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Polyphenol extracts from dried sugarcane inhibit inflammatory mediators in an *in vitro* colon cancer model

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ABSTRACT

Sugarcane is an important crop grown in tropical regions for sugar, and for ethanol production. Sugarcane is also a source of phytochemicals but its nutraceutical potential has been under-explored. We show that ethanol extracts of whole dried sugarcane (WDS) recovers a rich content of polyphenols, flavonoids and antioxidant activity that act on inflammatory mediator proteins. To investigate the mechanisms of this activity, we stimulated SW480 colon cancer cells with lipopolysaccharide, exposed cells to WDS and quantitated changes to the proteome and phosphoproteome using label-free mass spectrometry. The grape-derived anti-inflammatory polyphenol, resveratrol (RSV) was used as a control. Using SWATH-MS we quantitated ~3000 proteins showing that WDS significantly altered the expression of the oxidative stress regulator SELH. WDS induced changes in protein expression predicted the involvement of NFκB pathway members. Reduced NFκB phosphorylation and IL-8 secretion confirmed this effect. In contrast, RSV was predicted to act primarily through modulation of the PI3K/AKT pathway. Phosphoproteomics studies indicate that WDS interfered in the phosphorylation of cell stress regulators c-Jun, EGFR, PKA, PKCβ and SIRT1. Confirmed through pharmacological inhibition, kinase enrichment analysis presented C-Raf to modulate WDS activity. These results demonstrate the anti-inflammatory utility of WSD and define aspects of its mechanisms of action.

Significance: Despite the increasing interest of nutraceuticals in health promotion, scientific evidence proving the molecular mechanisms involved is still lacking. This study investigated some of the mechanistic aspects of *in vitro* use of whole dried sugarcane extracts in the context of regulating cellular inflammation by using proteomics and phosphoproteomics strategies. We determined that WDS extracts regulate key inflammatory pathways including NFκB, while kinase enrichment analysis from phosphoproteomics demonstrated a role for C-Raf in controlling this mechanism. We demonstrated that the mechanism of WDS extracts on controlling inflammation differs from that of the polyphenol, resveratrol. The results presented herein contribute towards unravelling the activity of nutraceuticals extracted from sugarcane.

1. Introduction

Sugarcane is a cultivar of great economic importance grown in the tropics for sugar and for ethanol production. Although major economic and social development has been associated with the farming of sugarcane, there is interest to explore other uses of this crop including its potential for nutraceutical purposes. Sugarcane is known to possess chemical agents associated with positive health benefits such as polyphenols, flavonoids, phytosterols and triterpenoids [1]. Sugarcane derivatives obtained during sugar production including juice, molasses and syrup are also important sources of phenolics with strong antioxidant activity [2].

Nutraceuticals and functional foods have gained attention in recent times with a particular focus on prevention and treatment of inflammatory conditions [3]. Many *in vitro* and *in vivo* studies have demonstrated anti-inflammatory activity of polyphenols and flavonoids linked in part to their antioxidant properties [4]. For example, the red grape derived polyphenol resveratrol (RSV) is well regarded for its potent anti-inflammatory and anti-carcinogenic properties due to its ability to effectively modulate signalling cascades associated with

Abbreviations: WDS, whole dried sugarcane; RSV, resveratrol; TPC, total polyphenol content; TFC, total flavonoid content; LPS, lipopolysaccharides; SWATH, sequential window acquisition of all theoretical fragment-ion spectra

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inflammation [5].

The inflammatory response is mediated by a range of sensors and effectors involving receptors, signalling cascades and secreted effector proteins [6]. Lipopolysaccharides (LPS) from bacterial membranes are sensed by toll-like receptors (TLR) invoking the master regulator NFκB pathway and activator protein 1 (AP-1) transcription factor. Phosphorylation and subsequent nuclear translocation of these transcription factors leads to the activation of pro-inflammatory target genes such as TNFα, IL-1, IL-8 among others [7,8]. There is much interest in therapeutic approaches to regulate the expression of these pro-inflammatory mediators [9].

Although widely used in biomedicine, the use of `omics techniques such as genomics, transcriptomics, and proteomics is still emerging for investigations of functional foods. Mass spectrometry (MS)-based proteomics is a useful tool for investigating cellular responses to compounds, including food and dietary products [10]. For example, omics studies have been used to examine the anti-inflammatory effect associated with using curcumin in an *in vivo* model of inflammatory bowel disease (IBD) [11]. Among MS approaches for quantitation of protein abundances, data independent acquisition (DIA) techniques such as sequential window acquisition of all theoretical fragment-ion spectra (SWATH) is gaining in use due its highly reproducible quantitative properties coupled with deep proteomic profiling of complex samples [12]. MS-based mapping of phosphoproteins has also demonstrated great potential in dissecting cell signalling pathways which rely on reversible protein phosphorylation to regulate function [13].

Two-dimensional gel electrophoresis and MS-based proteomics have shown some benefit in investigating the sugarcane proteome [14,15]. Proteomics studies have determined the protein expression of sugarcane as a result of water [16] and salt stress [17]; protein response to biotic agents such as fungal [18] and bacterial infection [19] has also been explored. Despite these recent advances in the sugarcane proteomics field, its nutraceutical utility on the human proteome has not been assessed.

In this study, we investigated the nutraceutical properties of WDS extracts using an *in vitro* model of gut inflammation. LPS-stimulated SW480 colon cells were exposed to WDS ethanol extracts rich in polyphenols, flavonoids and antioxidant activity. SWATH-MS and phosphoproteomics mapping were used to investigate the cellular mechanisms associated with the anti-inflammatory activity on the extracts, this was compared with the activity of the well-known polyphenol, RSV. This study revealed distinct responses of WDS and RSV to mediate anti-inflammatory activities in these cell and demonstrates the nutraceutical potential of WDS to elicit favourable changes to control inflammatory responses.

2. Methods

2.1. WDS extraction

WDS (provided by Gratuk Technologies Sydney, Australia) was obtained from whole sugarcane (strain Q208) grown in the Burdekin region of Queensland, Australia. Briefly, fresh sugarcane was collected from a controlled field, cut into billets and transferred to cool (< 10 °C) storage for processing. Billets were processed within 24 h of cutting. The billets underwent multiple size reduction steps using sheer force to disrupt the cell wall until particle size was approximately 10 mm. The sugarcane was steeped using a proprietary technology to remove sucrose while maintaining micronutrient content. Water/sugar solution was removed by centrifugation and the particles dried using proprietary technology by exposing the sugarcane to super-dried air. The dried particles were milled using a gap mill until particle size was < 50 μm. WDS and raisins (Coles, Australia) extraction was performed in 52% ethanol in an ultra-sonic water bath at 60 °C for 30 min as reported by Feng et al. [1]. Suspended WDS was centrifuged (5000 rpm, 5 min), supernatant was collected and immediately characterised. Cranberry juice (Ocean Spray Intl.) was used neat.

2.2. Total polyphenol content (TPC)

TPC was determined as described previously [20]. Briefly, 20 μL of sample mixed with 1.5 mL of water and 100 μL of the Folin-Ciocalteu reagent (Sigma Aldrich) was incubated for 6 min. After addition of 300 μL 7.5% Na_2CO_3 sample was incubated for 2 h. Gallic acid was used as a standard over the range of 25–500 mg/L. Optical density was read at 765 nm and results expressed in mg of gallic acid equivalents (GAE)/100 g extract.

2.3. Total flavonoid content (TFC)

TFC was determined as described previously [21]. Briefly, 250 μL of sample mixed with 1.2 mL of water and 75 μL 5% $NaNO_2$ was incubated for 6 min. After addition of 150 μL 10% $AlCl_3$ sample was incubated for 5 min and added 0.5 mL 1 M NaOH. (+)-catechin was used as a standard at over the range of 50–500 mg/L. Optical density was read at 510 nm and results expressed in mg of (+)-catechin equivalents (CE)/100 g extract.

2.4. Antioxidant potential, ferric reducing ability potential (FRAP) assay

FRAP assay was determined as described previously [22]. Briefly, FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridil-s-triazine (TPTZ) solution and 20 mM $FeCl_3$ at a 10:1:1 ratio. 20 μL of sample was mixed with 0.2 mL of water and 1.8 mL of FRAP reagent and incubated for 10 min at 37 °C. Ferrous sulphate served as a standard over the concentration range of 125–2500 μM. Optical density was measured at 593 nm and results were expressed in mM $FeSO_4$/kg.

2.5. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity

DPPH assay was determined as described previously [23]. Briefly, 80 μL of sample was mixed with 3 mL of 0.1 mM DPPH in 80% methanol and incubated for 10 min at room temperature. Ascorbic acid served as the standard over the concentration range of 10–100 mg/L. Optical density was determined at 517 nm and results were expressed in mg ascorbic acid equivalents (AAE)/100 g extract.

2.6. Cell culture

Colon carcinoma cell line SW480 cultured in RPMI 1640 medium supplemented with L-glutamine (Thermo Fisher Scientific), fetal bovine serum (Life Technologies) and 1% antibiotics (penicillin, 100 μg/mL and streptomycin, 100 μg/mL) was incubated at 37 °C and 5% CO_2.

Polyphenol content of WDS was adjusted to 50 μM GAE, dried and resuspended in medium before treatment. RSV (Sigma Aldrich) was dissolved in dimethyl sulphoxide (Sigma Aldrich). LPS (5 μg/mL) from *Escherichia coli* (Sigma Aldrich) was added to the medium for 4 h before addition of WDS or RSV for 20 h still in the presence of LPS. For phosphoproteomics analysis, LPS stimulation lasted for 2 h followed by WDS or RSV exposure for 4 h. When indicated, the specific C-Raf inhibitor GW5074 (Selleckchem) was co-incubated with WDS at varied concentrations (0.5, 1 and 5 μM).

After cell harvesting, lysis was performed on 1% sodium deoxycholate and heated at 95 °C for 3 min. Benzonase (0.5 μL) was added and incubated at room temperature for 30 min. Protein concentration was determined using the bicinchoninic acid assay (560 nm).

2.7. Western blot and ELISA

Extracted protein from cells (10–15 μg) were reduced in 10 mM DTT, loaded on a sodium dodecyl sulphate – polyacrylamide gel