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Cell-based screening assay for anti-inflammatory activity of bioactive compounds

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ABSTRACT

Excess dietary intake may induce metabolic inflammation which is associated with insulin resistance and cardiovascular disease. Recent evidence indicates that dietary bioactive compounds may diminish metabolic inflammation. To identify anti-inflammatory bioactives, we developed a screening assay using the human H293-NF-κB-RE-*luc2P* reporter cell line. Under optimised conditions we determined the anti-inflammatory activity of vegetables and purified bioactives, by monitoring their potency to inhibit TNF-α-induced NF-κB activity, as assessed by sensitive chemiluminescence detection in a 96-well assay format. Minced broccoli seedlings reduced NF-κB activity by 16%, while sulphoraphane, the dominant bioactive in broccoli seedlings, inhibited NF-κB activity with an IC_{50} of 5.11 μmol/l. Short-chain fatty acids also reduced NF-κB activity in the order butyrate > propionate ≫ acetate with IC_{50} of 51, 223, and 1300 μmol/l, respectively. The H293-NF-κB-RE-*luc2P* reporter cell line is a sensitive tool for rapid high-throughput screening for bioactives with anti-inflammatory activity.

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1. Introduction

An unbalanced diet, high in macronutrients and low in micronutrients, may lead to low-grade inflammation, acutely as well as chronically (Lopez-Garcia et al., 2004; Priebe, Wang, Weening, Schepers, Preston & Vonk, 2010). This metabolic inflammation is a risk factor for various pathological conditions, such as insulin resistance, type 2 diabetes and cardiovascular diseases. In contrast, both acute and chronic intake of several bioactive compounds, e.g. vitamins and phytochemicals, have been associated with anti-inflammatory effects, increased insulin sensitivity and decreased levels of vascular risk factors (Calder et al., 2011). Consumption of bioactive compounds has been associated with decreased adipose tissue inflammation and subsequent decreased risk of insulin resistance and atherosclerosis (Alves et al., 2012; Calder et al., 2011; Siriwardhana, Kalupahana, Cekanova, LeMieux, Greer & Moustaid-Moussa, 2013). These compounds comprise a wide range of biological molecules, such as flavonoids, carotenoids, plant sterols and isothiocyanates (Kris-Etherton, Lefevre, Beecher, Gross, Keen & Etherton, 2004). A rapid and convenient assay for screening this wide range of bioactives for anti-inflammatory activity and establishing dose–response curves, would be useful to select bioactive components as candidates for evaluation in human studies. Inflammation can be assessed by analysing the activity of the NF-κB pathway. Inactive NF-κB is normally sequestered in the cytosol by the inhibitor of κB, IκBα. Upon activation, e.g. by cytokines, such as TNF-α, the inhibitor of κB kinase (IKK) is phosphorylated, resulting in the degradation of Iκ-Bα, and thus dissociation of NF-κB. Unbound NF-κB translocates to the nucleus where it initiates the transcription of a wide range of pro-inflammatory cytokines, such as IL-6 and TNF-α (Pahl, 1999). The use of reporter cell lines to study NF-κB activation has been described before. Muriach, Bosch-Morell, Arnal, Alexander, Blomhoff and Romero (2008) and Paur et al. (2010) both used transfected human monocytic U937 cells with three κB response elements and Armoza, Haim, Bashiri, Wolak, and Paran (2013) used EA.hy296 cells (a hybrid of human endothelial HUVEC cells with human adenocarcinoma cells), transfected with a dual luciferase NF-κB reporter construct. Here, we study the commercially available H293-NF-κB-RE-*luc2P* reporter cell line for application as a screening assay for anti-inflammatory bioactives. This human embryonic kidney cell line (HEK293) contains five stably transfected NF-κB response elements in front of the promoter of an optimised luciferase reporter gene (*luc2P*). Induction of the *luc2P* gene leads to the subsequent production of the luciferase enzyme, which generates

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a proportional chemiluminescence signal when a substrate is added. Hence, the change in nuclear translocation of the NF-κB protein is directly proportional to the chemiluminescent signal and a measure for inflammatory activity. The advantages of this cell line for screening purposes are that it is readily available and easy to handle. It uses sensitive chemiluminescence detection and is applicable to a 96-well assay format, enabling high throughput screening. To determine the suitability of this cell line for our purpose, we studied the anti-inflammatory potency of sulphoraphane (SFN), lutein and short-chain fatty acids (SCFA), by screening for monitoring the inhibition of TNF-α-induced NF-κB activity. Moreover, we investigated the underlying mechanisms involved in SCFA inhibition of NF-kB signalling.

SFN is an isothiocyanate derived from hydrolysed glucoraphanin in cruciferous vegetables. Previously, we showed that the SFN precursor glucoraphanin is already present in broccoli seeds and dilutes in the growing plant, since there is no *de novo* biosynthesis during early plant growth (Gorissen, Kraut, de Visser, de Vries, Roelofsen & Vonk, 2011). SFN is widely studied for its antioxidant and anti-cancer effects (Guerrero-Beltrán, Calderón-Oliver, Pedraza-Chaverri, & Chirino, 2012; Juge, Mithen, & Traka, 2007). Additionally, SFN has been shown to exhibit anti-inflammatory effects, both indirectly via the nuclear factor E2-related factor (Nrf2) pathway (Lin, Wu, Wu, Khor, Wang & Kong, 2008) and directly by affecting the toll-like receptor 4 (TLR4) and the NF-κB pathway (Heiss, Herhaus, Klimo, Bartsch, & Gerhauser, 2001; Koo, Park, Kim, & Lee, 2013; Moon, Kim, Kang, Choi, & Kim, 2009; Youn et al., 2010). Lutein is a carotenoid found in green leafy vegetables, like spinach and kale. Lutein has antioxidant activity and is thought to be preventive against macular degeneration. It has been reported to reduce NF-κB activity more than 50% (Krinsky, Landrum, & Bone, 2003; Kim et al., 2008). Furthermore, it was shown that lutein was able to reduce combined LPS and high-glucose-induced NF-κB activation in a human macrophage reporter cell line (Muriach et al., 2008).

SCFA, such as acetate, butyrate and propionate, are bioactive compounds produced during colonic fermentation of dietary fibre. SCFA are studied as anti-cancer agents, but are also shown to activate PPAR-γ in colon cells (Alex et al., 2013). SCFA exert anti-inflammatory effects (Liu et al., 2012) and evidence suggests that they may play a preventive role in adipose tissue inflammation and insulin resistance (Meijer, de Vos, & Priebe, 2010; Roelofsen, Priebe, & Vonk, 2010). SCFA effects are mediated by signalling via the G-protein-coupled receptors GPR41, GPR43 and GPR109a (Brown et al., 2003; Thangaraju et al., 2009), but may also exert effects intracellularly, since SCFA can be transported into the cell by the sodium-coupled monocarboxylate transporter-1 (SLC5a8) (Ganapathy, Gopal, Miyauchi, & Prasad, 2005).

Establishing an accessible and rapid cell-based screening assay for the anti-inflammatory effects of bioactive compounds will help to identify promising bioactives for further testing in human intervention studies. We show that the H293-NF-κB-RE-*luc2P* reporter cell line is a sensitive tool for rapid screening for bioactives with anti-inflammatory activity.

2. Materials and methods

2.1. Assay materials

H293-NF-κB-RE-*luc2P* cells and the luciferase assay kit were obtained from Promega (Promega, Fitchburg, WI, USA). Low-glucose DMEM, hygromycin B, fetal bovine serum (FBS) and penicillin–streptomycin were obtained from Invitrogen (Life Technologies, Carlsbad, CA, USA). As model compounds we used TNF-α (R&D Systems, Minneapolis, MN, USA); sulphoraphane (SFN, Santa Cruz Biotechnology, Dallas, TX, USA); lutein (Extrasynthese, Genay, France); butyrate, propionate, acetate, and pertussin toxin (Sigma Aldrich, Saint-Louis, MO, USA); broccoli seedlings (BroccoCress, Koppert Cress, Monster, The Netherlands) and butter lettuce (Albert Heijn, Groningen, The Netherlands). The assay was performed in 96-well luminescence assay plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) and luminescence was determined with an FL_x800 microplate reader (BioTek, Winooski, VT, USA).

2.2. Cell culture and stimulation

Human Embryonic Kidney 293 (HEK293) cells – stably transfected with a luciferase gene (*luc2P*) under the control of a minimal TATA promoter with five NF-κB response elements – were maintained in low-glucose DMEM, supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C and 5% CO_2. The transfected cells contain a hygromycin resistance gene. Therefore, 50 μg/ml hygromycin B was added for selective pressure. After growing to confluency, cells were cultured in medium without hygromycin B for 24 h. The cells were then trypsinyzed and 6800 cells/well were seeded in the assay plate and stimulated with TNF-α for 3 h in medium without hygromycin B, with or without FBS. Simultaneously with TNF-α, cells were incubated with either minced vegetables or purified bioactive compounds. Minced broccoli seedlings and lettuce was prepared by mechanically chopping 8 g of broccoli seedlings or lettuce for 45 s in 250 ml tap water. These mixtures were centrifuged for 10 min at 18,000×*g* and diluted 1:1000. Purified bioactive compounds were incubated simultaneously with TNF-α in the following concentrations: 0.5, 1, 2.5, 5, 7.5, 10 or 15 μmol/l SFN; 0.1, 0.5, 1, 2, 5, 7.5, 10 or 15 μmol/l lutein; 0.01, 0.05, 0.1, 0.5, 1, 2 or 5 mmol/l butyrate or propionate; 0.1, 0.5, 1 or 5 mmol/l acetate. To test the involvement of $G_{i/o}$ signalling by GPCRs, $G_{i/o}$ signalling was blocked by incubating with 100 ng/ml pertussin toxin (PTX). After incubation, cells were lysed and luminescence was determined.

2.3. Quantitative reverse-transcriptase PCR

For analysis of gene expression of SCFA receptors and transporter, total RNA was isolated using an RNeasy kit (Qiagen, Venlo, The Netherlands) and cDNA was prepared using the Quantitect Reverse Transcription kit (Qiagen). Primers were obtained from Biolegio (Biolegio, Nijmegen, The Netherlands) and ready-to-use primer mixes were obtained from Applied Biosystems (Table 1). The qRT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) in a total reaction volume of 10 μl. The PCR conditions were 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative gene expression levels were determined in triplicate on an ABI9700HT (Applied Biosystems by Life Technologies Corp. Carlsbad, CA, USA).

2.4. LC–MS/MS analysis

SFN content of broccoli seedlings and lettuce preparations was determined using LC–MS/MS. SFN concentrations were determined against an external SFN calibration. A 1 ml aliquot of each preparation was centrifuged for 10 min at 16,000×*g* at room temperature. The supernatant was 1000 times diluted and 25 μl was injected into the LC–MS/MS. For liquid chromatography (Shimadzu Prominence UFLC; Shimadzu, Nakagyo-ku, Kyoto, Japan) the following solvents were used: solvent A, ultrapure water; solvent B, acetonitrile (Biosolve, Valkenswaard, The Netherlands), both containing 0.1% v/v formic acid (Fluka, Sigma–Aldrich, St.-Louis, MO, USA). The flow rate was 0.3 ml/min over a 150 × 2 mm 18C synergy 4μ hydro-RP-80a column (Phenomenex, Torrance, CA, USA). The gradient was as follows: 10% solvent B at 0 min to 90% solvent B at

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