Short Communication

Dihydroxylated phenolic acids derived from microbial metabolism reduce lipopolysaccharide-stimulated cytokine secretion by human peripheral blood mononuclear cells

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Oligomers and polymers of flavan-3-ols (proanthocyanidins) are very abundant in the Mediterranean diet, but are poorly absorbed. However, when these polyphenols reach the colon, they are metabolised by the intestinal microbiota into various phenolic acids, including phenylpropionic, phenylacetic and benzoic acid derivatives. Since the biological properties of these metabolites are not completely known, in the present study, we investigated the effect of the following microbial phenolic metabolites: 3,4-dihydroxyphenylpropionic acid (3,4-DHPPA), 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid and 4-hydroxyhippuric acid (4-HHA), on modulation of the production of the main pro-inflammatory cytokines (TNF-α, IL-1β and IL-6). The production of these cytokines by lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) pre-treated with the phenolic metabolites was studied in six healthy volunteers. With the exception of 4-HHA for TNF-α secretion, only the dihydroxylated compounds, 3,4-DHPPA and 3,4-DHPAA, significantly inhibited the secretion of these pro-inflammatory cytokines in LPS-stimulated PBMC. Mean inhibition of the secretion of TNF-α by 3,4-DHPPA and 3,4-DHPAA was 84.9 and 86.4 %, respectively. The concentrations of IL-6 in the culture supernatant were reduced by 88.8 and 92.3 % with 3,4-DHPPA and 3,4-DHPAA pre-treatment, respectively. Finally, inhibition was slightly higher for IL-1β, 93.1 % by 3,4-DHPPA and 97.9 % by 3,4-DHPAA. These results indicate that dihydroxylated phenolic acids derived from microbial metabolism present marked anti-inflammatory properties, providing additional information about the health benefits of dietary polyphenols and their potential value as therapeutic agents.

TNF-α: IL-1β: IL-6: Atherosclerosis: Phenolic acids: Microbial metabolism

Polyphenols are among the most abundant antioxidant compounds of the Mediterranean diet and may play a key role in the prevention of cardiovascular and neurodegenerative diseases, and cancer(1). Health effects derived from polyphenol consumption depend on their bioavailability, a factor that greatly varies from one compound to another(2). Among polyphenols, the oligomers and polymers of flavan-3-ols, also called proanthocyanidins, are not absorbed or degraded into monomers during their transit through the stomach(2,3). However, when they reach the colon, they are metabolised by the intestinal microbiota into various phenolic acids, including phenylpropionic, phenylacetic and benzoic acid derivatives(4). Hydroxycinnamic acid esters and polyphenols linked to rhamnose are also degraded into phenolic acids by the microbiota(2). Recently, it has been reported that these metabolites may also exert several biological activities, such...
as the inhibition of platelet aggregation and activation function\(^{5}\), inhibition of cyclo-oxygenase-2 in HT-29 colon cancer cells\(^{6}\), reduction in the synthesis of prostanoids in colon cells\(^{7}\), antiproliferative activity in prostate and cancer cells\(^{8}\) and, finally, influence cell proliferation, apoptosis and signalling pathways in human colon carcinoma cells\(^{9}\).

Atherosclerosis is now considered to be a low-grade chronic inflammatory process resulting from the interactions between plasma lipoproteins, cellular components (monocyte/macrophages, T lymphocytes, endothelial cells and smooth muscle cells) and the extracellular matrix of arterial wall\(^{10}\). Pro-inflammatory cytokines are involved in all phases of the atherosclerotic process: they stimulate chemokines and adhesion molecules, leading to early recruitment of monocytes and lymphocytes in the arterial intima, and later exert potential noxious effects promoting weakening of plaques that are more prone to rupture\(^{10}\). However, data concerning the effect of inflammatory cytokines up to 295·48 pg/ml (min 48·24, max 773·45) for IL-1\(\beta\) and 4-HBA significantly increased TNF-\(\alpha\) to 181·65 mg/d phytoestrogens.

Peripheral blood from the volunteers was collected and PBMC were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden)\(^{16}\). Harvested cells were washed with PBS 10 × buffer (Roche Diagnostics GmbH, Mannheim, Germany) and then counted in a haemocytometer chamber. Cell viability was estimated with trypan blue. PBMC were resuspended in RPMI-1640 (Biowhittaker, Verviers, Belgium) containing fetal bovine serum (10 %) and gentamicin (0·05 mg/ml) (RPMI-10 % fetal) up to a concentration of 1 × 10\(^6\) viable cells/ml.

For each of the following phenolic acids, a \(3 \mu\)M solution was prepared in RPMI-10 % fetal: 3,4-dihydroxyphenylpropionic acid (3,4-DHPPA); 3-hydroxyphenylpropionic acid (3-HPPA); 3,4-dihydroxyphenylacetic acid (3,4-DHPAA); 3-hydroxyphenylacetic acid (3-HPAA); 4-hydroxybenzoic acid (4-HBA; Sigma-Aldrich, St Louis, MO, USA); 4-hydroxyhippuric acid (4-HHA; PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany).

PBMC were cultured with the different phenolic acid solutions in the presence of LPS (Sigma-Aldrich). Five hundred microlitres of the 1 × 10\(^6\) cells/ml suspension (5 × 10\(^5\) cells, total number of cells) were pre-treated (16 h at 37°C, 5 % CO\(_2\)) with 250 µl of the 3 µM-phenolic acid solution (1 µM, final concentration with cells) in twenty-four-well plates. After the pre-treatment period, the cell viability was estimated with trypan blue and was higher than 95 %. LPS (1 µg/ml) was then added to the culture followed by incubation for 72 h at 37°C. Unstimulated and LPS-stimulated polyphenol-free cells were also cultured under the same conditions. Experiments were performed in duplicate. After the incubation period, the cultures were centrifuged and the supernatant collected and stored at −80°C until analysis. Pro-inflammatory cytokines IL-1\(\beta\), IL-6 and TNF-\(\alpha\) were determined in the culture supernatants by ELISA (Bender Med Systems GmbH, Vienna, Austria). Detection limits were as follows: 0·7 pg/ml for IL-1\(\beta\); 0·92 ng/ml for IL-6; 1·65 pg/ml for TNF-\(\alpha\).

For the statistical treatment of the data, \(t\) test for paired samples was performed using the PC software package SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Differences between values were expressed as the percentage of enhancement or inhibition. All statistical tests were two-tailed, and the significance level was 0·05.

**Results**

The effects of tested phenolic acids on the secretion of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 from the PBMC of healthy subjects are shown in Fig. 1 and Table 1. Stimulation of PBMC with LPS significantly increased the levels of the three pro-inflammatory cytokines up to 295·48 pg/ml (min = 48·34, max = 773·45) for TNF-\(\alpha\), 128·38 pg/ml (min = 44·24, max = 209·91) for IL-1\(\beta\) and 386·58 pg/ml (min = 331·36, max = 475·57) for IL-6. 3,4-DHPPA, 3,4-DHPAA and 4-HHA significantly reduced (\(P<0·01\) for 3,4-DHPPA and 3,4-DHPAA, \(P<0·05\) for 4-HHA) TNF-\(\alpha\) secretion in LPS-stimulated PBMC by 84·9, 86·4 and 30·4 %, respectively (Table 1). Contrarily, 4-HBA significantly increased TNF-\(\alpha\) secretion by 9·9 %. No significant
changes in TNF-α secretion were recorded after the addition of the remaining tested phenolic acids, 3-HPPA and 3-HPAA. In the case of IL-1β, only 3,4-DHPPA and 3,4-DHPAA significantly (P<0.001) reduced the secretion of this cytokine in LPS-stimulated PBMC by 93.1 and 97.9%, respectively (Fig. 1; Table 1). Similarly, a significant reduction (P<0.001) in IL-6 secretion from LPS-stimulated PBMC was also observed after treatment with 3,4-DHPPA and 3,4-DHPAA, resulting in
an inhibition of 88.8 and 92.3 %, respectively. However, no significant changes were found in IL-1β and IL-6 levels after the addition of the remaining tested phenolic acids.

Discussion

In the present study, we observed that dihydroxylated phenolic acids derived from microbial metabolism presented marked in vitro anti-inflammatory properties, reducing the secretion of TNF-α, IL-1β and IL-6 in LPS-stimulated PBMC from healthy subjects. Six different phenolic acids (3,4-DHPPA, 3-HPPA, 3,4-DHPAA, 3-HPA, and 4-HBA) derived from the microbial metabolism of polyphenols, in particular from monomeric flavanols and proanthocyanidins, were tested at a concentration level (1 μM) within the range (0.1–10 μM) found in plasma samples after the intake of a polyphenol-rich meal and recommended for in vitro studies. Tested compounds were chosen on the basis of their structural features (i.e., hydroxylation pattern and side-chain length of the functional group) and on their abundance in biological fluids after the ingestion of polyphenol-rich foods. To our knowledge, the effects of microbial-derived phenolic acids on the production and release of pro-inflammatory cytokines from human PBMC have not been published previously.

Previous studies based on the consumption of catechin by human subjects resulted in an increase in 3-HPPA. Consumption of catechins and proanthocyanidins from chocolate by human subjects resulted in an increased urinary excretion of 3,4-DHPPA, 3-HPPA, and 3-HBA. More recently, Ward et al. described that 3-HPPA was the main metabolite in urine when the human subjects were supplemented with grape-seed polyphenols. Studies on laboratory animals have shown similar results. Main urinary metabolites formed from rats fed a catechin diet were 3-HPPA, 3-HBA and 3-HBA. Besides these metabolites, 3-HPA and 4-HBA were detected in the urine of rats fed wine polyphenols. In vitro experiments also confirmed that 3-HPPA was the most abundant metabolite produced from proanthocyanidin polymers by human colonic microflora, whereas 3,4-DHPPA was the main metabolite from rutin, which was also found to be the major end product of the colonic metabolism of chlorogenic acid by human faecal microbiota. The yield of microbial metabolites could be high, in particular for the polyphenols that are poorly absorbed in the small intestine. For example, for chlorogenic and caffeic acids, it represented 57.4 and 28.1 % of the total intake, respectively. However, in order for these metabolites to be effective at a physiological level, they need to be absorbed and reach target tissues. In fact, once produced by the microbiota, some colonic metabolites could be further absorbed and reach the liver and the kidney where they could be methylated, hydroxylated or conjugated with glycine. Recently, the absorption mechanism of some of these colonic metabolites is beginning to be elucidated. Metabolites such as ferulic, p-coumaric, m-coumaric and 3-HPPA are absorbed by the

| Table 1. Summary results of the effect of phenolic acids (1 μM) derived from microbial metabolism on the secretion of TNF-α, IL-1β and IL-6 after stimulation with lipopolysaccharide (LPS) in peripheral blood mononuclear cells from six healthy subjects |
|-----------------|-----------------|---------------|--------------|-----------------|-----------------|---------------|
|                | Minimum         | Maximum        | Mean          | SD             | Enhancement or inhibition (%) | P*            |
| TNF-α (pg/ml)  | Non-stimulated  | 5.73           | 40.55         | 18.23          | 11.51           |                |
|                | LPS-stimulated  | 48.34          | 773.45        | 295.48         | 265.97          |                |
|                | LPS + 3,4-DHPPA | 16.55          | 128.15        | 44.51          | 35.66           | -84.9         | 0.009         |
|                | LPS + 3-HPPA    | 87.07          | 709.68        | 313.14         | 232.76          | +6.0          | 0.588         |
|                | LPS + 3,4-DHPAA | 17.95          | 72.51         | 40.06          | 18.35           | -86.4         | 0.007         |
|                | LPS + 3-HPA     | 76.07          | 781.47        | 288.48         | 249.69          | -2.4          | 0.728         |
|                | LPS + 4-HBA     | 86.51          | 819.10        | 324.87         | 270.58          | +9.9           | 0.021         |
|                | LPS + 4-HHA     | 69.86          | 672.0         | 205.67         | 216.82          | -30.4         | 0.026         |
| IL-1β (pg/ml)  | Non-stimulated  | 4.36           | 28.12         | 11.39          | 7.41            |                |
|                | LPS-stimulated  | 44.24          | 209.91        | 128.38         | 52.60           |                |
|                | LPS + 3,4-DHPPA | 0.00           | 22.28         | 8.85           | 7.94            | -93.1         | 0.000         |
|                | LPS + 3-HPPA    | 97.49          | 236.79        | 138.57         | 46.73           | +10.5         | 0.192         |
|                | LPS + 3,4-DHPAA | 0.00           | 12.00         | 2.72           | 4.65            | -97.9         | 0.000         |
|                | LPS + 3-HPA     | 65.05          | 202.09        | 128.54         | 44.20           | +0.1          | 0.982         |
|                | LPS + 4-HBA     | 59.15          | 218.71        | 122.92         | 60.68           | -4.3          | 0.583         |
|                | LPS + 4-HHA     | 68.54          | 195.74        | 113.70         | 42.28           | -11.4         | 0.323         |
| IL-6 (pg/ml)   | Non-stimulated  | 27.51          | 264.91        | 161.22         | 73.60           |                |
|                | LPS-stimulated  | 331.36         | 475.57        | 386.58         | 49.38           |                |
|                | LPS + 3,4-DHPPA | 2.94           | 102.68        | 43.29          | 31.14           | -88.8         | 0.000         |
|                | LPS + 3-HPPA    | 324.35         | 456.42        | 378.79         | 48.03           | -2.0          | 0.138         |
|                | LPS + 3,4-DHPAA | 2.94           | 99.42         | 29.59          | 33.18           | -92.3         | 0.000         |
|                | LPS + 3-HPA     | 331.36         | 458.06        | 393.63         | 44.24           | +1.9          | 0.482         |
|                | LPS + 4-HBA     | 343.01         | 478.96        | 396.05         | 54.08           | -0.1          | 0.946         |
|                | LPS + 4-HHA     | 333.34         | 480.36        | 385.20         | 46.68           | -0.4          | 0.880         |

3,4-DHPPA, 3,4-dihydroxyphenylpropanoic acid; 3-HPPA, 3-hydroxyphenylpropanoic acid; 3,4-DHPPA, 3,4-dihydroxyphenylacetic acid; 3-HPA, 3-hydroxyphenylacetic acid; 4-HBA, 4-hydroxybenzoic acid; 4-HHA, 4-hydroxyhippuric acid.

* Significant differences between LPS-stimulated cells and those in the presence of phenolic acids were determined by the t-test.
monocarboxylic acid transporter, whereas caffeine acid and 3,4-
DHPPA permeate across Caco-2 cells via the paracellular pathway (24–26). Using immunohistochemical tests, Kawai et al. (27) have recently confirmed that polyphenol metabolites could penetrate the tissues. Quercetin-3-glucuronide, a major metabolite of quercetin, was permeable in LPS-stimulated macrophages, and was converted into the more active aglycone, a part of which was further converted into the methylated form. These data suggest that microbial phenolic metabolites could also undergo a similar pathway in injured cells.

Atherosclerosis is now considered as an inflammatory dis-

ease (28). Recent epidemiological and clinical studies have shown that the Mediterranean diet or its main components, rich in polyphenols, are associated with a lower inflammatory status (29). However, in epidemiological and even in clinical studies, it is difficult to control the effects of the diet consumed and physical activity performed (30). Thus, in vitro studies allow us to obtain additional information in relation to the direct effect of some compounds (i.e. polyphenol metabolites) in biochemical pathways related to cardiovascular health, such as the production of pro-inflammatory cytokines that participate in the first stages of atherosclerosis. LPS is a bacterial protein and is used as a method to challenge immune cells to produce cytokines, including the inflamma-
tory cytokines. Some of the inflammatory cytokines that are produced are those that have been associated with chronic inflammation and atherosclerosis risk.

The results found in the present study indicate that the effects of the tested phenolic acids on cytokine secretion by PBMC were structure dependent. With the exception of the effects of 4-HHA on TNF-α secretion, only the dihydroxy-
lated phenolic acids, 3,4-DHPPA and 3,4-DHPAA, caused a statistically significant decrease in the secreted levels of the three different cytokines from LPS-stimulated PBMC (Table 1). The standard deviation of the data reflects large inter-individual difference in cytokine secretion among the volunteers. In addition, the degree of inhibition was found to be influenced by the cytokine family. The inhibition of IL-1β by both compounds was slightly higher than that for TNF-α and IL-6 (Table 1). Monohydroxylated phenolic acids (3-HPPA, 3-HPAA, 4-HBA and 4-HHA) did not produce significant changes in cytokine secretion with the excep-
tion of 4-HHA on TNF-α secretion, which produced a significant increase in this cytokine.

The present results on TNF-α, IL-1β and IL-6 secretion are in agreement with other studies performed with other polyphenols and cell types. Quercitin inhibited the expression of IL-8 and MCP-1 in TNF-α-stimulated synovial cells (31). Small oligo-
meric procyanidin fractions (monomer to tetramer) isolated from cocoa reduced the secretion of IL-1 from PHA-stimulated PBMC, whereas polymers (pentamer to decamer) produced an increase in the secreted levels (12). However, the same fractions promoted the secretion of TNF-α (13). Dimeric flavanols isolated from pine bark also enhanced TNF-α levels in stimulated macrophages, while monomers strongly inhibited its secretion (32). Ramiro et al. (33) found that epicatechin, isoorcitrin and cocoa extracts decrease the secretion of TNF-α by macrophages in a dose-dependent manner. Also in this line, it has also been reported that several flavones and flavanols inhibited TNF-α secretion by LPS-stimulated macrophages (34).

The superior effect of dihydroxylated phenolic acids in comparison with the monohydroxylated ones on the inhibition of pro-inflammatory cytokines has also been reported for other tested biological properties. Thus, 3,4-DHPAA showed more potent cytotoxicity against tumour cell lines than 4-hydroxy-
phenylacetic acid (8). 3,4-DHPPA was also among the phenolic acids inhibiting the expression of P-selectin in resting plate-
lets (5). According to Russell et al. (35), dihydroxylated phenolic acids present a better antioxidant capacity than monohydroxy-
lated ones due to their stabilisation into quinones.

The mechanism associated with the inhibitory or stimu-

latory activities of polyphenols on cytokine production may result from transcriptional and post-transcriptional events (14). In fact, NF-κB, a transcription factor responsible for the activation of a series of cytokines, including TNF-α and IL-1β, is redox sensitive, and it is well known that the antioxidants such as flavonoids can inhibit its activation (36). Other authors have recently suggested that, besides their antioxidant effects, poly-
phenols could also function as signalling molecules (36).

In this sense, Tedgui & Mallat (36) have suggested that future therapeutic approaches to treat atherosclerosis may include agents that block pro-inflammatory cytokine signalling or the transcription of inflammatory-mediating molecules, among others. The results found in the present study demon-
strate that due to their down-regulating effect on the pro-
duction of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, polyphenols such as dihydroxylated phenolic acids derived from microbial metabolism could be among the new generation of therapeutic agents for the management of immuno-inflammatory diseases such as atherosclerosis.

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References


