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Oxidative Status of Stressed *Caenorhabditis elegans* Treated with Epicatechin

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ABSTRACT: The aim of this work was to examine the mechanisms involved in the in vivo antioxidant effects of epicatechin (EC), a major flavonoid in the human diet. The influence of EC in different oxidative biomarkers (reactive oxygen species (ROS) production, intracellular glutathione, activity of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD)) was studied in the model organism *Caenorhabditis elegans*. Under thermal stress condition, exposure of the worms (wild type N2 strains) to EC (200 μM) significantly reduced ROS levels (up to 28%) and enhanced the production of reduced glutathione (GSH). However, no significant changes were appreciated in the activities of GPx, CAT, and SOD, suggesting that further activation of these antioxidant enzymes was not required once the concentration of ROS in the EC-treated worms was restored to what could be considered physiological levels.

KEYWORDS: *Caenorhabditis elegans, thermoresistance, epicatechin, ROS, glutathione, flavonoids*

INTRODUCTION

Oxidative stress is an imbalanced state in which excessive quantities of reactive oxygen and/or nitrogen species (ROS/RNS, e.g., superoxide anion, hydrogen peroxide, hydroxyl radical, peroxynitrite) overcome the endogenous antioxidant capacity of a biological system, leading to an accumulation of oxidative damage in a variety of biomacromolecules, such as enzymes, proteins, DNA, and lipids, which results in a progressive loss in functional cellular processes.[1] Oxidative stress has been related to an increased risk for cardiovascular disease, cancer, autism, and other diseases, as well as, at least partially, the process of aging.[2] On the other hand, whereas an excess of ROS can be considered toxic, certain levels of ROS are necessary as intracellular messengers and can also mediate the adaptive stress response of cells.[3] Intracellular antioxidant defenses and intake of dietary antioxidants may help maintain an adequate antioxidant status in the body. Antioxidants can decrease the oxidative damage directly via reacting with free radicals or indirectly by inhibiting the activity or expression of free radical generating enzymes or enhancing the activity or expression of intracellular antioxidant enzymes.[4]

Flavonoids are recognized as efficient antioxidants and free radical scavengers, as largely demonstrated in in vitro assays. This property is often argued as being responsible for the beneficial health effects of flavonoid-rich diets. Nevertheless, there are indirect effects that could be of greater biological importance for the in vivo effects of flavonoids than their direct involvement in redox reactions. It has been indicated that whereas high concentrations of polyphenols are required to induce a direct antioxidant activity, lower concentrations, as provided by a dietary intake, would induce indirect antioxidant effects or even act as pro-oxidants.[5,6]

Catechins are one of the most abundant flavonoids in the diet that are present in many fruits, tea, cocoa, and red wine. Antioxidant and radical scavenging properties of catechins are well established in in vitro studies,[7] and they have also been shown to be able to enhance the antioxidant defenses in cell systems exposed to pro-oxidants.[8−10] Although cell cultures are commonly used as model systems to study the effects of bioactive compounds on the redox status, it is important to take into account that the cell culture imposes itself a state of oxidative stress on cells. Thus, ROS-dependent signal transduction pathways identified in cultured cells should be validated in in vivo studies.[11] An interesting approach is the use of model organisms such as *Caenorhabditis elegans*. This nematode offers several advantages for the genetic and biochemical study of aging related to oxidative stress, which include (i) a short maximum life span; (ii) a 3 day life cycle; (iii) a robust genetic foundation that includes a completely sequenced genome and the opportunity to apply techniques such as transformation and RNAi; and (iv) a soma consisting of fewer than 1000 cells, all of which are postmitotic in adults, which offers the possibility to detect cumulative age-related cellular alterations.[12] *C. elegans* shows a strong conservation in molecular and cellular pathways in relation to mammals, and comparison between human and *C. elegans* genomes confirmed that the majority of human disease genes and disease pathways are present in the worm.[13] Therefore, it has been used to study a variety of biological processes including apoptosis, cell signaling, cell cycle, cell polarity, gene regulation, metabolism, and aging.

Different authors have used *C. elegans* to investigate the protective effects of several flavonoid-rich extracts or pure

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flavonoids on stress resistance.[14−17] Nevertheless, to our knowledge, none of them have studied in detail how epicatechin modulates the endogenous antioxidant defenses in *C. elegans*. Protective effects observed in assays carried out with *Ginkgo biloba* extracts were suggested to be due to the ability of flavonoids to decrease intracellular ROS accumulation[18] together with a parallel down-regulation of glutathione *S*-transferase and catalase.[19] A reduction in the intracellular ROS accumulation was also described for kaempferol,[14] fisetin,[14] and rutin and quercetin.[15] In the case of quercetin, one of the most studied flavonoids, different mechanisms of action have been proposed, from the modulation of genes *age-1*, *daf-2*, *sek-1*, or *unc-43*[20] to the involvement of the DAF-16 transcription factor.[15,21] Translocation of DAF-16 from the cytoplasm to the nucleus, and the subsequent modulation of the insulin-like signaling pathway, has been also described for epigallocatechin-3-*O*-gallate,[22] cocoa polyphenols,[23] or apigenin.[24] However, the implication of DAF-16 is controversial, and some authors point out that DAF-16 is not obligatorily required for flavonoid-mediated longevity and stress resistance.[25,26] In a recently published paper by our group,[27] catechins were shown to improve the resistance of *C. elegans* against thermal and juglone-induced oxidative stress, increasing the survival rates by 28−44% compared to untreated worms, with epicatechin (EC) being the compound that provided greater protection. The aim of this study is to delve into the biochemical mechanisms involved in these effects through the study of different biomarkers of the cellular redox status (ROS production; intracellular glutathione; activity of catalase, glutathione peroxidase, and superoxide dismutase). This work constituted a first screening to check whether EC induced effects on the redox status of the worm and decide about further studies using specific mutant worms.

MATERIALS AND METHODS

Standards and Reagents. Epicatechin, fluorodeoxyuridine (FUdR), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), NADPH, glutathione reductase, hydrogen peroxide, and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich (Madrid, Spain); dimethyl sulfoxide (DMSO) was from Scharlau Chemie (Barcelona, Spain); and sodium hypochlorite and *tert*-butylhydroperoxide (*t*-BOOH) were purchased from Panreac (Barcelona, Spain).

Strains and Maintenance Conditions. *C. elegans* strains wild type N2 were obtained from the *Caenorhabditis* Genetics Centre at the University of Minnesota (Minneapolis, MN, USA). All strains were routinely propagated at 20 °C on nematode growth medium (NGM) plates using heat-killed (30 min at 65 °C) *Escherichia coli* strain OP50 as a food source. Synchronization of worm culture was achieved by treating gravid hermaphrodites with bleach (12% aqueous solution of 10%, w/v, sodium hypochlorite). The suspension was shaken vigorously during 1 min and kept for a further minute in ice; this process was repeated five times. Eggs were resistant to bleach, whereas worms were dissolved in the bleach solution. The suspension was centrifuged (1 min, 2000*g*), and the complete process was repeated twice. The pellet containing the eggs was washed twice with an equal volume of buffer M9 (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 mL of 1 M $MgSO_4$, and H_2O to 1 L). The supernatant was removed, and the eggs were resuspended and kept in a small volume of M9. Around 100−200 μL of the M9 with eggs (depending on egg concentration) were transferred and incubated in NGM agar plates. Epicatechin solutions (200 mM) were prepared in DMSO and added to the NGM medium during its preparation. The final concentration of the EC in the treatment plates was 200 μM. Control plates were also prepared lacking EC but containing the same volume of DMSO (final concentration of 0.1% DMSO (v/v) in all plates).

Determination of ROS. Cellular ROS were quantified by the dichlorofluorescein assay using a microplate reader.[28] At the sixth day of adulthood, treated and control worms were individually transferred to the well of a 96-well plate containing 75 μL of PBS. Then the worms in the well plate were exposed to thermal stress (2 h at 35 °C), and subsequently 25 μL of DFCH-DA solution in PBS buffer was added to each well. The final concentration of DFCH in the well was 62.5 μM. After removal of the acetate groups in worm cells, the compound can be oxidized by the intracellular ROS to yield the fluorescent dye DCF. The fluorescence from each well was measured at 35 °C immediately after incorporation of the reagent and every 10 min for 30 min with a 1 s integration time, using 485 and 535 nm as excitation and emission wavelengths, respectively. Recording of the DCF fluorescence intensity with time was used as an index of the individual intracellular levels of ROS in single worms. Fluorescence from worms not exposed to stress was also measured. The response of the method was checked every day using a H_2O_2 curve. Five independent experiments were performed per treatment, and for each experiment ROS measurements were made in at less 36 individual worms. The measurements were performed in an Ultra Evolution Multi-functional Microplate Reader (Tecan, NC, USA).

Determination of Glutathione and Enzyme Activities. *Worm Homogenate.* Age-synchronized young larvae (L1) were transferred to fresh NGM agar plates (⌀ 100 mm) prepared with and without epicatechin and grown at 20 °C. When the worms reached the L4 stage, they were transferred to plates similar to the above-described but also containing FUdR at a final concentration of 150 μM to prevent reproduction and progeny overgrowth. The worms were transferred every 2 days to fresh plates with FUdR for the different treatments (with and without EC) until they reached the sixth day of adulthood. Then the worms were exposed to 35 °C for 5 h, and subsequently animals from three plates (⌀ 100 mm) of each treatment were collected to a flask and suspended in M9 buffer (around 1500−3000 individuals per homogenate). Worms not exposed to thermal stress were also assayed. Suspensions were centrifuged (12000*g*, 5 min), and the worm pellet was washed with PBST (PBS + 0.01% Tween 20) twice and finally with PBS. The remaining pellet was transferred to an eppendorf, resuspended in 500 μL of PBS, and kept at −20 °C for 12 h. Afterward, samples were stirred (Genius 3 vortex) and sonicated six times for 10 s in a Cell Disruptor (Microson XL2000 100) to obtain a homogenate. The homogenate was centrifuged (13000*g*, 5 min, 4 °C) and the supernatant collected for the determination of glutathione and enzyme activities. For each treatment three independent experiments were performed, and in each experiment the measurements of the different variables were made in triplicate using three different homogenates of worms.

The protein content was determined according to the Bradford method after digestion of the homogenate. The activities of all the enzymes and the content of GSH were scaled to protein content to correct for differences in biomass of the diverse homogenates.

Determination of Reduced Glutathione (GSH). The levels of GSH in the worms were determined according to the method of Beutler et al.,[29] based on the formation of 2-nitro-5-tiobenzoic acid from DTNB in the presence of GSH. In brief, 150 μL of trichloroacetic acid (15%) was added to 300 μL of the homogenate, following centrifugation at 13000*g* for 5 min at 4 °C. A supernatant aliquot (100 μL) was mixed with 400 μL of 3.4 mM EDTA dissolved in PBS, 1 mL of PBS, and 250 μL of DTNB in PBS (20 mg/mL). The absorbance was measured at 412 nm after 15 min and compared to a standard curve of GSH (0.5−0.010 mM).

Determination of Glutathione Peroxidase (GPx), Catalase (CAT), and Superoxide Dismutase (SOD) Activities. The activities of GPx, CAT, and SOD were measured in the supernatants. Determination of GPx activity was based on the oxidation of GSH by GPx using *t*-BOOH as a substrate, coupled to the disappearance of NADPH by glutathione reductase. The decrease in NADPH was measured at 340 nm during 2 min using 30 s intervals.[30]

CAT activity was determined by the rate of disappearance of hydrogen peroxide monitoring the decrease in absorbance at 240 nm during 2 min using 10 s intervals.[31]

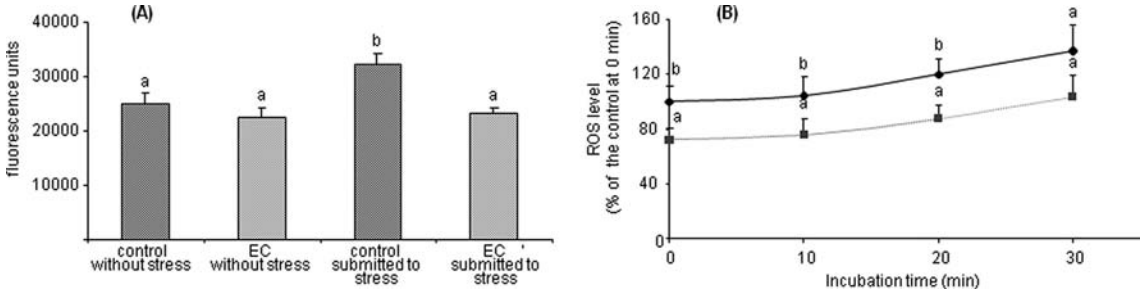

Figure 1. (A) Levels of intracellular ROS in *C. elegans* submitted or not to thermal stress (2 h, 35 °C) in the presence and absence of epicatechin (200 μM) in the culture media. ROS production is expressed as fluorescence units measured after incorporation of the reagent (0 min). Different letters indicate significant differences among groups ($p < 0.01$). (B) Evolution of ROS measured in worms submitted to thermal stress nontreated (upper trace) and treated with EC (lower trace). Results are expressed as percentage of fluorescence increase in relation to nontreated animals. Different letters indicate statistical significant differences between the EC-treated group and the control group ($p < 0.01$).

SOD activity was measured by an assay based on the inhibition of superoxide-induced lucigenin chemiluminescence.[32] Aliquots of 6.7 μL were taken from a serial sample dilution (100:0, 75:25, 50:50, 25:75, 0:100 in PBS) and added in triplicate to a 96-well plate; a blank reaction containing 6.7 μL of water was also included; 20 μL of xanthine oxidase diluted 1:5 in distilled water was then added, and afterward 174 μL of a reaction mixture (5.2 mL 0.1 M glycine, 1 mM EDTA, adjusted to pH 9.0 with NaOH, 10 mL 0.108 mM xanthine, 2.1 mL 1 mM lucigenin, and 1.2 mL water for a total of 18.5 mL) was quickly incorporated using a multichannel pipet. Luminescence was measured at 25 °C during the span required for 25 consecutive measurements in an Ultra Evolution Multi-functional Microplate Reader.

Statistical Analyses. Statistical analyses were performed by one-way analysis of variance (ANOVA) using the PC software package SPSS (version 13.0; SPSS Inc., Chicago, IL, USA). Significant differences were assessed with an LSD test at $p < 0.05$ and $p < 0.01$.

RESULTS

Intracellular ROS Levels. In a previous work by our group,[27] epicatechin was shown to increase the rates of survival of *C. elegans* exposed to thermal stress by 28% compared to untreated worms. Thermal stress is classically associated with damage caused by accumulation of ROS.[33] Thus, in the present work intracellular ROS levels were determined in *C. elegans* grown in NMG media with and without EC (200 μM) and exposed to thermal stress on the sixth day of adulthood.

As expected, in control worms not treated with EC, thermally stressed animals showed significantly higher levels of ROS than those not submitted to stress (Figure 1A), confirming that thermal stress induces ROS generation. Under stress conditions a significant reduction ($p < 0.01$) in the ROS levels was found in the EC-treated worms compared with nontreated worms (Figure 1A). ROS decrease ranged between 24 and 28% depending on the time of measurement (Figure 1B). Interestingly, some reduction in the ROS levels in EC-treated worms was also observed in the absence of stress (decrease of 10%, significant at $p < 0.05$ but not at $p < 0.01$), indicating that the response to EC was associated not only with a stress situation. Furthermore, the fact that similar ROS levels were determined in both EC groups, either submitted or not to thermal stress (Figure 1A), suggests that EC prevents an increase in ROS levels, maintaining them in physiological concentrations. This effect might be due to either a direct radical scavenging activity of EC or an indirect action through up-regulation of the antioxidant defenses, like those involving thiol-containing molecules (e.g., thioredoxin and glutathione systems) or the activity of enzymes such as superoxide dismutase, glutathione peroxidase, or catalase. Thus, further analyses of these antioxidant systems were performed to check whether they might account for the observed protection against thermal stress.

Reduced Glutathione Levels. Glutathione is one of the most abundant and important nonenzymatic defense molecules. In its reduced form (GSH), this molecule can directly reduce substrates but also act indirectly through the glutaredoxin system, as well as play a key role in protection against oxidative stress, intervening in detoxification and repair processes in combination with glutathione *S*-transferase.[34] It is assumed that GSH depletion reflects intracellular oxidation, whereas a balanced GSH concentration could be expected to prepare the cell against a potential oxidative insult.[10,35,36]

In this study, control worms submitted to thermal stress showed significantly lower levels of GSH (27 nmol/mg of worm protein) than those not submitted to thermal stress (48 nmol/mg protein), whereas treatment with EC restored GSH (45 nmol/mg protein) to levels similar to those existing in the worms grown in native conditions (Figure 2). Thus, it was

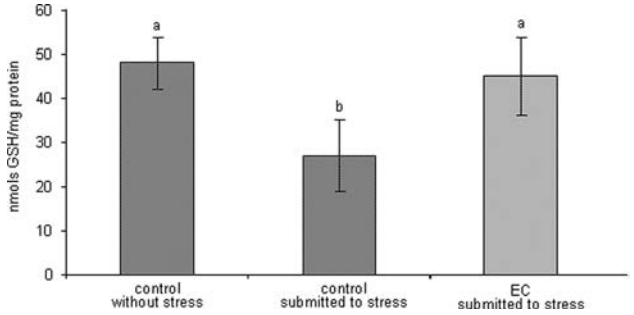

Figure 2. GSH concentrations (expressed as nmol GSH/mg protein) in worms not submitted to stress and in worms submitted to thermal stress (5 h, 35 °C) in the presence and absence of epicatechin (200 μM) in the culture media. Different letters indicate significant differences ($p < 0.01$). Values are the mean ± SD of three different homogenates of worms analyzed in triplicate.

confirmed that thermal stress induced GSH depletion, probably as a result of an increased oxidant status, which is counteracted by the presence of epicatechin. A similar observation was also made by Martin et al.[10] in stressed cell cultures treated with EC, although, to our knowledge, this effect has not been previously reported in a whole organism such as *C. elegans*.

GPx, CAT, and SOD Activities. Another mechanism to limit the cellular damage caused by ROS is the activation of a

complex antioxidant enzymatic system, in which GPx, CAT, and SOD would constitute a first defense line. SOD converts superoxide to H_2O_2, and CAT or peroxidases (e.g., glutathione peroxidase or peroxiredoxins) subsequently convert H_2O_2 to water; peroxidase activity can be further restored through reduction by either thioredoxin (TRX) or glutaredoxin. This antioxidant system could be activated in response to different stimuli, including thermal stress, and be modulated by chemical compounds.

In our study, stress conditions did not affect significantly the activity of GPx in *C. elegans*, whereas they increased the activity of CAT (compare controls in panels A and B of Figure 3),

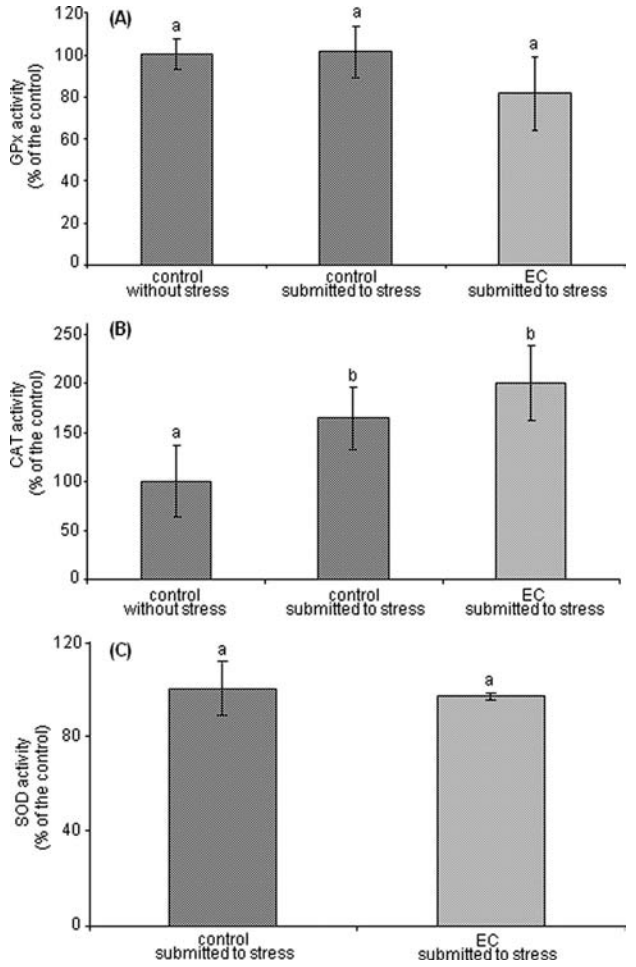

Figure 3. Activity of antioxidant enzymes: (A) glutathione peroxidase (GPx), (B) catalase (CAT), and (C) superoxide dismutase (SOD) in control worms not submitted to stress and in worms submitted to thermal stress (5 h, 35 °C) treated or not with epicatechin (200 μM in the culture media). The enzyme activities are expressed as percentages in relation to nonstressed control worms. Different letters indicate significant differences ($p < 0.01$) between groups in each assay. Values are the mean ± SD of three different homogenates of worms analyzed in triplicate.

suggesting that this latter enzymatic system was activated under stress conditions. Pretreatment with EC of thermally stressed worms produced a decrease (19% in relation to nontreated stressed worms) in the activity of GPx, whereas it increased the activity of CAT, although those changes were not found to be significant at $p < 0.05$. No modification was observed in the activity of SOD (Figure 3).

DISCUSSION

The obtained results showed that ROS accumulation inside the worms after thermal stress was prevented by EC. Furthermore, higher GSH levels were found in EC-treated worms (increase of 67% compared with untreated worms), whereas the activities of GPx, CAT, and SOD were not significantly modified ($p < 0.05$), which might suggest that the enhancement in GSH levels induced by EC could be sufficient to decrease ROS levels and increase worm survival under stress conditions. Nevertheless, although not significant, some apparent increase seemed to occur in the activity of CAT as well as a decrease in that of GPx. These observations partially agree with those reported by Martin and co-workers in HepG2 cell lines, who found that treatment with EC[10] or cocoa flavanols[9] decreased the activity of GPx and glutathione reductase and increased GSH levels in cells submitted to oxidative stress. Also, the simple phenol hydroxytyrosol, the flavonol quercetin, and green tea catechins have been reported to increase GSH levels and to decrease the activity of GPx in different cell lines subjected to oxidative stress.[36−38] On the other hand, a decrease in ROS levels in thermally stressed worms has also been observed following exposure to different flavonols such as quercetin, fisetin, rutin, or kaempferol.[14,15] It is important to note, however, that for fisetin or kaempferol >3−4 h of stress was required to observe a significant ROS decrease in treated worms compared to controls, whereas in our study the decrease was already significant after 2 h of thermal stress, suggesting greater ROS-reducing ability in the case of EC. This activity should be explained not only by the radical-scavenging properties of EC (largely demonstrated in in vitro studies) but also by an indirect activity through the activation of protective signaling pathways.

A particularly relevant pathway in the modulation of oxidative stress is the activation of the antioxidant response element (ARE), which promotes the expression of protective proteins including those required for glutathione synthesis (glutamate/cystine antiporter, γ-glutamylcysteine synthetase, and glutathione syntethase).[39] The expression of the majority of ARE-driven genes has been shown to be regulated by the nuclear factor-E2-related factor-2 (Nrf2).[40,41] Under basal conditions, Nrf2 is located in the cytoplasm bound to repressor protein Kelch-like ECH-associated protein 1 (Keap1). Upon activation, this complex is dissociated and Nrf2 translocates to the nucleus, interacting with ARE and triggering the transcription of phase II and antioxidant defense enzymes. Nrf2 activation is presumably sensitive to cellular oxidative stress and levels of thiol-reactive compounds and antioxidants, including polyphenols.[42] Up-regulation of ARE-mediated gene expression by flavonoids has been observed in different cell lines.[43−45] Flavonoids have been shown to increase the expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis, thus leading to a concomitant increase in the intracellular concentration of glutathione.[44,46] In particular, the ability of EC to activate Nrf2 and increase GSH levels was reported in astrocytes[47] and HepG2 cells.[48] Epicatechin was also seen to be a potent stimulator of the Nrf2/ARE pathway, leading to reduced $O_2^{\bullet-}$ vascular levels, in deoxycorticosterone acetate (DOCA) salt-treated hypertensive rats showing increased levels of oxidative stress.[49] Taking all of these observations into consideration, the increased glutathione (GSH) levels and reduced ROS concentrations observed in our assays in the EC-treated worms might be speculated as due to an activation of the ARE pathway. Glutathione is important

in many diseases, and regulation of intracellular glutathione concentrations may be one mechanism by which dietary flavonoids influence disease development. Our results show that EC could act as a modulator of GSH levels in *C. elegans*, which would also imply a regulation of GSH-dependent cellular processes, such as detoxification of xenobiotics, glutathionylation of proteins, and redox regulation.

Another pathway that has been related with the increase in thermal and oxidative resistance induced by flavonoids involves the modulation of the insulin/IGF-like signaling (IIS) cascade, a key regulator of thermotolerance and longevity in *C. elegans*. Thus, flavonols such as quercetin have been shown to increase the nuclear localization of DAF-16,[14,21,50] which could translate into elevated expression of defense enzymes. Nevertheless, Saul et al.[25] demonstrated prolonged lifespan induced by quercetin in daf-16 knockout strains, suggesting IIS-independent mechanisms of action. Redox-sensitive signaling cascades might also be a promising target because ROS were shown to be involved in signal transduction of different growth factors[51] and apoptosis.[52]

The present study does not allow us to conclude which particular molecular mechanisms are involved in the antioxidant effects of epicatechin. However, the changes that it induced in the studied redox-related variables in stressed worms constitute a helpful indication for designing further studies using suitable mechanistical approaches (i.e., with mutant worms and/or transcriptomics) to delve into the mechanisms by which EC exerts its antioxidant effects in vivo.

It is pertinent to note that the EC concentration used in our study (200 μM in the culture media, corresponding to approximately 6 mg/100 g) is in the same range of catechins concentration reported in different food items (e.g., around 10 mg/100 g in strawberries, 25 mg/100 g in apples, or 70 mg/100 g in tea). Obviously, the worm did not incorporate the whole of EC present in the medium, but the level of EC uptake by *C. elegans* seems rather low.[27] In these circumstances we may assume that the levels of exposure of the worm to the compound are below those of an average human dietary exposure.

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Notes

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