Activity of monomeric, dimeric, and trimeric flavonoids on NO production, TNF-α secretion, and NF-κB-dependent gene expression in RAW 264.7 macrophages

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Abstract Flavonoids are potent antioxidants and have been associated with lowering the risk of cardiovascular diseases. In this study, the effect of flavonoids (monomers, dimers and a trimer) as well as French maritime pine bark extract, Pycnogenol, on NO production, tumor necrosis factor-α (TNF-α) secretion and nuclear factor (NF)-κB activity was compared. Monomers and dimers repressed NO production, TNF-α secretion and NF-κB-dependent gene expression induced by interferon γ, whereas the trimeric procyanidin C2 and Pycnogenol enhanced these parameters. In addition, in unstimulated RAW 264.7 macrophages, both procyanidin C2 and Pycnogenol increased TNF-α secretion in a concentration- and time-dependent manner. These results demonstrate that procyanidins act as modulators of the immune response in macrophages.

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Key words: Tumor necrosis factor-α; Nitric oxide; Nuclear factor κB; Macrophage; Procyanidin

1. Introduction

Activated macrophages can generate large amounts of NO from L-arginine by the action of inducible NO synthase (iNOS). NO is an important intracellular and intercellular regulatory molecule of multiple biological functions, including macrophage-mediated cytotoxicity, neurotransmission, and smooth muscle relaxation [1,2]. Overproduction of NO has been associated with oxidative stress [3,4] and with the pathophysiology of various diseases such as arthritis, diabetes, stroke, septic shock, autoimmune disease, and chronic inflammation [5,6]. Cytokines such as interferon-γ (IFN-γ), interleukin (IL)-2, and other inflammatory stimuli such as bacterial lipopolysaccharide (LPS) regulate the activity of iNOS in macrophages [7,8]. It has also been shown that the production of tumor necrosis factor-α (TNF-α) is crucial for the synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated macrophages [9,10].

In macrophages, nuclear factor κB (NF-κB) in cooperation with other transcription factors coordinates the expression of genes encoding both iNOS and TNF-α. NF-κB plays a critical role in the activation of immune cells by upregulating the expression of many cytokines essential to the immune response [11]. In particular, NF-κB stimulates the production of IL-1, IL-6, TNF-α, lymphotoxin, and IFN-γ. Furthermore, some of these cytokines, e.g. IL-1 and TNF-α, activate NF-κB themselves, thus initiating an autoregulatory feedback loop. NF-κB activation by various stimuli occurs upon its dissociation from the inhibitory protein IκB and its subsequent nuclear translocation. Several lines of evidence, including the inhibition by various antioxidants, suggest that NF-κB activation is subject to redox regulation [12,13]. Because of the pivotal role of NO and TNF-α in the antimicrobial and tumoricidal activities of macrophages, a significant effort has focused on developing therapeutic agents that regulate NO production and TNF-α secretion [14].

Epidemiological reports have indicated that consumption of foods rich in flavonoids is associated with a lower incidence of degenerative diseases. Consistently, experimental data are accumulating regarding phenolic compounds as natural phytochemical antioxidants that possess antiinflammatory, antiviral, antiproliferative, and anticarcinogenic properties [15,16]. There is increasing interest in the biological activities of plant extracts such as that obtained from the bark of the French maritime pine, *Pinus maritima*. Pine bark extract (Pycnogenol, PYC) is a unique mixture of phenols and polyphenols, broadly divided into monomers (e.g. catechin, epicatechin and taxifolin), dimers (e.g. procyanidin B1, B2, B3, and B7), trimers (e.g. procyanidin C1, C2), and oligomers up to 5–7 units. PYC also contains phenolic acids such as caffeic, ferulic and *p*-hydroxybenzoic acid as minor constituents [17]. Bioflavonoids participate in an antioxidant network [18] and likely spare endogenous cellular vitamin E [19] and glutathione [20]. PYC also modulates NO metabolism in stimulated macrophages by affecting both iNOS mRNA expression and iNOS activity [21]. However, little is known about which particular constituents of PYC mediate its immunomodulatory properties. Therefore, the objective of the present study was to investigate whether purified monomeric, dimeric, and trimeric flavonoids in comparison to PYC can modulate NF-κB activation as well as NO production and TNF-α secretion in RAW 264.7 macrophages.

2. Materials and methods

2.1. Materials

Murine recombinant IFN-γ (1×10^6 U/mg), murine recombinant TNF-α (1×10^6 U/ml), rabbit anti-murine TNF-α polyclonal antibody

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Abbreviations: IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PCA, procyanidin; PYC, Pycnogenol; TNF-α, tumor necrosis factor-alpha

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(Ab), and hamster anti-murine TNF-α monoclonal Ab were from Genzyme (Munich, Germany). LPS (phenol-extracted *Salmonella enteritidis*), catechin, epicatechin, and taxifolin were from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum and antibiotics were from the University of California, San Francisco Cell Culture Facility. RPMI containing L-arginine (200 mg/l), Hanks' balanced salt solution, and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD, USA). Pycnogenol was a gift from Horphag Research Ltd. (Guernsey, UK). Procyanidins B1, B2 (both from apples, 98% purity), and C2 (from barley, 93% purity) were a gift from Tsukuba Research Laboratories (Kyowa Hakko Kogyo, Japan).

2.2. Macrophage culture

The murine monocyte/macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in 75-cm^2 plastic flasks (Falcon-Becton Dickinson Labwares, Franklin Lakes, NJ, USA) and maintained in RPMI 1640 supplemented with 10% (v/v) fetal calf serum and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin). For experiments, macrophages were detached by vigorous pipetting and, after centrifugation, incubated with fresh medium in either 96-well tissue culture plates (2×10^5 cells/well) or 24-well tissue culture plates (5×10^5 cells/well) at 37°C in an atmosphere of 5% CO_2 plus air. Cells were supplemented with the test compounds for 1 h followed by their stimulation with 10 U/ml IFN-γ and further incubated for various periods as described in Section 3.

2.3. Neutral red assay

Uptake of the dye neutral red was used as a measure of cell viability [22]. Cells were plated in 24-well plates, and samples were treated for 24 h in 500 μl of medium. Thereafter, the medium was removed and replaced with 500 μl of medium containing 60 μg/ml of neutral red (Fisher Scientific, Pittsburgh, PA, USA) for 3 h at 37°C. Following incubation with the neutral red dye, the medium was removed, and the cells were extracted with 500 μl of 50% ethanol, 49% H_2O, 1% glacial acetic acid, pH 4.2. Quadruplicate 100-μl samples from each well were transferred to a 96-well plate, and the absorbance at 510 nm was measured with a microplate reader (CS-931, Shimadzu Corporation, Columbia, MD, USA).

2.4. Assay of TNF-α secretion

TNF-α secretion was measured by modification of an enzyme-linked immunosorbent assay (ELISA), as described earlier [23]. For the ELISA, 96-well plates were coated with 6.25 ng/well of murine monoclonal Ab with specificity for murine TNF-α. Before use and between subsequent steps in the assay, coated plates were washed twice with phosphate-buufered saline (PBS) containing 0.05% (v/v) Tween-20 and twice with PBS alone. For the standard curve, rTNF-α was added to serum previously determined to be negative for endogenous TNF-α. After exposure to medium, assay plates were sequentially exposed to rabbit anti-TNF-α, phosphatase-conjugated goat anti-rabbit IgG, and *p*-nitrophenyl phosphate. Optical density readings at 410 nm were taken using a microplate reader.

2.5. Measurement of nitrite and nitrate concentration

NO secretion in cultured macrophages was measured by a microplate assay method, as described earlier [24,25]. To measure nitrite (NO_2^-), 100 μl of macrophage culture supernatant was collected, mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) and incubated for 10 min at room temperature. Nitrite concentration was determined by measuring the absorbance at 540 nm. $NaNO_2$ was used for external calibration. Cell-free medium alone contained 5–8 μM of nitrite; this value was determined in each experiment and subtracted from the value obtained for each cell sample. Nitrate (NO_3^-) was measured by reducing nitrate to nitrite with bacterial nitrate reductase, then measuring nitrite by using Griess reagent. There was no interference of the test compounds either with the ELISA or with Griess reagent.

2.6. Cell transfection and reporter gene assay

RAW 264.7 macrophages were plated at 2×10^5 cells per cm^2 in 12-well plates, and 24 h later transiently co-transfected with the plasmids pGL3-4κB-Luc and pRL-TK (reference plasmid containing a *Renilla* luciferase gene driven by a minimal thymidine kinase promoter, Promega, Madison, WI, USA) using Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.35 μg of pGL3-4κB-Luc and 0.15 μg of pRL-TK was mixed with the Fugene 6 reagent and added to the cells. Treatments with test compounds and IFN-γ were started 18 h after transfection. Cell lysis was performed 12 h after treatments and luciferase activities were measured using the dual-luciferase reporter assay system (Promega) with an LKB/Wallac luminometer 1250 (Wallac, Gaithersburg, MD, USA). The dual-luciferase system is based on the subsequent measurement of firefly (from pGL3-4κB-Luc) and *Renilla* (from pRL-TK) luciferase activities in the same tube with the same extract. The firefly luciferase activity was normalized in that system with the *Renilla* luciferase activity to correct for differences in the transfection efficiency [26].

2.7. Lipid A analysis

PYC was tested for the presence of lipid A. Samples and reference standard (deep rough chemotype LPS from *Escherichia coli* D31m4) were treated with EDTA, and purified by a DEAE-cellulose column chromatography as previously described [27]. Samples were hydrolyzed in 2 ml of 0.1 M HCl at 100°C for 30 min to yield monophosphoryl lipid A. Then 5 ml of chloroform/methanol (2:1, v/v) was added, mixed and centrifuged. The lower organic layer was recovered, filtered and dried. The extracted samples were applied in chloroform/methanol (4:1, v/v) to silica gel GHL plate (250 μm) and irrigated with chloroform/methanol/water/concentrated ammonium hydroxide (50:25:4:2, v/v). Spots were visualized by spraying the plate with dichromate/sulfuric acid reagent and charring.

2.8. Statistics or reproducibility

Data in figures are the mean ± S.D. of at least three different experiments performed in triplicate.

3. Results

3.1. TNF-α secretion by macrophages is modified by flavonoids in a structure-dependent manner

As a first step, the cytotoxic activity of the monomeric, dimeric and trimeric flavonoids as well as PYC in the absence and presence of IFN-γ was assessed using the neutral red assay. Pretreatment of both unstimulated and stimulated RAW 264.7 macrophages up to 100 μg/ml catechin, epicatechin as well as PCA B1, PCA B2 and PCA C2 did not significantly affect the cell viability (data not shown). However, in macrophages treated with 100 μg/ml taxifolin for 24 h a slight although significant cytotoxic effect could be observed.

In unstimulated macrophages only small amounts of TNF-α were secreted into the medium (Fig. 1A). Pretreatment of unstimulated cells with monomeric or dimeric flavonoids over 24 h did not result in any change in the secretion of TNF-α into the medium. However, in the presence of PCA C2 or PYC the secreted levels of TNF-α significantly increased. The stimulation of RAW 264.7 cells with IFN-γ resulted in a manifold increase in TNF-α secretion monitored 24 h after administration (Fig. 1B). All monomeric flavonoids tested (catechin, epicatechin, and taxifolin) decreased the secretion of TNF-α almost down to the baseline levels as measured in the unstimulated control cells. While the dimeric flavonoids PCA B1 and PCA B2 slightly inhibited IFN-γ-induced TNF-α secretion, the trimeric flavonoid PCA C2 induced about a two-fold increase in the secretion of TNF-α in IFN-γ-stimulated macrophages. Similar to PCA C2, PYC potentiated the effect of IFN-γ on TNF-α secretion.

The secretion of TNF-α due to PCA C2 and PYC increased both in a dose- and time-dependent fashion as shown in Fig. 2A,B, respectively.

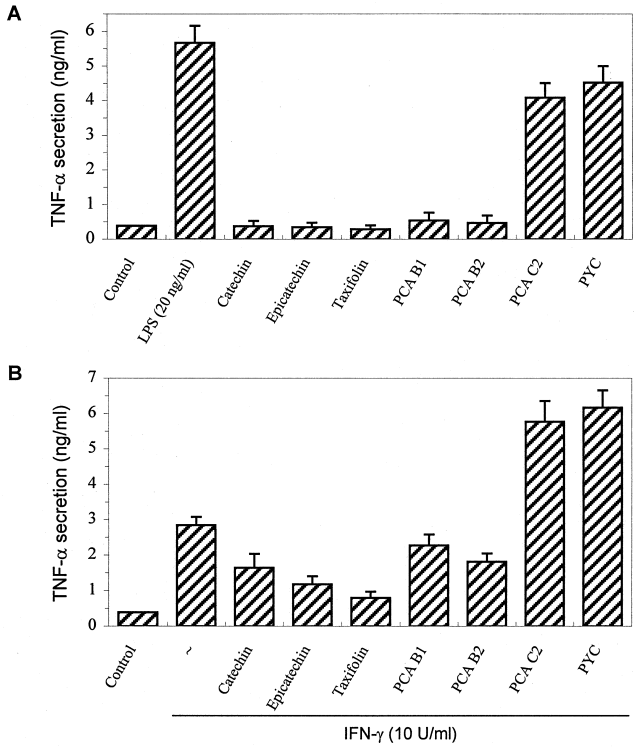

Fig. 1. Effect of structure-related flavonoids on TNF-α secretion in unstimulated (A) and IFN-γ-stimulated (B) RAW 264.7 macrophages. The cells were incubated for 24 h in the presence or absence of several pure flavonoids, PYC, or LPS. Then, supernatants were collected, and the amount of TNF-α secreted by macrophages was measured by specific ELISA. All flavonoids tested in this and all further experiments were supplemented at a concentration of 100 μg/ml (corresponds to the following concentrations in μM: catechin and epicatechin = 344, procyanidin B1 and B2 = 173, procyanidin C2 = 115) whereas taxifolin was supplemented at 50 μg/ml (164 μM). Values are means ± S.D. of three independent cell preparation.

3.2. Effect of monomeric, dimeric, and trimeric flavonoids on NO production in IFN-γ-stimulated macrophages

In parallel with the characterization of TNF-α secretion the effect of the different flavonoids on NO release into the medium measured by the Griess reaction was studied. Unstimulated control cells produced a negligible amount of NO (< 5 μM). However, in IFN-γ-stimulated cells a substantial increase in the production of NO was evident (Fig. 3). Pretreatment of macrophages with catechin, epicatechin, and taxifolin

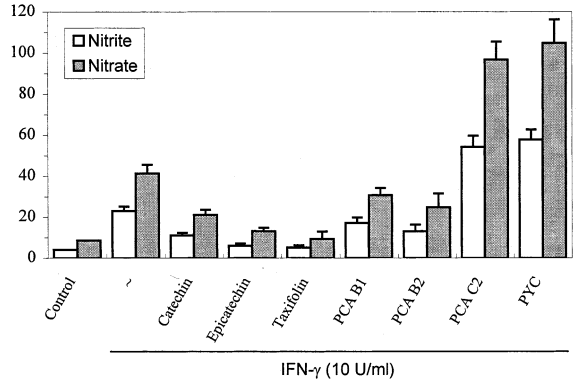

Fig. 3. Effect of structure-related flavonoids on NO production in IFN-γ-stimulated macrophages. The cells were incubated as described in Fig. 2. Then, the amounts of nitrite and nitrate released by macrophages were measured 24 h after stimulation by the method of Griess. Results are presented as means ± S.D. of three independent cell preparation.

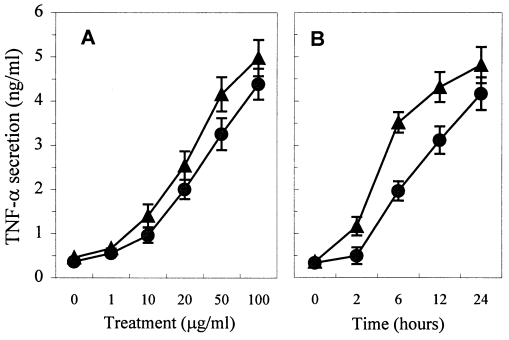

Fig. 2. A: Dose-dependent effect of PCA C2 (●) and PYC (▲) on TNF-α secretion in unstimulated macrophages. The cells were incubated for 24 h in the presence or absence of various concentrations of flavonoids. B: Time-dependent effect of PCA C2 and PYC on TNF-α secretion in macrophages. At the indicated time points after treatment of flavonoids supernatants were collected, and the amount of TNF-α secreted by macrophages was determined with an ELISA. Values are means ± S.D. of three experiments.

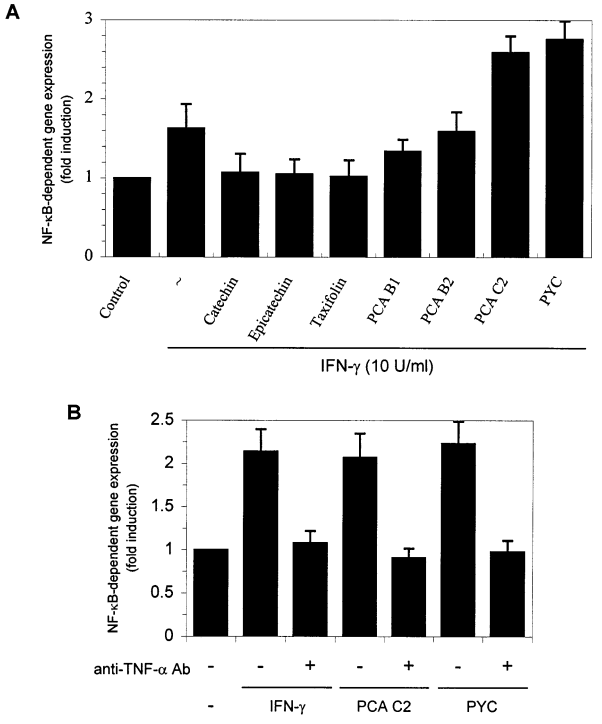

Fig. 4. A: Effect of structure-related flavonoids on NF-κB-dependent luciferase gene expression in IFN-γ-stimulated macrophages. After co-transfection, the cells were washed twice and incubated as described in Fig. 2. Then, the cells were lysed 24 h after stimulation. The dual-luciferase assay was performed and luciferase activity expressed as relative to that of the control (untreated cells). B: Effect of anti-TNF-α neutralizing Ab on rIFN-γ- or rIFN-γ+PCA C2-, or rIFN-γ+PYC-induced NF-κB-dependent gene expression in macrophages. After co-transfection, anti-TNF-α neutralizing Ab (dilution, 1:50) was added to the culture medium for 15 min. The cells were then treated with rIFN-γ (10 U/ml) or PCA C2 or PYC for 24 h prior to cell lysis. The data are presented as means from three independent experiments, each performed in duplicate.

significantly decreased IFN-γ-induced NO production by 40, 60, and 75%, respectively, as compared to IFN-γ-treated cells. The dimeric flavonoids PCA B1 and PCA B2 showed a moderate inhibitory effect on NO production. In contrast to the monomeric and dimeric procyanidins pretreatment of IFN-γ-stimulated macrophages with the PCA C2 as well as PYC significantly increased IFN-γ-induced NO production.

3.3. NF-κB-dependent gene expression in IFN-γ-stimulated macrophages is modulated by flavonoids

Induction of TNF-α and iNOS is modulated, in part through the activation of NF-κB. Consequently the effect of different flavonoids on NF-κB-dependent gene expression using the dual-luciferase reporter gene assay was studied. Nearly a two-fold increase in luciferase activity was observed when RAW 264.7 macrophages were stimulated with IFN-γ as compared to unstimulated control cells (Fig. 4A). Similar to the data for TNF-α secretion and NO production the monomeric flavonoids catechin, epicatechin and taxifolin completely counteracted IFN-γ-induced NF-κB transactivation. Pretreatment of macrophages with PCA B1 and PCA B2 resulted in a slight decrease in the luciferase activity. However, different to the monomers and dimers, PCA C2 and PYC significantly increased luciferase activity in IFN-γ-stimulated cells. In addition both PCA C2- and PYC-induced NF-κB activation was totally inhibited by the use of a anti-murine TNF-α-neutralizing Ab, indicating that PCA C2 or PYC-induced TNF-α secretion is crucial for NF-κB activation in macrophages (Fig. 4B).

4. Discussion

4.1. TNF-α secretion

TNF-α was produced in vanishingly small quantities in unstimulated cells as reported previously [28]. However, when macrophages were stimulated with IFN-γ, a manifold increase in the secretion of TNF-α into the medium was observed. Interestingly, both in the absence and in the presence of IFN-γ, pretreatment of macrophages with the trimer PCA C2 as well as PYC substantially elevated TNF-α release from undetectable levels up to 2–4 ng/ml. Similar to PCA C2 and PYC the flavonoid drug flavone-8-acetic acid (FAA), which augments mouse natural killer activity, has been shown to induce TNF-α secretion in J774 cells and thereby synergized with recombinant IL-2 for the treatment of murine renal cancer [29]. Moreover, the red wine polyphenol resveratrol has been reported to have a potent effect to increase basal TNF-α mRNA expression with a concomitant increase in TNF-α secretion in RAW 264.7 macrophages [30]. In this regard it is hypothesized that TNF-α induction appears to be one critical factor in the antitumor effects of this class of compounds. However, in contrast to PCA C2 and PYC the monomeric flavonoids (catechin, epicatechin, and taxifolin) tested in the present study had a strong and the dimeric flavonoids PCA B1 and PCA B2 a slight inhibitory effect on the secretion of TNF-α into the medium. Consistent with these findings green tea polyphenols also significantly decreased TNF-α and NO synthesis in elicited murine peritoneal macrophages as well as in the BALB/c mouse model [31]. The underlying mechanisms by which different flavonoids result in a different outcome in terms of TNF-α secretion and NO production have not been clarified yet.

4.2. NO production

The monomeric flavonoids catechin, epicatechin, and taxifolin significantly inhibited NO production in IFN-γ-stimulated cells. The cellular mechanisms by which flavonoids down-regulate NO production are not completely understood. It is suggested that this effect might be partially mediated due to the combination of several different biological activities, i.e. NO scavenging activity, inhibition of iNOS enzyme activity, and inhibition of iNOS mRNA expression [32]. It should also be taken into consideration that macrophages are often stimulated with LPS in order to ensure a high production of NO. However, stimulation of macrophages with LPS in the presence of flavonoids seems to be problematic since flavonoid-rich extracts have been recently found to suppress the activity of LPS and lipid A preparations regardless of the origin of the bacteria in the *Limulus* test [33] widely used for diagnosis of Gram-negative bacterial endotoxins. It is suggested that an inhibition in the production of NO is not exclusively mediated due to cellular effects of the flavonoids per se but might also be partially caused by a direct interaction of these compound with the LPS molecule. Therefore in the present investigation macrophages were stimulated with IFN-γ only.

In contrast to the monomeric and dimeric flavonoids, PCA C2 and PYC significantly increased the production of NO in IFN-γ-stimulated macrophages. Under the conditions investigated, treatment of unstimulated macrophages with PCA C2 and PYC also resulted in a considerable increase in TNF-α secretion into the medium known to be crucial for NO synthesis in macrophages. Based on monophosphoryl lipid A analysis PYC was completely devoid of lipid A. This finding suggests that PYC contains no significant amounts of Gram-negative bacterial-type LPS to contribute to its biological activities in terms of NO production, TNF-α secretion and NF-κB activation. In the absence of IFN-γ both treatment of macrophages with PCA C2 and PYC alone resulted in a considerable activation of NF-κB (Fig. 4B) that was significantly inhibited by the use of anti-murine TNF-α Ab. These data indicate that PCA C2- and PYC-induced changes in TNF-α secretion are at least partially responsible for the observed difference in NF-κB activation which again can affect NO synthesis.

4.3. NF-κB-dependent gene expression

The biosynthesis of TNF-α is tightly controlled by different molecular events regulating the TNF-α gene, mRNA and protein. At the transcriptional level, besides a TATA box promoter located 20 bp upstream of the transcription start site, a number of regulatory sequences are also found upstream of the TNF-α gene, including three NF-κB sites κ1, κ2, κ3 which are thought to be implicated in the mechanism of TNF-α induction. Furthermore, two upstream DNA regions of the iNOS promoter, the RI and RII domains, are required for the maximal promoter activation by LPS, and the RII domain mediates promoter *trans*-activation of IFN-γ. Both of these domains comprise multiple sequences homologous to those of *cis* elements involved in transcription activation, such as NF-κB binding sites, IFN-γ response elements, and IFN-γ-activated factor binding [34]. Therefore, we sought to study the effect of different structure-related flavonoids in terms of NF-κB-dependent gene expression using the dual-luciferase reporter gene assay.

Consistent with data for both TNF-α and NO, the mono-

meric flavonoids decreased whereas PCA C2 and PYC increased NF-κB-dependent gene expression indicating that differences in TNF-α secretion and NO production might be partially mediated due to difference in the activation or inhibition of NF-κB. Although no common second messenger has been identified, many NF-κB-activating signals have been shown to be inhibited by antioxidants. Plumb et al. [35] recently studied the antioxidant properties of catechin, epicatechin as well as structure-related dimeric and trimeric flavonoids similar to those used in the present study. Interestingly, the antioxidant activity in the aqueous phase increased from monomeric to trimeric flavonoids. However, in the present study the trimeric flavonoid PCA C2 as well as PYC induced a substantial activation of NF-κB. Hence changes in NF-κB-dependent gene expression due to flavonoids do not necessarily reflect differences in the antioxidant properties of these compounds. Virgili et al. [21] studied the effect of PYC on NF-κB activation and NO production in IFN-γ+LPS-stimulated murine macrophages using the same cell line. In IFN-γ+LPS-stimulated RAW 264.7 cells, PYC had no effect on NF-κB activation. PYC pretreatment was started 24 h before the IFN-γ+LPS administration. However, in the present experiment cells were pretreated with PYC only 1 h before the stimulation with IFN-γ. Possibly, both the duration of PYC pretreatment and the characteristic of the activating signal (IFN-γ+LPS vs. IFN-γ) are important in terms of PYC-induced changes in NF-κB-dependent gene expression in activated murine macrophages.

4.4. Conclusion

Flavonoids constitute a large group of phenolic phytochemicals with antioxidant properties in vitro. Despite an increasing number of publications concerning free radical-scavenging and hydrogen-donating activities of flavonoids [15], little is known about their biological activity in terms of cell signaling and gene expression. The present data show for the first time that monomeric, dimeric, and trimeric flavonoids as well as PYC display a selective activity in terms of TNF-α secretion, NO production and NF-κB-dependent gene expression in RAW 264.7 macrophages. In addition, the present findings suggest that the degree of polymerization of flavonoids seems to be important in determining the mechanism(s) by which flavonoids may exert their immunomodulatory and antiinflammatory activity. The concentrations of flavonoids used in the present study are higher than those readily available in food sources such as tea [31] or chocolate [36]. However, concentrated forms of plant extracts are now being made available thereby opening the possibility of achieving concentrations equivalent to those supplemented in the present investigation.

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