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Anti-inflammatory Properties of Clovamide and *Theobroma cacao* Phenolic Extracts in Human Monocytes: Evaluation of Respiratory Burst, Cytokine Release, NF-κB Activation, and PPARγ Modulation

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ABSTRACT: There is a great interest in the potential health benefits of biologically active phenolic compounds in cocoa (*Theobroma cacao*) and dark chocolate. We investigated the anti-inflammatory potential of clovamide (a *N*-phenylpropenoyl-L-amino acid amide present in cocoa beans) and two phenolic extracts from unroasted and roasted cocoa beans, by evaluating superoxide anion (O_2^-) production, cytokine release, and NF-κB activation in human monocytes stimulated by phorbol 12-myristate 13-acetate (PMA). The effects of rosmarinic acid are shown for comparison. Clovamide and rosmarinic acid inhibited PMA-induced O_2^- production and cytokine release (with a bell-shaped curve and maximal inhibition at 10−100 nM), as well as PMA-induced NF-κB activation; the two cocoa extracts were less effective. In all tests, clovamide was the most potent compound and also enhanced peroxisome proliferator-activated receptor-γ (PPARγ) activity, which may exert anti-inflammatory effects. These findings indicate clovamide as a possible bioactive compound with anti-inflammatory activity in human cells.

KEYWORDS: Clovamide, cocoa extracts, human monocytes, rosmarinic acid, cytokines, NF-κB, PPAR-γ

INTRODUCTION

Chocolate and cocoa products derived from the seeds of *Theobroma cacao* L. are popular foods in many countries, the pro capite consumption of chocolate in Europe ranging from 1.04 kg/year in Poland up to 11.85 kg/year in Ireland. Since 1750 B.C., the Olmec pre-Columbian civilization used cocoa as an "elite food" and a medicine; under the Emperor Montezuma, Aztecs assumed large amounts of a cocoa drink, called "chocolatl" in their language, which was later imported into Spain by Hernando Cortez.[1] Cocoa beans are rich in polyphenols, in particular catechins (flavan-3-ols, with the prevalence of epicatechin on catechin) and procyanidins (catechin/epicatechin monomers polymerize in *T. cacao*), the total polyphenol content being 6−8% by weight of the dry bean.[2,3]

In recent years, there has been a growing interest in the potential health-related benefits of antioxidant- and phytochemical-rich dark chocolate and cocoa. The high quantity of phenolic bioactive substances in cocoa has been positively correlated with its antioxidant and antiradical capacities.[4] Moreover, chocolate and cocoa-related products have been shown to decrease low-density lipoprotein oxidation and platelet activation, to enhance serum lipid profile, to favorably modify eicosanoid synthesis, to lower blood pressure, and to promote endothelium-dependent relaxation,[5−9] so supporting the beneficial role of cocoa in the cardiovascular system.[10−12] Moreover, the Kuna Indian people, who live on islands off of the coast of Panama and consume enormous amounts of cocoa daily, have lower blood pressure values and a reduced cardiovascular risk as compared with other Pan-American populations.[13]

Among the different polyphenols that have been identified in cocoa beans or cocoa-related products, clovamide (*N*-caffeoyl-L-dihydroxyphenylalanine) represents one of the less investigated. Clovamide, which belongs to the class of phenylpropenic acid amides, was first discovered in red clover (*Trifolium pratense*)[14] and, two decades later, in cocoa.[15] The clovamide content in cocoa beans ranges from 1.36 up to 2.64 mg/kg (unroasted fermented cocoa beans, dry weight), strictly depending on the origin. Also, the cocoa hulls (a cocoa byproduct released from the whole bean after the preroasting step) are rich in phenolic antioxidants and contain clovamide; indeed, the roasting process (used in cocoa technology to sanitize it and to develop its peculiar aroma, depending on the development of the Maillard reactions) strongly decreases the clovamide content (up to 50% in Ivory Coast cocoa).[16]

The structure of clovamide (Figure 1) is very similar to that of rosmarinic acid, an ester analogue occurring in various plants from the *Lamiaceae* family, especially in rosemary. Rosmarinic acid is often used as a standard "natural antioxidant", due to its antioxidant activity. Other clovamide-like structures have been identified in *T. cacao* and then synthesized.[17] Moreover, clovamide is structurally related to some β-adrenergic ligands (dobutamine, denopamine), and some clovamide derivatives have been shown to increase cAMP via β-2- adrenergic receptors in U937 cells.[18] Although clovamide is still not commercially available, its recent efficient synthesis[16] permitted us to direct some research on its

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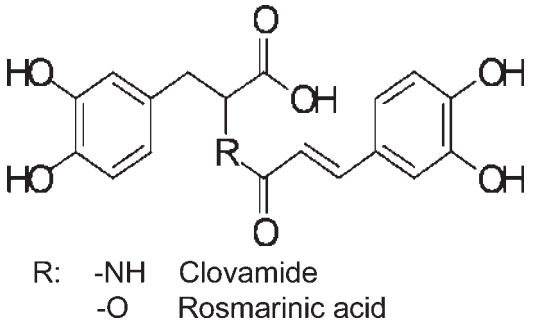

Figure 1. Chemical structure of clovamide and rosmarinic acid.

bioactivity as nutraceutical compound. Our previous data demonstrate that clovamide possesses antiradical activity[16] and exerts neuroprotective effects on different in vitro models of neuronal death.[19]

Human monocytes play a key role in inflammation and chronic diseases and represent an interesting model to evaluate the effects of potential anti-inflammatory drugs. In fact, when challenged with appropriate stimuli, they produce large amounts of oxy-radicals and release inflammatory cytokines and prostanoids etc. Moreover, they constitutively express PPARγ (peroxisome proliferator-activated receptor-γ) receptor,[20] whose role in regulating inflammatory processes and atherosclerosis is widely accepted. The anti-inflammatory potential of PPARγ agonists mainly resides in their ability to inhibit monocyte/macrophage activation and the expression of inflammatory molecules, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, iNOS, gelatinase B, and COX-2.[20,21]

Therefore, we have investigated the ability of clovamide (as well as two phenolic extracts from unroasted and roasted cocoa beans) to affect the respiratory burst, cytokine release, nuclear factor-κ B (NF-κB) activation, and PPARγ activity in human monocytes isolated from healthy donors, also in comparison with rosmarinic acid.

MATERIALS AND METHODS

Drugs and Analytical Reagents. Fetal bovine serum (FBS) was from Gibco (Paisley, United Kingdom). Phosphate buffer saline (PBS), Hystopaque, RPMI 1640 medium, glutamine, HEPES, streptomycin, penicillin, amphotericin B, protease inhibitors, superoxide dismutase (SOD), cytochrome C, phorbol 12-myristate 13-acetate (PMA), and Poly (dI-dC) were obtained from Sigma (St. Louis, MO); 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ) was from Alexis Corp. (Switzerland). All cell culture reagents, with the exception of FBS, were endotoxin-free according to details provided by the manufacturer. Rosmarinic acid [high-performance liquid chromatography (HPLC) grade] and all reagents and solvents used for synthesis of clovamide were obtained from Sigma-Aldrich (Milano, Italy); petrol ether and ethyl acetate were purchased from Carlo Erba (Rodano, Milano, Italy); silica 60 for gravimetric c.c. 70−230 mesh was from Delchimica Scientific glassware (Milano, Italy). Methanol, acetonitrile (all HPLC grade), and formic acid (50% v/v, LC-MS grade) were purchased from Sigma-Aldrich. Water was obtained by Milli-Q instrument (Millipore Corp., Bedford, MA).

Synthesis and Characterization of Clovamide. The synthesis of clovamide was obtained using the method previously described.[16] Briefly, L-3,4-dihydroxyphenylalanine was protected as methylester and subsequently condensed with caffeic acid to obtain crude clovamide methylester. This crude ester was hydrolyzed, and the residue was purified on silica gel column to obtain clovamide. The purified clovamide was characterized by means of NMR, IR, and mass analysis, and then, the purity was checked by HPLC (purity: 89%). Clovamide, as well as rosmarinic acid and the two cocoa extracts, was dissolved in ethanol just prior the experiments.

Cocoa Samples and Preparation of Cocoa Extracts. Fermented unroasted cocoa beans and their corresponding roasted nibs (geographical origin: Ghana, forastero) were kindly provided by a local enterprise. Cocoa samples were finely ground in a mixer and then extracted in the automatic Soxhlet Büchi Extraction System B-811 (Büchi Labortechnik AG, Flawil, Switzerland) for 12 h, using dichloromethane to remove the lipidic fraction; the phenolic fraction was extracted from the defatted cocoa powders in the same automatic Soxhlet apparatus, using methanol as the solvent for 4 h (up to complete discoloration). The solvent was finally evaporated to dryness (vacuum, 40 °C), and the dry extract was stored at −20 °C until use.

Isolation of Human Monocytes. Human monocytes were isolated from heparinized venous blood (30−40 mL) of healthy donors by standard techniques of dextran sedimentation, Hystopaque (density = 1.077 g/cm^3) gradient centrifugation (400*g*, 30 min, room temperature), and recovered by thin suction at the interface, as described elsewhere.[20,22,23] Cells were washed twice with PBS and resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 2 mM glutamine, 10 mM HEPES, and antibiotics (50 μg/mL streptomycin, 5 U/mL penicillin). Purified monocyte populations were obtained by adhesion (90 min, 5% CO_2, 37 °C); the nonadherent cells (mainly lymphocytes) were removed by three gentle washings with PBS.[20,22,23] Cell viability (trypan blue dye exclusion) was usually >98%; evaluation of surface markers CD14, MHCII, CD1a, and CD68 was performed as described.[22,23] A different number of monocytes was used according to the type of experiments [$0.5-1 \times 10^6$ cells for oxy-radical production, 5×10^6 cells for electrophoretic mobility shift assay (EMSA) assays, and 1×10^6 cells for cytokine release].

MTT Assay. To rule out toxic effects of the different drugs, we evaluated the cell viability by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay, in addition to the trypan blue dye exclusion evaluations. These experiments were conducted as described,[22] monocytes (1×10^5) were challenged with the maximal concentration of the compounds for 24 h. Thereafter, the medium was replaced by the MTT solution (1 mg/mL) after 2 h of incubation at 37 °C in the dark. The supernatant was removed, and DMSO was added to dissolve the purple formazan; the absorbance of the samples was read at 580 and 675 nm. All of the compounds did not reduce per se monocyte viability (absorbance values being always around 0.13 as control cells).

Assay of Superoxide Anion (O_2^-) Production. Adherent monocytes were stimulated, in the absence or presence (30 min) of clovamide (0.01−1000 nM) or other compounds (rosmarinic acid, unroasted or roasted cocoa extracts), by 1000 nM PMA for 30 min. In keeping with previous experience,[22] this PMA concentration was the optimal one to induce the respiratory burst. O_2^- production was evaluated by the SOD-inhibitable cytochrome C reduction, the absorbance changes being recorded at 550 nm in a Beckman spectrophotometer. O_2^- production was expressed as nmol cytochrome C reduced/10^6cells/30 min, using an extinction coefficient of 2.1×10^4 M^{-1} cm^{-1}.[22] To avoid interference with spectrophotometrical recordings of O_2^- production, cells were incubated with RPMI 1640 without phenol red, antibiotics, and FBS. The possibility that clovamide and other compounds interfere with the assay was assessed by evaluating compounds' ability to reduce per se cytochrome C in the medium. At the highest 1000 nM concentration, clovamide and rosmarinic acid reduced cytochrome C in the medium by 27 and 40%, respectively (data not shown); some interference (less than 10%) was observed with both clovamide and rosmarinic acid at 100 nM or roasted cocoa extract at the highest 10 μg/mL concentration (data not shown). As mentioned above, all of the compounds at study were dissolved in ethanol; at the maximal 0.1%

ethanol concentration, there was no significant interference with the spectrophotometrical recordings of O_2^- production.

Cytokine Release. Cytokine release was measured by enzyme-linked immunosorbent assay kits (Pelikine Compact; CBL, Netherlands), as described;[22,23] TNF-α was evaluated as the most relevant pro-inflammatory cytokine in human monocytes, and its amount was expressed in pg/mL, as indicated by the manufacturer's instructions. The release of IL-6, another relevant marker of cardiovascular risk, from the same samples, was also evaluated; interleukin-10 (IL-10) was measured as the most important anti-inflammatory cytokine. In these experiments, monocytes were pretreated for 1 h with clovamide (0.01−1000 nM), rosmarinic acid, or cocoa extracts and then stimulated by PMA 100 nM for 24 h. According to previous experiments,[22,23] the 24 h challenge period ensured the maximal cytokine release. The supernatants were collected and frozen until cytokine determination; a standard curve was performed for each plate and used to calculate the absolute concentrations of cytokines. The detection limits for TNF-α, IL-6, and IL-10 were 1.4, 0.5, and 2 pg/mL, respectively.

Preparation of Nuclear Extracts. Nuclear extracts were prepared by using "Nuclear Extraction Kit" (Active Motifs, Rixensart, Belgium). Briefly, adherent monocytes were resuspended in hypotonic buffer, lysed, and centrifuged (30 s, 14000*g*, 4 °C) according to manufacturer's instructions. The supernatant (cytoplasmic fraction) was recovered in a new tube; the pellet (nuclear fraction) was resuspended in lysis buffer and incubated for 30 min on ice on a rocking platform (150 rpm). The nuclear fraction was centrifuged (10 min, 14000*g*, 4 °C), and the supernatant (nuclear extract) was transferred and stored at −80 °C until use. The protein concentration was determined by using a protein assay, according to the manufacturer's instruction (Pierce, United States).

Evaluation of NF-κB Activation. The evaluation of NF-κB activation was performed by EMSA, as described.[22] Monocytes were pretreated for 1 h with clovamide, rosmarinic acid, or cocoa extracts and then stimulated by 1000 nM PMA for 1 h. According to previous experience, monocyte/macrophages challenged with 1000 nM PMA for 1 h presented a maximal NF-κB nuclear translocation.[22] Nuclear extracts (5 μg) were incubated with 2 μg of poly (dI-dC) and the [γ-^{32}P]ATP-labeled oligonucleotide probe (100000−150000 cpm; Promega, St. Louis, MO) in binding buffer (50% glycerol, 10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, and 1 mM DTT) in a final volume of 20 μL for 30 min at room temperature. The NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was from Promega. The nucleotide−protein complex was separated on a 5% polyacrylamide gel in 0.5× Bionic buffer (Sigma) at 150 V on ice. The gel was dried, and radioactive bands were detected by autoradiography.[23] Supershift assays for p65 subunit was performed using a commercial antibody (anti-NF-κB p65; final concentration 1 μL/mL) from Santa Cruz Biotechnology (CA).

Evaluation of PPARγ Activity. The activation of PPARγ was evaluated by measuring the nuclear migration by EMSA, as described.[23] In these experiments, human monocytes were stimulated for 6 h with 15d-PGJ (10 μM), a selective PPARγ agonist, or clovamide. Nuclear extracts (5 μg) were incubated with 2 μg of poly (dI-dC) and [^{32}P]ATP-labeled oligonucleotide probe (100000−150000 cpm; Promega) in binding buffer for 30 min at room temperature. The PPAR consensus oligonucleotide (5′-CAAAACTAGGTCAAAGGTCA-3′) was from Santa Cruz Biotechnology. The nucleotide−protein complex was separated on a polyacrylamide gel, the gel was dried, and radioactive bands were detected by autoradiography.[23]

Statistical Analysis. All statistical analyses were performed using SPSS statistical software (version 15.0, SPSS Inc., Chicago, IL). Data are means ± standard errors of the mean (SEMs) of "*n*" independent experiments on monocytes isolated from different healthy donors. Concentration−effect curves for clovamide, rosmarinic acid, and cocoa extracts were constructed, and their IC_{50} values (on PMA-induced O_2^- production or cytokine release) were interpolated from curves of best fit. Statistical evaluation was performed by analysis of variance between groups followed by Dunnett's multiple comparison test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Effect of Pure Compounds and Cocoa Extracts on Oxy-Radical Production in Human Monocytes. Because human monocytes are major phagocytes and release relevant amounts of oxy-radicals upon challenge with appropriate stimuli, we first checked clovamide ability to affect O_2^- production. PMA (1000 nM) is a potent stimulus in evoking O_2^- production that, in this set of experiments, amounts to 27.8 ± 3 nmol cytochrome C reduced/10^6 cells/30 min (Figure 2A−C; $n = 12$).

As reported in Figure 2A, in the range 0.01−1000 nM, clovamide inhibits PMA-induced O_2^- production with a bell-shaped curve. The maximal inhibition, observed at 10 nM concentration, is about 80%, clovamide IC_{50} value being 1.78 nM (Figure 2A; $n = 7$). Rosmarinic acid, used in the range 0.01−1000 nM, also inhibits PMA-induced O_2^- production ($IC_{50} = 135$ nM) in human monocytes, with a maximal 45% inhibition at 100 nM (Figure 2B; $n = 7$). Both cocoa extracts (unroasted and roasted cocoa samples), evaluated at different concentrations (0.1−10 μg/mL), inhibit PMA-induced O_2^- production, with maximal inhibition (about 60% for unroasted samples and 45% for roasted cocoa samples) at 1 μg/mL (Figure 2C; $n = 5$). As expected, both clovamide and rosmarinic acid reduce per se cytochrome C, at the highest 1000 nM concentration; such a direct effect (presumably to be ascribed to their antioxidant capability) is not observed at 10 nM concentration (data not shown).

Clovamide and Cocoa Extracts Inhibit the Release of Pro-inflammatory Cytokines in Human Monocytes. Clovamide inhibits, in a concentration-dependent manner (0.01−1000 nM) and with a bell-shaped curve, PMA-induced TNF-α release in human monocytes (Figure 3; $n = 8$), maximal inhibition (about 50%) being observed at 10−100 nM. Rosmarinic acid (10^{-11}−10^{-6} M; Figure 4A; $n = 8$) and the two cocoa samples (unroasted or roasted; 0.01−10 μg/mL; Figure 4B; $n = 6$) also reproduce the clovamide's inhibitory effects. Maximal inhibition and the concentration at which it was observed are as follows: rosmarinic acid (45 ± 7% inhibition at 10 −100 nM), unroasted cocoa sample (50 ± 12% inhibition at 10 μg/mL), and roasted cocoa sample (45 ± 15% inhibition at 10 μg/mL). In the experiments reported in Figures 3 and 4, PMA-evoked TNF-α release amounts to 910 ± 52 pg/mL ($n = 16$). By evaluating PMA-induced IL-6 release (Table 1), clovamide, rosmarinic acid, and the two cocoa extracts exert inhibitory effects with results similar to those reported above for TNF-α. In the case of IL-6, too, clovamide results the most potent compound (Table 1). In these experiments, PMA-induced IL-6 release in monocytes is 313 ± 47 pg/mL ($n = 6$). In keeping with previous observations,[22] 100 nM PMA releases very small amounts of IL-10 (46 ± 7 pg/mL; $n = 6$) in monocytes, which are significantly lower than the levels of pro-inflammatory cytokines (see above). All of the compounds under study exert some inhibitory effects on IL-10 release (Table 2); however, because of the low PMA-evoked IL-10 release, the quantitative effect is difficult to determine and does not reach statistical significance.

Clovamide and Cocoa Extracts Inhibit PMA-Induced NF-κB Translocation in Human Monocytes. In these experiments,

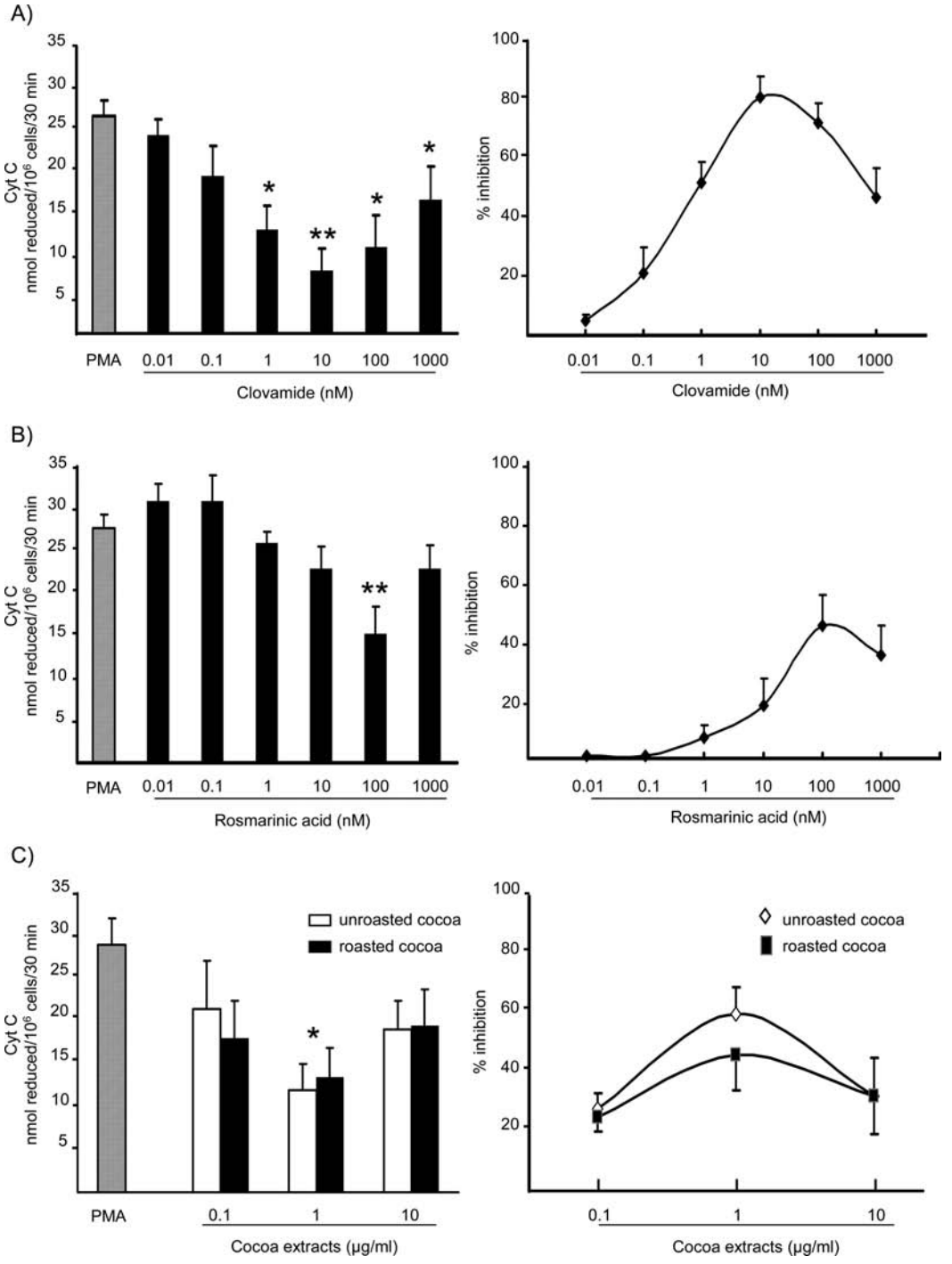

Figure 2. Effects of clovamide, rosmarinic acid, and the two cocoa extracts on O_2^- production from PMA-challenged human monocytes. On the left: O_2^- production in monocytes; on the right: % inhibition of PMA-induced O_2^- production. (A) Clovamide inhibits in a concentration-dependent manner O_2^- production in monocytes. Human monocytes were pretreated with or without clovamide (0.01−1000 nM) for 30 min and then stimulated by 1000 nM PMA for 30 min. Values are means ± SEMs; $n = 7$. *$P < 0.05$ vs PMA, and **$P < 0.01$ vs PMA. (B) Concentration-dependent effects of rosmarinic acid. Human monocytes were pretreated with or without rosmarinic acid (0.01−1000 nM) for 30 min and then stimulated by 1000 nM PMA for 30 min. Values are means ± SEMs; $n = 7$. **$P < 0.01$ vs PMA. (C) Concentration-dependent effects of unroasted cocoa extract (□) and roasted cocoa extract (■). Human monocytes were pretreated with or without the two cocoa extracts (0.1−10 μg/mL) for 30 min and then stimulated by 1000 nM PMA for 30 min. Values are means ± SEMs; $n = 5$. *$P < 0.05$ vs PMA.

cells are stimulated by 1000 nM PMA for 1 h, which ensures maximal effects.[22] In human monocytes, PMA induces a marked NF-κB nuclear translocation (Figure 5A,B), with a major involvement of the p65 subunit, as demonstrated by supershift assays (see later, Figure 5C). As depicted in Figure 5A, clovamide and rosmarinic acid, evaluated in the range 10−1000 nM, inhibit PMA-induced NF-κB activation. The endogenous PPARγ agonist 15d-PGJ, known to inhibit NF-κB activation,[22] has been

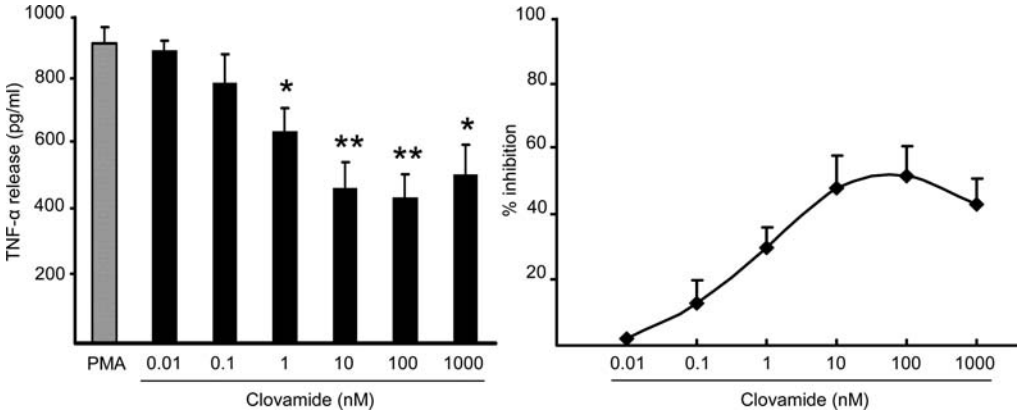

Figure 3. Clovamide inhibits PMA-induced TNF-α release in human monocytes. On the left: TNF-α release; on the right: % inhibition of PMA-induced TNF-α release. Human monocytes, pretreated for 1 h with or without clovamide (0.01−1000 nM), were stimulated by PMA 100 nM for 24 h. Values are means ± SEMs; $n = 8$. *$P < 0.05$ vs PMA; and **$P < 0.01$ vs PMA.

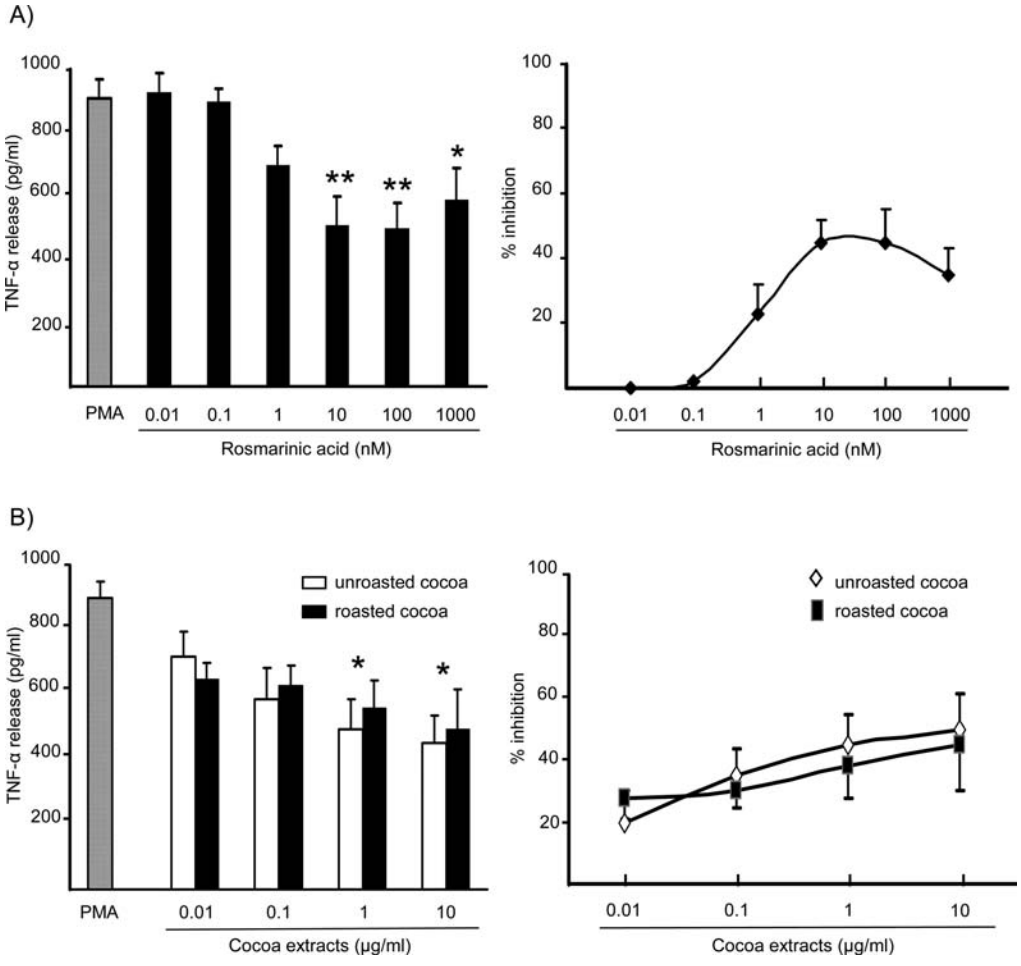

Figure 4. Rosmarinic acid and the two cocoa extracts inhibit PMA-induced TNF-α release in human monocytes. On the left: TNF-α release; on the right: % inhibition of PMA-induced TNF-α release. (A) Human monocytes, pretreated for 1 h with or without rosmarinic acid (0.01−1000 nM), were stimulated by PMA 100 nM for 24 h. Values are means ± SEMs; $n = 8$. *$P < 0.05$ vs PMA; and **$P < 0.01$ vs PMA. (B) Human monocytes, pretreated for 1 h with or without different concentrations (0.01−10 μg/mL) of unroasted (□) and roasted cocoa extract (■), were stimulated by PMA 100 nM for 24 h. Values are means ± SEMs; $n = 6$. *$P < 0.05$ vs PMA.

used for comparison (Figure 5A). Likewise, the two cocoa extracts inhibit PMA-induced NF-κB translocation with maximal effect at a 10 μg/mL concentration (Figure 5B). Supershift assays for NF-κB p65 subunit also have been conducted, to further confirm the effects of clovamide and rosmarinic acid (Figure 5C). PMA induces a marked NF-κB nuclear translocation with a major

Table 1. Effects of Clovamide, Rosmarinic Acid, and Two Cocoa Extracts on PMA-Induced IL-6 Release in Human Monocytes[a]

treatment	IL-6 release (pg/mL)	% inhibition
100 nM PMA	313 ± 47	
+ 1000 nM clovamide	150 ± 15*	52 ± 3
+ 100 nM clovamide	76 ± 5**	76 ± 4
+ 10 nM clovamide	56 ± 5**	82 ± 2
+ 1 nM clovamide	82 ± 5**	73 ± 5
+ 0.1 nM clovamide	225 ± 20	28 ± 10
+ 0.01 nM clovamide	293 ± 18	6 ± 3
+ 1000 nM rosmarinic acid	191 ± 12*	39 ± 4
+ 100 nM rosmarinic acid	176 ± 15*	44 ± 5
+ 10 nM rosmarinic acid	141 ± 10**	55 ± 3
+ 1 nM rosmarinic acid	250 ± 12	20 ± 5
+ 0.1 nM rosmarinic acid	297 ± 10	5 ± 3
+ 0.01 nM rosmarinic acid	320 ± 30	0
+ 10 μg/mL unroasted cocoa	103 ± 2**	67 ± 4
+ 1 μg/mL unroasted cocoa	144 ± 11*	54 ± 5
+ 0.1 μg/mL unroasted cocoa	270 ± 65	24 ± 5
+ 0.01 μg/mL unroasted cocoa	329 ± 70	0
+ 10 μg/mL roasted cocoa	109 ± 22*	65 ± 7
+ 1 μg/mL roasted cocoa	57 ± 3**	81 ± 3
+ 0.1 μg/mL roasted cocoa	82 ± 9**	74 ± 4
+ 0.01 μg/mL roasted cocoa	182 ± 43	41 ± 12

[a] Values are means ± SEMs; $n = 6$. *$P < 0.05$ vs PMA, and **$P < 0.01$ vs PMA.

Table 2. Effects of Clovamide, Rosmarinic Acid, and Two Cocoa Extracts on PMA-Induced IL-10 Release in Human Monocytes[a]

treatment	IL-10 release (pg/mL)	% inhibition
100 nM PMA	46 ± 7	
+ 1000 nM clovamide	40 ± 4	10 ± 4
+ 100 nM clovamide	30 ± 6	30 ± 9
+ 10 nM clovamide	35 ± 5	22 ± 4
+ 1 nM clovamide	41 ± 5	7 ± 5
+ 0.1 nM clovamide	50 ± 10	0
+ 1000 nM rosmarinic acid	41 ± 10	11 ± 4
+ 100 nM rosmarinic acid	30 ± 9	31 ± 12
+ 10 nM rosmarinic acid	32 ± 8	29 ± 10
+ 1 nM rosmarinic acid	45 ± 5	4 ± 2
+ 0.1 nM rosmarinic acid	48 ± 7	0
+ 10 μg/mL unroasted cocoa	40 ± 2	9 ± 3
+ 1 μg/mL unroasted cocoa	44 ± 11	4 ± 2
+ 0.1 μg/mL unroasted cocoa	50 ± 15	0
+ 10 μg/mL roasted cocoa	38 ± 7	15 ± 5
+ 1 μg/mL roasted cocoa	49 ± 12	0
+ 0.1 μg/mL roasted cocoa	50 ± 13	0

[a] Values are means ± SEMs; $n = 6$.

involvement of the p65 subunit, which is significantly inhibited by both clovamide and rosmarinic acid, evaluated at 1000 nM (Figure 5C). In this case, too, the higher effect of clovamide, as compared to rosmarinic acid, can be appreciated.

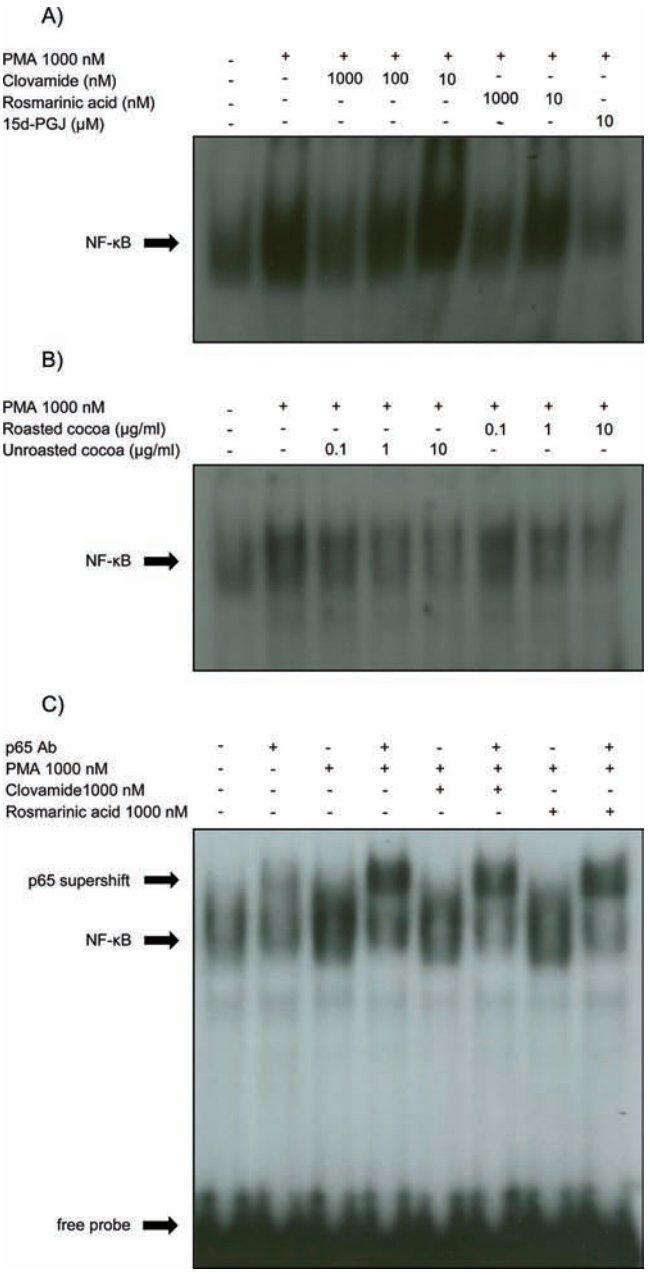

Figure 5. Clovamide, rosmarinic acid, and the two cocoa extracts inhibit PMA-induced NF-κB translocation in human monocytes. (A) Effects of clovamide (10−1000 nM) and rosmarinic acid (10 and 1000 nM). Human monocytes, pretreated for 30 min with clovamide or rosmarinic acid, were stimulated by 1000 nM PMA for 1 h. The effect of 15d-PGJ at 10 μM is shown for comparison. (B) Effects of unroasted and roasted cocoa extracts (0.1−10 μg/mL). Human monocytes, pretreated for 30 min with cocoa extracts, were stimulated by 1000 nM PMA for 1 h. (C) Supershift for p65 subunit. Human monocytes, pretreated for 30 min with 1000 nM clovamide or 1000 nM rosmarinic acid, were stimulated by 1000 nM PMA for 1 h. Each blot is representative of three other independent experiments.

Clovamide Enhances PPARγ Activity in Human Monocytes. Because PPARγ protein is constitutively expressed in human monocyte/macrophages[20,22,23] and clovamide has been reported to increase PPARγ expression in SH-SY5Y neuroblastoma cells,[19] we have evaluated whether clovamide affects PPARγ

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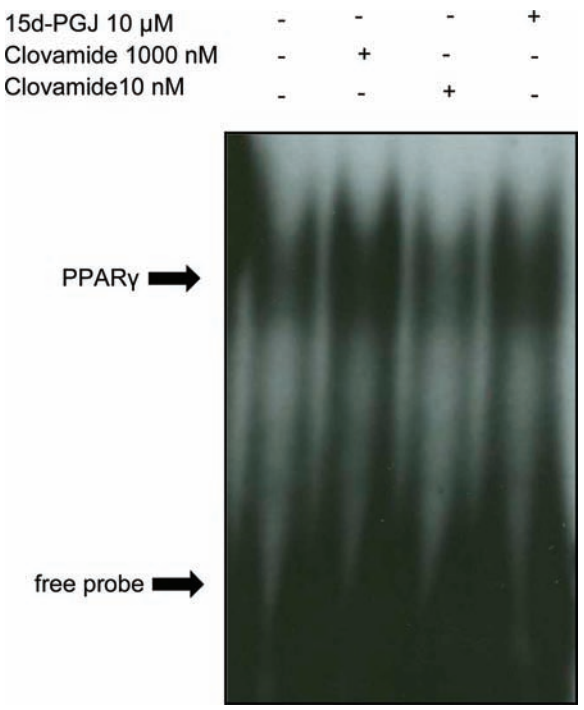

Figure 6. Clovamide enhances PPARγ activity in human monocytes. Human monocytes were stimulated for 6 h with the selective PPARγ agonist 15d-PGJ (10 μM) or clovamide at two different concentrations (10 and 1000 nM). This blot is representative of two others.

activity in human monocytes. As shown in Figure 6, clovamide, used at two different concentrations, potently enhances PPARγ activity (evaluated by EMSA assays); the effect of the PPARγ agonist 15d-PGJ is shown for comparison. Interestingly, at the highest 1000 nM concentration, clovamide is about as effective as the PPARγ agonist (Figure 6).

DISCUSSION

Over the past years, polyphenols have been the subject of numerous investigations for their several properties, often correlated to the antioxidant/antiradical capacity, and cocoa has been demonstrated to be rich in polyphenols, especially cathechins and proanthocyanidins.[2,24,25] Although still poorly investigated, clovamide, the amidic analogue of rosmarinic acid, is an interesting compound concerning the nutraceutical research, which has been shown to exhibit a stronger antioxidant activity than dideoxyclovamide in different in vitro tests.[15] According to Arlorio et al.,[16] the antioxidant activity of clovamide is comparable to that of rosmarinic acid, with EC_{50} values around 10 μM (DPPH$^{\bullet}$ method).

The antioxidant and antiradical properties of rosmarinic acid are well recognized, and this compound has been shown to inhibit O_2^- production, nitric oxide (NO) release, and iNOS protein synthesis, as well as peroxynitrite-mediated damage, in RAW264.7 murine macrophages.[26] In these cells, rosmarinic acid acts dose dependently and is more potent in inhibiting PMA-induced O_2^- production (evaluated by chemiluminescence, which provides a global measure of all of the oxy-radical species produced, and not only superoxide anions) than LPS-induced NO release: as an example, at 10 μM, it inhibits the respiratory burst to about 60%, while it exerts negligible effects on nitrite accumulation.[26]

At present, the anti-inflammatory potential of clovamide has not been fully investigated, and there is no information about its effects in human cells from healthy individuals. In this study, we show that clovamide inhibits PMA-induced oxy-radical production, cytokine release, and NF-κB activation in human monocytes and, at the highest 1000 nM concentration, also enhances PPARγ activity. Under the same experimental conditions, rosmarinic acid and two cocoa extracts obtained from unroasted and conventionally roasted beans, although exerting some effects, do not display similar levels of activity.

Cocoa extracts have been demonstrated to possess antiradical properties in cell-free systems [16] and to exert a protective effect against liver cytotoxicity.[25] In liver cells, both cocoa extracts (roasted and unroasted; evaluated at 0.5 mg/mL) reduce the cytotoxic effects of celecoxib, possibly by inducing autophagy mechanisms.[25] This observation could suggest that cocoa acts as a putative chemopreventing agent, useful in the diet of both healthy individuals and patients. In fact, the nutraceutical use of food to prevent damage, as well as to improve wellness, represents a fundamental issue in modern nutrition.

The results here reported corroborate these notions, since clovamide, rosmarinic acid, and the two cocoa extracts inhibit, with different potencies, PMA-induced O_2^- production, cytokine release, and NF-κB activation in human monocytes. Interestingly, clovamide, which also enhances PPARγ activity (see later), is the most potent compound in each assay, while the two cocoa extracts, although effective, demonstrate less potency. By evaluating the respiratory burst and cytokine release, we have observed that clovamide and rosmarinic acid demonstrate a bell-shaped curve. We have no definite explanations for this, but it is tempting to suggest that both compounds act in a biphasic manner, possibly affecting specific target(s) to be identified; at lower concentrations, they potently inhibit cytokine release and O_2^- production, whereas at higher concentrations, a diminished inhibitory effect is observed.

Particularly relevant is clovamide's ability to reduce the respiratory burst. In this assay, clovamide acts in the range 0.01−1000 nM, with maximal inhibition (about 80%) at 10 nM and an IC_{50} value of 1.78 nM, significantly lower than the one measured in cell-free tests (see ref 16 for comparison). This is in keeping with the fact that human monocytes, as major phagocytes, express an efficient NADPH oxidase, a multicomponent enzyme that is dormant in resting cells but is rapidly activated on exposure to appropriate stimuli, and suggests human monocytes as a valid in vitro human model to evaluate antiradical effects of phenolic compounds. Rosmarinic acid, too, reduces PMA-induced O_2^- production, with a maximal 43% inhibition at 0.1 μM and an IC_{50} of 135 nM, which is about 70-fold higher than clovamide. Anyway, in human monocytes, rosmarinic acid is more efficient than in murine RAW 264.7 cells.[26] On the contrary, the two cocoa extracts are significantly less potent, displaying a significant inhibition at 1 μg/mL only.

The compounds studied also affect the release of pro-inflammatory cytokines, TNF-α being reduced by about 50% in monocytes treated with clovamide 10−100 nM. At the same concentrations, rosmarinic acid displayed a maximal 40% inhibition, whereas the two cocoa extracts reduced PMA-evoked TNF-α release at higher (1−10 μg/mL) concentrations. Even more relevant effects are documented by measuring PMA-induced IL-6 release; in this case, an 80% inhibition is observed with 10 nM

clovamide, possibly due to the lower quantitative release of IL-6, as compared to TNF-α. It is worth reminding that TNF-α and IL-6 are relevant biomarkers for cardiovascular risk; therefore, clovamide (and, to a minor extent, the other compounds here evaluated) could be regarded as a potential tool in the prevention and/or treatment of cardiovascular diseases. Indeed, clovamide type phenylpropenoic acid amides significantly reduce P-selectin expression and platelet-leukocyte interactions in in vitro and in vivo models.[27,28] Cocoa flavanols and related procyanidins have been repetitively indicated to affect cytokine release and to improve endothelial function (see refs 9 and 10 for reviews). In the last years, beneficial results of cocoa ingestion have been reported in various clinical trials.[9,29,30] In a recent crossover trial enrolling 42 volunteers at high risk of cardiovascular disease, the intake of 40 mg cocoa powder for 4 weeks significantly reduced serum levels of P-selectin and intercellular adhesion molecule-1, but not IL-6, and resulted in a significantly lower expression of VLA-4, CD40, and CD36 in monocytes.[30]

The release of pro-inflammatory cytokines is generally the result of gene transcription, which is controlled by the activation of various transcription factors. Among the different signal transduction pathways involved in cytokine secretion, we have focused our attention on NF-κB and PPARγ, which are both functionally active in human monocytes.[20,22]

In this study, clovamide inhibits, in a concentration-dependent manner, PMA-induced NF-κB translocation, with maximal inhibition at 100−1000 nM. At these concentrations, clovamide effects are significantly higher than equimolar concentrations of rosmarinic acid, while cocoa extracts are less effective. Previous observations demonstrate that rosmarinic acid (evaluated at 10 μM), but not clovamide, significantly reduces NF-κB activity in SH-SY5Ycells, which constitutively express high levels of the transcription factor.[19] In our opinion, these contrasting results can be ascribed to the different cell populations, a neuroblastoma cell line in the former experiments and human monocytes from healthy donors in the actual experiments. Both clovamide and rosmarinic acid have been previously shown to increase PPARγ protein expression in SH-SY5Y cells.[19]

As reported in the present work, clovamide significantly enhances PPARγ activity in human monocytes, with a profile similar to that of the endogenous PPARγ agonist, 15d-PGJ. In our opinion, this represents an interesting finding of the study, since PPARγ activation is largely regarded as a relevant anti-inflammatory mechanism.

Although we have not yet assessed clovamide bioavailability, it is interesting to note that, in mice, oral administration of *N*-caffeoyltyramine (a clovamide type phenylpropenoic amide; 0.1 mg/30 g body weight) ensures a plasma concentration around 50 nM,[31] very similar to the concentrations at which clovamide acts in our in vitro models.

Concerning all of these findings, we suggest clovamide as a novel bioactive compound endowed with anti-inflammatory properties. All together, these findings indicate that clovamide exerts greater effects than rosmarinic acid and cocoa extracts on three anti-inflammatory mechanisms (inhibition of respiratory burst, pro-inflammatory cytokine release, and NF-κB activation), so regulating human monocyte activity. Although clovamide is present in low amounts in cocoa powder, its nutraceutical value and its possible beneficial effects for the treatment of cardiovascular inflammatory disorders could be evidenced by adding it to cocoa-derived products.

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ABBREVIATIONS USED

EMSA, electrophoretic mobility shift assay; IL-6, interleukin-6; IL-10, interleukin-10; NF-κB, nuclear factor-κB; O_2^-, superoxide anion; 15d-PGJ, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PMA, phorbol 12-myristate 13-acetate; PPARγ, peroxisome proliferator-activated receptor-γ; TNF-α, tumor necrosis factor-α

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