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Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells☆

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Abstract

Catechins and their polymers procyanidins are health-promoting flavonoids found in edible vegetables and fruits. They act as antioxidants by scavenging reactive oxygen species and by chelating the redox-active metals iron and copper. They also behave as signaling molecules, modulating multiple cell signalling pathways and gene expression, including that of antioxidant enzymes. This study aimed at determining whether catechins and procyanidins interact with the redox-inactive metal zinc and at assessing their effect on cellular zinc homeostasis. We found that a grape-seed procyanidin extract (GSPE) and the green tea flavonoid (−)-epigallocatechin-3-gallate (EGCG) bind zinc cations in solution with higher affinity than the zinc-specific chelator Zinquin, and dose-dependently prevent zinc-induced toxicity in the human hepatocarcinoma cell line HepG2, evaluated by the lactate dehydrogenase test. GSPE and EGCG hinder intracellular accumulation of total zinc, measured by atomic flame absorption spectrometry, concomitantly increasing the level of cytoplasmic labile zinc detectable by Zinquin fluorescence. Concurrently, GSPE and EGCG inhibit the expression, evaluated at the mRNA level by quantitative reverse transcriptase-polymerase chain reaction, of zinc-binding metallothioneins and of plasma membrane zinc exporter ZnT1 (SLC30A1), while enhancing the expression of cellular zinc importers ZIP1 (SLC39A1) and ZIP4 (SLC39A4). GSPE and EGCG also produce all these effects when HepG2 cells are stimulated to import zinc by treatment with supplemental zinc or the proinflammatory cytokine interleukin-6. We suggest that extracellular complexation of zinc cations and the elevation of cytoplasmic labile zinc may be relevant mechanisms underlying the modulation of diverse cell signaling and metabolic pathways by catechins and procyanidins.

Keywords: Epigallocatechin gallate; Procyanidins; Flavonoids; Labile zinc; Metallothionein; Zinc transporters

1. Introduction

The flavan-3-ols (+)-catechin and (−)-epicatechin, their gallated derivatives and their polymeric forms, procyanidins, constitute the most abundant polyphenols of flavonoid type present in edible fruits, red wine, chocolate and tea. They are considered bioactive micronutrients whose consumption entails benefits for human health as they may reduce the risk of cardiovascular diseases and metabolic disorders such as hypertriglyceridemia, diabetes and inflammation, and cancer [1–7]. Many beneficial effects of flavonoids have been ascribed to their antioxidant activity, which they exert directly by scavenging reactive oxygen species (ROS) and by chelating the redox-active transition metals iron and copper, that may act as ROS generators [8,9]. Flavonoids also act indirectly as antioxidants by inhibiting redox-sensitive transcription factors and pro-oxidant enzymes as well as through induction of phase II and antioxidant enzymes [10–12]. Beyond their antioxidant actions, flavonoids modulate multiple cell signaling pathways and, ultimately, gene transcription and metabolic fluxes [13–16].

Some flavonoids have been shown capable of complexation with the redox-inactive metal zinc [9,17–19], an essential micronutrient whose deficiency causes multiple dysfunctions, including alterations of glucidic and lipid metabolisms [20,21]. The majority of cellular zinc is tightly bound to proteins, functioning as a catalytic or structural component of an estimated 300 mammalian enzymes and proteins involved in virtually all cellular processes; some intracellular zinc exists in its free ionic form or loosely bound to proteins, and acts as a second messenger that modulates multiple signaling and metabolic pathways [22–26]. In mammalian cells, zinc from the extracellular milieu and from intracellular compartments enters the cytoplasm through 14 specialized transmembrane proteins of the ZIP/SLC39 family, whereas cytoplasmic extrusion of zinc is performed by 10 transporters of the ZnT/SLC30 family, being ZnT1, located at the plasma membrane, the primary regulator

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of cellular zinc efflux [27–29]. Within the cytoplasm, zinc is bound by metal-free apo-metallothionein (apo-MT) and by protonated glutathione to generate Zn-MT and G-SZn, respectively; the apo-MT/MT ratio controls free and labile zinc concentrations [30,31]. MT also serves as ROS scavenger and as heavy metal chelator, and its transcription responds, in addition to zinc, to stress stimuli such as ROS, heavy metals and proinflammatory cytokines [32,33]. The zinc-sensor transcription factor MTF1 (metal response element-binding transcription factor 1) coordinately up-regulates the transcription of MTs, ZnT1 and glutamate-cysteine ligase catalytic subunit (GCLC), the rate-limiting enzyme in glutathione biosynthesis, in response to elevated extracellular and intracellular labile zinc concentrations [34]. Dysfunctions of MT and zinc transporters act as promoting factors in several chronic pathologies including diabetes and cancer [35–37].

Research of flavonoids effects on zinc homeostasis has been scarce and focused on MT expression, regarded as an antioxidant enzyme. For instance, in human intestinal Caco-2 cells, genistein enhances expression of MT, whereas quercetin has the opposite effect, albeit enhances copper induction of MT [38–40]. Also, long-term consumption of flavonoids may affect mineral status, and for instance, iron, copper and zinc levels are diminished in the liver of rats fed with rutin and baicalin [41]. Conversely, zinc has been shown to influence the bioactivity of some flavonoids. Thus, zinc yields (−)-epigallocatechin-3-gallate (EGCG) effective in protecting cultured rat hepatocytes against hepatotoxin-induced cell injury [17] and enhances its antiproliferative effects on prostate cancer cells [18]. Zinc also stimulates the apoptotic effect of genistein in osteoclastic cells [42]. Therefore, bioactivity of flavonoids and zinc metabolism might be interconnected.

The aim of this work was to gain insight into the modulation of zinc homeostasis by catechins and procyanidins in the human hepatocarcinoma HepG2 cells, used as a model of hepatic cell. We found that a grape-seed procyanidins extract (GSPE) and the green tea polyphenol EGCG chelate zinc in solution and dose-dependently prevent zinc toxicity in HepG2 cells. GSPE and EGCG hindered the induction of MT genes and ZnT1 expression and the accumulation of total intracellular zinc elicited by treatment of cells with zinc and interleukin 6 (IL-6) but, on the contrary, enhanced the expression of plasma membrane ZIP transporters and the elevation of cytoplasmic labile zinc elicited by zinc and IL-6. Given the relevance of zinc in cell signaling and control of metabolic pathways, we forward the hypothesis that modulation of zinc homeostasis might underlay some of the health-promoting actions of catechins and procyanidins.

2. Materials and methods

2.1. Chemicals

GSPE was from Les Dérives Résiniques et Térpeniques (Dax, France). This extract consist of monomeric catechins (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%) and oligomeric (5–13 U) (35.7%) procyanidins and phenolic acids (4.22%). Pure procyanidin C1 was provided by Prof. Jean-Michel Mérillon and Dr. Xavier Vitrac (Polyphénols Biotech, Bordeaux, France). All other procyanidins, catechins, TPEN [*N*,*N*,*N*′,*N*′-tetrakis(2-phridylmethyl) ethylenediamine], $ZnCl_2$, dexamethasone, Zinquin ethyl ester and dimethyl sulfoxide (DMSO) were from Sigma, and IL-6 was from Roche.

2.2. Interaction of catechins and procyanidins with zinc in solution

UV-Vis absorption spectra of flavonoids (10 μM) in phosphate-buffered saline (PBS) at pH 7.4, was recorded before and after addition of 5 μM $ZnCl_2$, as previously reported [38], using a Hitachi U-1900 Spectrophotometer, with a 4-nm slit width. Zinc-dependent fluorescent emission of Zinquin (485-490 nm) dissolved in 50 mM Tris-HCl buffer and 0.1 M NaCl, pH 7.4 [43], was recorded in a Perkin Elmer LS 50 spectrofluorimeter, with excitation set at 365–370 nm, at 25°C. Quenching of zinc-dependent Zinquin fluorescence by flavonoids was monitored 5 min after addition of different amounts of the flavonoids to the solution containing 10 μM Zinquin and 1 μM zinc, or 25 μM Zinquin and 100 μM zinc.

2.3. Cell cultures, treatments and cytotoxicity assays

HepG2 cells (ATCC code HB-8065) were grown in Dulbecco's modified Eagle medium (DMEM; BioWittaker) supplemented with 10% fetal bovine serum (BioWittaker), 2 mM glutamine and 1% nonessential amino acids. This medium contains 4.9±0.2 μM zinc as determined by flame atomic absorption spectrometry (FAAS). Cells were incubated at 37°C in a humidified, 5% CO_2-enriched atmosphere and routinely splitted at a 1:5 ratio upon reaching 80% confluence. For treatments, cultures at 80% confluence were trypsinized and resuspended at a density of $0.5 \cdot 10^6$ cells/ml, and 1-ml aliquots were seeded per well in 12-well plates (Orange Scientific). Twenty-four hours later, medium was replaced with 1 ml of fresh medium containing the different test substances or vehicle (final 0.2% ethanol). Cytotoxicity of GSPE, EGCG and $ZnCl_2$ was assessed by measuring lactate dehydrogenase (LDH) leakage in cells treated 24 h with different concentrations of the test substances as previously described [12].

2.4. Measurements of intracellular total and labile zinc

To quantify total intracellular zinc, cells were thoroughly washed with PBS and lysated with 0.01 M NaOH and 0.01% sodium dodecyl sulfate. Aliquots of the cell lysates were used to quantify zinc by FAAS as previously described [44], using a Hitachi Z-8200 Polarized Zeeman AA Spectrophotometer, and protein content, by the Bradford method. To measure changes in intracellular labile zinc, cells were washed with PBS, incubated 30 min at 37°C in 25 μM Zinquin ethyl ester in PBS, washed again and finally suspended in PBS; protein content and Zinquin fluorescence were determined in cell aliquots. Background fluorescence of Zinquin-unloaded cells was substracted from readings to derive Zinquin-dependent fluorescence as described [22].

2.5. Gene expression analysis

Total RNA was isolated from HepG2 cells using NucleoSpin RNA 2 kit (Macherie-Naegel, Germany). To quantify relative mRNA levels of specific genes in different RNA samples, cDNAs were generated from total RNAs using TaqMan Reverse Transcription Reagents, and quantitative reverse transcriptase-polymerase chain reactions (RT-PCRs) were performed using the TaqMan PCR Core Reagent Kit, specific TaqMan Assay-on-Demand Probes and the Real-Time 7000 PCR System, all from Applied Biosystems. Cyclophilin peptidylprolyl isomerase A (cyclophilin A) (PPIA) was used as reference gene. For microarray analysis, RNAs were obtained from four independent control or GSPE-treated cells and pooled. Integrity of pooled RNA was assessed with the Agilent 2100 Bioanalyzer and the RNA 6000 LabChipR. For microarray hybridization, Cy3- or Cy5-labeled cRNA was obtained from each RNA pool using the Agilent Low RNA Input Fluorescent Linear Amplification Kit. Fluorescent probes of each labeled cRNA were pooled and hybridized against Agilent Whole Human Genome Microarrays following the Agilent 60-mer Oligo Microarray processing protocol. Fluorescence signals of hybridized microarrays were acquired with the Agilent G2505B scanner and quantified with the Agilent G2567AA Feature Extraction Software. Duplicate hybridizations with a dye-swap labeling were performed for the pair of RNA samples being compared.

2.6. Statistical analysis

For statistical analysis in cytotoxicity assays, zinc and protein quantification, quantitative RT-PCRs and fluorescence measurements, *t* test and one-way analysis of variance (ANOVA) analyses were performed using SPSS software. Except for microarray analysis, all data are the result of at least three independent experiments. Differences were considered significant for $P \leq .05$.

3. Results

3.1. Catechins and procyanidins interact with zinc in solution

In order to test whether catechins and procyanidins may interact with zinc cations in solution, we monitored the modification of the UV-Vis absorption spectrum of GSPE and EGCG in the presence of $ZnCl_2$. Based on the previously described 2:1 stoichiometry in flavonoid-Fe(II) complexes [38], the concentration of GSPE and EGCG was set at 10 μM, and that of Zn(II) was 5 μM (Fig. 1A). GSPE and EGCG rapidly changed their spectral properties upon addition of $ZnCl_2$ to the solution, implying that they complex with zinc cations when these are free in solution. Subsequently, we tested the ability of GSPE; (+)-catechin (C); (−)-epicatechin; (−)-epicatechin gallate; EGCG; dimeric procyanidins B1, B2, B3 and B4 and the procyanidin trimmer C1 to quench the zinc-dependent fluorescence of Zinquin, a zinc-specific fluorescent chelator widely employed to measure concentrations of labile (free plus loosely bound) zinc within cells and in biological fluids [22,43,45]. GSPE and the

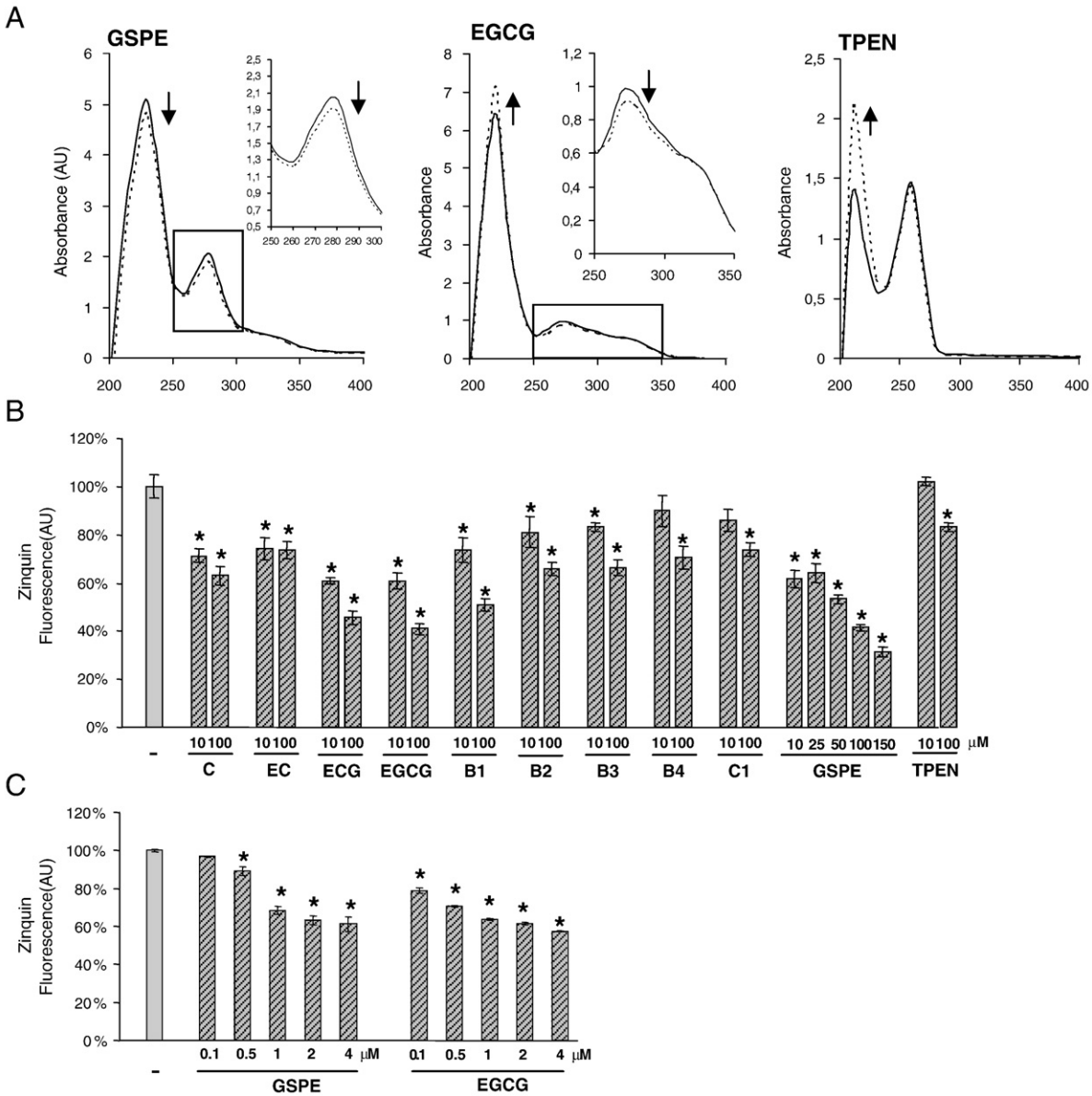

Fig. 1. Interaction of catechins and procyanidins with zinc cations in solution. (A) UV-Vis absorption spectra of GSPE and EGCG coexisted in solution with Zn(II). Continuous lines represent the UV-Vis spectra of 10 μM GSPE, EGCG or the zinc chelator TPEN dissolved in PBS, recorded 5 min after the substances have been added to the buffer. Dashed lines represent the corresponding spectra when the substances were dissolved in PBS containing 5 μM $ZnCl_2$. AU, arbitrary units. (B) Quenching of zinc-dependent fluorescence of Zinquin by catechins and procyanidins in solution. Fluorescent emission of zinc-Zinquin complexes formed in 10 mM Tris-HCl buffer, pH 7.4, 25°C, at 25 μM Zinquin ethyl ester and 100 μM $ZnCl_2$ (B1) or 10 μM Zinquin ethyl ester and 1 μM $ZnCl_2$ (B2) was measured before (control values, 100 arbitrary units of fluorescence) and 5 min after addition of different catechins and procyanidins at the indicated micromolar concentrations. Asterisks indicate significant difference ($P<.05$) versus control sample (−) using independent-samples *t* test.

individual catechins and procyanidins quenched zinc-dependent Zinquin fluorescence rapidly and dose-dependently, with higher efficacy than the zinc chelator TPEN, at flavonoid to Zinquin ratios of 10:25 in the presence of 100 μM zinc (Fig. 1B) and even at 0.1:10 flavonoid to Zinquin ratio in the presence of 1 μM zinc, for both GSPE and EGCG (Fig. 1C). These results imply that these flavonoids bind zinc cations in solution with enough affinity to cause its displacement from Zinquin-zinc complexes.

3.2. GSPE regulates the expression of MT and zinc transporter genes in HepG2 cells in a time- and dose-dependent manner

Zinc chelators are known to affect the expression of genes involved in the cellular uptake, storage and extrusion of zinc cations [43,46,47]. In order to asses whether the ability of catechins and procyanidins to complex with zinc may affect cellular zinc homeostasis, we monitored changes in the expression profile of genes involved in zinc transport and storage in HepG2 cells grown 12 h in standard culture medium (5 μM zinc) supplemented with 150 mg/L GSPE, using oligonucleotide microarray hybridization. Expression of MT genes and zinc transporters of the ZnT and ZIP families were profoundly affected by GSPE (Table 1). mRNA levels of all MT genes expressed in HepG2 were down-regulated by GSPE. Among the zinc transporters of the ZnT family (efflux of zinc from the cytoplasm), only ZnT1 and ZnT10 (extrusion of zinc out of the cell) were clearly down-regulated, whereas the expression of ZnT5 and ZnT7 (uptake of zinc into the Golgi network) were up-regulated. Regarding the ZIP family of zinc transporters (import of zinc into the cytoplasm), mRNA

Table 1
Effect of GSPE on mRNA levels of genes involved in zinc homeostasis in HepG2 cells

Genebank ID	Gene symbol/s; name	Mean fold change GSPE vs. control	
		Microarray	RT-PCR
Metallothioneins			
NM_005946	MT1A	0.54	0.34
NM_005947	MT1B	0.61	0.26
NM_175617	MT1E	0.45	0.48
NM_005949	MT1F	0.64	
NM_005950	MT1G	0.69	0.34
NM_005951	MT1H	0.74	
NM_175622	MT1J	0.65	
NM_176870	MT1K	0.46	
NR_001447	MT1L	0.77	
NM_005952	MT1X	0.54	0.26
NM_005953	MT2A	0.70	0.64
SLC30A family of zinc transporters (cytoplasmic zinc export)			
NM_021194	SLC30A1/ZnT1	0.46	0.68
NM_001004434	SLC30A2/ZnT2	-	
NM_003459	SLC30A3/ZnT3	1.23	
NM_013309	SLC30A4/ZnT4	-	
NM_022902	SLC30A5/ZnT5	1.80	
NM_017964	SLC30A6/ZnT6	2.09	
NM_133496	SLC30A7/ZnT7	4.19	
NM_173851	SLC30A8/ZnT8	-	
NM_006345	SLC30A9/ZnT9	0.93	
NM_018713	SLC30A10/ZnT10	0.51	
SLC39A family of zinc transporters (cytoplasmic zinc import)			
NM_014437	SLC39A1/ZIP1	1.77	2.25
NM_014579	SLC39A2/ZIP2	-	
NM_144564	SLC39A3/ZIP3	0.51	
NM_130849	SLC39A4/ZIP4	1.45	1.60
NM_173596	SLC39A5/ZIP5	0.61	
NM_012319	SLC39A6/ZIP6	2.04	
NM_006979	SLC39A7/ZIP7	0.65	
NM_022154	SLC39A8/ZIP8	-	
NM_018375	SLC39A9/ZIP9	1.57	
NM_020342	SLC39A10/ZIP10	4.97	
NM_139177	SLC39A11/ZIP11	0.51	
NM_152725	SLC39A12/ZIP12	-	
NM_152264	SLC39A13/ZIP13	1.80	
NM_015359	SLC39A14/ZIP14	1.10	0.90
Glutathione biosynthesis			
NM_001498	GCLC; glutamate-cysteine ligase, catalytic subunit	3.73	3.35
NM_002061	Glutamate-cysteine ligase, modifier subunit	2.73	
Plasma zinc carriers			
NM_000477	ALB; albumin	2.62	
NM_000014	A2M; alpha-2-macroglobulin	1.34	
Transcription factors			
NM_005955	MTF1; metal-regulatory transcription factor 1	0.98	
Antioxidant enzymes			
NM_000454	SOD1; Cu/Zn-SOD; superoxide dismutase 1, soluble	0.89	
NM_000636	SOD2; Mn-SOD; superoxide dismutase 2, mitochondrial	7.07	
NM_001752	CAT; catalase	0.67	

HepG2 cells cultivated in standard culture medium (5 μM zinc) were incubated 12 h with 150 mg/L GSPE. Control cells where given only vehicle (final 0.1% ethanol). Total RNA from each group of cells (4 samples per group) were pooled and processed to obtain microarray hybridization data. Mean fold change refers to the mRNA levels of each gene in GSPE-treated cells relative to that in untreated cells, and are the mean of two independent hybridizations with dye-swap labeling of RNA samples.
–, signal is similar to background fluorescence. MFC in bold characters were obtained by quantitative RT-PCR performed with non-pooled RNA samples.

levels of ZIP1, ZIP4, ZIP6, ZIP10 and ZIP13 (located in the plasma membrane) became elevated upon GSPE treatment, whereas ZIP3, ZIP5, ZIP7 (which extrude zinc form the Golgi apparatus) and ZIP11 (unknown location) were down-regulated. GCLC and glutamate-cysteine ligase, modifier subunit, which encode the catalytic and regulatory subunit, respectively, of the rate-limiting enzyme in glutathione biosynthesis, were strongly up-regulated by GSPE treatment. Also, albumin and alpha-2-macrogobulin, the main carriers of zinc in plasma, became up-regulated by GSPE. Therefore, the expression of the main genes involved in zinc homeostasis is modulated by catechins and/or procyanidins present in GSPE.

Next, in order to confirm microarray data and asses time and dose dependency of the response of zinc-related genes to GSPE, we cultured HepG2 cells in the presence of 15, 75 or 150 mg/L GSPE, and monitored changes in gene expression of six MT genes and four plasma membrane zinc transporters at various times, using quantitative RT-PCR. The results show (Fig. 2A) that all six MT genes tested were progressively down-regulated by 150 mg/L GSPE with respect to control cells, reaching relative minimal values between 9 and 12 h after addition of GSPE. Only mRNA levels of MT2A returned to control expression after 24 h of treatment. Time-dependent expression of ZnT1 closely paralleled that of MT2A, suggesting a common regulatory mechanism for the down-regulation by GSPE, whereas the levels of ZIP1 and ZIP4 mRNAs, which where up-regulated by GSPE, progressed with an inverse tendency to that of MT2A and ZnT1. The expression of ZIP14 resulted unaffected by GSPE, and mRNA levels of GCLC increased steadily in GSPE-treated cells. Dose dependency was shown for changes in the expression of MT1A, MT1X, MT2A, ZnT1, ZIP1, ZIP4 and GCLC in HepG2 cells treated 12 h with 15, 75 or 150 mg/L GSPE (Fig. 2B).

3.3. GSPE diminishes the accumulation of zinc in HepG2 cells cultured in basal zinc conditions

The modification elicited by GSPE in the expression of MTs, ZnT1, ZIP1 and Zip4, as well as ZnT5 and ZnT7, in HepG2 cells is remarkably similar to that described in other cell lines grown in conditions of reduced zinc availability, i.e., treated with the zinc chelator TPEN [43,46–48] or in zinc-depleted medium [49] that results in a reduction of total intracellular zinc. To address this point, we measured the total zinc content of control and GSPE-treated cells at different times (Fig. 2C). In control cells, total intracellular zinc increased steadily from 3.355 (S.D. 0.125) to 5.511 (S.D. 0.373) nmol of zinc per milligram of protein 15 h after the addition of fresh medium to the cells. In contrast, in cells treated with 150 mg/L GSPE, total intracellular zinc remained roughly constant from the time of GSPE administration until the end of cultivation. Therefore, GSPE hinders the normal entrance of zinc cations into HepG2 cells.

3.4. GSPE counteracts the effect of excess zinc on expression of MT and zinc transporters, on intracellular zinc accumulation and on cell viability

Next, we tested the capacity of GSPE to inhibit MT and ZnT1 expression and intracellular zinc accumulation in conditions of zinc overload. Zinc in excessive amounts is toxic to all types of cells and induction of MT and ZnT1 expression, mediated by the zinc-sensing transcription factor MTF1, followed by chelation of zinc by the newly synthesized apo-thionein and extrusion of zinc out of the cell, is recognized as a defense mechanism against zinc toxicity [33,34]. As shown in Fig. 3A, addition of 100 μM zinc to the culture media, a concentration that is not toxic to HepG2 cells (Fig. 3C), resulted in a 25-fold induction of MT1X and a 13-fold induction of MT2A, 12 h after addition of zinc. Concomitantly, expression of ZnT1 was enhanced twofold, whereas ZIP4 was down-regulated to 60% of control value. Expression of ZIP1 and ZIP14 was unaffected by addition of zinc. Total intracellular zinc was 3.2-fold higher in zinc-treated cells than in

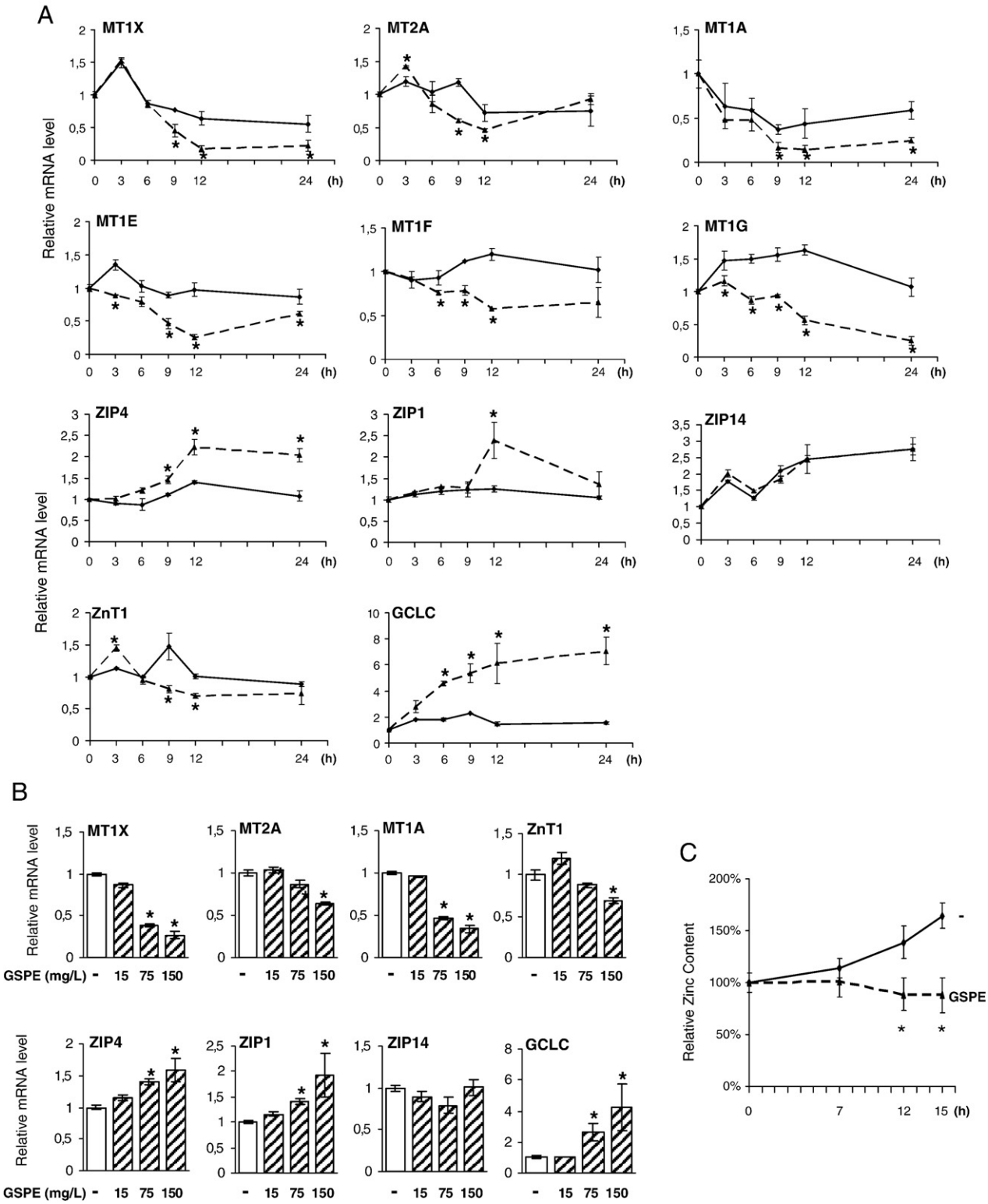

Fig. 2. GSPE modulates the expression of zinc-homeostasis genes and total zinc content in HepG2 cells cultured in standard medium. (A) Kinetics of mRNA levels of MT, GCLC and plasma membrane zinc transporters. Black lines represent the evolution of the mRNA level of each gene in control cells, relative to the mRNA level of that gene in untreated cells just at the beginning of the treatment with 150 mg/L GSPE (dashed lines), determined by quantitative RT-PCR. (B) Dose-dependency of the effects of GSPE on expression of zinc-homeostasis genes. HepG2 cells were treated with either vehicle (−), 15, 75 or 150 mg/L GSPE for 12 h, and relative mRNA levels of the indicated genes determined by RT-PCR. (C) Progression of total intracellular zinc content. The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. The zinc to protein ratio in the cells just before the beginning of the treatment with 150 mg/L GSPE was 3.355 nanomoles of zinc per mg of protein (0.125 S.D.), and is assigned the arbitrary value of 100. Asterisks indicates significant difference ($P<.05$) in treated cells versus control cells at the same time point using independent-samples t test.

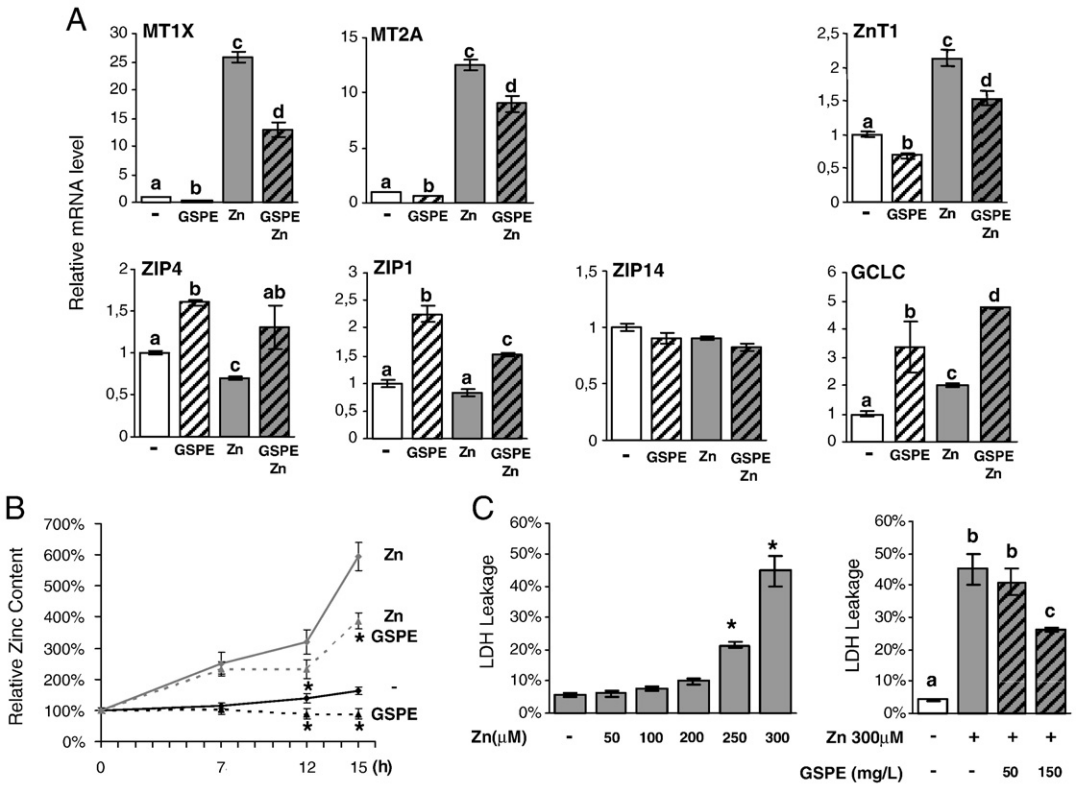

Fig. 3. Effects of GSPE on zinc homeostasis in HepG2 cells treated with excess zinc. (A) mRNA levels of MT and zinc transporter genes upon 12-h incubation with 150 mg/L GSPE (GSPE), 100 μM $ZnCl_2$ (Zn) or both (GSPE Zn), relative to the levels in untreated cells (−). (B) Evolution of total intracellular zinc, normalized per protein content, in the same cell cultures. (C) Dose-dependent toxicity of zinc on HepG2 cells and prevention of toxicity of 300 μM zinc in the culture medium by the addition of GSPE, assessed by the LDH test after 24 h of treatment. Numbers indicate the percentage of total LDH activity of a cell culture which is present in the culture medium. Asterisks indicate significant difference ($P<.05$) from control cells. Different letters denote different values after one-way ANOVA test ($P<.05$).

control cells. Addition of GSPE (150 mg/L) to the media significantly hindered the zinc-induced up-regulation of MT1X and MT2A, whereas it was unable to impede induction of ZnT1. In the presence of excess zinc, GSPE still up-regulated ZIP1 and ZIP4 as in control cells, but to a lesser extent. Therefore, GSPE counteracts the effects of additional zinc on MTs, ZnT1 and ZIP4 expression, whereas zinc counteracts the effects of GSPE on MT, ZnT1, ZIP1 and ZIP4. Concomitantly, zinc-stimulated accumulation of intracellular zinc was severely inhibited by GSPE (Fig. 3B). These results suggested that GSPE should be able to counteract toxics effects of zinc. To test this, we performed LDH tests to evaluate the effect of increasing amounts of zinc on cell viability (Fig. 3C). Zinc concentrations above 200 μM resulted in significant LDH leakage, reaching 80% upon 24 h incubation of cells with 300 μM zinc. Addition of 150 mg/L GSPE to the cells prevented the noxious effects of 300 μM zinc by 50% after the LDH test. Thus, even if inhibiting MT expression, nontoxic amounts of GSPE are able to counteract the toxic effects of excessive zinc, concomitantly reducing intracellular accumulation of zinc, strongly suggesting that GSPE renders zinc in the culture medium unavailable and, hence, nontoxic, to cells.

3.5. GSPE hinders the induction of MTs and ZIP14 and the intracellular zinc accumulation elicited by IL-6

Subsequently, we tested whether GSPE may affect de expression of MT and zinc transporters and intracellular zinc accumulation when cells are stimulated to take up zinc by stimuli different from zinc itself. In murine hepatocytes, IL-6 induces the expression of MT and Zip14 signaling through the STAT (signal transducer and activator of transcription) pathway; subsequently, uptake of zinc mediated by Zip14 further increases the activation of MT expression via MTF1; this response has been associated to the hypozincemia that accompanies the acute-phase response in infectious and inflammatory processes [27,50]. In HepG2 cells, IL-6 induced a marked increase in the expression of MT1X, MT2A, ZIP14 and ZIP1 (Fig. 4A), concomitantly increasing intracellular zinc levels to 150% of untreated cells (Fig. 4B). ZnT1 and ZIP4 mRNA levels were not affected by IL-6 at this time. When GSPE was added to HepG2 cells together with IL-6, induction of MT genes was completely blocked (Fig. 4A), and so was intracellular zinc accumulation (Fig. 4B). IL-6 induction of ZIP14 was unaffected by GSPE, consistent with direct regulation of ZIP14 by IL-6 in a way independent of zinc availability. As in standard zinc conditions, ZnT1 was down-regulated, and Zip4 was up-regulated by GSPE independently of IL-6. Effect of GSPE on ZIP1 was not evident at this time. Therefore, inhibition of zinc uptake and MT expression by GSPE is relevant in standard and zinc-overload conditions but also in the cellular response to extracellular signals, such as those that mediate the acute-phase response in inflammatory processes.

3.6. GSPE elevates intracellular labile zinc in HepG2 cells

We next monitored the effect of GSPE treatment on cytoplasmic levels of labile zinc, measured as zinc-dependent Zinquin fluorescence. As shown in Fig. 5A, GSPE increased the cytoplasmic levels of Zinquin-detectable zinc in HepG2 cells in all conditions tested. After 12 h of GSPE treatment, Zinquin fluorescence was enhanced by 12-fold compared with cells cultured in standard conditions. Addition of 100 μM zinc resulted in a 7-fold increase in Zinquin fluorescence, and coincubation with 100 μM zinc and 150 mg/L GSPE produced a further increase of up to 120-fold compared to untreated cells. IL-6 treatment

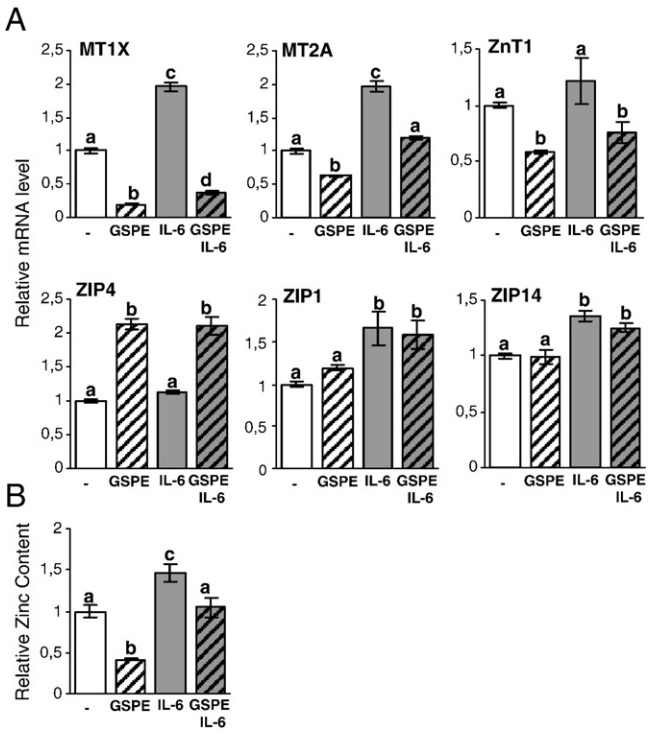

Fig. 4. Effects of GSPE on zinc homeostasis in HepG2 cells treated with IL-6. (A) mRNA levels of MT and zinc transporter genes after 15-h incubation with 150 mg/L GSPE, 1000 U/ml IL-6, or both treatments simultaneously, relative to control cells. Dexamethasone 1 μM was added to all cultures. (B) Total intracellular zinc content, normalized per protein content, in the same cell cultures. Different letters denote different values after one-way ANOVA test (P<.05).

of HepG2 cells cultured in standard zinc conditions enhanced the labile pool of zinc by 1.4-fold, and addition of GSPE further increased it up to 18-fold. Microscopic observations of Zinquin-loaded HepG2 cells were done to visualize this effect of GSPE on cytoplasmic labile zinc in HepG2 cells grown in basal zinc concentrations as well as in conditions of zinc excess (Fig. 5B). Thus, in spite of hindering intracellular zinc accumulation, GSPE produced an increase in the cytoplasmic pool of labile zinc in HepG2 cells, in standard growth conditions as well as when cells are stimulated to accumulate zinc.

3.7. EGCG reproduces the major effects of GSPE on zinc homeostasis

GSPE consist of a mixture of dozens of different catechins and procyanidins which, expectedly, will display different affinities for zinc. For the convenience of characterizing an individual flavonoid, we tested the effect of EGCG on the different parameters of zinc homeostasis previously tested with GSPE in HepG2 cells. The results show that EGCG behaved essentially as GSPE in all conditions studied. Thus, in basal zinc conditions (Fig. 6A), EGCG repressed MT1X, MT2A and ZnT1 expression while enhancing that of ZIP1 and ZIP4; concomitantly, EGCG hindered the normal augmentation of total intracellular zinc but enhanced the pool of cytoplasmic labile zinc. In conditions of zinc excess (Fig. 6B), EGCG reverted the zinc-induced up-regulation of MT and ZnT1 genes and the zinc-induced down-regulation of ZIP1 and ZIP4, concomitantly slowing down the associated accumulation of intracellular zinc. Likewise, EGCG dose-dependently reverted zinc-induced toxicity. EGCG also impeded the induction of MT1X, MT2A and ZIP14 by IL-6 and hampered the associated accumulation of total intracellular zinc (Fig. 6C). Finally, like GSPE, EGCG produced a large increase in the intracellular, Zinquin-detectable, labile pool of zinc in all conditions tested (Fig. 6A-C).

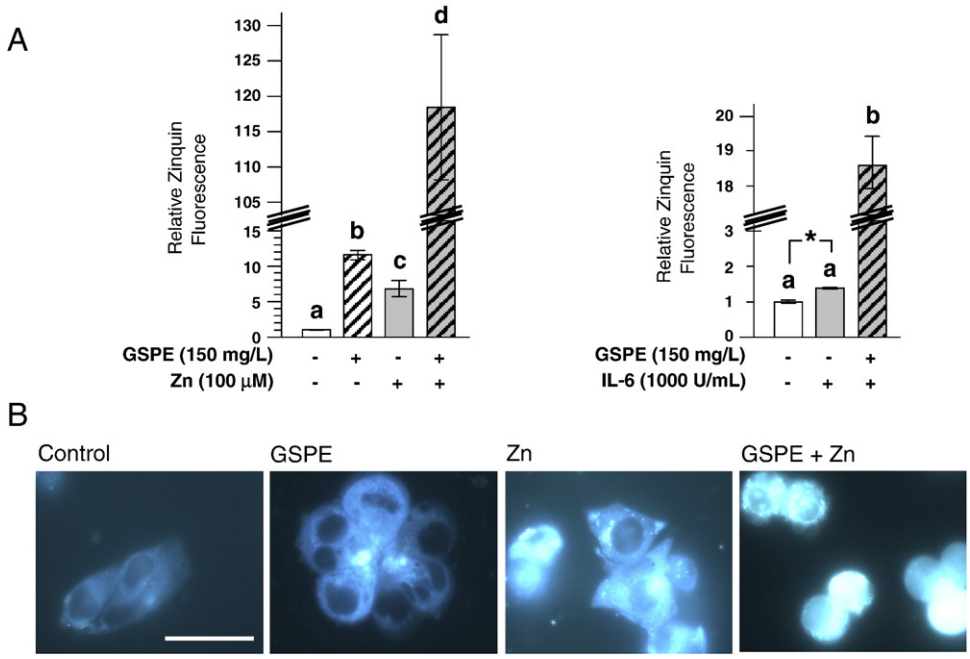

Fig. 5. Effect of GSPE on cytoplasmic labile zinc in HepG2 cells. (A) Fluorescence (arbitrary units) of Zinquin-loaded cells was normalized to protein content in each sample, and untreated cells were assigned the arbitrary value of 1. Treatments with GSPE, zinc and IL-6 were as in Figs. 3 and 4, respectively. (B) Images of Zinquin-loaded HepG2 cells. To visualize intracellular Zinquin fluorescence, cells were seeded in wells containing glass coverslips, treated as above, and loaded with Zinquin ethyl ester as described [22]; images at 1000× magnification were acquired with a Leica DM 4000B microscope using UV light illumination (λ_{ex}=340–380 nm) and a blue emission filer (λ_{em}≥425nm). Bar indicates 50 μm.

4. Discussion

We have shown here that GSPE, a mixture of catechins and procyanidins, as well as individual catechins and procyanidins, display an affinity for zinc cations in solution high enough to make them dissociate from the zinc-specific chelator Zinquin, even at very low concentrations and at molar ratios of 0.1 μM flavonoid to 10 μM Zinquin and 1 μM zinc. This strongly suggests that these flavonoids will

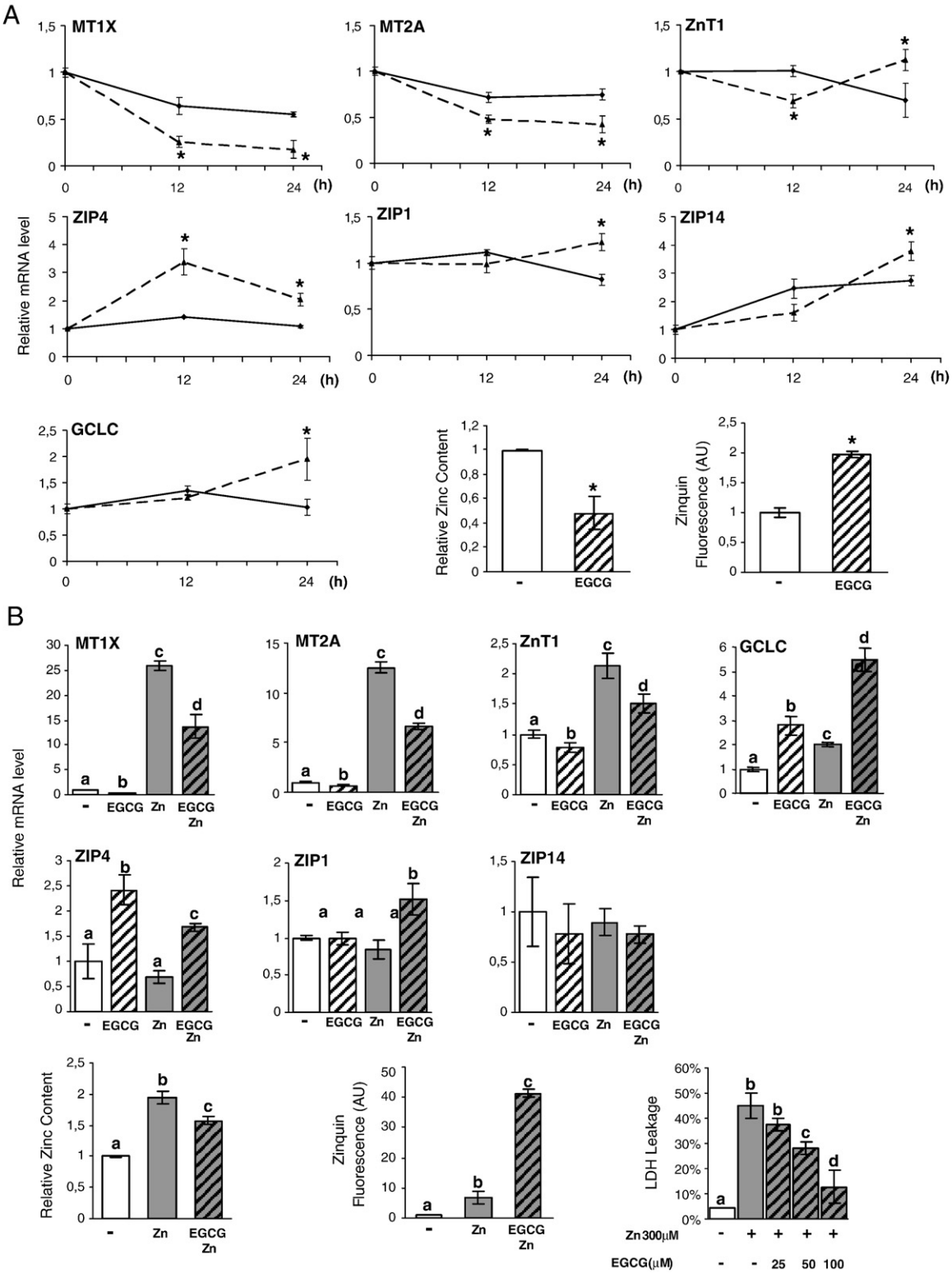

Fig. 6. Effects of EGCG on zinc homeostasis in HepG2 cells. Effect of EGCG on gene expression, total intracellular zinc and cytoplasmic labile zinc in HepG2 cells cultured in standard conditions (A), in cell cultures treated 12 h with additional (100 μM) zinc (B) and in cells treated 15 h with IL-6 (C). Asterisks indicates significant difference from control value with $P \leq .05$. Different letters denote different values after one way ANOVA test ($P < .05$).

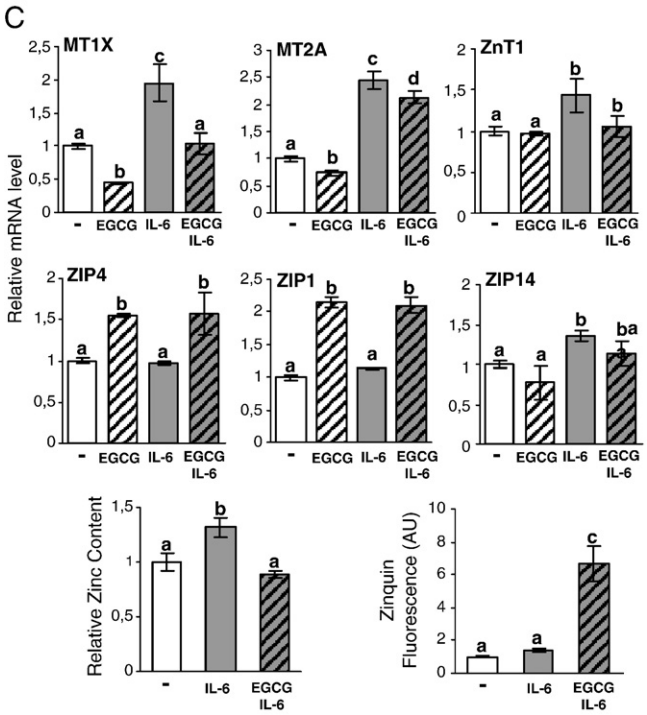

Fig. 6. (*continued*)

be able to displace zinc loosely bound to proteins in cell culture media and, once internalized, within the cell cytoplasm, as does Zinquin itself [22,45]. In the cytosol of most cell types, free zinc concentrations are in the picomolar to nanomolar range, and those of Zinquin-detectable, labile zinc are in the range of 1–10 μM, whereas the concentration total intracellular zinc, which includes the fixed or structural pool of zinc within metalloproteins, is several hundred (usually 100–300) μM [22,24–26]. In human plasma, Zinquin-chelatable zinc is roughly 8 μM, constitutes about >90% of albumin-bound zinc and is considered in route for uptake by the different tissues [45]. Thus, chelation of labile zinc by catechins and procyanidins of GSPE is likely to be relevant in vivo since, following oral administration of 1 g GSPE per kilogram of body weight to rats, parent unmetabolized catechins and dimeric and trimeric procyanidins reach plasma concentrations of 2, 2 and 8 μM, respectively, 2 h after GSPE administration. Glucuronidated catechin and epicatechin reach plasma concentrations of 23.9 and 20.5 μM, respectively, in these animals [51]. These metabolites are expected to retain the ability to bind zinc cations as they still have a strong bidentate metal binding site (two adjacent hydroxyl groups) in the B ring and a hydroxyl group in the A ring (see Ref. [9]). Tetrameric and pentameric procyanidins are also absorbed in rats and may reach plasma concentrations of 7 μg/ml each one [52]. Likewise, EGCG may reach concentrations of up to 1.5 μM in human plasma after oral intake of a single 800 mg dose of EGCG [53]. In this context, it is noteworthy that 5 h after GSPE administration, MT mRNA levels are drastically reduced to 30% of control values in the liver of rats fed a single oral dose of 250 mg/GSPE per kilogram of body weight [54], a dose that effectively lowers postprandial triglyceridemia [4].

We have shown that accumulation of total intracellular zinc in HepG2 cells was hindered upon addition of 150 mg/L GSPE or 100 μM EGCG to the standard culture medium (5 μM zinc) as well as when cells were stimulated to accumulate zinc by the addition of 100 μM zinc to the medium or by treatment with IL-6 in basal zinc conditions. In addition, toxic effects of 300 μM zinc in the medium were prevented by non-toxic amounts of GSPE and EGCG. Reversion of excess zinc toxicity occurred despite the fact that zinc-induced expression of MT, a major defense against zinc toxicity [32,33], was hindered by GSPE and EGCG. Taken together, these results supports the concept that EGCG and catechins/procyanidins of GSPE form complexes with zinc in the culture medium, thereby preventing the entrance of zinc into the cells through plasma membrane zinc transporters. In this regard, it is known that metal complexation may cause the concatenation of monomeric flavonoids with the metal cations, yielding the flavonoids less prone to partition into membranes [9]. It may be inferred that the metal cations that link together the flavonoid subunits will also be unable to enter the cell through transmembrane metal ion transporters. Actually, catechins and procyanidins have been shown to inhibit the entrance of iron in human intestinal Caco-2 cells [8], and long-term consumption of high doses of GSPE may lower serum iron levels in rats [55]. Also consistent with this mechanism, it has also been reported that the ratio of zinc to EGCG, as well as its way of administration, determines the rate of EGCG uptake by PC-3 cells: when EGCG is complexed with zinc by precipitation of saturated solutions of the flavonoid and the metal, the entrance of EGCG (80 μM) in the cells is less than half that when the same amount of only EGCG is provided to the cells; on the contrary, when EGCG is supplied in solution with and equimolar amount of zinc, intracellular accumulation of EGCG is enhanced more than twofold [18]. The authors inferred that the variable structure and stoichiometry of EGCG-zinc complexes determines different permeability of the flavonoids to the cell membrane.

The changes elicited by GSPE and EGCG in the expression of MT, ZnT and ZIP genes in different conditions are also consistent with a diminished availability of extracellular zinc for HepG2 cells, as they closely resemble the changes elicited by zinc deprivation described in different cell models. Thus, GSPE and EGCG exerted an effect contrary to those of zinc supplementation on the expression of MT1X, MT2A and zinc efflux transporter ZnT1, which where simultaneously up-regulated by addition of zinc and repressed by GSPE in basal and zinc overload conditions. The expression of zinc importers ZIP1 and ZIP4 was also simultaneously repressed by GSPE and EGCG in conditions of basal and increased zinc concentrations in the medium. Similarly, in cultured mouse fibroblasts, mRNA levels of MT and ZnT1 are elevated upon addition of zinc to the medium, a response mediated by MTF1 and are down-regulated when cells are cultured in zinc depleted medium [49]. Also, Caco-2 cells respond to supplementation of zinc elevating the expression of MT and ZnT1, whereas treatment with the zinc chelator TPEN results in enhanced expression of ZIP4 and down-regulation of ZnT1 and MT1 [47]. In HeLa cells, supplementation of zinc enhances expression of ZnT1, whereas administration of TPEN up-regulates ZnT7 and ZnT5 [43], as shown here in HepG2 cells treated with GSPE in basal zinc conditions. These expression changes have been interpreted as a homeostatic response of the cells to compensate for reduced zinc availability and directed to maintain adequate zinc levels in the cytoplasm and within the Golgi network [43,47,48]. Strikingly, incubation of HepG2 cells with GSPE and EGCG, in spite of diminishing total intracellular zinc concentrations relative to control cells in conditions of basal (5 μM) and excess (100 μM) zinc concentrations, as well as in cells treated with IL-6, always produced an augmentation of Zinquin-detectable labile pool of intracellular zinc. To our knowledge, only two reports have described the effect of dietary polyphenolic compounds on labile zinc. The glycone isoflavone genistin, applied at 100 μM, enhances the proapoptotic effects of zinc in HepG2 cells and up-regulate the expression of MT and ZnT1 concomitantly increasing the labile zinc pool detectable by FluoZin-3 [56]. The effect of genistin on total zinc content and on zinc toxicity was not reported. The stilbene resveratrol, at physiological concentrations (10 μM), efficiently chelates zinc in solution and, when applied to normal human prostate epithelial cells cultured in 16 or 32 μM Zn(II), arrests cell growth and enhances Zinquin-detectable zinc, while not affecting total zinc nor MT expression [57]. The authors

propose that the increment of labile zinc elicited by resveratrol is due to the cellular uptake of resveratrol-zinc complexes, followed by the intracellular dissociation of the complexes [57]. Considering these published data and those presented here, it appears that flavonoids may enhance intracellular labile zinc levels independently of their effect on MT and ZnT1 expression, which would rather correlate with total intracellular zinc content. A plausible explanation for the increment of cytoplasmic labile zinc is that membrane-permeable flavonoid-zinc complexes are always formed in addition to membrane-impermeable flavonoid-zinc concatenamers. Those zinc atoms that enter the cells complexed with the flavonoids, that would be, in this way, acting as ionophores, will add to the pool of labile zinc and latter, once the flavonoid has been metabolized, to the pool of free zinc. This increment in free/labile zinc should not suffice to produce significant increments of total zinc content, as seen in HepG2 cells treated with EGCG and GSPE. An alternative, though not excluding, possibility is that, once internalized, the flavonoids mobilize zinc from intracellular zinc stores such as MT, endoplasmic reticulum, vesicles or zincosomes, as has been shown for Zinquin itself, that is able to retrieve some of the MT-bound zinc cations [22]. Mobilization of copper ions from the nuclear compartment has been shown in lymphocytes treated with EGCG [58]. In any case, the increment in labile zinc elicited by GSPE and EGCG should not be immediately available or sufficient to stimulate the transcriptional activity of MTF1 on the promoters of MT and ZnT1, given the observed down-regulation of MT and ZnT1 mRNA levels in GSPE-treated cells. It could, however, suffice to enhance the transcription of GCLC, also under the control of MTF1. MTF1 discerns between different metal response element (MRE) in response to different zinc load [34]. Alternatively, up-regulation of GCLC by GSPE and EGCG may be independent of MTF1. EGCG is known to induce the expression of GCLC and manganese superoxide dismutase by activating the transcriptional activity of the redox-sensitive nuclear factor erythroid 2 p45-related factor which recognizes the antioxidant response element in the promoter of these antioxidant genes [11]. A known mechanism for repression of MT expression in hepatocarcinoma cell lines, including HepG2, lays on the activation of phosphatidylinositol 3-kinase (PI3K) and the serine/threonine kinase Akt, also called protein kinase B (PKB), that in turn-inactivates glycogen synthase kinase 3; this kinase activates MT expression by phosphorylating the CCAAT/enhancer binding protein α that interacts with MTF1 and/or basal transcription factors in the promoter of MT genes [59]. On the other hand, increased levels of cytoplasmic free/labile zinc, brought about by zinc ionophores such as pyrithione, enhance phosphorylation and activation of PI3K and Akt/PKB in many cell types, including hepatic cells [60,61]. By combining these two mechanisms, it appears that increments in labile zinc may occur simultaneously with repression of MT expression, a situation that should help the cell to keep intracellular zinc available for essential functions when extracellular zinc becomes temporally unavailable, and is consistent with a homeostatic response of cells to low zinc availability. In this context, it is remarkable that GSPE has been recently shown to elicit phosphorylation of PI3K and Akt/PKB, a mechanism that might underlie the known insulinomimetic effect of procyanidins [5].

Whatever the underlying mechanism, the increment of labile, Zinquin-detectable cytoplasmic zinc described here for GSPE and EGCG may be relevant to explain the bioactivity of these flavonoids. Zinquin-detectable zinc is considered a measure of the pool of this metal that can be exchanged between proteins and is endowed with a regulatory and signaling function, i.e., it may modulate the activity of components of signal transduction pathways and key enzymes of multiple metabolic pathways [22–26]. Thus, increments within the nanomolar range in the levels of free cytoplasmic zinc inhibit the activity of cyclic nucleotide phosphodiesterases (PDE) and protein tyrosine phosphatases (PTP), and activate mitogen-activated protein kinase (MAPK), protein kinase C and calcium-calmodulin activated protein kinase-2, leading to changes in the phosphorylation state of numerous downstream cell signaling and transcription factors. For instance, inhibition of PTP 1B by zinc (IC_{50} 17 nM) results in enhanced net phosphorylation of the insulin receptor and activation of downstream signalling cascades pathways such as MAPK, PI3K and Akt/PKB and is thought to contribute to the insulin-mimetic effects of zinc and zinc complexes [60–62]. Likewise, inhibition of PDE by zinc (IC_{50} 20 nM) enhances cGMP and cAMP signalling [23]. Free zinc may also directly activate transcription factors, as is the case for MTF1, or inhibit its transcriptional activity, as for nuclear factor-kappa B (NF-κB) [23]. It is remarkable that many actions described for diverse flavonoids on signaling pathways overlap with those described for fluctuations of free and labile zinc. As mentioned above, GSPE has been recently shown to elicit phosphorylation of PI3K and Akt/PKB independently of insulin, a mechanism that might underlie the known insulinomimetic effect of procyanidins [5]. Likewise, many flavonoids, including EGCG and procyanidins inhibit the transcriptional activity of NF-κB [6,7,10]; similarly, many flavonoids have been shown to inhibit PDE activity, consequently elevating cytoplasmic cAMP levels [15].

In summary, we have presented evidence supporting that interaction of catechins and procyanidins with zinc cations modulate zinc absorption and metabolism, resulting in increased levels of cytoplasmic labile zinc. We forward the hypothesis that modulation of labile zinc by these flavonoids may be a relevant mechanism by which flavonoids affect multiple metabolic and cell signalling pathways that respond to intracellular fluctuations of labile zinc. Further research is necessary to asses the mechanisms by which these flavonoids enhance cytoplasmic labile zinc and the consequences of this enhancement on modulation of zinc signaling and metabolic pathways, as well as to asses the relevance that zinc chelation by individual catechin/procyanidins and their metabolites may have in vivo.

Acknowledgments

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