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The cocoa flavanol (−)-epicatechin protects the cortisol response

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ABSTRACT

Various health benefits of the cocoa flavanol (−)-epicatechin (EC) have been attributed to its antioxidant and anti-inflammatory potency. In the present study we investigated whether EC is able to prevent deterioration of the anti-inflammatory effect of the glucocorticoid (GC) cortisol in the presence of oxidative stress. It was found that cortisol reduces inflammation in differentiated monocytes. Oxidative stress extinguishes the anti-inflammatory effect of cortisol, leading to cortisol resistance. EC reduces intracellular oxidative stress as well as the development of cortisol resistance. This further deciphers the enigmatic mechanism of EC by which it exerts its anti-inflammatory and antioxidant action. The observed effect of the cocoa flavanol EC will especially be of relevance in pathophysiological conditions with increased oxidative stress and consequential GC resistance and provides a fundament for the rational use of dietary antioxidants.

1. Introduction

Flavanols are a group of flavonoids abundant in foods (e.g. apples and cocoa beans) and beverages (e.g. tea and wine). These flavanols are known to display anti-inflammatory as well as antioxidant effects and their intake is associated with beneficial health effects [1,2]. While the antioxidant effect is well documented, the mechanism of the anti-inflammatory effect is enigmatic.

Inflammation is a vital process to maintain tissue in a healthy condition and to fight off infections. During this process, monocytes are attracted to the site of inflammation, transformed into macrophages and an array of inflammatory mediators, such as the cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) and the chemokine interleukin-8 (IL-8), is produced [3–5]. These mediators further fuel inflammation by attracting more inflammatory cells and inducing the production of more inflammatory mediators. This feed-forward process is prone to overreact and progress into an unbalanced inflammatory pathological condition as seen in rheumatoid arthritis, inflammatory bowel disease, and chronic obstructive pulmonary disease (COPD).

Reactive oxygen species (ROS) play a role in signal transduction and are also produced during inflammation, e.g. to kill microorganisms. Unbalanced levels of ROS, however, cause oxidative stress which induces tissue damage, accelerated aging, lipid peroxidation and impairment of various cellular functions [6]. Oxidative stress can also trigger the immune response via the NF-kB pathway and activator protein-1 (AP1) resulting in the production of pro-inflammatory cytokines [7]. In particular the lungs are exposed to various oxidants and pollutants, especially when smoking, leading to even higher levels of inflammation and oxidative stress, which may develop into COPD [5].

Inflammation can be mitigated by glucocorticoids (GCs). The endogenous GC cortisol is produced by the adrenal gland and binds to the glucocorticoid receptor (GCR). The cortisol-GCR complex translocates to the nucleus where it activates the transcription of anti-inflammatory genes and suppresses pro-inflammatory gene transcription [8,9]. In COPD and patients with severe asthma, the anti-inflammatory potency of GCs is often drastically reduced. This is known as GC resistance, which aggravates the level of

Abbreviations: AP1, activator protein-1; COPD, chronic obstructive pulmonary disease; DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; EC, (−)-epicatechin; GC, glucocorticoid; GCR, glucocorticoid receptor; IL-8, interleukin-8; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; MH, monoHER, 7-mono-O-(β-hydroxyethyl)-rutoside; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PMA, Phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SRB, sulforhodamine B; TNF, tumor necrosis factor.

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inflammation and bears major clinical implications [10]. One of the clinical features of COPD is the occurrence of oxidative stress, which is best evidenced by the high levels of ROS in exhaled air [11,12]. Moreover, oxidative stress is known to impair receptor function [13,14].

In the present study we investigated if (−)-epicatechin is able to prevent the decline of the anti-inflammatory cortisol action induced by oxidative stress in human monocytes. The ability of epicatechin to preserve the endogenous glucocorticoid (GC) response of cortisol links its antioxidant activity with its anti-inflammatory properties.

2. Materials and methods

2.1. Chemicals

Phorbol 12-myristate 13-acetate (PMA), cortisol, lipopolysaccharides (LPS), 2′,7′-dichlorofluorescein-diacetate (DCFH-DA), sulforhodamine B (SRB), pyruvate, nicotinamide adenine dinucleotide (NADH), 2,4-dinitrophenylhydrazine (DNPH) and trichloric acid (TCA) were purchased from Sigma (St. Louis, MO, USA) and (−)-epicatechin (EC) was purchased from Extrasynthese (Genay, France). 7-Mono-O-(β-hydroxyethyl)-rutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). Roswell Park Memorial Institute 1640 (RPMI 1640) medium, fetal calf serum (FCS), phosphate buffered saline (PBS), penicillin, and streptomycin were obtained from Gibco (Life Technologies, Carlsbad, CA, USA).

2.2. Cell culture

The human monocyte cell line U937 (LGC Standards, Teddington, UK) was cultured in RPMI 1640 medium supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. Before exposure, cells were differentiated to macrophage like cells using 50 ng/ml PMA treatment for 4 h. After incubation, cells were plated in a 24 well plate (4×10^5 cells/well) and allowed to differentiate for 48 h. Medium was changed to 1% FCS for a further overnight incubation. Cells were washed with PBS and all exposures were performed in medium without FCS. Cell viability was determined after each experiment.

2.3. Cell viability

To investigate the potential cytotoxic effect of the exposures, LDH leakage was measured in the medium and the amount of cell protein was determined. Twenty microliters of medium was transferred to a 96 well plate to measure lactate dehydrogenase (LDH) activity. Fifty microliters of 10 mg/ml NADH in 0.75 mM sodium pyruvate was added to the medium and incubated for 30 min at 37 °C. To determine the remaining amount of pyruvate, 50 μl 0.2 mg/ml DNPH was added to the wells and incubated at room temperature in the dark for 20 min. To develop the color, 50 μl 4 M NaOH was added and absorbance was measured after 5 min at λ = 540 nm using a Spectramax plate reader (Molecular Devices, Sunnyvale, CA, USA) and LDH activity determined using a range of standards.

Cell amount was determined using the SRB assay [15]. Cells were washed and fixed with 7% (m/V) TCA for 1 h at 4 °C. After 5 wash steps with water, cells were stained with SRB (0.057%, m/V) for 20 min at room temperature. Cells were washed with 1% (V/V) acetic acid to remove unbound SRB and the SRB bound to cells was dissolved in 0.8 ml TRIS buffer (10 mM, pH 10.5) and quantified by measuring absorbance at λ = 540 nm. Viability was calculated relative to unexposed cells (100% viability) and cells exposed to 0.1% (V/V) triton X-100 (0% viability).

2.4. Inflammation

Oxidative stress was induced for 1 h by incubating differentiated monocytes with H_2O_2 (1 pmol/cell, which equals the amount of H_2O_2 epithelial lung cells are exposed to, based on the concentration found in the exhaled air in COPD patients [12]). EC (1, 10, and 50 μM) or monoHER (10 μM) was added during the incubation with H_2O_2. Control experiments demonstrated that there is no direct reaction between EC and H_2O_2 [2]. Cells were washed with PBS and incubated with LPS (1 ng/ml) ± cortisol (100 nM, unless otherwise stated) for 16 h. The cytokine concentrations in the medium were measured using a commercially available ELISA kit (Sanquin, Amsterdam, Netherlands) according to manufacturer's protocol.

2.5. Intracellular oxidative stress

The fluorescent probe of 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) was used to quantify intracellular oxidative stress in differentiated monocytes. In the presence of ROS, the non-fluorescent DCFH is oxidized to the highly fluorescent dichlorofluorescein (DCF). The intensity of DCF fluorescence corresponds to the level of intracellular ROS formation. Cells were differentiated in a black/clear bottom 96-wells plate and grown for 72 h. Fifty micromolar DCFH-DA was added to the cells and incubated for 30 min at 37 °C, 5% CO_2. After washing, cells were exposed to 100 μl serum-free medium containing H_2O_2 ± EC or monoHER and DCF fluorescence was measured with excitation and emission wavelengths of 485 nm and 535 nm, respectively, at 37 °C over a period of 60 min. Incubating cells with LPS (1 ng/ml) did not produce a significant increase in oxidative stress (data not shown).

2.6. Statistics

Data are expressed as mean ± SD or mean ± SEM (n = 3–12). Statistical comparisons were performed using one-way ANOVA with Bonferroni post hoc test with Prism v5 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. The anti-inflammatory effect of cortisol

First, the anti-inflammatory potential of cortisol was determined in monocytes differentiated to macrophages. The inflammatory response was induced by adding LPS (1 ng/ml) to the cells and quantified by measuring the concentration of IL-8 and TNF-α in the medium. Cortisol efficiently repressed the IL-8 and TNF-α production in a concentration dependent manner (Fig. 1). At a concentration of 100 nM, cortisol completely inhibited the LPS response.

3.2. Oxidative stress induces GC resistance

The cells were subjected to oxidative stress before the anti-inflammatory effect of cortisol was assessed. It was found that oxidative stress reduced the anti-inflammatory potency of cortisol over the entire concentration range tested (1–300 nM). Hundred nanomolar of cortisol only partially reduced the LPS induced IL-8 release (52%) after oxidative stress, while in the control experiment, without oxidative stress, the same concentration of cortisol fully blocked the pro-inflammatory LPS response (Fig. 1A). Without oxidative stress, 30 nM cortisol completely inhibited TNF-α release. When oxidative stress was induced, 30 nM cortisol only partially