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Epigallocatechin activates haem oxygenase-1 expression via protein kinase Cδ and Nrf2

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ABSTRACT

The Nrf2/anti-oxidant response element (ARE) pathway plays an important role in regulating cellular anti-oxidants, including haem oxygenase-1 (HO-1). Various kinases have been implicated in the pathways leading to Nrf2 activation. Here, we investigated the effect of epigallocatechin (EGC) on ARE-mediated gene expression in human monocytic cells. EGC time and dose dependently increased HO-1 mRNA and protein expression but had minimal effect on expression of other ARE-regulated genes, including NAD(P)H:quinone oxidoreductase 1, glutathione cysteine ligase and ferritin. siRNA knock down of Nrf2 significantly inhibited EGC-induced HO-1 expression. Furthermore, inhibition of PKC by Ro-31-8220 dose dependently decreased EGC-induced HO-1 mRNA expression, whereas MAP kinase and phosphatidylinositol-3-kinase pathway inhibitors had no significant effect. EGC stimulated phosphorylation of PKCαβ and δ in THP-1 cells. PKCδ inhibition significantly decreased EGC-induced HO-1 mRNA expression, whereas PKCα- and β-specific inhibitors had no significant effect. These results demonstrate for the first time that EGC-induced HO-1 expression occurs via PKCδ and Nrf2.

Catechins, a family of plant polyphenols, exert anti-oxidant, anti-inflammatory and anti-proliferative effects in vitro and in vivo [1,2]. This may be in part due to their activation of Phase II enzymes and cellular anti-oxidants, including NAD(P)H:quinone oxidoreductase 1 (NQO1), catalase and enzymes involved in glutathione synthesis [3–5]. These and other cytoprotective molecules, including haem oxygenase-1 (HO-1, the rate-limiting enzyme in haem catabolism) and the iron-binding protein ferritin, contain anti-oxidant response elements (ARE) in the regulatory regions of their genes. The transcription factor NF-E2-related factor 2 (Nrf2) binds to this regulatory element and plays a key role in ARE-driven gene transcription [6]. Nrf2 knockout mice are deficient in cellular anti-oxidants and are susceptible to inflammation [7,8], demonstrating the importance of this pathway in cellular defence. Several kinase pathways have been implicated in activation of Nrf2 including the ERK and p38 MAP kinase pathways, phosphatidyl inositol 3 kinase and protein kinase C (PKC) [9–14].

Green tea is a rich source of catechins, comprising over 30% of its dry weight [15]. Green tea extract activates ARE-mediated reporter activity [16]. EGCG is the most abundant catechin in green tea and studies to date have focused on this compound. EGCG stimulates expression of many Nrf2-dependent genes in liver and intestine in mice and activates HO-1 expression in B lymphoblasts, epithelial and endothelial cells [13,14,17,18]. Epigallocatechin (EGC) is also a major catechin in green tea [19]. Although EGCG and EGC are structurally similar except for the addition of a 3-gallate group in EGCG, their biological effects differ extensively [3,20–26]. EGC is also more bioavailable than EGCG and other catechins and oral administration of EGC, but not EGCG, results in a significant increase in plasma anti-oxidant activity [27].

Monocytes play an essential role in the host response to oxidative stress and inflammation. In the present study, the effects of EGC on ARE-mediated gene expression were examined in THP-1 cells, a human monocytic cell line that we have previously used to examine ARE-mediated gene expression [9–11]. In addition, the role of Nrf2 and the various potential kinase pathways leading to Nrf2 activation were investigated.

Materials and methods

Materials. LY333531 was purchased from Alexis Biotechnologies (Nottingham, UK). SB203580, Ro-31-8220, rottlerin, LY294002, Go6976 and PD98059 were obtained from Calbiochem (Nottingham, UK). (−)Epigallocatechin and all other chemicals were purchased from Sigma (Poole, UK).

Cell culture. THP-1, a human monocytic leukaemia cell line [28], was purchased from ECACC (Porton Down, UK) and cultured in

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RPMI 1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine (Biowhittaker, Wokingham, UK) and 2-mercaptoethanol. Cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Western immunoblotting. Cells (1×10^6) were unstimulated or stimulated with EGC and whole cell lysates prepared, proteins separated and immunoblotting carried out as previously described [11]. Antibodies were purchased from the following: mouse anti-human HO-1 antibody (Stressgen Biotechnologies Corporation, Victoria, Canada); rabbit anti-human phosphorylated PKCαβ and PKCδ antibodies (Cell Signalling Technology, Beverley, USA); mouse anti-human endogenous PKCδ antibody (BD Biosciences, CA, USA); goat anti-mouse and goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA); mouse anti-human β-actin antibody (Sigma).

Real-time PCR. Cells (5×10^5) were unstimulated or stimulated with EGC for various times at 37 °C. In some experiments, cells were pre-treated with kinase inhibitors for 30 min prior to EGC stimulation. RNA extraction, reverse transcription, and real-time PCR were carried out as previously described [10]. Relative quantitative mRNA expression of HO-1, NQO1, GCLM or ferritin was normalized to 18s ribosomal unit mRNA expression.

Nrf2 siRNA transfection. Nrf2 siRNA sense sequences 5′-GAGUAU GAGCUGGAAAAACtt-3′ (siNrf2 A) [29], 5′-CCUUAUAUCUCGAAG UUUUtt-3′ (siNrf2 B), their complementary antisense sequences and negative controls were obtained from Ambion as purified annealed duplexes. THP-1 cells (5×10^4/well) were transfected in serum-free media with control siRNA or Nrf2-targeted siRNA (30 nM final concentration), using Oligofectamine transfection reagent according to the manufacturer's instructions (Invitrogen). Transfected cells were incubated for 48 h, with addition of 10% FCS at 4 h. Following this, cells were stimulated with EGC for 4 h before RNA extraction and real-time PCR analysis.

PKCδ antisense oligodeoxyribonucleotide (ODN) transfection. THP-1 cells (5×10^4) were transfected in serum-free media with sense or antisense ODN to PKCδ, using Oligofectamine transfection reagent (Invitrogen), as previously described [10]. Following transfection, cells were unstimulated or stimulated with EGC for 4 h, total RNA extracted and real-time PCR performed.

Statistical analyses. Where indicated, statistical analyses were performed using paired Student's t test. Results are means ± SD of three independent experiments. Results with $p < 0.05$ were considered statistically significant.

Results

EGC increases HO-1 expression in THP-1 cells

EGC increased HO-1 mRNA expression in THP-1 cells, peaking at 4 h ($p < 0.01$), remaining elevated at 8 h ($p < 0.01$) and returning to baseline by 24 h (Fig. 1A). This correlated with an elevation in HO-1 protein expression by 4 h, which further increased by 8 h and decreased, but was still evident, at 24 h (Fig. 1B). EGC also time and dose dependently increased GCLM mRNA expression in THP-1 cells with maximal induction at 4 h. However, the induction was much weaker than that seen with HO-1 (GCLM at 4 h, 50 μM EGC, 2.5 ± 0.5, $p < 0.01$; 100 μM EGC, 3.0 ± 1.30, $p < 0.01$, mean fold increase above control ± SD, $n = 7$). EGC (12.5–100 μM) had no significant effect on either NQO1 or ferritin gene expression up to 24 h (data not shown). To ensure that EGC-induced HO-1 expression was not the result of toxicity, THP-1 cells pre-incubated with EGC for 24 h were analysed by MTT assay. At concentrations up to 125 μM THP-1 cells remained 96% (±2.3) viable compared to control cells, suggesting that the EGC concentrations used in this study did not exert cytotoxic effects.

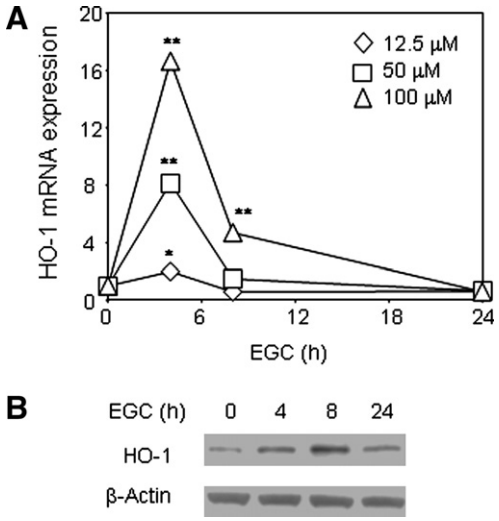

Fig. 1. EGC increases HO-1 expression. (A) THP-1 cells were treated with 0–100 μM EGC for 4, 8 or 24 h. RNA was extracted, reverse transcribed and real-time PCR performed for HO-1 mRNA expression. Mean ± SD, three independent experiments. Student's two-tailed t test, $^{*}p < 0.05$, $^{**}p < 0.01$. (B) THP-1 cells were treated with 100 μM EGC for indicated times, whole cell extracts prepared and Western blotting performed. Representative of three independent experiments.

Role of Nrf2 in EGC-induced HO-1 expression

Nrf2 plays a key role in HO-1 regulation. The role of Nrf2 in EGC-induced HO-1 expression was confirmed by use of siRNA. THP-1 cells were transiently transfected with control or two different Nrf2 siRNAs and Nrf2 mRNA expression measured by real-time PCR. Fig. 2A demonstrates that both Nrf2 siRNAs significantly inhibited Nrf2 mRNA expression to a similar extent when compared with the negative control (black bars, $p = 0.004$, $p = 0.0002$, respectively). These siRNAs also inhibited Nrf2 protein expression (data not shown). In addition, they significantly inhibited EGC-induced HO-1 mRNA expression (grey bars, A, 60.7 ± 8.9, $p = 0.01$; B, 66.1 ± 2.6, $p = 0.0007$; mean ± SD% inhibition, $n = 3$), confirming that Nrf2 plays a key role in this pathway in THP-1 cells.

Investigation of kinase pathways regulating EGC-induced HO-1 expression

THP-1 cells were pre-treated with LY294002 (a PI3K inhibitor), SB203580 (a p38 MAPK inhibitor), PD98059 (an ERK MAPK pathway inhibitor) or Ro-31-8220 (a pan-PKC inhibitor) prior to stimulation with EGC. LY294002, SB203580 and PD98059 had no

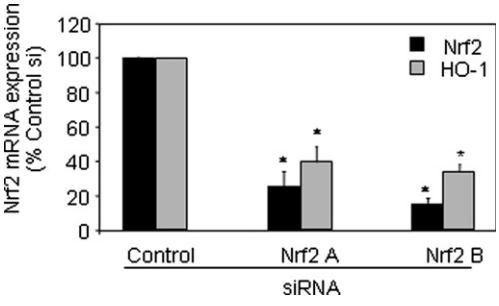

Fig. 2. Nrf2 regulates EGC-induced HO-1 mRNA expression. THP-1 cells were transfected with control or Nrf2-targeted siRNA in serum-free media for 4 h followed by addition of 10% FCS for 44 h. Following this, cells were stimulated with EGC for 4 h before RNA extraction, reverse transcription and real-time PCR analysis for Nrf2 or HO-1 mRNA expression. Mean ± SD three independent experiments. Student's two-tailed t test, $^{*}p < 0.05$.