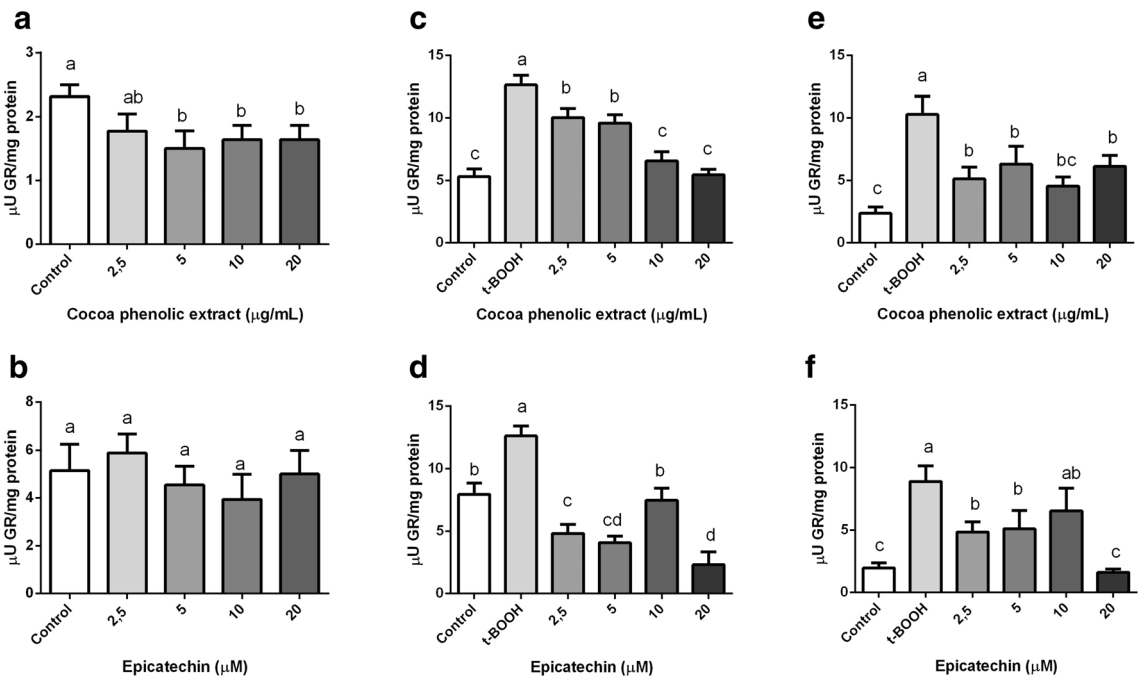

Fig. 4 Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on glutathione reductase activity in EA.hy926 cells. (**a**, **b**) direct effect of CPE and EC; (**c**, **d**) effect of co-treatment of 100 μM t-BOOH plus noted CPE or EC concentrations for 18 h; (**e**, **f**) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 μM t-BOOH. Results are means ± SD (n=3 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data

Table 1 Effect of co-treatment and pre-treatment of EA.hy926 cells with EC (2.5, 5, 10 and 20 μM) and CPE (2.5, 5, 10 and 20 μg/mL) on MDA concentration expressed as nmol/mg protein

	nmol MDA/mg prot	
	Co-treatment	Pre-treatment
Control	$1.06^{a} \pm 0.21$	$0.69^{b} \pm 0.09$
tBOOH	$3.96^{e} \pm 0.31$	$3.38^{e} \pm 0.53$
2.5 μM EC	$2.75^{c} \pm 0.19$	$0.12^{a} \pm 0.08$
5 μM EC	$2.28^{b} \pm 0.19$	$0.45^{b} \pm 0.02$
10 μM EC	$2.32^{b} \pm 0.41$	$0.67^{b} \pm 0.08$
20 μM EC	$3.70^{d} \pm 0.21$	$1.74^{d} \pm 0.34$
2.5 μg/mL CPE	$2.42^{b} \pm 0.20$	$1.10^{c} \pm 0.12$
5 μg/mL CPE	$2.70^{c} \pm 0.47$	$1.83^{d} \pm 0.35$
10 μg/mL CPE	$3.30^{c,d} \pm 0.11$	$1,.41^{d} \pm 0.05$
20 μg/mL CPE	$3.16^{c} \pm 0.71$	$1.02^{c} \pm 0.10$

MDA, a three-carbon compound formed by scission of peroxidized polyunsaturated fatty acids, mainly arachidonic acid, is the main product of lipid peroxidation [18] and has been found elevated in several diseases thought to be related to free radical injury; thus, it is widely used as an index of lipoperoxidation in biomedical sciences [18]. A four-fold increase in MDA concentration was found in endothelial cells after treatment with t-BOOH, but most tested doses of CPE and EC were able to significantly restrain the t-BOOH-induced raise of MDA both in co-treatment and pre-treatment assays (Table 1). The significant protection by EC or CPE against an induced lipid peroxidation in endothelial cells is in agreement with previous studies that showed a comparable effect of other compounds [17].

Carbonyl groups are considered as consistent biomarkers of oxidative damage to proteins, a crucial event in the development of cellular toxicity [18]. The significant increase in the cellular concentration of carbonyl groups during oxidative stress induced by t-BOOH in cultured endothelial cells confirmed extensive damage to cellular proteins. Nevertheless, the lowest CPE and EC doses tested in this study significantly abolished the protein damage induced by the stressor recovering carbonyl group concentration to values similar to those of control unstressed cells (supplementary data, fig. 1). This protective effect against protein oxidation has been previously reported for CPE and EC in pancreatic beta cells [13] and colonic metabolites of flavonoids in endothelial cells [8].

Since both CPE and EC were involved in the regulation of the antioxidant defense mechanisms necessary to face the oxidative challenge, their protective effect

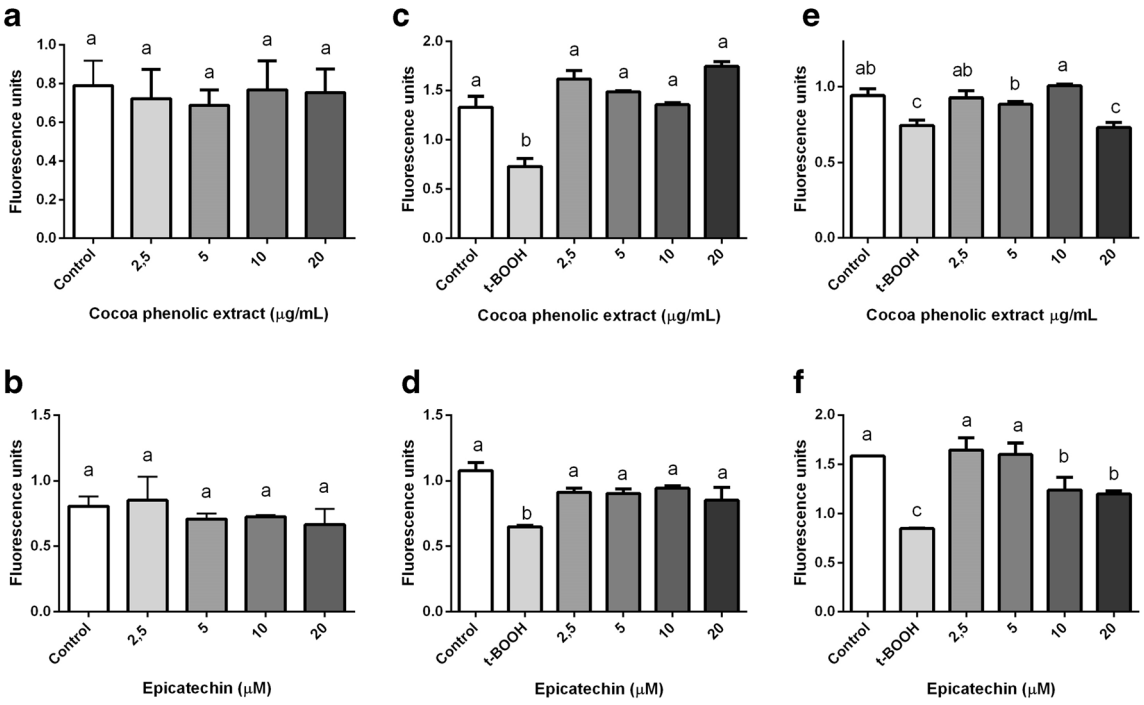

Fig. 5 Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on EA.hy926 cell viability. (**a**, **b**) direct effect of CPE and EC; (**c**, **d**) effect of co-treatment of 100 μM t-BOOH plus noted CPE or EC concentrations for 18 h; (**e**, **f**) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 μM t-BOOH. Results are means ± SD (n=8 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data

should be physiologically exposed in terms of increased cell viability; thus, a potent oxidative challenge induced by t-BOOH severely compromised cell viability but co-treatment or pre-treatment of cells with CPE or EC showed a significant recovery of cell viability in almost all cases (Fig. 5). Consequently, realistic doses of both antioxidant compounds were capable of preserving cell life and function. Thus, the protective mechanism of CPE and EC on endothelial cells submitted to an oxidative stress could be explained in terms of regulation of the cellular redox status: a decrease of ROS production during stress reduces the need of peroxide detoxification through GPx as well as of GSH and, consequently, its recovery from oxidized glutathione through GR. Furthermore, decreased ROS level reduces oxidative damage to macromolecules, lipids and proteins, resulting in diminished cell injury and death. Interestingly, EC being the most abundant flavanol of cocoa suggests that a great percent of the protecting effect of CPE in endothelial cells may be due to EC, but the potential effect of other compounds besides EC should not be ruled out.

Conclusions

Our studies in cultured endothelial cells demonstrate, for the first time, that flavanol rich extract from cocoa, CPE, and its main flavanol, EC, display positive health effects against an oxidative stress through an efficient regulation of cell redox status; limiting ROS generation and strengthening antioxidant defense response. The present data propose a prominent role for cocoa and its flavanols in the protection afforded by fruits, vegetables and plant-derived beverages against pathologies such as CVD, for which excessive production of ROS has been implicated as a causal or contributory factor.

Acknowledgements Tatiane Ferreira-Martins is a fellow of the University of São Paulo. D. Álvarez-Cilleros is a FPI fellow from the predoctoral program of MINECO (BES-2016-076721).

Availability of data and material (data transparency). Not applicable.
Code availability (software application or custom code). Not applicable.

Authors' contributions TFM and LG carried out most of the experimental and data analysis. OP carried out and analyzed MDA assay. SR carried out and analyzed data from carbonyl groups. DAC and MAM contributed to the critical revision of the manuscript. LG conceived and designed the study and wrote the manuscript with significant contributions from all other authors.

Funding This work was supported by the grant AGL 2015–67087-R (MINECO/FEDER, UE) from the Spanish Ministry of Science and Innovation.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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