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Flavonoids from *Theobroma cacao* Down-Regulate Inflammatory Mediators

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In the present study, we report the effects of a cocoa extract on the secretion and RNA expression of various proinflammatory mediators by macrophages. Monocyte chemoattractant protein 1 and tumor necrosis factor α (TNFα) were significantly and dose-dependently diminished by cocoa extract, and this effect was higher than that produced by equivalent concentrations of epicatechin but was lower than that produced by isoquercitrin. Interestingly, cocoa extract added prior to cell activation resulted in a significantly greater inhibition of TNFα secretion. Both cocoa extract and epicatechin decreased TNFα, interleukin (IL) 1α, and IL-6 mRNA expression, suggesting that their inhibitory effect on cytokine secretion is produced, in part, at the transcriptional level. Cocoa extract also significantly decreased NO secretion in a dose-dependent manner and with a greater effect than that produced by epicatechin. In conclusion, our study shows that cocoa flavonoids not only inhibit NO release from macrophages but also down-regulate inflammatory cytokines and chemokines.

KEYWORDS: Chemokine; cytokine; epicatechin; cocoa

INTRODUCTION

Macrophages, pivotal cells in the innate immune response, participate in microbe phagocytosis, the generation of microbicidal reactive oxygen and nitrogen intermediates, and the production of immune-modulating cytokines and chemokines (*1*). Proinflammatory cytokines and chemokines produced by stimulated macrophages include tumor necrosis factor α (TNFα), interleukin (IL) 1, IL-6, and monocyte chemoattractant protein 1 (MCP-1) among others. TNFα is considered a key mediator of innate immunity since it activates neutrophils, promotes adhesion molecule expression on vascular endothelial cells, and enhances the secretion and synthesis of other proinflammatory cytokines and acute-phase proteins. TNFα also acts as an endogenous pyrogen (*2*). Moreover, IL-1 participates in the stimulation of T helper cells, B cell proliferation, and the enhancement of arachidonic acid metabolism. Furthermore, IL-6 is mainly involved in macrophage and osteoclast differentiation, T and B cell proliferation, synthesis of acute-phase proteins, suppression of albumin production, and enhancement of tumor cell growth (*3*). Finally, MCP-1 chemokine has a potent monocyte/macrophage chemotactic activity and acts as an activator of basophils, inducing histamine release playing a pivotal role in certain pathologies, such as in allergic and autoimmune processes (*4*).

In response to endotoxins and cytokines, macrophages and neutrophils synthesize nitric oxide (NO), which plays a major host defense role function due to its microbicidal properties. However, its overproduction could be hazardous to healthy tissue and has been implicated in the pathophysiology of autoimmune and inflammatory diseases (*5*). Extensive prior studies have demonstrated the efficacy of TNFα and IL-1 blocking therapies in various inflammatory and autoimmune diseases, such as rheumatoid arthritis and Crohn's disease (*6*, *7*).

Cocoa is a rich source of such flavonoids as (−)-epicatechin, (+)-catechin, and procyanidins, which are oligomers derived from these monomers (*8*, *9*). Other minor polyphenols have been identified, such as quercetin, isoquercitrin (quercetin 3-*O*-glucoside), quercetin 3-*O*-arabinose, hyperoside (quercetin 3-*O*-galactoside), naringenin, luteolin, apigenin, and others (*10*). Cocoa flavonoids are considered potent antioxidants, and their radical scavenging capacity is much higher in cocoa than in black tea, green tea, or red wine (*11*), an attribute common to the large oligomers contained in cocoa but not in tea (*12*). In fact, such oligomers are more effective than monomers in protecting against peroxynitrite-dependent oxidation (*13*, *14*). While the antioxidant effects of cocoa have been widely reported, little is known about the interaction of their flavonoids with the immune system. Recently, we demonstrated the effects of cocoa in down-regulating IL-2 secretion and IL-2 receptor surface expression on a lymphoid cell line (*15*). Moreover, Sanbongi et al. (*16*) reported that cacao liquor inhibits hydrogen peroxide and superoxide anion on stimulated granulocytes and

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lymphocytes, while Mao et al. (*17*) found that isolated procyanidins from cocoa have different effects on some cytokines secreted by peripheral blood mononuclear cells. These findings prompted us to extend our research to the effects of cocoa on inflammatory mediators synthesized by stimulated macrophages.

MATERIALS AND METHODS

Chemicals. (−)-Epicatechin, lipopolysaccharide from *Escherichia coli* (055:B5), propidium iodide (PI), fluorescein diacetate (FDA), *o*-phenylendiamine dihydrochloride (OPD), and ethidium bromide were obtained from Sigma-Aldrich (Madrid, Spain). Isoquercitrin was provided by Extrasynthese (Genay, France). Culture media, fetal calf serum (FCS), glutamine, and a mixture of penicillin−streptomycin−amphotericin B were purchased from PAA (Pasching, Austria). Recombinant rat IFNγ was obtained from BD Biosciences (Madrid, Spain). An Ultraspec RNA isolation system was obtained from Biotecx Laboratories (Houston, TX). RNAsin and oligo(dT) were obtained from Promega (Madison, WI), and Superscript-II (RT-MMLV) was obtained from Gibco Life Technologies (Scotland, U.K.). Deoxynucleotides triphosphate (dNTPs) and Taq DNA polymerase were provided by Ecogen (Barcelona, Spain). A rat inflammatory cytokine set 1 multiplex PCR kit was purchased from Biosource (Nivelles, Belgium). The rat alveolar NR8383 and the murine RAW 264.7 macrophage cell lines were provided by ATCC (American Type Culture Collection, Wiltshire, U.K.). The rat NR8383 cell line was used as a macrophage model with the exception of NO secretion, which was studied in RAW 264.7 cells because low amounts of NO were produced by the former cell line.

Preparation of Cocoa Extract and Epicatechin and Isoquercitrin Solutions. Natural Forastero cocoa powder from Malaysia was provided by Nutrexpa (Barcelona, Spain). Cocoa was subjected to an extraction of phenols following the method described by Andrés-Lacueva et al. (*18*) with some modifications. Briefly, 10 g of cocoa was mixed with 8 mL of deionized water at the boiling point. Then 32 mL of ethanol was added, and the solution was vortexed. After centrifugation, the supernatant was concentrated under vacuum to a final volume of 4 mL, avoiding light exposure. To further purify the extract, the sample was applied to a Water Oasis HLB extraction cartridge and washed with water and 5% ethanol in water. The phenols were eluted with ethanol, and the extract was evaporated under a stream of nitrogen, avoiding dryness, and reconstituted with ethanol−water (80% v/v). The total phenol content in the extract was determined according to the Folin−Ciocalteu method (*19*). The cocoa extract used in this study contained 20.4 mg/mL total polyphenols (expressed as catechin). Qualitative analysis of the cocoa extract showed a wide range of phenolic compounds (*10*), but HPLC analysis revealed that it contained 1.9 mg/mL epicatechin and 0.575 mg/mL isoquercitrin. This extract was pooled and stored at −80 °C until later use.

Stock solutions of (−)-epicatechin and isoquercitrin were prepared in a minimum volume of ethanol and were pooled and stored at −80 °C. Just before the treatment of cell lines, cocoa polyphenol extract and (−)-epicatechin and isoquercitrin ethanol solutions were diluted in fresh culture medium to obtain the desired concentrations in cell suspensions.

Treatment of NR8383 and RAW 264.7 Cell Lines with Cocoa Flavonoids. The NR8383 cell line was maintained in Ham's medium supplemented with 15% FCS, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 250 μg/mL amphotericin B. RAW 264.7 cells were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 250 μg/mL amphotericin B.

The study design consisted of treating NR8383 macrophages with either cocoa extract or epicatechin and, in some cases, isoquercitrin. Flavonoids were added either simultaneously with the stimuli or 14 h prior. Cocoa extract was used at final concentrations ranging from 5 to 100 μg/mL total polyphenols. (−)-Epicatechin was used at final concentrations of 10−400 μM, which corresponded to 2.9−116 μg/mL, and isoquercitrin was tested at 5−40 μM (equivalent to 2.32−18.6 μg/mL).

To examine the effects of cocoa flavonoids on cytokine synthesis and release, NR8383 cells (1×10^6 cells/mL in medium containing 1% FCS) were activated by the addition of LPS (10 ng/mL). Cells were immediately treated with cocoa extract, (−)-epicatechin, or isoquercitrin. Flavonoids were added in low light, and controls included unstimulated and flavonoid-treated cells and stimulated cells incubated with a flavonoid vehicle (ethanol). After 6 h of stimulation, cells were harvested to determine cell viability and to extract total RNA and supernatants collected and stored at −80 °C for TNFα and MCP-1 quantification. To enhance the effects of flavonoids on TNFα secretion, NR8383 macrophages were pretreated in some experiments with cocoa extract or epicatechin 14 h before cell stimulation.

To study the effects of cocoa flavonoids on NO secretion, RAW 264.7 cells (5×10^5 cells/mL in medium containing 1% FCS) were stimulated by LPS (10 ng/mL) and IFNγ (50 U/mL). RAW 264.7 cells were immediately treated with cocoa extract or (−)-epicatechin. Flavonoids were used under the same settings as described above using the same kind of controls. Following 24 h of activation, cells were harvested to determine cell viability and supernatants were frozen at −20 °C for the nitrite assay.

Cell Viability by Flow Cytometry. Cell viability was determined on the basis of FDA hydrolysis by cell enzymes and PI exclusion (*20*). Cells were immediately analyzed by a Coulter Epics XL2 Corp. fluorescence-activated cell sorter.

Percentages of PI- and FDA-stained cells were assessed by cytometer software (System II Software v.3.0, Coulter Corp.). Only supernatants of viable cells (>80%) were used to quantify cytokines.

MCP-1 and TNFα Quantification by ELISA. Levels of MCP-1 and TNFα secreted by NR8383 cells were quantified using rat MCP-1 and TNFα OptEIA sets from BD Pharmingen (Madrid, Sapin). ELISAs were carried out as specified by the manufacturer.

To study the effect of flavonoids on TNFα secretion, the cytokine levels secreted by untreated stimulated cells were considered as 100%. The results obtained from resting and activated cells incubated in the presence of flavonoids were compared to those of untreated resting and stimulated cells, respectively. In the case of MCP-1, the chemokine was secreted under basal conditions; thus, the levels secreted by untreated unstimulated cells were used as 100%. Results from untreated and treated stimulated cells were compared to those of unstimulated cells.

RNA Isolation and Semiquantitative RT-PCR. Total RNA was isolated from 5×10^6 NR8383 cells in a single-step method using Ultraspec RNA reagent according to the manufacturer's protocol. The purity and concentration of RNA were determined using a spectrophotometer at 260 and 280 nm, and RNA integrity was verified by denaturing-gel electrophoresis.

A 4 μg sample of total RNA was reverse-transcribed at 42 °C for 60 min in a reaction volume of 20 μL of buffer containing first strand buffer 1×, 0.2 mM dNTPs, 20 U of RNAsin, 0.5 μg of oligo(dT), and 200 U of Superscript-II (RT-MMLV). Reactions were halted by heat inactivation at 95 °C for 3 min.

Levels of TNFα, IL-1α, and IL-6 gene expression were examined using the multiplex PCR kit. The GAPDH housekeeping gene was used to correct variations between cDNA samples. Amplification was performed in a PTC-100 programmable thermal controller (MJ Research, Watertown, MA), with the thermocycle profile consisting of denaturation at 95 °C for 30 s, primer annealing at 65 °C for 30 s, and primer extension at 72 °C for 30 s during 35 amplification cycles.

PCR products were resolved by 5% acrylamide gel electrophoresis and stained with ethidium bromide. Bands were analyzed with Quantity One software, version 4 (Bio-Rad Laboratories, Hercules, CA). Results were compared to those of the GAPDH housekeeping gene and were expressed as percentages, with the values of untreated LPS-stimulated cells treated as 100%.

Nitrite Quantification by Griess Colorimetric Reaction. Supernatant nitrite concentrations were quantified as a marker of NO secretion using a commercial kit (R&D Systems, Minneapolis, MN) based on the Griess colorimetric assay. Concentrations of nitrites secreted by untreated stimulated cells were regarded as 100%, and nitrite concentrations secreted by treated stimulated cells were compared with those secreted by untreated stimulated cells.

Statistical Analysis. An SPSS 10.0 software package was used for statistical analysis. Conventional ANOVA was conducted with the

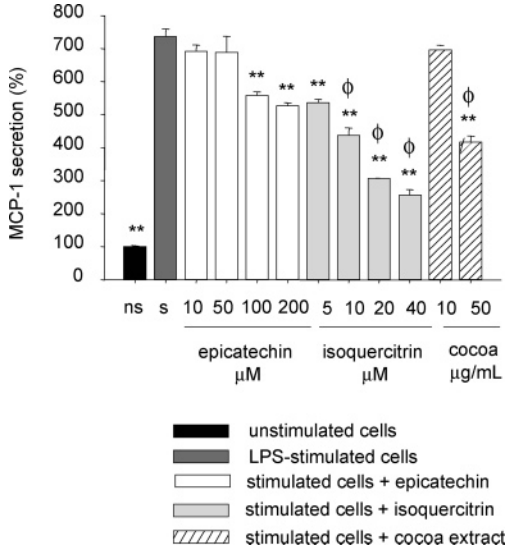

Figure 1. Effect of epicatechin, isoquercitrin, and cocoa polyphenol extract on the MCP-1 secretion of activated NR8383 cells. Flavonoids were added simultaneously with LPS. Each bar represents the mean of 10 values + SEM obtained from three individual experiments. Significant differences between LPS-stimulated cells and stimulated cells treated with flavonoids are represented by "**" ($P < 0.01$). Significant differences between stimulated cells treated with 200 μM epicatechin and stimulated cells treated with isoquercitrin or cocoa extract are represented by "ϕ" ($P < 0.01$).

flavonoid concentration as the independent variable, with viabilities and levels of MCP-1, TNFα, and NO as dependent variables. When the flavonoid content had a significant effect on the dependent variable, a Student's *t* test was performed. Epicatechin and cocoa extract treatments were also compared by means of a Student's *t* test. Results were considered significant at $P < 0.05$.

RESULTS

Effect of Flavonoids on Cell Viability Following LPS Activation. Cocoa extract (5−100 μg/mL), epicatechin (10−400 μM), and isoquercitrin (5−40 μM) did not modify NR8383 or RAW 264.7 cell viability with respect to that of untreated activated cells (data not shown).

Effects of Flavonoids on MCP-1 Secretion after LPS Activation. In a resting state, NR8383 cells secreted approximately 7.1 ± 0.3 ng/mL MCP-1 after 6 h of incubation. These levels were not significantly affected by the addition of cocoa extract, epicatechin, or isoquercitrin (data not shown). After 6 h of LPS activation, MCP-1 release increased about 7-fold over baseline levels (49.5 ± 0.2 ng/mL). All cocoa flavonoids tested were capable of reducing MCP-1 secretion in LPS-activated cells (**Figure 1**). Thus, epicatechin (100−200 μM, corresponding to 29−58 μg/mL) reduced MCP-1 secretion, exhibiting an inhibition of up to 28% ($P < 0.01$). Cocoa extract (50 μg/mL) also diminished MCP-1 secretion ($P < 0.01$). This inhibitory effect was significantly stronger than that produced by epicatechin (200 μM, corresponding to 58 μg/mL) ($P < 0.01$). Isoquercitrin (5−40 μM, which represents 2.32−18.6 μg/mL) was the most effective in lowering MCP-1 levels, causing a 28−65% dose-dependent decrease ($P < 0.01$) (**Figure 1**).

Effects of Flavonoids on TNFα Secretion Following LPS Activation. After 6 h of incubation, resting NR8383 cells produced undetectable amounts of TNFα which remained unmodified by the addition of cocoa extract, epicatechin, or isoquercitrin (data not shown). LPS stimulation induced 11.41 ± 0.5 ng/mL TNFα secretion after 6 h of incubation. Both epicatechin (10−200 μM, corresponding to 7.25−58 μg/mL) and isoquercitrin (5 and 40 μM, representing 2.32 and 18.6 μg/mL) significantly reduced TNFα secretion, achieving an inhibition of approximately 35% and 20%, respectively ($P < 0.01$) (**Figure 2A**). Cocoa extract (50 μg/mL) also significantly decreased TNFα secretion by about 15% ($P < 0.05$) (**Figure 2A**).

To enhance the effects of flavonoids on TNFα secretion, cells were pretreated with cocoa extract or epicatechin 14 h prior to cell stimulation. Epicatechin (200 μM) reduced TNFα levels by about 15% ($P < 0.01$) (**Figure 2B**), an effect lower than that occurring with simultaneous addition of flavonoids. Interestingly, cocoa extract (12.5−50 μg/mL) exerted a drastic reduction of TNFα of up to 60% ($P < 0.01$) (**Figure 2B**), which was significantly higher than that produced when the extract was added with the stimuli ($P < 0.01$).

Effects of Flavonoids on TNFα, IL-1α, and IL-6 mRNA Following LPS Activation. To determine whether the inhibitory effects of cocoa flavonoids on TNFα secretion were produced at the transcriptional level, TNFα mRNA levels were analyzed in NR8383 cells. IL-1 and IL-6 mRNA levels were also examined. The study was carried out adding flavonoids 14 h prior to cell activation. Levels of TNFα and IL-1α, but not IL-6, were present in resting cells and increased after 6 h of LPS stimulation (**Figure 3**). A 200 μM (58 μg/mL) concentration of epicatechin reduced TNFα and IL-6 mRNA levels, having a higher effect on IL-1α mRNA expression, and one which was very close to basal levels (**Figure 3**). A 25 μg/mL concentration of cocoa extract produced a greater down-regulation of TNFα, IL-1α, and IL-6 mRNA. Following cocoa extract treatment, IL-1α mRNA remained at basal levels and LPS-induced TNFα mRNA overexpression was reduced (**Figure 3**).

Effects of Flavonoids on NO Secretion Following LPS Activation. Resting RAW 264.7 cells produced undetectable levels of nitrites after 24 h of incubation. Under this setting, the addition of cocoa extract or epicatechin did not promote NO secretion (data not shown). However, the stimulation of cells with LPS and IFNγ induced nitrite release (177.77 ± 10.4 μmol/L). **Figure 4** summarizes the effects of cocoa extract and epicatechin on NO secretion of stimulated cells. Both cocoa extract (5−100 μg/mL) and epicatechin (200−400 μM) significantly reduced NO secretion in a dose-dependent manner ($P < 0.01$). Interestingly, cocoa extract reduced nitrite levels more than epicatechin. Thus, 100 μg/mL cocoa extract inhibited NO release by approximately 70% ($P < 0.01$), while 400 μM epicatechin (corresponding to 116 μg/mL) only limited NO secretion by 50% ($P < 0.01$).

DISCUSSION

In this paper, the down-regulating effects of cocoa extract containing flavonoids on the synthesis and release of pro-inflammatory mediators from macrophages are established. Epicatechin and isoquercitrin were used as controls. Both compounds constitute the main flavonoids in the cocoa, the amount of epicatechin being 3-fold higher than that of isoquercitrin (*21*).

Cocoa flavonoids decreased LPS MCP-1 chemokine induction in NR8383 cells in a dose-dependent manner. We are not aware of any previous studies reporting the effects of cocoa extract, epicatechin, or isoquercitrin on macrophage MCP-1 secretion. Cocoa extract effected a 1.5-fold greater reduction in MCP-1 levels than epicatechin, which may be attributable to the synergism between the wide spectrum of flavonoids present in this extract (*10*) and/or the role of polymeric procyanidins, which

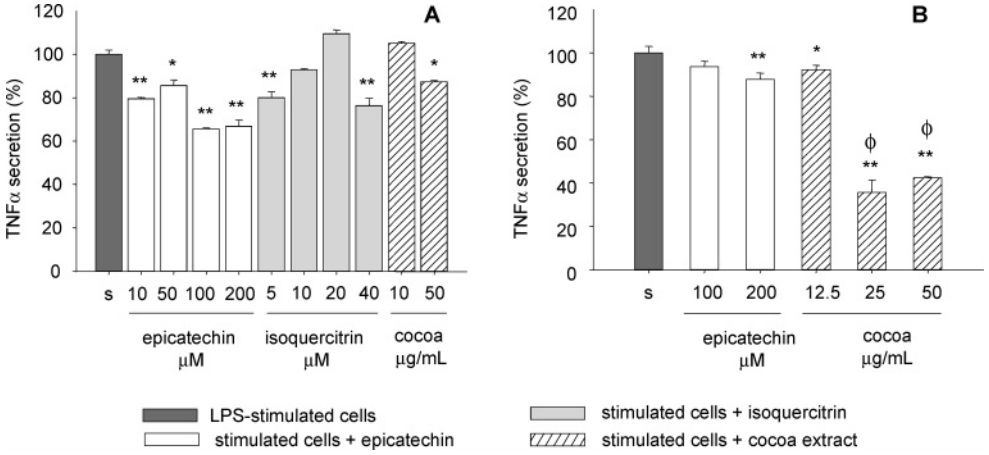

Figure 2. Effect of epicatechin, isoquercitrin, and cocoa polyphenol extract on the TNFα secretion of activated NR8383 cells: (**A**) flavonoids added simultaneously with LPS; (**B**) flavonoids added 14 h before cell stimulation. Each bar represents the mean of 10 values + SEM obtained from three individual experiments. Significant differences between LPS-stimulated cells and stimulated cells treated with flavonoids are represented by "*" ($P < 0.05$) and "**" ($P < 0.01$). Significant differences between stimulated cells treated with 200 μM epicatechin and stimulated cells treated with cocoa extract are represented by "ϕ" ($P < 0.01$).

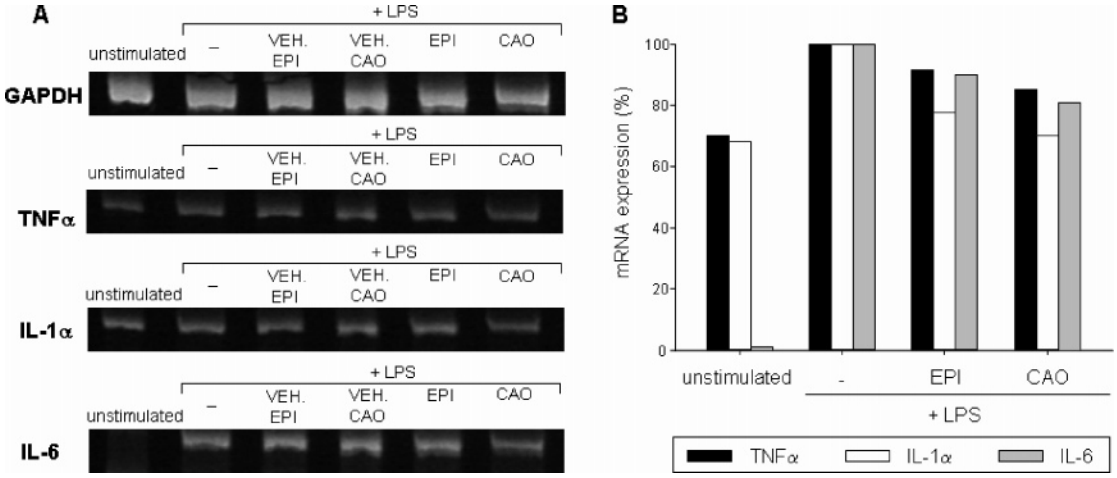

Figure 3. Effect of epicatechin (EPI) (200 μM = 58 μg/mL) and cocoa polyphenol extract (CAO) (25 μg/mL) on TNFα, IL-1α, and IL-6 mRNA expression in activated NR8383 cells. Flavonoids were added 14 h before LPS activation. Results were related to those of the GAPDH housekeeping gene. (**A**) Representative acrylamide gel of PCR-amplified products. (**B**) Quantification of the corresponding bands. Normalized TNFα, IL-1α, and IL-6 mRNA expression levels from LPS-stimulated cells were considered as 100%. mRNA expression levels of each cytokine from resting cells and stimulated cells treated with epicatechin or cocoa extract were related to their corresponding cytokine mRNA expression levels from LPS-stimulated cells.

appear to be more potent. As isoquercitrin has been shown to be the most potent MCP-1 inhibitor, this flavonoid may be an important contributor to the inhibitory actions of cocoa on MCP-1 release. Indeed, the inhibitory effect of cocoa on this chemokine suggests a comparison with such antiinflammatory flavonoids as quercetin, genistein, and daidzein, all of which induce a decrease in MCP-1 in various cell types (*22*, *23*).

Both cocoa extract and isoquercitrin were notably more active in diminishing MCP-1 than in decreasing TNFα when added simultaneously with LPS. Interestingly, cocoa flavonoids produced a much stronger effect, about 4 times greater, when they were added before LPS stimulation. This may be due to an underlying mechanism present in cocoa flavonoids which acts by modulating the early steps of cell activation. Moreover, such a period could be necessary for smaller fractions of hydrolyzing polymeric procyanidins to prepare for cellular uptake or to become converted into more active compounds.

Our results on TNFα secretion appear to diverge from those of Mao et al. (*24*), who reported that isolated procyanidins from cocoa (monomers to decamers) promoted TNFα secretion in resting and stimulated human peripheral blood mononuclear cells. This may be due to differences in the compounds tested, the cells used, and/or the experimental design. Indeed, their study only tested certain flavonoid fractions isolated from cocoa, and not the entire extract. In addition, this prior study was conducted using human blood mononuclear cells, including monocytes and lymphocytes, whereas we used a homogeneous macrophage cell line. Supporting this, the effects of cocoa flavonoids on TNFα secretion reported here are similar to those described for other polyphenols. Thus, monomeric flavonoids purified from pine bark strongly inhibit TNFα secretion in stimulated macrophages, while dimers and whole extract enhance TNFα levels (*25*). Furthermore, apigenin, luteolin, quercetin, chrysin, naringenin, and genistein have been found to reduce TNFα levels in LPS-activated macrophages (*26*−*28*). The relationship between flavonoid structure and certain activities has been the subject of debate. In this sense, TNFα inhibition appears to be related to the presence of the basal structure of flavanol, which contains a double bond at position C_2-C_3 of the C ring and an oxo function at position 4. Hydroxyl groups at positions A-5, A-7, and B-4′ are also important contributors to TNFα reduction (*28*, *29*). Consistent with these findings, we found that isoquercitrin,

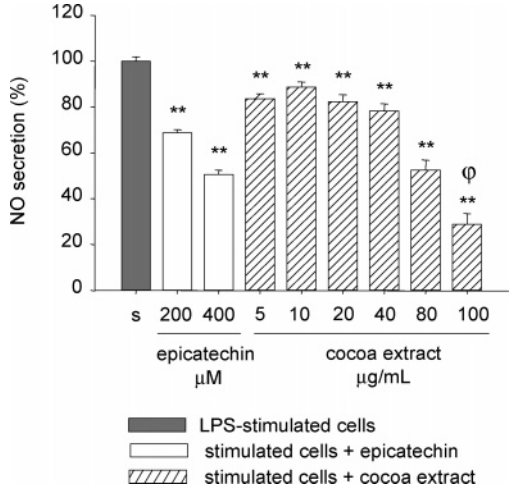

Figure 4. Effect of epicatechin and cocoa polyphenol extract on the NO release of activated RAW 264.7 cells. Flavonoids were added at the same time as LPS. Each bar represents the mean of 10 values + SEM obtained from three individual experiments. Significant differences between LPS-stimulated cells and stimulated cells treated with flavonoids are represented by "**" ($P < 0.01$). Significant differences between stimulated cells treated with 400 μM epicatechin and stimulated cells treated with cocoa extract are represented by "φ" ($P < 0.01$).

which possesses optimal structure, was more potent in blocking TNFα than epicatechin.

Although the underlying mechanisms by which flavonoids modulate cytokine secretion have not yet been clarified, our results show the down-modulating effects of cocoa flavonoids on IL-1α, TNFα, and IL-6 at the transcriptional level. The reduction of TNFα mRNA levels suggests that the effects of flavonoids on TNFα secretion may be caused, at least partially, by a transcriptional effect. Similar studies were carried out by Mao et al. (*30*), who found that isolated procyanidins (monomers to tetramers) from cocoa reduced the transcription of IL-1β while the larger fractions enhanced IL-1β gene expression in peripheral blood human mononuclear cells. On the other hand, nobiletin, a citrus polymethoxy flavonoid, was found to decrease the gene expression of TNFα, IL-1, and IL-6 in murine macrophages (*31*). A recent study also demonstrated the inhibitory effects of quercetin, chrysin, and kaempferol on IL-1β mRNA expression in activated macrophages (*32*).

The precise mechanism by which flavonoids inhibit cytokine expression remains unclear. Some transcriptional factors, such as NF-κB and AP1, are known to be redox-sensitive. Therefore, flavonoids could modify the cell redox status and inhibit the activation of these signaling cascades. However, recent studies have speculated that the antioxidant activity of flavonoids could not be the sole explanation for their effects (*33*). In this sense, we found that isoquercitrin, whose antioxidant activity is lower than that of epicatechin (*34*), produced a stronger inhibition on MCP-1 and TNFα secretion.

The effects of both cocoa extract and epicatechin on cytokine secretion were accompanied by a decreased NO production by LPS-activated macrophages. Cocoa extract once again had a greater effect than epicatechin alone, which supports the hypothesis describing the synergism between various flavonoids in cocoa extract such as catechin, epicatechin, isoquercitrin, and luteolin among others (*10*). These results are consistent with those of Ono et al (*35*), who found that a crude extract of cocoa reduced NO release from activated murine macrophages. Karim et al. (*36*) found that polymeric procyanidins (pentamers to decamers) from cocoa produced endothelial relaxation derived from the induction of NOS activity. Moreover, it has recently been reported that the ingestion of flavonol-rich cocoa by humans with diminished endothelial function restores plasma NO levels (*37*). The differences among these recent results regarding increased endothelial NOS activity and both the former results cited above and our own demonstrating decreased NO release from macrophages may be due to the different NO synthases targeted. Thus, under stress conditions cocoa extract seems to strengthen the constitutive enzyme while down-regulating the inducible enzyme.

In conclusion, cocoa flavonoids modulate macrophage activation by means of down-regulating the most representative proinflammatory mediators, MCP-1, TNFα, IL-1α, IL-6, and NO. These findings demonstrate the potential value of cocoa extract in the diet of patients with inflammatory and autoimmune diseases characterized by chronic hyperactivity of the immune system.

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