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Effect of Flavonoids on Stress Responses in Myotube Cultures

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Effects of flavonoids on stress response of myotube cultures was studied by monitoring the release of [^{14}C] taurine, leukotriene production, and 2′,7′-dichlorodihydroflourescein ($DCFH_2$) oxidation. Stress was induced by hypotonic shock, which was accompanied by cell swelling leading to increased leukotriene production and a concomitant increase in reactive oxygen species and osmolyte release. In this model system, addition of the flavonoids catechin and quercetin decreased leukotriene production, $DCFH_2$ oxidation, and taurine efflux, indicating a reduction of cellular stress. High concentrations of epigallocatechin gallate (EGCG) and tea extract increased leukotriene production and initial $DCFH_2$ oxidation, indicating an increased cellular stress (possibly toxicity). However, taurine efflux was reduced, and also longer exposure time as well as lower concentrations of EGCG and tea reduced $DCFH_2$ oxidation. Trolox and α-tocopherol did not significantly affect taurine efflux or leukotriene production, and it was therefore concluded that suppression of these responses was not confined to redox activity in a myotube culture.

KEYWORDS: Cell culture; myotubes; stress; flavonoids; tea; leukotriene; taurin efflux; $DCFH_2$ oxidation

INTRODUCTION

Epidemiological studies indicate a number of beneficial health effects of increased intake of fruits and vegetables, and a major group of compounds in fruits and vegetables, the flavonoids, has been suggested to contribute to these beneficial effects (*1*). Quercetin is one of the most abundant flavonoids in fruits and vegetables (*2*), and catechins (e.g., epigallocatechin gallate, EGCG) are the most abundant in tea (*3*). Bioavailability is a prerequisite for a biological effect on a cellular level, and experiments on rodents have shown that quercetin (*4*) and the catechin EGCG (*5*) are absorbed and transported to the tissues; in human subjects both catechins (*6*−*8*) and quercetin (*9*−*11*) and metabolites thereof have been found in urine and plasma (*12*). Catechins and quercetin are thus present in the blood after the ingestion of catechin- and quercetin-rich foods or drinks.

Studies on various effects of naturally occurring antioxidants often result in limited effects (*13*, *14*) because of limitations in dosage, duration, and severity of the stress imposed on the subjects. Humans at rest with no nutritional deprivation have a very well balanced system; to increase the chances of revealing effects of naturally occurring compounds, several studies have either depleted fruit and vegetable intake before intervention and throughout the experimental period (*15*, *16*) or tried to increase the stress, for example, by using smokers (*15*, *17*, *18*).

Various experimental systems, for example, membrane systems, cell culture, animal models, and human intervention studies, have been used to study the biological activity of compounds in fruits and vegetables, with accompanying advantages and limitations of model systems. Cell culture models have a number of advantages including the ability to conduct mechanistic studies and the ease with which the experimental environment can be controlled.

The most abundant cell type in the human body is muscle cells, constituting ∼40% of the body weight. Under physical exercise the muscle cells are exposed to stress, which may be affected by the environment/compounds in the circulating system.

The aim of the present study was to investigate effects of flavonoids on cellular stress responses in a model system of myotube cultures. Stress was induced on the myotubes by hypotonic shock and monitored as release of [^{14}C]taurine, leukotriene production, and 2′,7′-dichlorodihydroflourescein ($DCFH_2$) oxidation.

MATERIALS AND METHODS

Chemicals. Antibiotics, fetal calf serum (FCS, no. 10106-169), and Dulbecco's modified Eagle's medium (high glucose) with glutaMAX I (DMEM, no. 32430-027) were obtained from Life Technologies (Naperville, IL). [^{14}C]Taurine (NEC-439) was used from NEN Life Science Products, Inc. (Boston, MA), and the leukotriene kit (RPN224) was from Amersham Biosciences (Uppsala, Sweden). Trolox was obtained from Acros Organics (Geel, Belgium), trypsin−EDTA from Invitrogen Life Technologies (Carlsbad, CA), and 2′,7′-dichlorodihydroflourescein diacetate (H_2DCFDA) from Molecular Probes, Inc. (Eugene, OR). Tea extract was an ethanol extract from tea extract

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capsules (VitaViva, Simpelweld, Holland). All other compounds were from Sigma Chemical Co. (St. Louis, MO).

Inorganic Media. Hypotonic KCl solution contained 95 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM *N*-2-hydroxyethylpiperazin-*N*′-ethanesulfonic acid (Hepes). Isotonic KCl solution was obtained by adding KCl to the hypotonic KCl solution to 150 mM, keeping all other components unchanged. Isotonic Krebs−Hepes buffer (KHB) contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM Hepes, and 10 mM D-glucose. Hypotonic KHB was obtained by diluting the KHB 1:1 with water containing only 10 mM Hepes. The pH was adjusted to 7.40 in all solutions. Water used for all solutions was from an ELGA purification/deionization system (18.2 mΩ) (Maxima, USF ELGA).

Muscle Cell Culture. Myotube cultures were established from a mouse myoblast line (C2C12) originally derived from a mouse thigh muscle (*19*) (American Type Culture Collection, Manassas, VA). A clone from this cell line, which effectively fused and formed myotubes, was isolated (*20*). The clone was grown in an 80 cm^2 culture flask in 10 mL of growth medium consisting of DMEM, 10% (v/v) FCS and supplemented with 1% antibiotics giving 100 IU/mL penicillin, 100 μg/mL streptomycin sulfate, 3 μg/mL amphotericin B, and 20 μg/mL gentamycin (growth medium). Cells were maintained in an atmosphere of 95% air and 5% CO_2 at 37 °C. Prior to confluence, cells were harvested in 0.25% trypsin and seeded in laminin-coated (0.5 μg/mL) plates at a density of 10000 cells/cm^2. Six-well plates (Nunc 150229) were used for leukotriene experiments and microtiter plates for measuring either taurine efflux (Nunc 167008) or $DCFH_2$ oxidation (Nunc 165306). Cells were grown to confluence in growth medium and left to fuse in differentiation medium containing 4% FCS. After ∼4 days, the cultures contained differentiated multinuclear myotubes and were ready for experimental use.

Taurine Efflux and $DCFH_2$ Oxidation. KHB was used for $DCFH_2$ oxidation experiments and KCl buffer for taurine experiments. For taurine experiments myotubes were loaded with [^{14}C]taurine (10 nCi/mL) added to differentiation medium for 24 h. For $DCFH_2$ oxidation experiments myotubes were loaded with H_2DCF-DA in KHB (10 μM) for 2 h at 37 °C (95% air, 5% CO_2). Medium/buffer was aspirated, and myotubes were washed twice with 0.2 mL/well isotonic buffer to remove excess extracellular taurine and H_2DCF-DA, respectively. Cells were incubated (0.1 mL/well) in thermostated hypotonic buffer at 37 °C in the presence of antioxidants at various concentrations. For taurine efflux measurements, samples were transferred to a scintillation plate (Isoplate 1450-514, Wallac, Turku, Finland) after 30 min for determining ^{14}C activity as disintegrations per minute (dpm) (1450 Microbeta, Wallac). Intracellular $DCFH_2$ oxidation was determined every 4 min directly in the culture plate by fluoresence from 2,7-dichlorofluorescein (DCF) at excitation and emission wavelengths of 490 and 515 nm, respectively, at 34 °C (Perkin-Elmer LS50B fluorometer, Beaconsfield, U.K.), fitted with a microtiter plate reader and a custom-made thermostating element from Mikrolab, (Århus, Denmark) up to 6734 s (∼112 min). Data were corrected for background signal (taurine or $DCFH_2$ oxidation) from wells without cells but otherwise treated similarly. All concentrations of the antioxidants were determined in triplicate in each of three independent experiments.

Leukotriene LTC_4. Medium was aspirated, and myotubes were washed three times in isotonic KHB and incubated (1 mL/well) in thermostated hypotonic KHB at 37 °C in the presence of antioxidants at various concentrations. After 30 min, 1 mL of cold ethanol was added to each well, and the whole content of the well (cells plus media/ethanol) was transferred to glass containers; the well was washed with 1 and 0.5 mL of ethanol, which was also added to the containers. Samples were vacuum-centrifuged to dryness, resuspended in 100 μL of KHB, washed with 50 μL of KHB, and centrifuged at 4000*g* for 10 min. Samples of 50 μL were analyzed for leukotriene LTC_4 as recommended by the manufacturer. Briefly, a microplate precoated with primary antibody was incubated for 2 h at 4 °C with standards and samples together with an antiserum including a secondary antibody specific for leukotriene. Peroxidase-labeled leukotriene C_4 was added and incubated for 3 h at 4 °C, competing for binding sites with unlabeled leukotriene C_4 in the samples. Wells were washed four times and incubated for 30 min with shaking at room temperature with the substrate tetramethylbenzidine/hydrogen peroxide, stopped by the addition of sulfuric acid; optical density was determined at 450 nm. The amount of peroxidase-labeled leukotriene bound to the antibody will be inversely proportional to the concentration of added unlabeled leukotriene. Leukotriene C_4 concentrations in the samples were calculated according to a standard curve as recommended by the manufacturer. The leukotriene response from each concentration of the antioxidants was determined in duplicate in each of two independent wells.

Table 1. Multiple Reaction Monitoring Conditions for the LC-MS/MS Detection of the Catechins

compound	parent → daughter	cone (V)	collision (eV)
epicatechin	290.91 → 122.93	20	20
epicatechin gallate	443.04 → 122.93	20	19
epigallocatechin	306.94 → 138.89	19	15
epigallocatechin gallate	459.0 → 138.95	17	30

Catechins in Tea Extract. Catechins were quantified by liquid chromatography−tandem mass spectrometry (LC-MS/MS). Green tea capsules were extracted with ethanol and diluted 1:500 with methanol/water/formic acid (100:899:1, v/v/v). The catechins were analyzed by LC-MS/MS on a Quattro LC (Micromass, Manchester, U.K.) using a Symmetry C18 3.5 μm column from Waters (Milford, MA) (150 mm × 2.1 mm i.d.). The catechins were separated by a binary gradient consisting of 0.1% HCOOH in water and CH_3OH going from 10 to 30% CH_3OH in 15 min, flow rate = 0.4 mL min^{-1}. The MS system was operated in the electrospray positive mode with the capillary at 3.0 kV, desolvation gas (N_2) at a flow rate of 610 L h^{-1}, and a temperature of 250 °C. A multiple reaction monitoring (MRM) method using Ar as collision gas was constructed using the mass transitions outlined in **Table 1** for detection. Linearity was checked from 0.01 to 0.25 mmol L^{-1}.

Data Analyses. Data were analyzed using the mixed procedure in Statistical Analysis System version 8.00 (SAS Institute, Cary, NC). The model on taurine release and leukotriene LTC_4 included the fixed effect of concentrations of the antioxidants and the random effect of experiment and replicate by experiment by concentration interaction. The model on CFH_2 oxidation was similar to that of trauine release except that induction time (three time points) was included as a repeated measurement, and appropriate interactions containing time were also included. Data are presented as LS means and, when overall effects are present, separated by least significant difference tests.

RESULTS

Catechins in Tea Extract. The four major catechins in tea (*21*) were determined in the ethanol-extracted tea extract (mean of two determinations); epigallocatechin gallate (EGCG), 50.6 mM; epigallocatechin (EGC), 5.1 mM; epicatechin gallate (ECG), 13.0 mM; and epicatechin (EC), 5.4 mM. The extract was used at a 100-fold dilution in myotube assays. The concentration of tea extract as used is given as EGCG equivalents.

Taurine Efflux. Efflux of hypotonic-induced [^{14}C]taurine is shown in **Table 2**. Tea extract and EGCG inhibited taurine efflux dose dependently, and catechin reduced taurine efflux slightly at the highest concentration used. Quercetin showed inhibition at a concentration of 0.11 mM and possibly toxicity at 1.0 mM. Trolox and α-tocopherol did not affect taurine efflux at the tested concentrations.

$DCFH_2$ Oxidation. $DCFH_2$ oxidation is shown in **Table 2** for three fixed time points (0, 57, and 115 min), and **Figure 1** shows continuous measurements every 250 s. Catechin and α-tocopherol suppressed $DCFH_2$ oxidation dose dependently. Trolox and quercetin displayed strong inhibition of $DCFH_2$ oxidation at the tested concentrations. EGCG and tea showed the most efficient suppression of $DCFH_2$ oxidation at the lowest

Table 2. LS Means of Leukotriene LTC_4 ($n = 4$), Intracellular Oxidation of 2,7-Dichlorofluorescein (DCF) ($n = 9$), and Taurine Efflux ($n = 9$) from C2C12 Myotubes Exposed to Hypotonic Buffer in the Presence of Quercetin, Catechin, Epigallocatechin Gallate (EGCG), Tea Extract, α-Tocopherol, and Trolox

				reactive oxygen species (fluorescence from DCF)					
		leukotriene (LTC_4)		time				taurine efflux ([^{14}C] taurine)	
antioxidant	concn (mM)	pg/well	SEM	0 min	57 min	115 min	SEM	counts/min	SEM
control	0	33.6a[a]		12.8a	103.6a	216.2a		1733a	
quercetin	0.037	ND[b]	1.2	–0.6a	24.4b	32.0b	18.0	1579ab	415
	0.111	23.4c		3.1a	25.5b	35.8b		1410b	
	0.333	19.7b		4.5a	26.2b	39.2b		1558ab	
	1.000	19.3b		6.0a	27.2b	42.2b		1111c	
catechin	0.012	ND	1.2	11.0a	55.3b	93.1c	20.7	1785ab	460
	0.037	25.8b		11.0a	38.9b	64.6bc		1773ab	
	0.111	29.0b		9.9a	27.9b	44.6b		1689abc	
	0.333	25.7b		13.4a	26.6b	36.9b		1552c	
EGCG	0.008	43.3c	1.8	9.6a	43.7b	53.8c	28.5	1678a	362
	0.024	49.2c		15.4a	65.2ab	75.4bc		1516a	
	0.073	77.7b		34.0a	105.0a	107.1b		1097b	
	0.218	ND		46.1a	104.4a	86.6bc		741b	
tea extract	0.006	30.2a	1.6	6.8a	41.3c	50.9b	25.1	1572a	324
(EGCG equiv)	0.019	29.3a		15.0a	61.2bc	70.1b		1315a	
	0.056	44.0b		36.5a	85.4ab	82.8b		999b	
	0.169	ND		50.7b	84.8ab	72.1b		703b	
α-tocopherol[c]	0.032	ND	1.9	12.9a	97.3a	212.4a	39.2	1808a	514
	0.096	35.9a		13.0a	87.8a	176.6d		1830a	
	0.289	ND		12.2a	75.4ab	146.1c		1721a	
	0.867	27.3a		8.1a	57.6b	113.6b		1829a	
Trolox	0.074	ND	1.2	3.7a	37.7b	68.2b	18.7	1790a	464
	0.222	ND		5.0a	47.3b	71.2b		1836a	
	0.667	33.0a		3.1a	44.0b	65.3b		1668a	
	2.000	35.3a		1.4a	42.3b	63.4b		1496a	

[a] Within a column and specific antioxidant compared to control, means without a common letter differ ($P < 0.05$). [b] Not determined. [c] 27.3 pg/well was significantly different from 35.9 pg/well, but neither differed from the control.

concentrations (0.008 mM EGCG and 0.006 mM EGCG equivalents, respectively), and the highest concentrations of tea showed higher $DCFH_2$ oxidation even from the first measuring point and quickly reached a plateau, ending up lower than the control, that is, suppressed $DCFH_2$ oxidation relative to the control. These effects increased over time (diverting curves), as indicated by significant ($P < 0.001$) time by dose interactions for all of the tested antioxidants.

Leukotriene Production. Leukotriene production was decreased by all tested concentrations of quercetin and catechin and increased by all tested concentrations of EGCG and the highest green tea extract concentration tested. Trolox and α-tocopherol did not affect leukotriene production at the tested concentrations.

DISCUSSION

Several groups of plant compounds including flavonoids have been suggested to contribute to the beneficial health effects of fruits and vegetables (*1*). Because these compounds possess antioxidative activities (*22*), they have often been evaluated for biological effects in model systems on the basis of their antioxidative activity or effects on the antioxidative system of animals or humans (*23*). However, compounds showing antioxidative effects may, in addition, have other biological effects such as enzyme regulation (*24*, *25*) or signaling functions (*26*, *27*).

In the present test system in which myotube cultures are exposed to hypotonic buffer, cell swelling is induced, which activates phospholipase A_2, and in turn increases the production of reactive oxygen species (ROS), 5-lipoxygenase (5-LO) products (e.g., leukotriene), and osmolyte release (*28*). The cellular redox tone has been suggested to regulate both taurine release (*29*) and 5-LO product formation (*30*), but also no-redox-type inhibitors have been shown to affect the cellular 5-LO activity in rat peritonal leukocytes (*22*). Earlier studies have also shown that the ring structure (*27*) and iron chelation/iron reducing capacity of flavonoids are important properties regarding inhibition of mammalian 5-LO activity (*31*).

Some of the flavonoids deemed to be quantitatively important in the diet, quercetin and catechins (*32*), were tested in the present model system together with the antioxidants α-tocopherol and Trolox. The concentrations of each compound were selected from preliminary studies of a wider concentration range on the basis of their effects on osmolyte release (taurine efflux), leukotriene production, and $DCFH_2$ oxidation because we wanted to test all three responses of some of the same concentrations of the specific compound.

Some researchers have warned that effects obtained using quercetin, catechins, and related phenolic compounds on cell culture may be an artifact (*33*, *34*), because addition of these compounds to, for example, DMEM, leads to generation of substantial amounts of hydrogen peroxide. In the present study DMEM is used as culture medium, but the artifacts should be of no significance, because the compounds are added to buffers as the cells are stressed by hypotonic conditions for limited time periods.

The flavonoids catechin and quercetin decreased leukotriene production and $DCFH_2$ oxidation at all of the tested concentrations, and taurine efflux was reduced, although less pronouncedly (only 0.33 mM cantechin and 0.11 mM quercetin decreased taurine efflux). Catechin and quercetin thus seem to reduce the stress-induced cellular responses tested. The decreased taurine efflux at 1 mM quercetin is likely to represent a toxic effect, because flavonoids such as quercetin (*35*) and

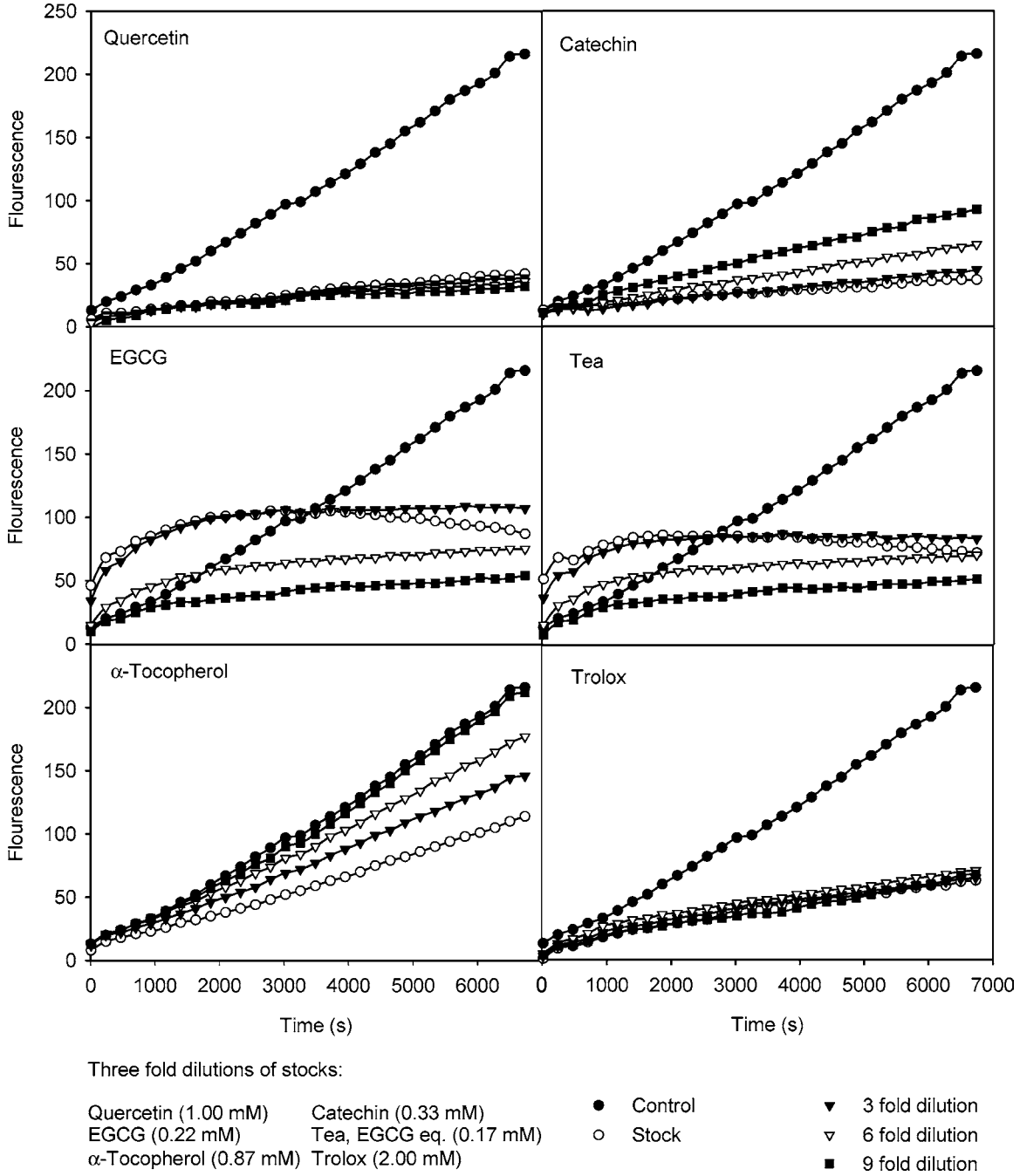

Figure 1. Means ($n = 9$) of oxidation of intracellular 2,7-dichlorofluorescein in C2C12 myotubes exposed to hypotonic buffer in the presence of quercetin, catechin, epigallocatechin gallate (EGCG), tea extract, α-tocopherol, and Trolox. LS means and SEM of three time points for each antioxidant are given in **Table 2**.

EGCG (*36*, *37*) are known to be toxic at high concentrations depending on the cell system and response criteria.

High concentrations of EGCG and tea extract increased leukotriene production and initial $DCFH_2$ oxidation, indicating an increased cellular stress. The third stress indicator, taurine efflux, pointed in the opposite direction. Taurine efflux was reduced at high concentrations, which indicates a reduction in the stress, and also longer exposure time as well as lower concentrations of EGCG and tea reduced $DCFH_2$ oxidation.

EGCG and tea at similar concentrations based on EGCG content showed similar responses regarding taurine efflux and $DCFH_2$ oxidation, indicating that EGCG in the tea extract is the main compound involved in the overall effect of the tea extract. This was not the case regarding leukotriene production, which was increased more by the pure EGCG samples compared to the tea extracts. Other components in the tea extract, for example, catechin, which showed a reducing effect on leukotriene production, do to some extent counteract the strong inducing effect of EGCG.

Trolox and α-tocopherol did not significantly affect taurine efflux or leukotriene production at any of the tested concentrations, whereas $DCFH_2$ oxidation was decreased. The DCF assay (*38*) is widely used, as an overall index of oxidative stress in cells, even though it remains unclear which oxidative species or other redox-active compounds are responsible for the oxidation of $DCFH_2$ to the fluorescent compound DCF. Compounds in cell culture medium have been reported to oxidize DCFH (*39*), and it has even been suggested that horseradish peroxidase (*40*) and other redox-active compounds (*41*) may oxidize DCFH. In the present study oxidation of

$DCFH_2$ is assayed in a buffer and should be independent of potential culture medium effects. However, if part of the DCF signal from the control cells without antioxidant addition is due to horseradish peroxidase activity, it is likely that part of the flavonoid-induced signal reduction is due to inactivation of this enzyme, because flavonoids are known to bind to proteins (*42*) and affect enzyme activities (*43*−*45*). The decreased oxidation of $DCFH_2$ caused by the flavonoids may thus not exclusively be ascribed to antioxidative activity, but because the antioxidants α-tocopherol and Trolox also inhibited oxidation of $DCFH_2$, the antioxidative properties of the compounds were of significant importance.

Trolox and α-tocopherol did not significantly affect taurine efflux or leukotriene production, and we therefore conclude that suppression of these responses was not confined to redox activity in a myotube culture.

ABBREVIATIONS USED

5-LO, 5-lipoxygenase; DCF, 2,7-dichlorofluorescein; DMEM, Dulbecco's modified Eagle's medium; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; FCS, fetal calf serum; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; Hepes, *N*-2-hydroxyethylpiperazin-*N*′-ethansulfonic acid; KHB, Krebs−Hepes buffer; LC-MS/MS, liquid chromatography−tandem mass spectrometry; MRM, multiple reaction monitoring; ROS, reactive oxygen species.

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