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ORIGINAL ARTICLE

Evaluation of antioxidant properties of major dietary polyphenols and their protective effect on 3T3-L1 preadipocytes and red blood cells exposed to oxidative stress

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Abstract

Obesity has been associated with a marked risk of metabolic diseases and requires therapeutic strategies. Changes in redox status with increased oxidative stress in adipose tissue have been linked with obesity-related disorders. Thus, the biological effect of antioxidants such as polyphenols is of high interest. We aimed to measure antioxidant capacities of 28 polyphenols representative of main dietary phenolic acids, flavonoids, stilbenes and curcuminoids. Then, 14 molecules were selected for the evaluation of their effect on 3T3-L1 preadipocytes and human red blood cells exposed to oxidative stress. Analysis of reducing and free radical-scavenging capacities of compounds revealed antioxidant properties related to their structure, with higher activities for flavonoids such as quercetin and epicatechin. Their effects on preadipocytes' viability also depended on their structure, dose and time of exposure. Interestingly, most of the compounds exhibited a protective effect on preadipocytes exposed to oxidative stress, by reversing H_2O_2-induced anti-proliferative action and reactive oxygen species production. Polyphenols also exerted an anti-inflammatory effect on preadipocytes exposed to H_2O_2 by reducing IL-6 secretion. Importantly, such antioxidant and anti-inflammatory effects were observed in co-exposition (polyphenol and prooxidant during 24 h) or pretreatment (polyphenol during 24 h, then prooxidant for 24 h) conditions. Moreover, compounds protected erythrocytes from AAPH radical-induced lysis. Finally, these results led to demonstrate that antioxidant and anti-inflammatory properties of polyphenols may depend on structure, dose, time of exposure and cell conditioning with oxidative stress. Such findings should be considered for a better understanding of polyphenols' benefits in strategies aiming to prevent obesity-related diseases.

Keywords: obesity, oxidative stress, inflammation, dietary antioxidants, polyphenols

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Introduction

According to the World Health Organization, overweight and obesity, which are defined as abnormal or excessive fat accumulation that may impair health, are the fifth leading risk for global deaths and worldwide obesity has nearly doubled since 1980. Excess of adiposity is closely linked to the pathogenesis of major health issues such as type 2 diabetes [1,2], hypertension [3,4], metabolic syndrome [5,6] and cardiovascular diseases [7,8]. High-fat and carbohydrate diet is the major cause of an elevation of energy storage in the white adipose tissue, inducing an increase in preadipocytes' proliferation, differentiation into adipocytes as well as the size of mature adipocytes [9,10]. The processes of adipose hypertrophia and hyperplasia are associated with intracellular abnormalities of adipocyte function, particularly endoplasmic reticulum and mitochondrial stress. Abnormal lipid accumulation causes enhanced endoplasmic reticulum activity, which ultimately can alter the capacity of the reticulum to properly fold nascent proteins [11]. This reticulum stress associated with the presence of free fatty acids is responsible for oxidative stress in the mitochondria, which is defined as imbalance in levels of reactive oxygen species (ROS) versus the reducing substances that protect against damaging free radicals and peroxides. Whereas low levels of ROS are required to support natural cellular function and regulate intracellular signaling, excess of ROS production can cause damage to all cellular macromolecules, including nucleic acids, lipids and proteins [12,13]. High ROS production also leads to a dysregulation of adipokine secretory patterns: adipocytes and cells from the stroma vascular fraction including preadipocytes, macrophages and adipose stem cells contribute to the production of pro-inflammatory cytokines in obesity, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and the reduction in anti-inflammatory molecules, such as adiponectin [14]. This pro-inflammatory state as well as excessive ROS production participates in insulin resistance by several mechanisms, such as the reduction of insulin sensitivity by influencing the insulin receptor phosphorylation state

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[15]. Ultimately, insulin resistance induces ectopic fat deposits and may contribute to explain obesity-related cardiovascular diseases [7]. As oxidative stress appears as a critical event between obesity and related chronic diseases such as type 2 diabetes, the biological effect of natural micronutrients that may increase the antioxidant capacity of the body is of high interest.

Plant polyphenols constitute the most abundant antioxidants present in the human diet. They are mainly provided by fruits, vegetables, cereals, as well as some beverages such as tea, coffee, cocoa and juice. More than 5,000 molecules have been identified and classified into major chemical families, namely flavonoids, phenolic acids, stilbenes, lignans and curcuminoids [16]. Among phenolic acids, gallic, vanillic and chlorogenic acids have been intensively studied for their biological effects on adipocytes [17]. Concerning flavonoids, quercetin, epicatechin and epigallocatechin gallate have been reported to have strong antioxidant capacities [18,19]. Several biological properties have been attributed to dietary polyphenols, such as antioxidant and anti-inflammatory effects as well as actions on cell cycle [20,21]. Few years ago, much attention has been paid to the understanding of polyphenol bioavailability as their metabolic fate constitutes a major factor which governs their ability to reach target tissues and to exert biological effects. We demonstrated the crucial role of the gut microflora in the degradation of polyphenols that are not easily absorbed through the proximal part of the gut barrier [22–24]. Such a microbial metabolism leads to the production of specific metabolites including both 3,4-dihydroxyphenylacetic and hippuric acids which may contribute to explain the biological properties attributed to some native polyphenols not easily absorbed [25].

Numerous studies have been reported about the impact of polyphenols on adipose tissue, mainly on preadipocyte differentiation and adipokine secretion from mature adipocytes [20,26]. However, their effects on preadipocytes have been poorly evaluated despite preadipocytes constitute major cells governing adipose tissue development. Another cell type particularly exposed to oxidative stress due to its function in the organism, particularly its high tension of oxygen, is red blood cells [27]. Our objective was to compare for the first time the antioxidant properties of 26 major dietary polyphenols from flavonoids, phenolic acids, stilbenes, curcuminoids families and 2 of their circulating microbial metabolites (Figure 1), as well as their biological effect on 3T3-L1 preadipocytes and human red blood cells exposed to oxidative stress. Importantly, polyphenols' action on preadipocytes was evaluated in both co-exposition (polyphenol and prooxidant during 24 h) and pretreatment (polyphenol during 24 h, then prooxidant for 24 h) conditions.

Materials and methods

Reagents

All polyphenols and microbial metabolites were purchased from Sigma-Aldrich.

Antioxidant-capacity assays

The reducing capacity of compounds was determined by Folin–Ciocalteu test and the free radical-scavenging activity was assessed by 2,2-diphenyl-2-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) methods.

For Folin–Ciocalteu assay [28], 50 µM sample, Folin–Ciocalteu's phenol reagent (Sigma-Aldrich) and sodium carbonate were added in a 96-well microplate and incubated at 54°C for 5 min and then at 4°C for 5 min. The absorbance was measured at 765 nm (FLUOstar Optima, Bmg Labtech). A calibration curve was prepared using a standard solution of gallic acid (0–300 µM). The total reducing capacity of the compound tested was expressed as mole gallic acid equivalent (GAE)/mole compound.

The free radical-scavenging activity on DPPH radical was measured according to the method described by Yang et al. with slight modifications [29]. Briefly, 0.25 mM DPPH (Sigma-Aldrich) diluted in methanol was incubated with 50 µM sample. After 25 min at 25°C, the optical density (OD) was read at 517 nm. The percentage of free radical-quenching activity of DPPH was determined according to the following formula:

$$\text{Antioxidant capacity}\,(\%) = \left(\frac{(\text{OD control} - \text{OD sample})}{\text{OD control}}\right) \times 100$$

The free radical-scavenging activity was also evaluated by ORAC test which is based on the decrease in fluorescein fluorescence in the presence of the chemical oxidant 2,2′-azobis[2-methyl-propionamidin] dihydrochloride (AAPH) (Sigma-Aldrich), according to the method of Huang et al. with some modifications [30]. Briefly, 0.5 µM sample and 150 µL of 8.38×10^{-5} mM fluorescein were placed in a 96-well black microplate, and after 15 min at 37°C, 25 µL of 153 mM AAPH radical was added to each well. Then, the fluorescence was measured for 1 h 40 min at a wavelength of excitation and emission of 485 nm and 530 nm, respectively (Infinite 200, Tecan). The results were based on the Area Under the Curve of fluorescence decay over time and compared with Trolox calibration curve ranging from 6.25 to 75 µM. The free radical-scavenging activity of the compound tested was expressed as mole Trolox equivalent/mole compound.

Cell culture

The mouse embryo 3T3-L1 murine preadipocyte cell line was obtained from the American Type Culture Collections (ATCC, USA). The culture medium included Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% heat-inactivated fetal bovine serum (FBS), L-glutamin (5 mM), streptomycin (2 µg/mL) and penicillin (50 µU/mL). The cell culture condition was in a humidified 5% CO_2 incubator at 37°C.

Cell viability measurement

The 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to the

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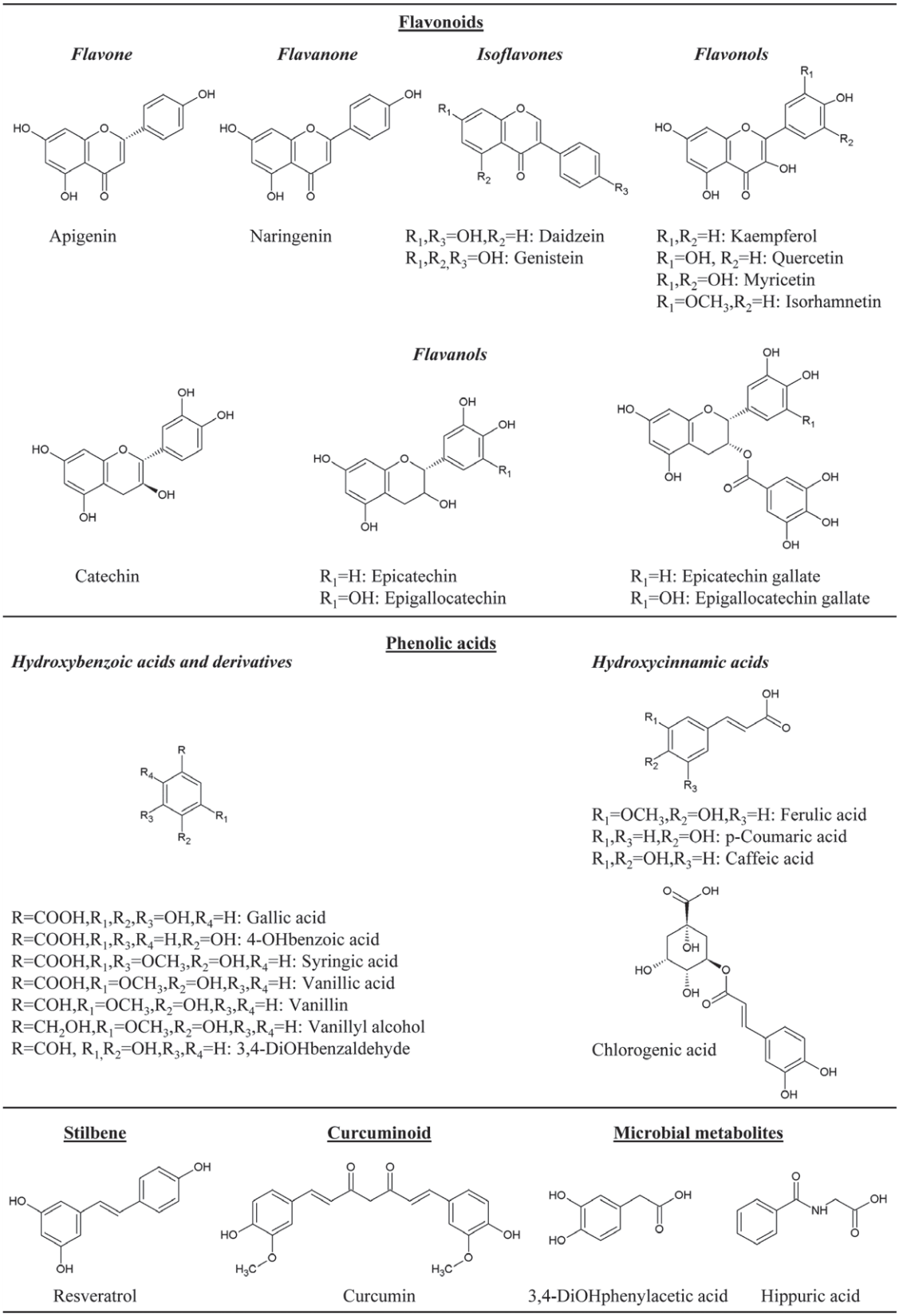

Figure 1. Chemical structures of 26 polyphenols and 2 microbial metabolites selected for the study.

method of Mosmann [31]. To assess polyphenols' dose-dependent effect, cells cultured in 96-well microplates (5×10^3 cells/well) were exposed to compounds (25, 50, 100 µM) during 24 or 48 h. For co-exposition experiments, cells were treated with 500 µM H_2O_2 and 50 µM polyphenols for 24 h. For pretreatment experiments, cells were pretreated with 50 µM polyphenols during 24 h, then antioxidants were washed out and cells were exposed to H_2O_2 during 24 h. Five hours before the end of each experiment, 20 µL of sterile filtered MTT solution (5 mg/mL)

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(Sigma-Aldrich) in phosphate-buffered saline (PBS, pH 7.4) was added to each well and the plate was incubated at 37°C. Then, the unreacted dye was removed by centrifugation, the insoluble formazan crystals were dissolved in 200 µL/well dimethyl sulfoxide and the absorbance was measured at 560 nm (FLUOstar Optima, Bmg Labtech).

Concerning cell counting, preadipocytes were treated with 50 µM polyphenols for 24 h and then counted with a hemocytometer using the trypan blue exclusion method [32].

To assess cell death, LDH leakage measurement was performed using a commercial kit (Sigma-Aldrich) [33]. Cells were treated with 50 µM polyphenols for 24 h and then analyzed for LDH leakage into the culture medium. Then, the percentage of LDH leakage was calculated, as compared with untreated cells (0% of LDH leakage).

Determination of ROS production

The level of intracellular ROS was assessed by measuring the oxidation of DCFH-DA. DCFH-DA diffuses through the cell membrane and is deacetylated by cellular esterases to the non-fluorescent DCFH. Intracellular ROS are able to oxidize DCFH to the fluorescent 2,7-dichlorofluorescein (DCF), whose intensity of fluorescence is directly proportional to the levels of intracellular ROS. Briefly, cells were cultured in 96-well black microplates (5×10^3 cells/well) for 24 h. Then, the medium was removed and replaced by PBS containing 10 µM of DCFH-DA (Sigma-Aldrich) and cells were kept in a humidified atmosphere (5% CO_2, 37°C) for 45 min. Next, cells were co-exposed to 500 µM H_2O_2 and 50 µM polyphenols for 1 h. Fluorescence was measured at an excitation wavelength of 492 nm and an emission wavelength of 520 nm (FLUOstar Optima, Bmg Labtech).

Measurement of IL-6 and TNF-α secretion

Culture media collected from 3T3-L1 cells co-exposed to polyphenols and H_2O_2 during 24 h, or pretreated with polyphenols for 24 h and then treated with H_2O_2 for 24 h, were analyzed using Mouse IL-6 or TNF-α ELISA kits (eBioscience). Absolute values were normalized according to total cellular-protein content assessed by Bradford test [34].

Hemolysis assay

The capacity of the compounds to inhibit free-radical-induced hemolysis was assessed according to Prost with modifications [35]. Red blood cells were obtained from ten subjects aged 20–40 years, according to the authorization from the Committee for the Protection of Persons and Guidelines from La Réunion Hospital. The cells were washed and suspended in 0.15 M NaCl, at pH 7 and incubated with 61 mM AAPH and 50 µM of the compounds for 18 h at 37°C. Cell lysis was determined by measuring the absorbance at 450 nm at 10-min intervals (FLUOstar Optima, Bmg Labtech).

Statistical analysis

Data were expressed as means ± SEM. All assays were performed in three independent experiments, with triplicate for each of them. Statistical analysis was achieved using Prism software. Significant differences ($p < 0.05$) between the means were determined by analysis of variance procedures followed by a multiple comparison test (Dunnet's or Dunn's test).

Results

Antioxidant properties of polyphenols evaluated by Folin–Ciocalteu, DPPH and ORAC assays

Antioxidants can reduce free radicals by two mechanisms which are hydrogen atom transfer (HAT) and electron transfer (ET). In this study, two tests measuring the antioxidant activity were implemented, namely DPPH (ET) and ORAC (HAT) assays. Folin–Ciocalteu method measuring the reducing capacity of polyphenols was also performed.

The results obtained through Folin–Ciocalteu assay showed that some flavonoids including epigallocatechin gallate, epicatechin gallate, quercetin and epicatechin as well as one member of phenolic acid family, namely 3,4-dihydroxybenzaldehyde, had the strongest reducing capacity which was 2–3 fold greater than that of gallic acid, with values ranging from 3.91 to 2.09 mole GAE/mole compound (Table I). A second group of molecules showing high reducing capacity was distinguished, with values ranging from 1.99 to 1.28 mole GAE/mole compound. This group included the microbial metabolite 3,4-dihydroxyphenylacetic acid, catechin, caffeic acid, isorhamnetin, vanillyl alcohol, myricetin, resveratrol, 4-hydroxybenzoic acid, naringenin, genistein, vanillin and apigenin. Epigallocatechin, ferulic and chlorogenic acids exerted a moderate reducing activity close to that of gallic acid with values reaching 1.18, 1.13 and 1.06 mole GAE/mole compound, respectively, while the other polyphenols (p-coumaric, vanillic, syringic and hippuric acids, daidzein, kaempferol and curcumin) exhibited a lower reducing capacity from 1.02 to 0.16 mole GAE/mole compound.

Concerning DPPH assay, gallic acid, epigallocatechin gallate, 3-4-dihydroxybenzaldehyde, epicatechin gallate, quercetin and myricetin exerted the strongest scavenging activity, with values ranging from 69.84% to 57.04%. Chlorogenic acid, caffeic acid, epicatechin, syringic acid, catechin, 3,4-dihydroxyphenylacetic acid, isorhamnetin, epigallocatechin, vanillyl alcohol, ferulic acid, kaempferol, curcumin and resveratrol also presented a high scavenging activity with values yielding 54.33–29.98%. The other polyphenols showed moderate activities with values less than 17.72%, as found for p-coumaric acid.

Additionally, ORAC method showed that catechin, epicatechin, genistein and epicatechin gallate which are compounds from flavonoids family exhibited a strong antiradicalar activity with respective values of 34.70, 33.26,

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Table I. Antioxidant activities of 26 polyphenols and 2 microbial metabolites assessed by Folin–Ciocalteu, DPPH and ORAC methods.

	Reducing capacity	Radical-scavenging activity	
Phenolic compounds	mole GAE/mole compound	DPPH reduced (%)	mole Trolox equivalent/ mole compound
Epigallocatechin gallate	3.91 ± 0.03	63.85 ± 0.78	14.86 ± 0.35
Epicatechin gallate	3.45 ± 0.01	61.63 ± 0.73	25.58 ± 0.63
Quercetin	2.54 ± 0.03	60.15 ± 0.73	15.32 ± 0.58
Epicatechin	2.54 ± 0.03	51.45 ± 0.78	33.26 ± 0.53
3,4-DiOHbenzaldehyde	2.09 ± 0.02	63.54 ± 0.66	16.28 ± 0.74
3,4-DiOHphenylacetic acid	1.99 ± 0.04	47.32 ± 0.70	19.44 ± 0.91
Catechin	1.88 ± 0.02	47.36 ± 0.73	34.70 ± 0.71
Caffeic acid	1.87 ± 0.02	53.43 ± 1.08	11.78 ± 0.48
Isorhamnetin	1.74 ± 0.03	45.71 ± 0.77	20.50 ± 0.45
Vanillyl alcohol	1.69 ± 0.02	44.35 ± 0.95	16.56 ± 0.29
Myricetin	1.68 ± 0.02	57.04 ± 0.81	8.27 ± 0.64
Resveratrol	1.65 ± 0.02	29.98 ± 0.74	23.21 ± 0.74
4-OHbenzoic acid	1.56 ± 0.02	2.73 ± 0.48	19.61 ± 0.42
Naringenin	1.43 ± 0.02	6.14 ± 0.78	18.84 ± 0.74
Genistein	1.37 ± 0.01	3.52 ± 0.63	26.80 ± 0.77
Vanillin	1.32 ± 0.03	10.42 ± 0.73	5.25 ± 0.58
Apigenin	1.28 ± 0.02	3.47 ± 0.25	9.38 ± 0.23
Epigallocatechin	1.18 ± 0.02	45.65 ± 0.78	4.33 ± 0.63
Ferulic acid	1.13 ± 0.03	39.01 ± 0.52	9.97 ± 0.46
Chlorogenic acid	1.06 ± 0.03	54.28 ± 1.21	7.36 ± 0.51
Gallic acid	1.03 ± 0.02	69.84 ± 0.91	3.68 ± 0.27
p-Coumaric acid	1.02 ± 0.03	17.72 ± 1.29	11.32 ± 0.20
Vanillic acid	0.99 ± 0.02	9.15 ± 0.59	ND
Daidzein	0.98 ± 0.02	3.53 ± 0.52	12.89 ± 0.27
Syringic acid	0.73 ± 0.02	48.28 ± 0.45	2.01 ± 0.12
Kaempferol	0.72 ± 0.01	38.37 ± 0.55	1.81 ± 0.20
Curcumin	0.37 ± 0.02	35.13 ± 0.49	3.24 ± 0.18
Hippuric acid	0.16 ± 0.01	1.93 ± 0.62	ND

Each compound was tested at 50 μM for Folin–Ciocalteu and DPPH tests and at 0.5 μM for ORAC assay. Results are means ± SEM of three independent experiments with triplicate for each compound (ND: not detected).

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26.80 and 25.58 mole Trolox equivalent/mole compound. Except gallic acid, curcumin, syringic acid and kaempferol which had an activity less than 4.00 mole Trolox equivalent/mole compound and vanillic and hippuric acids without any detected activity, other phenolic compounds exerted a high antioxidant capacity (23.21–4.33 mole Trolox equivalent/mole compound). Regarding vanillic and hippuric acids, ORAC data were in agreement with results described above showing that both these compounds were characterized by weak antioxidant effects.

Finally, results obtained from Folin–Ciocalteu, DPPH and ORAC assays highlighted three groups of compounds: i) a first group of molecules exhibiting a strong antioxidant activity, ii) a second one exerting a high antioxidant activity, iii) the last one presenting a moderate antioxidant activity. Thus, most of the molecules tested were able to act as antioxidants; and more particularly, epicatechin gallate whose strong antioxidant properties were detected through the three assays used. Epigallocatechin gallate and quercetin also exerted strong reducing and radical-scavenging activities through Folin–Ciocalteu and DPPH assays. Moreover, epicatechin was characterized by a high antioxidant capacity by using Folin–Ciocalteu and ORAC assays. It is noteworthy that all these polyphenols are members of the flavonoids family.

Effect of polyphenols on preadipocytes and red blood cells exposed to oxidative stress

Among the 28 molecules tested, 13 polyphenols and 1 of their microbial metabolites were selected in order to investigate further their protective effects on 3T3-L1 preadipocytes and human red blood cells exposed to oxidative stress. Compounds were chosen based on their antioxidant properties measured above, as well as according to literature data on their abundance in the diet and bioavailability extent [22,36]. The selected polyphenols were gallic, caffeic, chlorogenic and ferulic acids, 3,4-dihydroxybenzaldehyde, quercetin, genistein, daidzein, resveratrol, epicatechin, epicatechin gallate, naringenin and curcumin. We also selected 3,4-dihydroxyphenylacetic acid, as it was the microbial metabolite exhibiting the highest antioxidant capacity in the present study. Even if in vivo the bioavailability of polyphenols differs greatly from one polyphenol to another, plasma concentrations are in the range of μM. The concentrations selected in the present study were 25,

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50 and 100 µM. Although these doses are rather high with respect to physiological concentrations, such pharmacological concentrations are broadly used in similar studies in the literature [17,37].

Effect of polyphenols on preadipocytes' viability

Dose-dependent effects of 14 compounds on preadipocytes during 24 or 48 h were determined by MTT assay. This colorimetric assay is based on the mitochondrial activity of metabolizing cells. Tetrazolium salts (MTT) are reduced to a blue colored formazan only by metabolizing cells [31]. After 24 h of treatment, significant changes in mitochondrial activity were observed for polyphenol concentrations at 25, 50 and 100 µM (Figure 2). Depending on the concentration, three groups of phenolic compounds could be distinguished. One group of compounds, including gallic acid, daidzein, resveratrol and curcumin induced a significant decrease yielding 15–20% for doses ranging from 25 to 100 µM. Gallic acid and resveratrol were the most effective compounds with an increasing effect from 25 to 100 µM. Another group of molecules

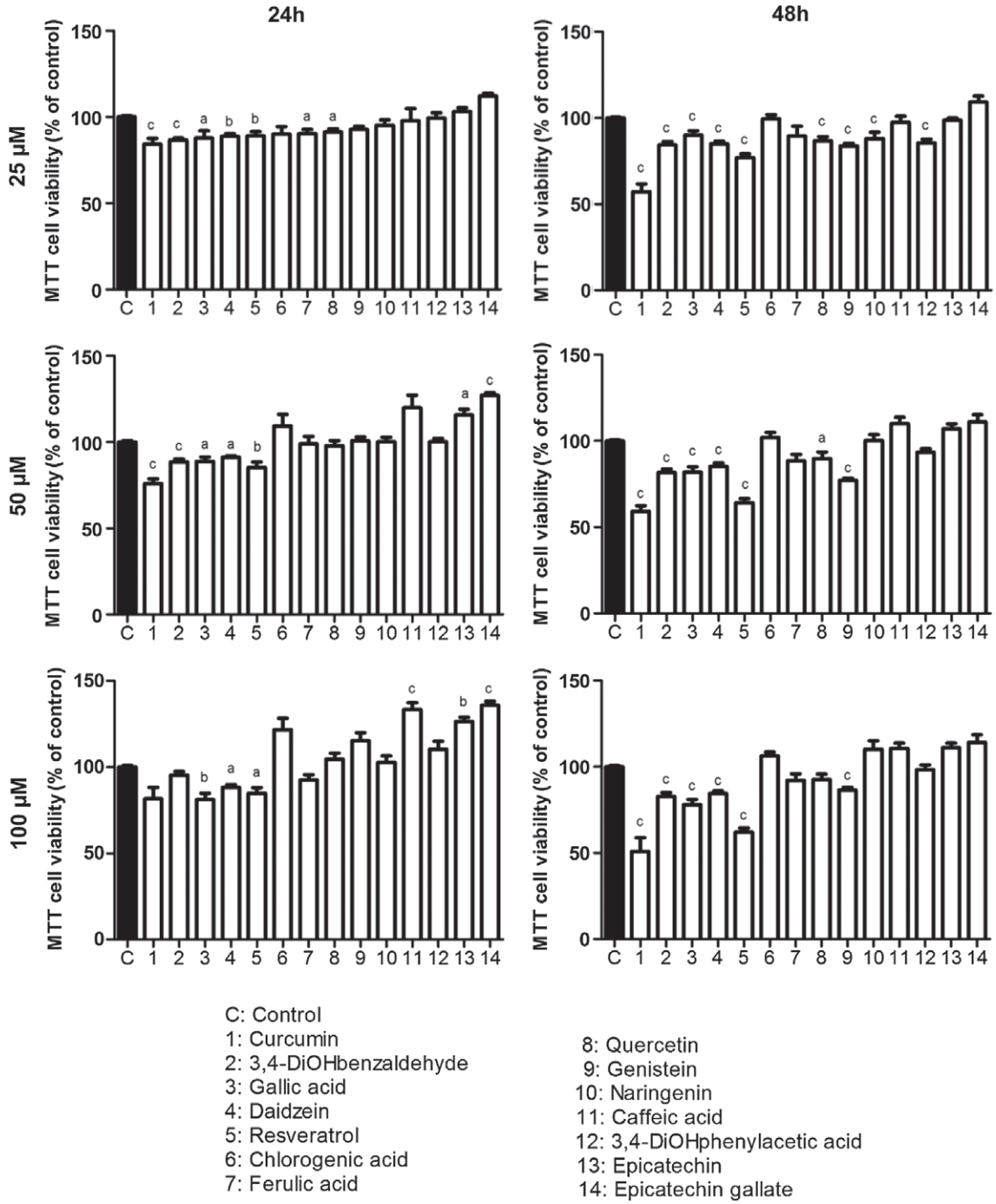

Figure 2. Effect of polyphenols on preadipocytes' mitochondrial metabolic activity determined by MTT assay. Cells were incubated with 25, 50 and 100 µM polyphenols for 24 and 48 h. Reported values are means ± SEM of three independent experiments. p value was calculated using the Dunn's multiple comparison test (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ as compared with control).

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elevated the metabolic activity, such as epicatechin and epicatechin gallate which induced an enhancement in preadipocytes' metabolic activity reaching 115.6% and 127.0% at 50 μM, respectively. These effects were more pronounced at 100 μM. Finally, some molecules such as chlorogenic acid, genistein and naringenin did not exert any statistically significant effect. Figure 2 also indicates that changes in mitochondrial activity induced by most of the inhibitory compounds were more pronounced after 48 h, as compared with 24 h for all concentrations tested. As observed for resveratrol, a dose-dependent effect appeared, with values ranging from 76.9% at 25 μM to 62.1% at 100 μM. Conversely, for molecules which increased metabolic activity after 24 h of exposure, namely epicatechin and epicatechin gallate, the elevation induced was no longer observed after 48 h.

As MTT assay reflected mitochondrial activity of living cells, we wondered if the compounds had an effect on the number of living cells or on cell metabolism itself. For the following experiments, polyphenols' concentration chosen was 50 μM and exposure time was 24 h, as significant changes induced by polyphenols were mainly observed at these conditions.

First, we investigated whether the variation in mitochondrial metabolic activity induced by the compounds was due to their influences on preadipocytes' viability, by using the trypan-blue-exclusion method. As shown in Figure 3, curcumin and 3,4-dihydroxyphenylacetic acid showed the highest reducing effect on cell viability, with cell number values reaching 7.4% and 26.2%, respectively. Quercetin, genistein and resveratrol also exerted a reduction of cell viability, as well as 3,4-dihydroxybenzaldehyde, ferulic, gallic and chlorogenic acids. Comparatively, as observed by using MTT assay, epicatechin raised the number of living preadipocytes until 123%, whereas naringenin, daidzein, caffeic acid and epicatechin gallate did not exert any significant effect. Altogether, these results indicated a link between the effect on preadipocytes measured by MTT assay and by cell counting, for only some compounds, such as 3,4-dihydroxybenzaldehyde, resveratrol and curcumin. In contrast, although epicatechin gallate at 50 μM for 24 h of exposition increased mitochondrial metabolic activity, this compound did not affect preadipocytes' number. Accordingly, the microbial metabolite 3,4-dihydroxyphenylacetic acid decreased cell number, without affecting mitochondrial metabolic activity. These results suggest that epicatechin gallate may modulate mitochondrial metabolism and not cell proliferation, whereas the opposite effect may occur for the microbial metabolite. Thereby, results obtained by MTT assay must be analyzed very carefully and not studied only as data for viability assessment.

In order to investigate whether cell-growth reduction mediated by polyphenols and the microbial metabolite cited above, was linked to cell death, cellular membrane permeability was next assessed by measuring LDH release in cell culture medium. As shown in Figure 4, only curcumin and gallic acid induced a significant LDH release at 50 μM for 24 h whereas other compounds did not induce any cytotoxic effect. Thus, these results revealed three groups of phenolic compounds: i) molecules which did not affect the number of cells, such as epicatechin gallate; ii) molecules which increased cell growth, such as epicatechin and iii) molecules which were able to induce cell death, as observed for curcumin.

Antioxidant effect of polyphenols on preadipocytes exposed to H_2O_2-induced oxidative stress

To investigate the potential protective effects of the 14 selected compounds (50 μM) against H_2O_2-induced

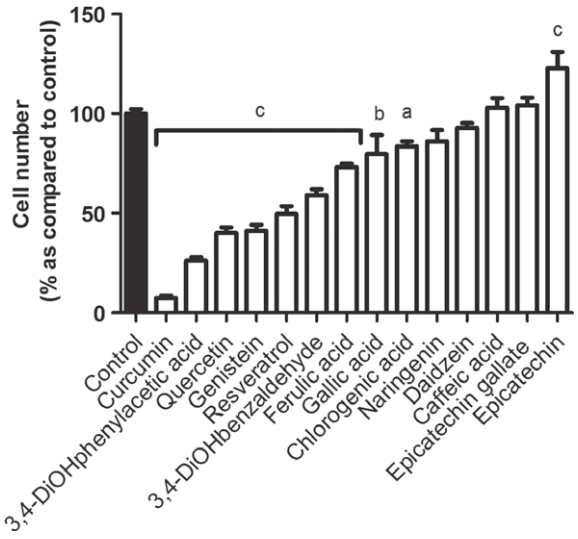

Figure 3. Effect of polyphenols on preadipocytes' growth assessed by cell counting. Cells were treated with 50 μM polyphenols for 24 h and then counted with the trypan blue exclusion method. Reported values are means ± SEM of three independent experiments. p value was calculated using the Dunnet's multiple comparison test (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ as compared with control).

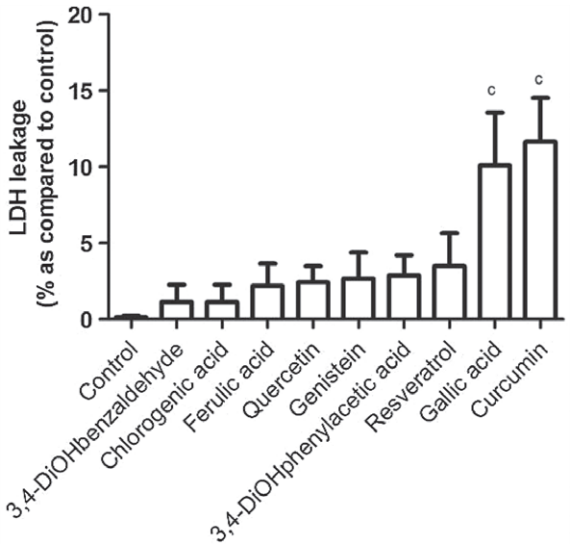

Figure 4. Effect of polyphenols on preadipocytes' death assessed by LDH leakage. Cells were treated with 50 μM polyphenols for 24 h and then analyzed for LDH leakage into the culture medium. Reported values are means ± SEM of three independent experiments. p value was calculated using the Dunnet's multiple comparison test (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ as compared with control).

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oxidative stress, H_2O_2 dose-effect on preadipocytes viability and ROS production was explored. The MTT metabolic activity and the LDH leakage assays performed demonstrated that H_2O_2 exerted an inhibitory dose-dependent effect on cell viability with an induction of cell death for doses more than 200 µM (Figure 5A). Moreover, there appeared a significant increase in ROS production depending on H_2O_2 dose (Figure 5B). This is in accordance with previous data showing that until 200 µM, H_2O_2 treatment induced a transient proliferation arrest in 3T3-L1 preadipocytes [38,39]. Barnouin et al. have also shown that dividing cells exposed to sublethal doses of H_2O_2 undergo detoxification or repair, and reinitiate cell cycle progression [38]. Here, to assess the protective effect of polyphenols, the cytotoxic dose of 500 µM H_2O_2 was selected, as reported for 3T3-L1 adipose cells in previous studies [40]. First, MTT assay showed that H_2O_2 decreased mitochondrial activity (from 100% to 81% as compared with untreated cells, $p < 0.01$), whereas 8 of the 14 molecules tested were able to significantly modulate H_2O_2 effect, in co-exposition condition (Figure 6A). Epicatechin and caffeic acid exhibited the highest protective effect by elevating mitochondrial metabolic activity beyond 116%. This result can be explained by the strong antioxidant activity of both compounds shown on Table I.

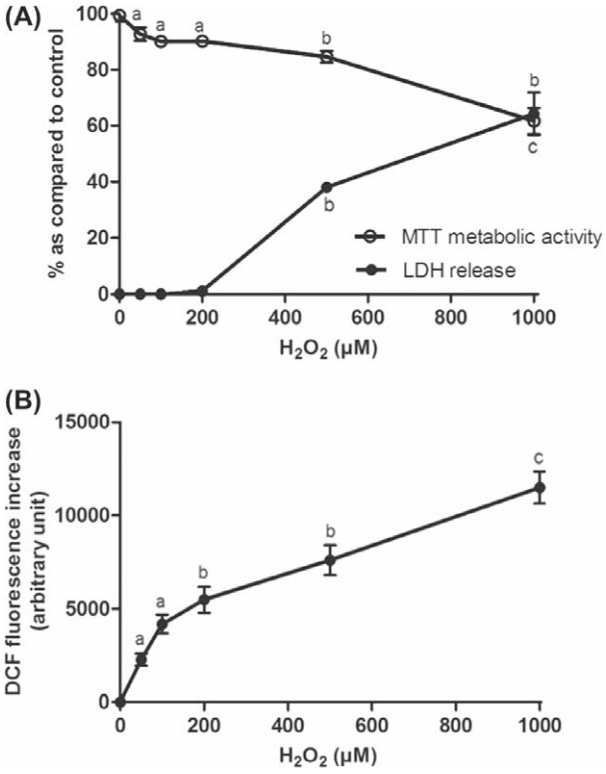

Figure 5. H_2O_2 dose-effect on preadipocytes' viability and ROS production. Cells were exposed to 0–1000 µM H_2O_2 during 24 h. (A) Mitochondrial metabolic activity was determined by MTT assay and LDH leakage was measured in the medium. (B) Cells were exposed to 10 µM of DCFH-DA for 45 min at 37°C and then were treated with H_2O_2 for 1 h. Reported values are means ± SEM of three independent experiments. p value was calculated using the Dunn's multiple comparison test (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ as compared with control).

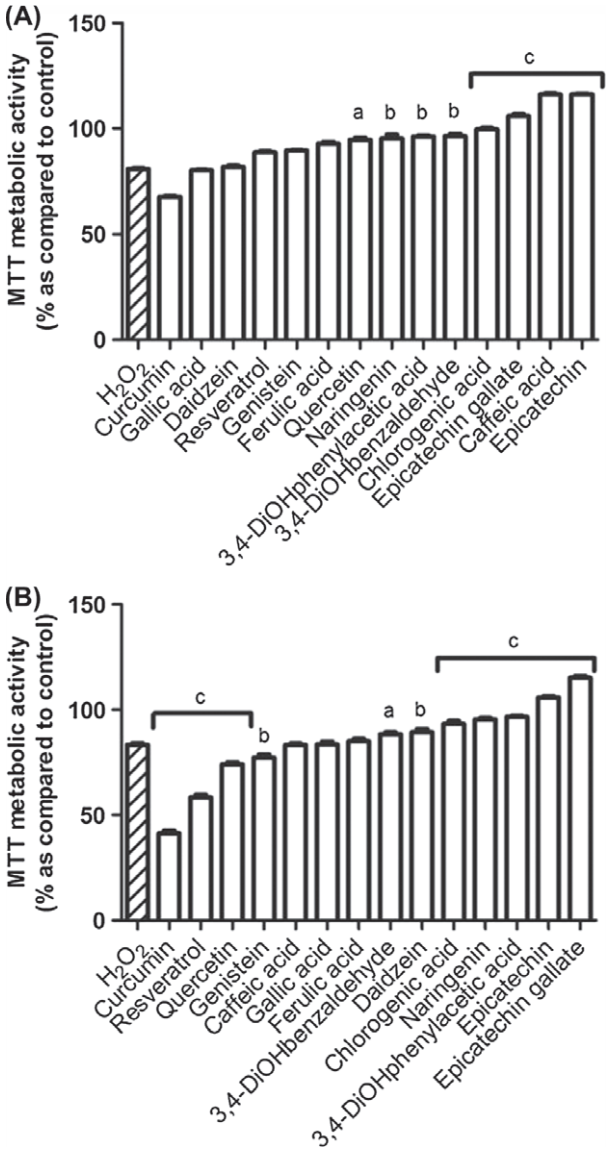

Figure 6. Effect of polyphenols on mitochondrial metabolic activity of preadipocytes exposed to H_2O_2. (A) Cells were co-exposed to 50 µM polyphenols and 500 µM H_2O_2 during 24 h. (B) Cells were pretreated with 50 µM polyphenols for 24 h and then exposed to 500 µM H_2O_2 for 24 h. Reported values are means ± SEM of three independent experiments. p value was calculated using the Dunn's multiple comparison test (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ as compared with H_2O_2).

Epicatechin gallate, chlorogenic acid, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxyphenylacetic acid, naringenin and quercetin also reversed effects of H_2O_2 (105.8–94.7%). Other compounds did not protect themselves against detrimental effect of H_2O_2 but did not intensify it either. Interestingly, most of these protective polyphenols also reversed cytotoxic effect of H_2O_2 when cells were pretreated during 24 h, before being exposed to the prooxidant agent (Figure 6B). The decrease in mitochondrial metabolic activity mediated by curcumin, resveratrol, quercetin and genistein could be explained through their reducing effect on cell viability demonstrated above. Surprisingly, quercetin and caffeic acid which reversed effect of H_2O_2 in co-exposition condition were not able to act as antioxidants in preconditioning. Such results raised the

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question about the impact of compounds on ROS production from preadipocytes exposed to H_2O_2.

When ROS production is not compensated by cellular antioxidant defense system, oxidative stress occurs. In order to investigate the potential protective effect of polyphenols against oxidative stress, we measured ROS production after co-treating preadipocytes with polyphenols (50 µM) and H_2O_2 (500 µM) during 1 h. Whereas all compounds had no effect on ROS basal production as compared with untreated cells (data not shown), H_2O_2 treatment induced a significant increase in ROS generation (from 100% to 129%, $p < 0.001$) (Figure 7). Except the isoflavones genistein and daidzein that were also characterized by a low antioxidant capacity according to DPPH assay, all polyphenols tested significantly reduced ROS production with the highest effects depicted for curcumin, epicatechin and epicatechin gallate.

Anti-inflammatory effect of polyphenols on preadipocytes exposed to H_2O_2-induced oxidative stress

As previously described, adipocytes exposed to oxidative stress are characterized by an impairment in adipokine secretion [14]. Thus, we investigated IL-6 and TNF-α production from preadipocytes exposed to oxidative stress. TNF-α was not detectable in any condition in our study (data not shown). Concerning IL-6, the basal level detected was 22.7 ± 1.0 pg/mg proteins and was not increased by any polyphenol (Figure 8A). Interestingly, some compounds including epicatechin gallate, epicatechin, genistein, naringenin, curcumin and 3,4-dihydroxyphenylacetic acid even reduced IL-6 secretion. When cells were exposed to H_2O_2, IL-6 level increased from 22.7 ± 1.0 to 48.9 ± 2.9 pg/mg proteins ($p < 0.001$) (Figure 8B). In co-exposition condition, all polyphenols significantly reduced this up-regulation of IL-6 production, with the highest effects observed for naringenin, quercetin, the microbial metabolite 3,4-dihydroxyphenylacetic acid, epicatechin gallate and resveratrol (6.8, 7.4, 13.6, 15.2 and 16.3 pg/mg proteins, respectively). When cells were pretreated with polyphenols and then exposed to H_2O_2, all polyphenols also exerted an anti-inflammatory activity, except ferulic acid (Figure 8C). In such pretreatment condition, the most pronounced anti-inflammatory action was observed for curcumin, resveratrol and daidzein, which significantly reduced IL-6 secretion (from 81% to 96%).

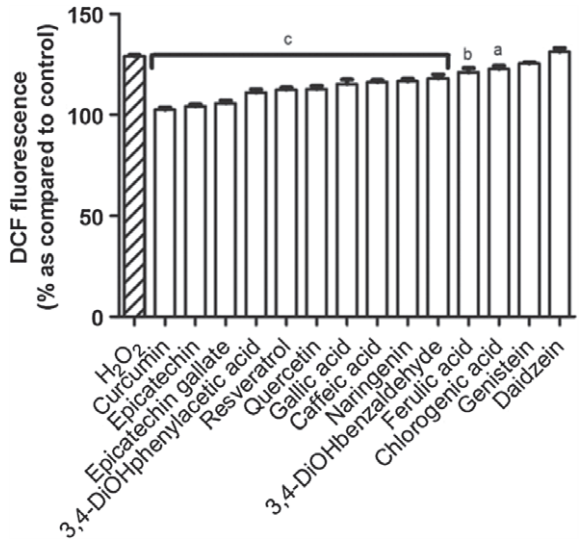

Figure 7. Effect of polyphenols on ROS production from preadipocytes exposed to H_2O_2-induced oxidative stress. Cells were exposed to 10 µM of DCFH-DA for 45 min at 37°C. Then they were co-treated with 50 µM polyphenols and 500 µM H_2O_2 for 1 h. Reported values are means ± SEM of three independent experiments. p value was calculated using the Dunnet's multiple comparison test (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ as compared with H_2O_2).

Protective effect of polyphenols against AAPH radical-induced lysis of human red blood cells

To assess the antioxidant property of phenolic compounds on another cellular type, we explored their effect on human erythrocyte hemolysis induced by AAPH radical. The phenolic compounds themselves did not induce hemolysis (data not shown). As shown in Figure 9, when erythrocytes were incubated with AAPH radical for 18 h, a hemolysis yield of 50% corresponding to the red blood cell half-life was induced at 1 h 55 min. The highest protective effect was observed for quercetin and 3,4-dihydroxybenzaldehyde which delayed hemolysis to 14 h 08 min and 12 h 35 min, respectively. Gallic acid exhibited the lowest inhibitory effect, corresponding nonetheless to a red blood cell half-life which was 4.7 fold higher than the control. Finally, all other compounds also exerted a significant antioxidant action by delaying AAPH radical-induced hemolysis (9 h 09–11 h 20 min).

Discussion

The antioxidant capacity of polyphenols can be mediated through their ability to scavenge free radicals, donate electrons or hydrogen atoms [41]. Such different mechanisms could explain the differences between the three antioxidant-capacity assays obtained for some compounds in the present study. The structure of these compounds is known to determine their antioxidant activity, and is referred to as "structure–activity relationship" [42]. Our results showed that flavonoids had greater reducing and radical-scavenging capacities in ORAC and Folin–Ciocalteu assays than those of the other families, and also a good antioxidant capacity in DPPH test. Indeed, in contrast to phenolic acids and stilbenes which have one or two aromatic ring(s), members of flavonoids family have three of them. It is well established that the antioxidant potential is related to structure in terms of delocalization of the aromatic nucleus. Where these compounds react with free radicals, the phenoxyl radicals produced are stabilized by the resonance effect of the aromatic nucleus [43]. Here, the compound with the most effective reducing capacity was epigallocatechin gallate, followed by epicatechin gallate. Both these polyphenols also exhibited a strong radical-scavenging activity. This agrees with previous published results indicating that flavanols such

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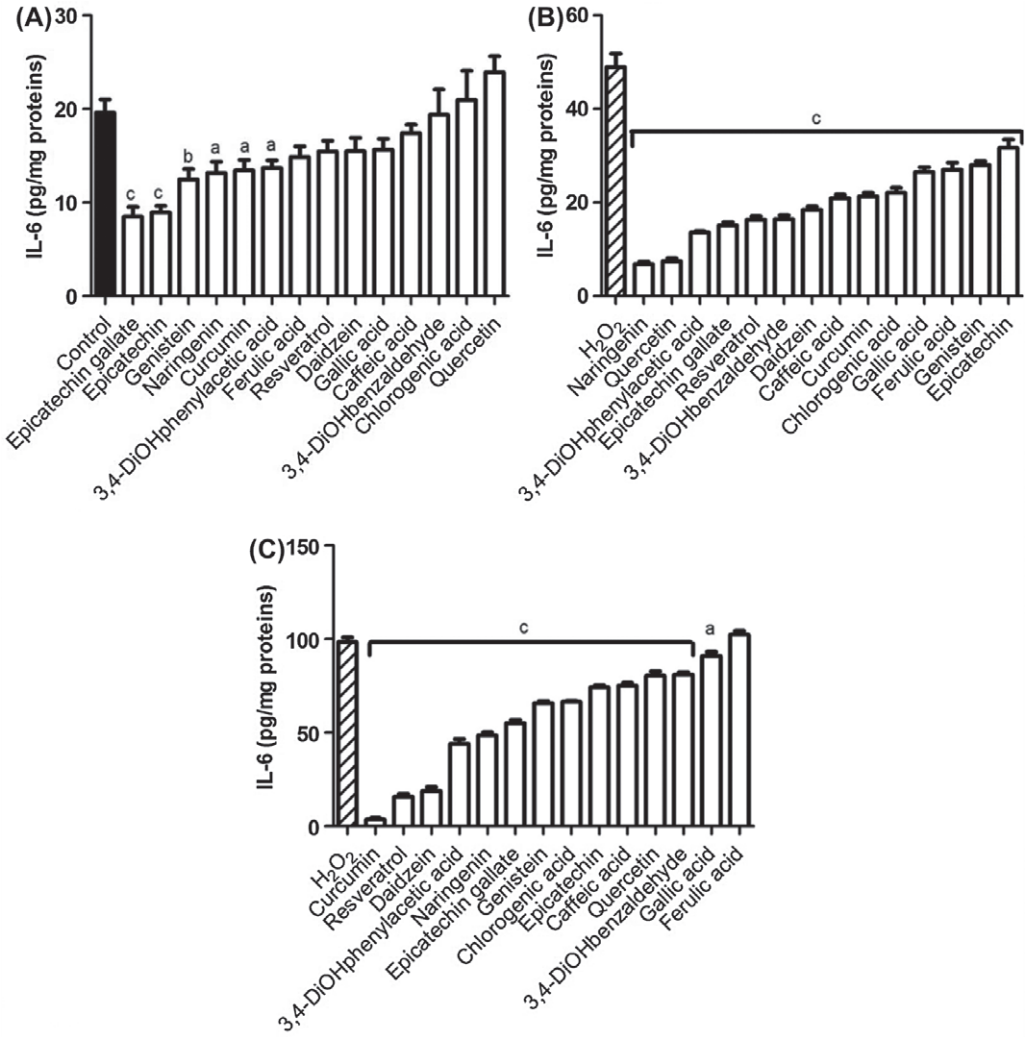

Figure 8. Effect of polyphenols on IL-6 production from 3T3-L1 preadipocytes. IL-6 content was measured in the three following conditions. (A) Cells were exposed to 50 μM polyphenols for 24 h. (B) Cells were co-exposed to 50 μM polyphenols and 500 μM H_2O_2 during 24 h. (C) Cells were pretreated with 50 μM polyphenols for 24 h and then exposed to 500 μM H_2O_2 for 24 h. Reported values are means ± SEM of three independent experiments. p value was calculated using the Dunnet's multiple comparison test (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$ as compared with control in Fig. A, and with H_2O_2 in Fig. B and C).

as epicatechin, epicatechin gallate and epigallocatechin gallate are better radical scavengers than many monomeric flavones and flavonols, due to the presence of catecholic and pyrogallic moieties as privileged radical-scavenging sites [44]. The major consideration for flavonoids' activity is hydroxylation of the B-ring, which confers higher stability to the radical form and participates in electron delocalization [42]. Other properties confer to some flavonoids a more effective radical scavenging. For instance, the 2–3 double bond in conjugation with a 4-oxo function in the C-ring is responsible for electron delocalization from the B-ring. Moreover, the 3- and 5-OH groups with 4-oxo functions in A and C rings are required for a maximal radical-scavenging potential [43]. Interestingly, quercetin exhibits all properties cited above, and was one of the three most effective antioxidants among the 28 compounds tested in this study whereas kaempferol, which differs from quercetin structure for the lack of the 3′-OH group from the B-ring, presented only 28% of quercetin's reducing capacity. Some phenolic acids also exerted good antioxidant capacities, such as 3,4-dihydroxybenzaldehyde and caffeic acid. The antioxidant activity of phenolic acids and their esters depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl function groups [43,45]. This could explain the highest radical-scavenging activity in DPPH assay (69.8%) obtained for gallic acid, which is the phenolic acid characterized by the highest number of hydroxyl groups. The microbial metabolite, 3,4-dihydroxyphenylacetic acid, also exhibited a strong antioxidant activity, which could be attributed to the structure derived from its major known polyphenolic precursors, namely flavonoids [24,36].

Thus, biochemical experiments reported in the present work showed that the compounds exerted an antioxidant capacity depending on their structure. We wondered if this capacity could also be related to their cellular effects, as it has already been suggested for phenolic compounds [17]. Depending on the concentration and exposition time used on 3T3-L1 preadipocytes, mitochondrial metabolic activity of living cells was increased, decreased or unchanged.

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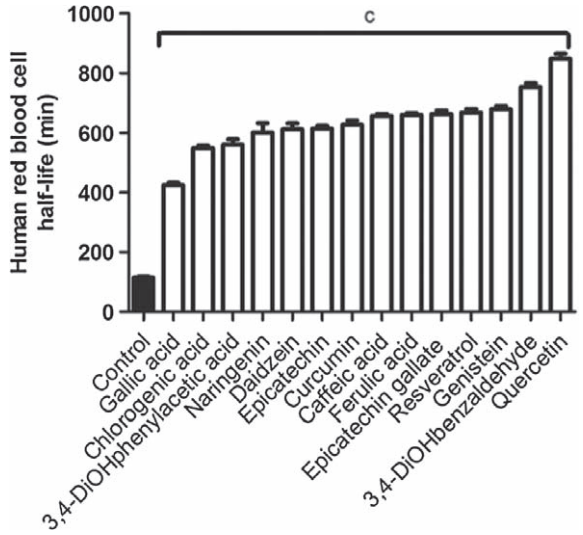

Figure 9. Protective effect of polyphenols against AAPH radical-induced lysis of human red blood cells. Red blood cells were incubated with 2,2′-azobis(2-amidinopropane) (AAPH) and 50 μM polyphenols for 18 h at 37°C, and cell lysis was determined by spectrophotometric measurements at 450 nm at 10-min intervals. Reported values are means ± SEM. p value was calculated using the Dunn's multiple comparison test (c: $p < 0.001$ as compared with control).

Moreover, a dose and time effect appeared for most of the polyphenols which decreased cell's metabolic activity, such as curcumin, gallic acid or resveratrol. As MTT assay reflects the metabolic activity of cells, we investigated if the compounds had an effect on this activity or on preadipocytes' viability itself. Fourteen compounds were selected on the basis of their different cellular effects to investigate further their impact on preadipocytes' proliferation. Some compounds induced an increase both in mitochondrial metabolic activity and number of preadipocytes, such as epicatechin. Further experiments on cell cycle analysis will be required to understand the signaling pathways involved. Other compounds had an anti-proliferative effect, demonstrated by the decrease in mitochondrial metabolic activity and number of preadipocytes. Curcumin that showed a low reducing- and radical-scavenging capacity was the compound which decreases cell viability the most. Moreover, epicatechin gallate which exerted a stronger reducing- and radical-scavenging property than quercetin, did not change preadipocytes viability, in contrast to quercetin. In addition, the results of this study demonstrated that resveratrol and genistein decreased 3T3-L1 preadipocytes' viability, and interestingly, combination of both compounds enhanced this effect according to Rayalam et al. [46]. The cell-growth reduction mediated by chlorogenic acid could be explained through a cell-cycle arrest at the G1 phase, as it was previously described on 3T3-L1 preadipocytes by Hsu et al. [17]. Altogether, these results suggest that the antioxidant capacity of compounds does not appear to be associated with their impact on cell viability. This agrees with data from a published study showing that the antioxidant capacity of flavonoids is not related to their inhibitory action on the growth of human colon adenocarcinoma cells [47].

The observed decrease in preadipocytes' number induced by polyphenols cited above could be attributed to a cell-cycle arrest (modification of the cell cycle) or to the induction of cell death, apoptosis or necrosis. The LDH assay led us to conclude that only gallic acid and curcumin induced 3T3-L1 preadipocytes' death at 50 μM for 24 h. It has been previously described that the induction of apoptosis in 3T3-L1 preadipocytes by gallic acid is mediated through the Fas and mitochondrial-mediated pathway [17,48]. Moreover, in accordance with our results, Kim et al. demonstrated that curcumin inhibited proliferation of 3T3-L1 preadipocytes at 50 μM. It is interesting to consider that the authors showed an opposite effect on cell proliferation, with concentrations of curcumin ranging from 0.01 to 20 μM [49]. Once again, this emphasizes possible different cellular effects exerted by the same compound depending on exposure conditions such as the dose used.

Then, we evaluated the possible protective role of dietary polyphenols against H_2O_2-induced oxidative stress. Excess of ROS production can severely impair the cell and lead to macromolecular damage, dysfunction and death. Overloaded ROS in adipocytes induce insulin resistance, which contributes to obesity-associated diabetes [50,51]. In our previous study, exposition of 3T3-L1 preadipocytes to 200 μM H_2O_2 induced a proliferation arrest, associated with an increase in mitochondrial biogenesis. Preconditioning cells with some phenolic acids or flavonoids totally or partially reversed H_2O_2-induced mitochondrial alterations [39]. Here, in co-exposition condition, all polyphenols tested either improved H_2O_2-altered mitochondrial metabolic activity or did not significantly decrease it, despite the anti-proliferative action reported above for some of them such as curcumin. When cells were pretreated with polyphenols, most of the compounds also reversed H_2O_2 impact, with the highest effect observed for epicatechin and epicatechin gallate. Moreover, except genistein and daidzein, all polyphenols tested significantly decreased ROS production enhanced by H_2O_2. This is in agreement with previous data reporting a suppression of TNF-α-induced ROS production by quercetin and resveratrol, at the same concentration of polyphenols than that used in our study [20]. Thus, most of the polyphenols protected preadipocytes against H_2O_2-induced detrimental effects and this property could be attributed to their direct antioxidant capacities against free radicals in co-exposition conditions. The effects observed in pretreatment conditions could be explained by the interaction of polyphenols with the cell membranes. Indeed, besides the free radical-scavenging activity of the compounds, other mechanisms have been proposed to explain their biological action, such as their interactions with membrane lipids and proteins. It has been demonstrated that the constitution of polyphenols in terms of hydrophilic and hydrophobic domains can determine their interaction with lipid bilayers [52]. Interestingly, curcumin lipophilicity may lead it to easily enter the cell and may contribute to explain its strong protective effect against ROS production despite its low radical-scavenging activity. The protective effects could also be

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explained by the uptake of phenolic compounds into the cells, where it has been demonstrated that they are able to interact with proteins such as enzymes or transcription factors. These interactions have different biological effects, including the modification of enzymatic activities, receptor–ligand binding and transcription factors binding to their specific sites in DNA [52,53]. Inside the cells, polyphenols could also exert their protective effect with mitochondrial alterations induced by H_2O_2, as we recently published [39]. Due to all these intracellular actions, the differences observed for the polyphenols in the pretreatment conditions could be due to their different levels of uptake into the cells. Very few studies have explored the cellular absorption of polyphenols. Interestingly, Salucci et al. have reported different uptakes of flavonoids in relation to their chemical structure in colon adenocarcinoma cells (Caco2) [47]. Thus, it would be relevant to evaluate the level of the compounds in adipose cells after the incubation period to determine if their cellular uptake significantly contributes to explain their protective activity. The absence of protective effect against ROS production observed for genistein and daidzein could be explained by their low antioxidant capacity as shown above, or also due to their bioaccessibility degree.

Obesity-induced chronic inflammation is a key component in the pathogenesis of insulin resistance. Numerous evidences suggest that this chronic inflammation resulting from cytokines secreted by adipose tissue may play a significant role in desensitizing cells to insulin [54]. Thus, reducing or preventing the inflammatory properties of adipose tissue may constitute a promising therapeutic approach to prevent obesity-related diseases. In this study, we were interested in the effect of polyphenols on the production of two major pro-inflammatory cytokines, namely TNF-α and IL-6. However, TNF-α was not detectable in 3T3-L1 preadipocytes. This agrees with previous data from authors who detected TNF-α mRNA in 3T3-L1 preadipocytes, but were unable to measure any secreted TNF-α [55]. A similar observation was reported by Fain et al., who found significant amounts of TNF-α secreted by stroma vascular cells, with little or no detectable TNF-α secreted by adipocytes obtained from human adipose explants [56]. The hypothesis suggested by the authors is that TNF-α secretion by adipocytes would depend on signaling events from their in vivo environment, where they are exposed to macrophage-derived TNF-α [55]. Elevated plasma levels of IL-6 are strongly linked to insulin resistance and this cytokine impairs insulin signaling in mouse adipocytes [57,58]. Here, interestingly, all polyphenols tested significantly inhibited H_2O_2-induced secretion of IL-6 from 3T3-L1 preadipocytes, in co-exposition condition. When cells were pretreated with polyphenols, most compounds also exerted an anti-inflammatory effect. Naringenin, quercetin, curcumin, resveratrol and 3,4-dihydroxyphenylacetic acid exerted the highest inhibitory action on IL-6 secretion from H_2O_2-exposed 3T3-L1 cells. This is in agreement with a previous report indicating that quercetin and resveratrol inhibited TNF-α-induced increase in the production of pro-inflammatory molecules, such as IL-6 and MCP-1, in mouse and human adipocytes [20,59]. Anti-inflammatory activities of curcumin have also been previously shown on numerous cellular types, including mouse adipocytes [60]. Curcumin, resveratrol and naringenin actions were explained by their inhibitory effect on the pro-inflammatory transcription factor nuclear factor-kappaB (NF-κB)-mediated cytokine expression in adipocytes [55,61]. To our knowledge, the present work demonstrates for the first time the anti-inflammatory effect of the microbial metabolite 3,4-dihydroxyphenylacetic acid. Further experiments will be needed to precise signaling pathways involved.

Importantly, this work also demonstrates the relevance of polyphenol preconditioning in order to prevent H_2O_2-induced oxidative stress and pro-inflammatory state, similarly to co-exposition condition. As this protective action of polyphenol preconditioning was observed for most compounds tested, except quercetin, caffeic acid and ferulic acid, it will be of high interest to elucidate molecular mechanisms implicated. A better understanding of these mechanisms should help to emphasize the interest of dietary polyphenol intake to prevent complications of obesity-related oxidative stress. Moreover, it should lead to improve preventive nutritional strategies as well as therapeutic pharmacological approaches.

Finally, we explored the benefits of polyphenols to protect against oxidative stress on another cell type, namely human red blood cells. Indeed, erythrocytes constitute a relevant model to assess antioxidant capacities of molecules: they are known to be constantly exposed to ROS due to their rich oxygen supply and iron-rich hemoglobin level, and thus are susceptible to be highly exposed to oxidative damage [27]. In our study, all polyphenols tested protected red blood cells against AAPH radical-induced lysis. Quercetin was identified as the most efficient antioxidant. This agrees with previous reports showing quercetin's ability to inhibit oxidative hemolysis [62,63]. Such an antioxidant effect was attributed to several mechanisms including quercetin action on the preservation of membrane integrity, modification of the membrane status through interaction with membrane lipids and proteins, as well as quercetin-induced increase in membrane resistance to destruction by free radicals [64–66]. Resveratrol also protected efficiently oxidative stress-exposed red blood cells, and its effect could be attributed to its ability to enter in erythrocytes and to activate plasma membrane redox system, leading to an antioxidant effect [67]. Therefore, anti-hemolytic effects of quercetin and resveratrol may be due to altered cellular membrane or uptake, rather than by directly scavenging peroxyl radicals. This is consistent with the protective effects induced by compounds exhibiting low antioxidant capacities such as daidzein or curcumin, on adipocytes and red blood cells in oxidative stress. Indeed, curcumin protected efficiently AAPH radical-induced hemolysis in our test, as it has been demonstrated in other studies [68,69]. Thus these protective effects on red blood cells could be explained by different mechanisms, including the interaction with membrane lipids and proteins. It could also be due to the peroxyl radicals scavenging outside the cells that

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prevents membrane damage. This mode of action is in agreement with the radical-scavenging activity demonstrated by DPPH assay.

To conclude, this study conducted on different polyphenols and microbial metabolites led to demonstrate that their antioxidant capacities were related to their structure. Members of the flavonoids family, such as epigallocatechin gallate, epicatechin gallate and quercetin, exerted stronger reducing and radical-scavenging activities than phenolic acids, stilbenes and curcuminoids. Interestingly, one of the microbial metabolites tested, namely 3,4-dihydroxyphenylacetic acid, showed a similar antioxidant capacity than its major known flavonoid precursors. We also demonstrated that even if polyphenols exhibited various effects on 3T3-L1 preadipocytes' viability in native conditions, depending on their chemical nature, dose and time of exposure, they reduced ROS production and protected cells in oxidative-stress condition. Polyphenols also exerted an anti-inflammatory action by reducing H_2O_2-induced IL-6 production. Finally, all polyphenols tested protected red blood cells against oxidative stress-induced hemolysis. Thus, the biological effect of polyphenols may depend on various factors such as chemical structure, concentration, time of exposure, presence of oxidative stress or not and exposure condition (preconditioning or co-treatment). We should further investigate in vivo benefits of these compounds to assess their antioxidant and anti-inflammatory effects, as well as their bioavailability extent. These results should be considered for the development of nutritional and pharmacological strategies aiming to reduce oxidative stress and inflammation associated with obesity and its metabolic disorders such as type 2 diabetes.

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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References

[1] Abdullah A, Peeters A, de Courten M, Stoelwinder J. The magnitude of association between overweight and obesity and the risk of diabetes: a meta-analysis of prospective cohort studies. Diabetes Res Clin Pract 2010;89:309–319.

[2] Stefanovic A, Kotur-Stevuljevic J, Spasic S, Bogavac-Stanojevic N, Bujisic N. The influence of obesity on the oxidative stress status and the concentration of leptin in type 2 diabetes mellitus patients. Diabetes Res Clin Pract 2008;79:156–163.

[3] Mikhail N, Golub MS, Tuck ML. Obesity and hypertension. Prog Cardiovasc Dis 1999;42:39–58.

[4] Chapman MJ, Sposito AC. Hypertension and dyslipidaemia in obesity and insulin resistance: pathophysiology, impact on atherosclerotic disease and pharmacotherapy. Pharmacol Ther 2008;117:354–373.

[5] Nakamura H, Ito H, Egami Y, Kaji Y, Maruyama T, Koike G, et al. Waist circumference is the main determinant of elevated C-reactive protein in metabolic syndrome. Diabetes Res Clin Pract 2008;79:330–336.

[6] Ritchie SA, Connell JM. The link between abdominal obesity, metabolic syndrome and cardiovascular disease. Nutr Metab Cardiovasc Dis 2007;17:319–326.

[7] Abate N. Obesity and cardiovascular disease. Pathogenetic role of the metabolic syndrome and therapeutic implications. J Diabetes Complications 2000;14:154–174.

[8] Chu NF, Spiegelman D, Hotamisligil GS, Rifai N, Stampfer M, Rimm EB. Plasma insulin, leptin, and soluble TNF receptors levels in relation to obesity-related atherogenic and thrombogenic cardiovascular disease risk factors among men. Atherosclerosis 2001;157:495–503.

[9] Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature 2006;444: 847–853.

[10] Ailhaud G. Adipose tissue as a secretory organ: from adipogenesis to the metabolic syndrome. C R Biol 2006;329: 570–577; discussion 653–655.

[11] de Ferranti S, Mozaffarian D. The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. Clin Chem 2008;54:945–955.

[12] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44–84.

[13] Styskal J, Van Remmen H, Richardson A, Salmon AB. Oxidative stress and diabetes: what can we learn about insulin resistance from antioxidant mutant mouse models? Free Radic Biol Med 2012;52:46–58.

[14] Goossens GH. The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol Behav 2008;94:206–218.

[15] Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. Proc Natl Acad Sci U S A 1994;91:4854–4858.

[16] Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J Nutr 2000;130:2073S–2085S.

[17] Hsu CL, Huang SL, Yen GC. Inhibitory effect of phenolic acids on the proliferation of 3T3-L1 preadipocytes in relation to their antioxidant activity. J Agric Food Chem 2006;54:4191–4197.

[18] Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. Free Radic Biol Med 1996;21:895–902.

[19] Ishimoto H, Tai A, Yoshimura M, Amakura Y, Yoshida T, Hatano T, Ito H. Antioxidative properties of functional polyphenols and their metabolites assessed by an ORAC assay. Biosci Biotechnol Biochem 2012;76:395–399.

[20] Yen GC, Chen YC, Chang WT, Hsu CL. Effects of polyphenolic compounds on tumor necrosis factor-alpha (TNF-alpha)-induced changes of adipokines and oxidative stress in 3T3-L1 adipocytes. J Agric Food Chem 2011;59:546–551.

[21] Hsu CL, Yen GC. Induction of cell apoptosis in 3T3-L1 preadipocytes by flavonoids is associated with their antioxidant activity. Mol Nutr Food Res 2006;50:1072–1079.

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[22] Gonthier MP, Verny MA, Besson C, Remesy C, Scalbert A. Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. J Nutr 2003;133: 1853–1859.
[23] Gonthier MP, Cheynier V, Donovan JL, Manach C, Morand C, Mila I, et al. Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. J Nutr 2003;133:461–467.
[24] Gonthier MP, Donovan JL, Texier O, Felgines C, Remesy C, Scalbert A. Metabolism of dietary procyanidins in rats. Free Radic Biol Med 2003;35:837–844.
[25] Spencer JP, Abd-el-Mohsen MM, Rice-Evans C. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. Arch Biochem Biophys 2004;423:148–161.
[26] Hsu CL, Yen GC. Effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes. J Agric Food Chem 2007;55:8404–8410.
[27] Claster S, Chiu DT, Quintanilha A, Lubin B. Neutrophils mediate lipid peroxidation in human red cells. Blood 1984; 64:1079–1084.
[28] Folin O, Denis W. A colorimetric estimation of phenols and phenol derivatives in urine. J Biol Chem 1915;22:305–308.
[29] Yang H, Protiva P, Cui B, Ma C, Baggett S, Hequet V, et al. New bioactive polyphenols from Theobroma grandiflorum ("cupuacu"). J Nat Prod 2003;66:1501–1504.
[30] Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J Agric Food Chem 2002;50:4437–4444.
[31] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[32] DeRenzis FA, Schechtman A. Staining by neutral red and trypan blue in sequence for assaying vital and nonvital cultured cells. Stain Technol 1973;48:135–136.
[33] Legrand C, Bour JM, Jacob C, Capiaumont J, Martial A, Marc A, et al. Lactate dehydrogenase (LDH) activity of the cultured eukaryotic cells as marker of the number of dead cells in the medium [corrected]. J Biotechnol 1992;25:231–243.
[34] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
[35] Prost M. Process for the determination by means of free radicals of the antioxidant properties of a living organism or potentially aggressive agents patent US 5135850 A. 1992.
[36] Ito H, Gonthier MP, Manach C, Morand C, Mennen L, Remesy C, Scalbert A. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages. Br J Nutr 2005;94:500–509.
[37] Yang JY, Della-Fera MA, Rayalam S, Ambati S, Hartzell DL, Park HJ, Baile CA. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. Life Sci 2008;82:1032–1039.
[38] Barnouin K, Dubuisson ML, Child ES, Fernandez de Mattos S, Glassford J, Medema RH, et al. H2O2 induces a transient multi-phase cell cycle arrest in mouse fibroblasts through modulating cyclin D and p21Cip1 expression. J Biol Chem 2002;277:13761–13770.
[39] Baret P, Septembre-Malaterre A, Rigoulet M, Lefebvre d'Hellencourt C, Priault M, Gonthier MP, Devin A. Dietary polyphenols preconditioning protects 3T3-L1 preadipocytes from mitochondrial alterations induced by oxidative stress. Int J Biochem Cell Biol 2013;45:167–174.
[40] Kobayashi H, Matsuda M, Fukuhara A, Komuro R, Shimomura I. Dysregulated glutathione metabolism links to impaired insulin action in adipocytes. Am J Physiol Endocrinol Metab 2009;296:E1326–E1334.
[41] Afanas'ev IB, Dorozhko AI, Brodskii AV, Kostyuk VA, Potapovitch AI. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. Biochem Pharmacol 1989;38:1763–1769.
[42] Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res 1995;22: 375–383.
[43] Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996;20:933–956.
[44] Bors W, Michel C. Antioxidant capacity of flavanols and gallate esters: pulse radiolysis studies. Free Radic Biol Med 1999;27:1413–1426.
[45] Robards K, Prenzler PD, Tucker G, Swatsitang P, Glover W. Phenolic compounds and their role in oxidative processes in fruits. Food Chem 1999;66:401–436.
[46] Rayalam S, Della-Fera MA, Yang JY, Park HJ, Ambati S, Baile CA. Resveratrol potentiates genistein's antiadipogenic and proapoptotic effects in 3T3-L1 adipocytes. J Nutr 2007; 137:2668–2673.
[47] Salucci M, Stivala LA, Maiani G, Bugianesi R, Vannini V. Flavonoids uptake and their effect on cell cycle of human colon adenocarcinoma cells (Caco2). Br J Cancer 2002;86: 1645–1651.
[48] Hsu CL, Lo WH, Yen GC. Gallic acid induces apoptosis in 3T3-L1 pre-adipocytes via a Fas- and mitochondrial-mediated pathway. J Agric Food Chem 2007;55:7359–7365.
[49] Kim CY, Le TT, Chen C, Cheng JX, Kim KH. Curcumin inhibits adipocyte differentiation through modulation of mitotic clonal expansion. J Nutr Biochem 2011;22:910–920.
[50] Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 2006;440:944–948.
[51] Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004;114: 1752–1761.
[52] Fraga CG, Galleano M, Verstraeten SV, Oteiza PI. Basic biochemical mechanisms behind the health benefits of polyphenols. Mol Aspects Med 2010;31:435–445.
[53] Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000; 52:673–751.
[54] Kalupahana NS, Moustaid-Moussa N, Claycombe KJ. Immunity as a link between obesity and insulin resistance. Mol Aspects Med 2012;33:26–34.
[55] Gonzales AM, Orlando RA. Curcumin and resveratrol inhibit nuclear factor-kappaB-mediated cytokine expression in adipocytes. Nutr Metab (Lond) 2008;5:17.
[56] Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 2004;145:2273–2282.
[57] Rotter V, Nagaev I, Smith U. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. J Biol Chem 2003; 278:45777–45784.
[58] Zou C, Shao J. Role of adipocytokines in obesity-associated insulin resistance. J Nutr Biochem 2008;19:277–286.
[59] Chuang CC, Martinez K, Xie G, Kennedy A, Bumrungpert A, Overman A, et al. Quercetin is equally or more effective than resveratrol in attenuating tumor necrosis factor-{alpha}-mediated inflammation and insulin resistance in primary human adipocytes. Am J Clin Nutr 2010;92:1511–1521.
[60] Gupta SC, Patchva S, Koh W, Aggarwal BB. Discovery of curcumin, a component of golden spice, and its miraculous

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biological activities. Clin Exp Pharmacol Physiol 2012;39: 283–299.
[61] Yoshida H, Takamura N, Shuto T, Ogata K, Tokunaga J, Kawai K, Kai H. The citrus flavonoids hesperetin and naringenin block the lipolytic actions of TNF-alpha in mouse adipocytes. Biochem Biophys Res Commun 2010;394:728–732.
[62] Ferrali M, Signorini C, Caciotti B, Sugherini L, Ciccoli L, Giachetti D, Comporti M. Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity. FEBS Lett 1997;416:123–129.
[63] Chen Y, Deuster P. Comparison of quercetin and dihydroquercetin: antioxidant-independent actions on erythrocyte and platelet membrane. Chem Biol Interact 2009;182:7–12.
[64] Lopez-Revuelta A, Sanchez-Gallego JI, Hernandez-Hernandez A, Sanchez-Yague J, Llanillo M. Membrane cholesterol contents influence the protective effects of quercetin and rutin in erythrocytes damaged by oxidative stress. Chem Biol Interact 2006;161:79–91.
[65] Pawlikowska-Pawlega B, Gruszecki WI, Misiak LE, Gawron A. The study of the quercetin action on human erythrocyte membranes. Biochem Pharmacol 2003;66:605–612.
[66] Hapner CD, Deuster P, Chen Y. Inhibition of oxidative hemolysis by quercetin, but not other antioxidants. Chem Biol Interact 2010;186:275–279.
[67] Rizvi SI, Pandey KB. Activation of the erythrocyte plasma membrane redox system by resveratrol: a possible mechanism for antioxidant properties. Pharmacol Rep 2010;62: 726–732.
[68] Banerjee A, Kunwar A, Mishra B, Priyadarsini KI. Concentration dependent antioxidant/pro-oxidant activity of curcumin studies from AAPH induced hemolysis of RBCs. Chem Biol Interact 2008;174:134–139.
[69] Deng SL, Chen WF, Zhou B, Yang L, Liu ZL Protective effects of curcumin and its analogues against free radical-induced oxidative haemolysis of human red blood cells. Food Chem 2006;98:112–119.

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