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Effects of pelargonidin-3-O-glucoside and its metabolites on lipopolysaccharide-stimulated cytokine production by THP-1 monocytes and macrophages

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Abbreviations: 4-HBA, 4-hydroxybenzoic acid; CVD, cardiovascular disease; FCS, fetal calf serum; IL, interleukin; LPS, lipopolysaccharide; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde; PMA, phorbol-12-myristate-13-acetate; TNF-α, tumor necrosis factor-α;

Abstract

Epidemiological evidence suggests cardioprotective effects of anthocyanin consumption. This study examined the predominant strawberry anthocyanin, pelargonidin-3-O-glucoside (Pg-3-glc), and three of its plasma metabolites (protocatechuic acid [PCA], 4-hydroxybenzoic acid, and phloroglucinaldehyde [PGA]) for effects on the production of selected cytokines by lipopolysaccharide-stimulated THP-1 monocytes and macrophages. Concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8 and IL-10 were determined using a cytometric bead array kit. PCA at 0.31, 1.25 and 20 μM and PGA at 5 and 20 μM decreased the concentration of IL-6 in the monocyte cultures, but there were no effects on TNF-α, IL-1β, IL-8 and IL-10 and there were no effects of the other compounds. In the macrophage cultures, PGA at 20 μM decreased the concentrations of IL-6 and IL-10, but there was no effect on TNF-α, IL-1β and IL-8 and there were no effects of the other compounds. In conclusion, while the effects of PGA were only observed at the higher, supraphysiological concentration and are thus considered of limited physiological relevance overall, the anti-inflammatory properties of PCA were observed at both the lower, physiologically relevant, and the higher concentrations; however, effects were modest and limited to IL-6 and monocytes. These preliminary data suggest potential for physiologically attainable PCA concentrations to modulate IL-6 production by monocytes.

Keywords: Anthocyanin, Cytokine, Inflammation, Pelargonidin-3-O-glucoside, Strawberry

2.1 Introduction

Epidemiological evidence links anthocyanin consumption with lower risk of cardiovascular disease (CVD) [1] and CVD risk factors [2]. The mechanisms are not yet fully elucidated, but evidence suggests modulation of vascular function, platelet aggregation and inflammation [3-9]. TNF-α, IL-1β, IL-6, IL-8 and IL-10 are inflammatory cytokines that play a critical role in atherosclerosis, the underlying cause of most CVDs [10].

Strawberries are particularly rich in anthocyanins, predominantly pelargonidin-3-O-glucoside (Pg-3-glc). Glucuronidated pelargonidin has been reported as the predominant metabolite in three pharmacokinetic studies [11-13], but there is ambiguity regarding the position of glucuronidation and glucuronidated pelargonidin compounds are currently commercially unavailable and hence cannot be tested in cellular models. 4-hydroxybenzoic acid (4-HBA) and protocatechuic acid (PCA) have also been reported in plasma following strawberry consumption in low micromolar concentrations (0.1-2 μM) [13-15]. In addition, it is likely that phloroglucinaldehyde (PGA) might appear in plasma following strawberry consumption, as it is a A-ring degradant, reported in plasma upon anthocyanin consumption in low to high nanomolar concentrations (20-600 nM) [16, 17]. However, most studies exploring the effect of anthocyanins to modulate cytokine secretion used unmetabolized parent anthocyanins, often at supraphysiologically high doses [3, 4]. In addition, although macrophages are an important source of inflammatory cytokines [18], there are no studies exploring the potential modulation of cytokine production by anthocyanins or their metabolites by human-derived macrophages, or studies comparing the effects in human-derived monocytes versus macrophages. Thus, the aim of this study was to examine the parent anthocyanin Pg-3-glc and three physiologically relevant plasma metabolites for effects on the production of selected

pro- and anti-inflammatory cytokines (tumor necrosis factor-α [TNF-α], interleukin [IL]-1β, IL-6, IL-8 and IL-10) in lipopolysaccharide (LPS)-induced THP-1 cells.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Pg-3-glc was purchased from Extrasynthese (Genay, France). PCA (3,4-dihydroxybenzoic acid), 4-HBA, PGA (2,4,6-trihydroxybenzaldehyde), LPS from *Escherichia coli*, phorbol 12-myristate 13-acetate (PMA), methanol and formic acid were purchased from Sigma-Aldrich (Dorset, United Kingdom). RPMI 1640 culture medium, fetal calf serum (FCS), penicillin and streptomycin were purchased from Lonza (Basel, Switzerland). The cytometric bead array kit to analyze cytokine concentrations was purchased from BD Biosciences (Oxford, United Kingdom).

2.2.2 Preparation and culture of THP-1 cells

THP-1 cells (human monocytic leukemia, ECACC 88081201) were cultured in RPMI 1640 culture medium supplemented with 100 UI/mL streptomycin, 100 µg/mL penicillin and 10% (v/v) FCS at 37 °C in a humidified atmosphere of 5% CO_2 and kept at a density of 2-9 x 10^5 cells/mL. For the experiments, cells were seeded in 24 well plates at a density of 1 x 10^6 cells/mL. For differentiation into macrophages, cells were exposed to PMA at a final concentration of 0.1 µM for 72 h [19]. Polyphenols were added to provide final concentrations of 0.08, 0.31, 1.25, 5 and 20 µM. Polyphenols were added in 20 µL of 10.98% methanol and 0.22% formic acid to produce a final concentration of 0.22% methanol and 0.004% formic acid in culture. The final culture volume was 1 mL. LPS (20 µL; 1 µg/mL final concentration) was added to stimulate cytokine production.

After 24 h incubation at 37 °C in a humidified atmosphere of 5% CO_2, plates were centrifuged at 260 x g for 5 min and culture supernatants were collected and stored in aliquots at −20 °C until analysis.

2.2.3 Measurement of cytokine concentrations

Concentrations of TNF-α, IL-1β, IL-6, IL-8 and IL-10 in the culture supernatants were measured using a cytometric bead array kit from BD Biosciences (Oxford, United Kingdom) according to the manufacturer's instructions. Data were acquired on a BD FACS Canto™ II flow cytometer and analyzed using the BD FCAP Array v3 software. Limits of detection of the cytokine assays were 0.13 pg/mL (IL-10), 1.2 pg/mL (TNF-α and IL-8), 1.6 pg/mL (IL-6) and 2.3 pg/mL (IL-1β).

2.2.4 Cytotoxicity

To determine whether test compounds had any cytotoxic effects, cell viability of THP-1 monocytes and macrophages was assessed using Trypan blue staining.

2.2.5 Statistical analyses

Results are expressed as percentage of cytokine production versus control (no polyphenols) and shown as means with their standard deviations (SD). One-way ANOVA was performed to determine whether test compounds affected the cytokine production, followed by Dunnett as post hoc analysis versus control group. Statistical analysis was performed using SPSS 21 (IBM Corporation, New York, USA) and a lowered $P<0.01$ was considered significant to account for multiple comparisons.

2.3 Results

2.3.1 Effects of Pg-3-glc, PCA, 4-HBA and PGA on viability of THP-1 cells

There were no cytotoxic effects of the studied compounds on THP-1 monocytes or macrophages at any of the tested doses (data not shown).

2.3.2 Effects of Pg-3-glc, PCA, 4-HBA and PGA on cytokine production by THP-1 monocytes

Stimulation with LPS increased IL-1β production 135-fold, TNF-α production 105-fold, IL-6 production 470-fold, IL-8 production 640-fold and IL-10 production 5-fold. PCA significantly reduced IL-6 production at 0.31, 1.25 and 20 μM compared to the control cultures (all $P<0.01$, Table 1). PGA also significantly inhibited IL-6 production and while the effects were only significant at 5 and 20 μM ($P<0.01$ and $P<0.001$ respectively, Table 1), they were slightly more potent (production was lowered by 25-35% compared to 20% for PCA) and appeared to be concentration-dependent. There was no significant effect of any tested compound, at concentrations up to 20 μM, on the production of IL-1β, TNF-α, IL-8 or IL-10 by THP-1 monocytes (Table 1).

2.3.3 Effects of Pg-3-glc, PCA, 4-HBA and PGA on cytokine production by THP-1 macrophages

Stimulation with LPS increased IL-1β production 10-fold, TNF-α production 45-fold, IL-6 production 220-fold, IL-8 production 80-fold and IL-10 production 5-fold. PGA was the only compound that induced significant changes in cytokine production. PGA at the highest dose

125 tested (20 μM) significantly lowered IL-6 production (P<0.001, Table 2), similar to the
126 monocyte results and with a similar magnitude of effect. Furthermore, it also significantly
127 decreased IL-10 production (20 μM; P<0.01; Table 2), an effect not observed in THP-1
128 monocytes. There was no significant effect of any of the tested compounds, at concentrations
129 up to 20 μM, on the production of IL-1β, TNF-α or IL-8 by THP-1 macrophages (Table 2).

Table 1 Effect of Pg-3-glc, PCA, 4-HBA and PGA on IL-1β, TNF-α, IL-6, IL-8 and IL-10 production by THP-1 monocytes

	Cytokine production (% of control)				
	IL-1β	**TNF-α**	**IL-6**	**IL-8**	**IL-10**
Pg-3-glc					
0 µM	100	100	100	100	100
0.08 µM	91.8 ± 18.9	114.2 ± 20.5	85.6 ± 17.5	108.6 ± 15.6	113.4 ± 12.4
0.31 µM	92.7 ± 10.7	110.8 ± 20.3	78.2 ± 13.2	102.3 ± 19.6	104.0 ± 8.9
1.25 µM	93.2 ± 8.4	112.9 ± 20.1	78.4 ± 11.8	96.1 ± 13.0	102.9 ± 10.4
5.00 µM	92.3 ± 9.6	106.4 ± 19.4	84.5 ± 10.4	101.9 ± 12.1	102.5 ± 8.0
20.00 µM	96.4 ± 13.9	101.1 ± 20.0	88.5 ± 18.6	103.9 ± 19.4	98.0 ± 4.9
PCA					
0 µM	100	100	100	100	100
0.08 µM	94.5 ± 7.8	103.5 ± 18.9	87.3 ± 7.0	110.3 ± 13.0	107.0 ± 6.9
0.31 µM	97.7 ± 7.5	93.4 ± 15.3	77.8 ± 9.7 **	98.2 ± 7.1	104.6 ± 5.9
1.25 µM	104.9 ± 5.2	92.9 ± 10.7	77.2 ± 10.2 **	106.0 ± 9.0	105.4 ± 7.0
5.00 µM	95.2 ± 12.8	85.7 ± 7.9	88.3 ± 15.9	100.5 ± 14.9	99.0 ± 9.5
20.00 µM	89.3 ± 19.3	85.9 ± 11.0	79.8 ± 14.1 **	102.4 ± 10.1	93.6 ± 7.6
4-HBA					
0 µM	100	100	100	100	100
0.08 µM	83.4 ± 7.6	104.2 ± 21.2	99.1 ± 14.5	94.0 ± 18.2	99.9 ± 8.6
0.31 µM	97.7 ± 10.4	109.3 ± 22.5	90.9 ± 5.1	91.3 ± 15.7	104.8 ± 8.9
1.25 µM	97.3 ± 15.3	110.4 ± 26.4	91.9 ± 3.6	90.9 ± 16.5	101.8 ± 6.9
5.00 µM	100.2 ± 8.6	107.2 ± 19.5	85.1 ± 21.6	93.5 ± 17.8	103.7 ± 8.0
20.00 µM	105.7 ± 13.0	114.2 ± 16.5	93.4 ± 25.0	90.2 ± 22.2	102.3 ± 7.1
PGA					
0 µM	100	100	100	100	100
0.08 µM	97.2 ± 11.6	111.4 ± 21.3	90.0 ± 16.2	103.2 ± 13.7	110.6 ± 13.1
0.31 µM	102.1 ± 7.4	107.5 ± 21.4	91.9 ± 17.2	96.8 ± 21.1	104.6 ± 11.0
1.25 µM	106.5 ± 11.8	112.9 ± 18.9	79.7 ± 10.6	97.0 ± 13.8	101.2 ± 9.0
5.00 µM	110.4 ± 12.3	117.0 ± 22.1	73.6 ± 6.9 **	95.2 ± 20.6	97.1 ± 9.2
20.00 µM	106.2 ± 12.6	111.6 ± 21.7	65.9 ± 17.6 ***	94.6 ± 19.3	95.6 ± 6.1

THP-1 monocytes (1 x 10^6 cells/mL) were treated with Pg-3-glc, PCA, 4-HBA, PGA or vehicle control at concentrations of 0-20 µM, prior to lipopolysaccharide stimulation (1 µg/mL) and incubated for 24 h at 37 °C. Cytokine concentrations in the culture supernatants were measured using a cytometric bead array kit. Results are expressed as percentage of cytokine concentration vs. control (no polyphenols). Data are represented as the mean ± SD of six independent experiments. Data were analyzed by one-way ANOVA and Dunnett post hoc analysis, where applicable, and a lowered $P<0.01$ was considered significant to account for multiple comparisons. Statistically significant differences are denoted as $^{**}P<0.01$ vs. control; $^{***}P<0.001$ vs. control. 4-HBA, 4-hydroxybenzoic acid; IL, interleukin; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde; TNF-α, tumor necrosis factor-α.

Table 2 Effect of Pg-3-glc, PCA, 4-HBA and PGA on IL-1β, TNF-α, IL-6, IL-8 and IL-10 production by THP-1 macrophages

	Cytokine production (% of control)				
	IL-1β	**TNF-α**	**IL-6**	**IL-8**	**IL-10**
Pg-3-glc					
0 μM	100	100	100	100	100
0.08 μM	113.2 ± 18.8	117.3 ± 31.0	113.5 ± 24.7	111.4 ± 12.8	100.9 ± 17.4
0.31 μM	111.6 ± 15.1	115.8 ± 22.2	108.0 ± 21.6	106.5 ± 12.2	87.3 ± 15.1
1.25 μM	108.9 ± 12.3	112.4 ± 16.0	101.4 ± 24.1	105.8 ± 16.9	88.0 ± 16.8
5.00 μM	107.8 ± 16.7	110.5 ± 24.5	101.6 ± 20.1	103.4 ± 16.1	80.1 ± 13.5
20.00 μM	99.2 ± 14.7	106.0 ± 18.1	107.7 ± 19.8	102.9 ± 11.8	84.3 ± 16.0
PCA					
0 μM	100	100	100	100	100
0.08 μM	102.7 ± 9.3	117.2 ± 23.2	116.2 ± 25.2	106.5 ± 16.1	100.8 ± 12.0
0.31 μM	103.8 ± 10.0	101.0 ± 18.6	111.7 ± 22.9	104.4 ± 14.1	85.0 ± 24.9
1.25 μM	100.8 ± 6.8	96.5 ± 26.0	101.8 ± 23.6	101.7 ± 12.5	84.1 ± 22.2
5.00 μM	100.9 ± 10.8	101.7 ± 17.5	107.2 ± 23.9	98.7 ± 12.0	83.2 ± 19.6
20.00 μM	99.1 ± 9.9	100.6 ± 22.9	88.6 ± 19.4	103.7 ± 12.0	77.8 ± 27.2
4-HBA					
0 μM	100	100	100	100	100
0.08 μM	108.7 ± 17.9	115.3 ± 28.7	107.9 ± 21.4	111.3 ± 13.6	106.2 ± 13.5
0.31 μM	107.4 ± 17.1	112.2 ± 27.7	104.2 ± 17.0	103.4 ± 16.6	93.1 ± 15.0
1.25 μM	110.2 ± 18.2	109.6 ± 29.3	101.1 ± 14.4	102.6 ± 19.5	90.8 ± 19.0
5.00 μM	109.9 ± 16.8	97.8 ± 27.3	97.4 ± 27.4	103.0 ± 15.2	88.5 ± 16.5
20.00 μM	112.2 ± 18.4	109.7 ± 27.9	94.7 ± 25.8	95.2 ± 16.1	94.8 ± 11.6
PGA					
0 μM	100	100	100	100	100
0.08 μM	106.6 ± 7.4	113.9 ± 21.7	109.5 ± 17.6	110.9 ± 16.4	110.3 ± 21.2
0.31 μM	111.4 ± 11.9	107.6 ± 18.0	95.7 ± 18.6	104.8 ± 11.9	77.6 ± 15.3
1.25 μM	112.3 ± 8.7	100.5 ± 18.7	79.7 ± 16.0	103.1 ± 21.2	72.1 ± 16.0
5.00 μM	110.9 ± 16.3	98.5 ± 21.4	71.8 ± 19.1	106.3 ± 12.3	70.9 ± 19.1
20.00 μM	113.7 ± 17.9	110.5 ± 23.7	58.8 ± 25.6 ***	115.6 ± 23.2	65.4 ± 26.2 **

THP-1 monocytes (1 x 10^6 cells/mL) were converted to macrophages through exposure to 0.1 μM phorbol-12-myristate-13-acetate for 72 h. THP-1 macrophages were treated with Pg-3-glc, PCA, 4-HBA, PGA or vehicle control at concentrations of 0-20 μM, prior to lipopolysaccharide stimulation (1 μg/mL) and incubated for 24 h at 37 °C. Cytokine concentrations in the culture supernatants were measured using a cytometric bead array kit. Results are expressed as percentage of cytokine concentration vs. control (no polyphenols). Data are represented as the mean ± SD of six independent experiments. Data were analyzed by one-way ANOVA and Dunnett post hoc analysis, where applicable, and a lowered $P<0.01$ was considered significant to account for multiple comparisons. Statistically significant differences are denoted as $^{**}P<0.01$ vs. control; $^{***}P<0.001$ vs. control. 4-HBA, 4-hydroxybenzoic acid; IL, interleukin; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde; TNF-α, tumor necrosis factor-α.

2.4 Discussion

This study aimed to compare the effects of Pg-3-glc and its plasma metabolites PCA, 4-HBA and PGA on cytokine secretion by LPS-stimulated THP-1 cells. There were modest anti-inflammatory effects of some of the tested compounds in THP-1 monocytes, with PCA and PGA inhibiting IL-6 production, but there were no effects on TNF-α, IL-1β, IL-8 and IL-10 and there were no effects of the other compounds. The effects in macrophages were slightly different. Whilst PGA inhibited IL-6 production by THP-1 derived macrophages, it also inhibited IL-10 production, which was not observed in THP-1 monocytes.

IL-6 is generally classified as a pro-inflammatory cytokine and the inhibition of LPS-stimulated IL-6 secretion by PCA and PGA in the monocyte cultures and PGA in the macrophage cultures is a novel observation. Importantly, the inhibitory effect of PCA was observed at physiologically attainable [14, 15, 17] low micromolar concentrations. Taken together with previous investigations, the current results contribute to the suggestion that PCA acts on multiple cell types involved in IL-6 secretion during atherosclerosis, including endothelial [7] and dendritic cells [4], although the latter experiment applied a supraphysiological PCA dose of 25 μM. It is important to note in this context that the presence of PCA in plasma has also been reported following ingestion of other anthocyanins [42, 53, 54], the flavonol quercetin [55] and it is also naturally present in several other dietary sources, such as chicory, olives, raspberries, dates and onions [2, 56, 57], but it is currently not known what proportion in plasma comes from these sources and what proportion indirectly as a metabolite. Whilst the bioactivity of PGA on IL-6 was a novel observation, the effect was only significant at the higher 5 and 20 μM concentrations. As levels in circulation remain below micromolar concentration (even after anthocyanin consumption), this finding is likely of limited physiological relevance [16, 17].

IL-10 is generally classified as an anti-inflammatory cytokine. PGA decreased the production of IL-10 in THP-1 macrophages, suggesting a pro-inflammatory tendency and this seems to contrast its IL-6-reducing (i.e. anti-inflammatory) effect. However, the IL-10 decreasing effect was observed only at high concentrations that are unlikely to be achieved *in vivo.* To our knowledge, no other studies have investigated the effect of PGA on IL-10 production by monocytes or macrophages. There is evidence to suggest that the inhibition of IL-6 and IL-10 production could be linked in a way that inhibition of IL-6 production could indirectly inhibit IL-10 production [20]. There were no effects of any of the other tested compounds on IL-10 levels, which is in line with experiments on the effect of PCA in human monocyte-derived dendritic cells [4] and Pg-3-glc, PCA and 4-HBA in THP-1 monocytes [6]. Beneficial effects on IL-10 production were observed in human leukocytes with tea-derived polyphenols [21], suggesting differential bioactivity of different polyphenol classes, although effects were only observed at the higher, supraphysiological polyphenol doses (10 and 20 µM).

4-HBA had no effect on the secretion of any of the cytokines. Consistent with this, 4-HBA at 0.1-10 µM was previously reported to have no effect on TNF-α secretion by LPS-stimulated THP-1 monocytes. Interestingly, however, this study is indicative of additive/synergistic effects between polyphenols as an inhibitory effect on TNF-α was reported when 4-HBA was coincubated with PCA or PCA plus vanillic acid, none of which was bioactive in isolation [6]. Furthermore, the reported IL-1β-reducing effect of 4-HBA is in contrast to the present study, despite both studies employing similar concentrations of 4-HBA, the same cell line and similar culture conditions, but the current experiment conducted no preincubation with test compounds. In peripheral blood mononuclear cells, 1 µM 4-HBA had no effect on IL-1β or IL-6 levels in accordance with the present data, but a modest 10% increase in TNF-α levels was observed, which could be related to the differences in cell type and culture conditions.

The discrepant findings regarding the effect of 4-HBA on IL-1β and TNF-α merit further investigation.

In this study only PCA and PGA modulated cytokine secretion, whose effects were targeted towards IL-6 and IL-10, while having no impact on the other cytokines. In contrast, 4-HBA and Pg-3-glc did not affect any of the cytokines tested. These results suggest that at least two hydroxyl groups (either *ortho* or *para* to each other) might be required for bioactivity. In line with this suggestion, potent radical scavenging activity of polyphenols was previously linked to the presence of an *ortho*-dihydroxy group [22] and was furthermore correlated with the number of hydroxyl groups on the B-ring [23]. In order to identify chemical structures or properties required for anti-inflammatory effects, screening studies with a larger number of related compounds are required.

2.5 Conclusion

In conclusion, the data suggest that PCA may possess anti-inflammatory properties through modulation of IL-6 production, which could contribute to protective effects in inflammatory diseases. Importantly, this action was observed at physiologically attainable concentrations, although the effect was modest and limited to monocytes. The IL-6 and IL-10-reducing effects of PGA were only observed at the higher, supraphysiological concentration and are thus considered of limited physiological relevance. Pg-3-glc and 4-HBA had no effect on any of the tested cytokines. Future studies should focus on screening a larger number of related compounds in order to identify chemical structures or properties required for anti-inflammatory effects and underlying mechanisms.

2.6 Acknowledgments

The authors declare no conflicts of interest related to this experiment.

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