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Inhibitory Effects of Grape Seed Procyanidins on Foam Cell Formation in Vitro

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Human and animal studies have demonstrated that procyanidin-rich diets reduce the risk of cardiovascular diseases and atherosclerosis. Some beneficial effects have been attributed to the well-known antioxidant activity of procyanidins. This study investigated another potential corrective role of procyanidins in cholesterol flux and inflammation in macrophage-derived foam cells. RAW 264.7 macrophages were cultured with moderately oxidized LDL (oxLDL), minimally oxidized LDL (moxLDL), or LPS (0.5 μg/mL) and oxLDL (LPS + oxLDL) to induce foam cells. Then, cells were treated with procyanidins derived from grape seed (PE, 45 μg/mL) for the last 12 h of incubation with the different lipoproteins (25 μg/mL). After lipid extraction, it was determined that total and esterified cholesterol and triglyceride accumulations in foam cells were increased by lipoprotein treatment but reduced by PE incubation. To asses the effect of PE on gene expression, the relative mRNA levels of CD36, ABCA1, iNOS, COX-2, and IκBα were determined by RT-PCR. It was shown that PE reduced the oxLDL scavenger receptor expression (CD36) and enhanced ATP-binding cassette A1 (ABCA1) expression, a key regulator of macrophage cholesterol efflux. PE also down-regulated inflammatory-related genes such as inducible nitric oxide synthase (iNOS) and kappa beta inhibitor-alpha (IκBα) without modifying COX-2 expression. In conclusion, evidence is provided that procyanidins may attenuate the development of foam cell formation by reducing cholesterol accumulation and modulating the expression of key genes in cholesterol flux and inflammation.

KEYWORDS: Procyanidins; atherogenesis; CD36; ABCA1; cholesterol; oxidized lipoproteins

INTRODUCTION

Procyanidins are natural phenolic compounds from the flavonoid class that comprise the oligomeric forms of monomeric catechins and epicatechins (see **Figure 1**). They are fairly abundant in several foods and drinks of plant origin such as grapes, cocoa, various berries, apples, nuts, red wine, chocolate, and tea. The beneficial effects of procyanidins have been largely attributed to their well-known antioxidant activity. Yet, their bioactivity is not limited to their antioxidant actions. We have previously reported that procyanidins can reduce the inflammatory response in LPS-activated macrophages by inhibiting the NF-κB pathway (*1*). Some recent human trials have demonstrated that procyanidins are cardioprotective, decreasing total cholesterol, LDL levels, and oxidized LDL (*2*), reversing the endothelial vasomotor dysfunction in patients with proven coronary artery disease (*3*), and reducing lipid peroxides in human plasma (*4*). Strong in vivo and in vitro evidence now exists to indicate that procyanidins could reduce cardiovascular disease resulting from atherosclerosis, which is the leading cause of morbidity and mortality in Westernized countries (*2, 3, 5*). Accumulation of modified LDL, such as oxidized LDL (oxLDL), and the recruitment of monocytes in the arterial subendothelial spaces are early events in atherogenesis. Macrophages take up oxLDL through the scavenger receptor pathways and become foam cells. Foam cells are well-known to play an important role in the development and progression of atherosclerosis, through the production of various bioactive molecules, such as growth factors and cytokines (*6*).

The primary cellular event that drives early atherosclerotic lesion formation is the unregulated accumulation of cholesterol ester by intimal-associated macrophages (*7*). As CD36 and other scavenger receptors are not subjected to negative regulation by high levels of intracellular cholesterol, massive accumulation of cholesterol esters can occur in macrophages, resulting in foam cell formation (*8*). It has been demonstrated that exposure to oxLDL resulted in a marked induction of CD36 mRNA expression, implying that CD36 mRNA was transcriptionally regulated by lipoproteins. Induction of CD36 mRNA expression was accompanied by an increase in CD36 protein (*9*). Thus,

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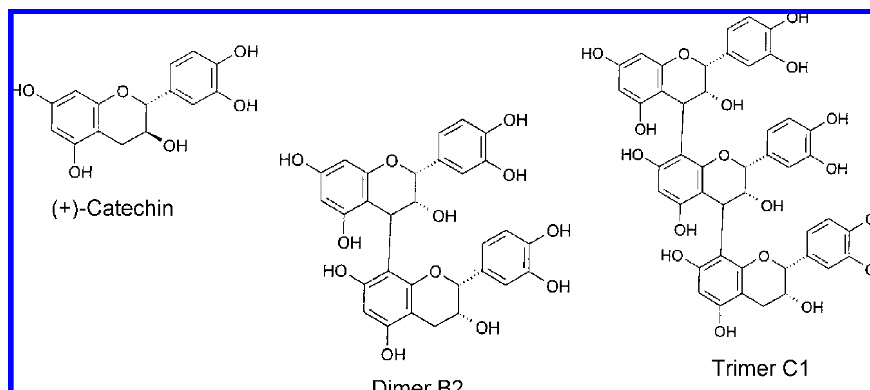

Figure 1. Structures of procyanidins derived from the three phenolic ring-basic structure of flavonoids. Procyanidins are highly hydroxylated polymers, composed of the monomeric structure flavanol (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate.

macrophage expression of CD36 and foam cell formation may be perpetuated by a cycle in which oxLDL drives its own uptake.

Although there are multiple mechanisms involved in the efflux of cellular cholesterol, the first step in the reverse cholesterol transport is linked to ABCA1 (*10*), a transmembrane protein mediating lipid efflux from cells to HDL, which then facilitates direct and indirect transport to the liver for biliary excretion (*11*). It has been concluded that ABCA1 plays an important role in cholesterol homeostasis and atherogenesis (*12*).

Several lines of evidence strongly support the hypothesis that oxidation of LDL is an essential step in its conversion to an atherogenic particle in animals (*13*). Although the precise mechanisms responsible for LDL oxidation are not known with certainty, lipooxygenases, myeloperoxidase, inducible nitric oxide synthase, and NADPH oxidases have been proposed as possible contributing enzymes because they can lead to LDL oxidation in vitro and they are expressed in human atherosclerotic lesions, where macrophages express each of these enzymes. Although macrophages may not be required to initiate LDL oxidation, they are likely to amplify oxidative reactions in macrophage-rich areas of atherosclerotic lesions (*14*). Activated macrophages also produce arachidonate metabolites that are synthesized by the cyclooxygenase (COX) enzyme. COX-2 is the inducible form of the enzyme that is rapidly induced by various proinflammatory stimuli to result in prostaglandin synthesis associated with inflammation, atherogenesis, and carcinogenesis (*15*).

Broadly speaking, atherosclerosis can be considered as a form of chronic inflammation resulting from interaction between modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall (*16*). The transcription factor NF-κB is a central mediator of gene expression induced by proinflammatory and proatherogenic stimuli, including inflammatory cytokines, oxidative stress, LPS, and bacterial products. Toll-like and interleukin receptor families deliver signals from a wide spectrum of ligands through downstream signaling to activate NF-κB translocation, inducing the coordinated expression of specific genes (*6, 17*).

Among the many genetic and environmental risk factors that have been identified by epidemiologic studies, infectious agents are recognized as being involved in the development of atherosclerosis. Recent studies have demonstrated that lipopolysaccharide (LPS) induces immune, proinflammatory, and other as yet undetermined mechanisms that may be important in triggering atherogenesis (*18*). Specialized functions of macrophages have evolved to protect the body from infection. However, the same mechanisms that enable the phagocytosis of pathogens and activation of leukocytes also permit the uptake of lipoproteins and release of reactive oxygen species and immune mediators that collectively contribute to atherosclerosis.

Most human trials and in vitro studies are focused on the preventive effects of procyanidins driven by its antioxidant capacity. Now, we want to investigate the corrective effects of procyanidins in cholesterol flux and inflammation in three models of macrophage-derived foam cells. New approaches to inhibiting lipid accumulation in macrophage foam cells and reducing inflammatory responses may be of therapeutic value in preventing coronary artery disease.

MATERIALS AND METHODS

Chemicals. According to the manufacturer, the grape seed procyanidin extract (PE) contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5−13 units) (31.7%) procyanidins and (4.7%) phenolic acids. LPS (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich, Inc. Human LDL were purchased from Calbiochem, U.K.

Oxidation of Lipoproteins. LDL was oxidized using 200 μg/mL in sterile PBS containing 10 μM $CuSO_4$ and incubated at 37 °C for 24 h (oxLDL; moderate oxidation) or 5 h (moxLDL; minimal oxidation) with shaking. Oxidation was arrested with 150 mM NaCl and 0.01% EDTA, pH 7.4, and the modified LDL was then washed and concentrated to about 1 mg/mL using Amicon Centriplus ultrafilters (Millipore). The extent of oxidation was determined by measuring the level of thiobarbituric acid-reactive substances (TBARs). Concentration of LDL protein was determined according to the Bradford method. All lipoproteins were used within 24 h of preparation.

Cell Culture Conditions and Treatments. Murine macrophage cell line RAW 264.7 (European Tissue Culture Collection ECACC, ref 91062702, London, U.K.) was cultured in DMEM containing 10%(v/v) fetal bovine serum, 2 mM D-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 25 mM HEPES. Cells were grown at 37 °C and with 5% CO_2 in fully humidified air and used for experiments between passages 5 and 14. Uptake of the neutral red dye was used as a measure of cell viability in response to procyanidin treatment (*1*). Nontoxic concentration of oxidized LDL was obtained from the bibliography (*19, 20*). We used fully supplemented medium with 10% of FBS to perform the experiments. Four different models were assayed: (1) Cells were incubated with native LDL (nLDL, 25 μg/mL) for 24 h. (2) Cells were incubated with moderately oxidized LDL (oxLDL, 25 μg/mL) for 24 h. (3) Cells were activated with 0.5 μg/mL LPS for 12 h and then incubated with oxLDL (25 μg/mL) for 24 h in fresh medium. (4) Cells were incubated with minimally oxidized LDL (moxLDL, 25 μg/mL) for 24 h. The PE treatment was performed during the last 12 h of incubation with the different lipoproteins. We used nontreated macrophages as a control group. The culture medium for control and treated cells was collected and tested. TBARs of the culture medium after the treatments were measured using the TBARs assay. It was normalized to the total protein content.

Table 1. TC, TG, and TBARs Contents of nLDL, oxLDL, and moxLDL[a]

parameter	nLDL	oxLDL	moxLDL
TC (μg/μg of protein)	2.12 ± 0.11	2.45 ± 0.21	2.24 ± 0.23
TG (μg/μg of protein)	0.25 ± 0.01	0.28 ± 0.02	0.26 ± 0.01
TG/TC ratio	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.02
TBARs (pmol/μg of protein)	0.75 ± 0.15	4.11 ± 0.38*	1.84 ± 0.28*

[a] oxLDL and moxLDL were prepared and oxidized by exposure to $CuSO_4$ (10 μM), and the total cholesterol (TC), triglyceride (TG), and thiobarbituric acid-reactive substances (TBARs) levels were determined. nLDL, native LDL; oxLDL, moderately oxidized LDL; moxLDL, minimally oxidized LDL. Data shown are the mean ± SEM of three independent preparations.

Quantification of Lipid Accumulation. The extent of foam cell formation was determined by monitoring the cell's lipid uptake using two different methods: oil red O staining and measurement of the internalized cholesterol and triglycerides. After the treatments, cells were washed twice with PBS, and total cellular lipids were extracted by incubating them for 30 min with hexane/2-propanol (3:2, v/v). The lipid extracts were dried under nitrogen and redissolved in 200 μL of 2-propanol. Total (TC) and free cholesterol (FC) and triglyceride (TG) contents of the extracts were measured using enzymatic colorimetric tests, following the manufacturers' instructions (QCA, Barcelona, Spain; and Wako Clinical Diagnostics Reagents, respectively). Cholesterol ester (CE) content was calculated as the difference between total and free cholesterol. All results were normalized to total protein content measured using the Bradford method.

CD36, ABCA1, iNOS, COX-2, and IκBα mRNA Analysis by RT-PCR Real Time. RNA from treated cells was isolated with a High Pure RNA Isolation Kit from Roche. cDNA was synthesized from 1 μg of total RNA using oligo-dT and Superscript II Rnase Reverse Transcriptase (Life Technologies). Twenty nanograms of cDNA was subjected to quantitative RT-PCR amplification using Sybr Green Master Mix (Applied Biosystems). The forward and reverse primers are as follows: CD36, F, 5′ TCGGAACTGTGGGCTCATTG 3′, R, 5′ CCTCGGGGTCCTGAGTTATATTTTC 3′; ABCA1, F, 5′ CAACTACAAAGCCCTCTTTG 3′, R, 5′ CTTGGCTGTTCTCCATGAAG 3′; iNOS, F, 5′ GGATCTTCCCAGGCAACCA 3′, R, 5′ AATCCACAACTCGCTCCAAGATT 3′; IκBα, F: 5′ CTTGGTGACTTTGGGTGCTGAT 3′, R, 5′ GCGAAACCAGGTCAGGATTC 3′; COX-2, F, 5′ GGAGAGACTATCAAGATAGT 3′, R, 5′ ATGGTCAGTAGACTTTTACA 3′; GAPDH, F, 5′ CATGGCCTTCCGTGTTCCT 3′, R, 5′ CCTGCTTCACCACCTTCTTGA 3′. Reactions were run on a quantitative PCR system, the thermal profile settings were 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 2 min. All results were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Calculations and Statistical Analysis. Results are expressed as mean value ± SEM. Effects were assessed using ANOVA or Student *t* test. We used Tukey's test of honestly significant differences to make pairwise comparisons. All calculations were performed using SPSS 14.0 software.

RESULTS

Characteristics of Native and Oxidized LDL. Total cholesterol and triglyceride contents were not significantly changed after oxidation of the lipoprotein particles. The extent of oxidation of the lipoprotein, as measured by the levels of TBARs present, was markedly increased after exposure to $CuSO_4$ (10 μM) by approximately 5.6-fold for oxLDL and 2-fold for moxLDL. The total cholesterol and triglyceride contents of nLDL, oxLDL, and moxLDL are given in **Table 1**.

Estimation of Lipid Peroxidation after PE Treatment. Concentrations of TBARs are an index of lipid peroxidation and oxidative stress. In the present study, malonaldehyde, a final product of the oxidative degradation of polyunsaturated fatty acids, was detected in the culture medium after treatment with different oxidized lipoproteins using the TBARs assay. As

Table 2. Estimation of Lipid Peroxidation in the Culture Medium after PE Treatment[a]

treatment	TBARs (pmol/μg of protein)
none	25.4 ± 3.8 (1.00)
nLDL	23.2 ± 1.1 (0.91)
oxLDL	48.5 ± 7.1* (1.91)
oxLDL + PE	46.4 ± 6.3* (1.83)
LPS + oxLDL	67.1 ± 3.7* (2.80)
LPS + oxLDL + PE	76.2 ± 3.7* (3.00)
moxLDL	37.9 ± 4.5* (1.49)
moxLDL + PE	36.2 ± 6.9* (1.42)

[a] Cells were incubated for 24 h with native LDL (nLDL; 25 μg/mL), moderately oxidized LDL (oxLDL; 25 μg/mL), pretreated for 12 h with LPS (500 ng/mL), and then incubated with oxLDL (LPS + oxLDL; 25 μg/mL) or incubated with minimally oxidized LDL (moxLDL; 25 μg/mL), in the absence or presence of the procyanidin extract (PE; 45 μg/mL) for 12 h. The extent of culture medium lipid peroxidation was determined by TBARs assay. Data are the mean ± SEM from three separate experiments. *, $P < 0.05$ compared to untreated macrophages. Values in parentheses are percent with respect to the culture medium of the untreated cells.

shown in **Table 2**, lipid peroxidation after oxLDL incubation increased 2-fold and in the LPS + oxLDL model increased 3-fold compared to the control and nLDL media. Treatment with moxLDL slightly increased lipid peroxidation. At the concentrations tested, we did not show any effect of PE (45 μg/mL) on lipid peroxidation in oxLDL, moxLDL, or LPS + oxLDL models.

Procyanidin Extract Reduces Lipid Accumulation in Foam Cells. First, the effects of PE on lipid accumulation in RAW 264.7 macrophages were assessed by staining with oil red O. Qualitative determination of the extent of staining showed that exposure of macrophages to oxLDL, moxLDL, or LPS + oxLDL for 24 h led to an increase of lipid accumulation in cells, whereas nLDL had little effect. Furthermore, PE treatment reduced total lipid accumulation in all treatments (**Figure 2**).

When we measured the lipid content of the cells, we obtained results consistent with those obtained from oil red O staining (**Table 3**). No increase in lipid content was observed with nLDL. A marked rise of TC and CE was found after incubation with oxLDL, where PE was able to reduce both ester and total cholesterol contents. Although TG content was not increased, PE provoked a reduction of nearly 40% of its content. Incubation with LPS + oxLDL induced lipid accumulation, thus increasing TC, CE, and TG contents. After PE incubation, these parameters were significantly reduced. Addition of moxLDL increased CE and TG contents. In this treatment PE again reduced lipid accumulation. As shown in **Table 3**, any LDLox treatment or PE produced variations in FC levels.

Procyanidin Extract Modulates the Expression of Cholesterol Flux Related Genes. To further investigate PE activity, we assessed its effect on the expression of genes implicated in atherosclerosis development. CD36 expression levels are of particular importance for cholesterol influx as CD36 is implicated in oxidized lipid uptake. As shown in **Figure 3A**, CD36 mRNA was increased when macrophages were incubated with oxidized lipoproteins, up to 3-fold in moxLDL treatment. Moreover, PE reduced CD36 expression in all of the foam cell models tested. ABCA1 is characterized as the rate-limiting unidirectional cellular cholesterol exporter. Interestingly, PE was able to increase ABCA1 expression (**Figure 3B**).

PE Down-regulates Inflammatory-Related Genes as iNOS and IκBα. The inflammatory response production of NO by iNOS contributes to LDL oxidation in vivo. We show that iNOS

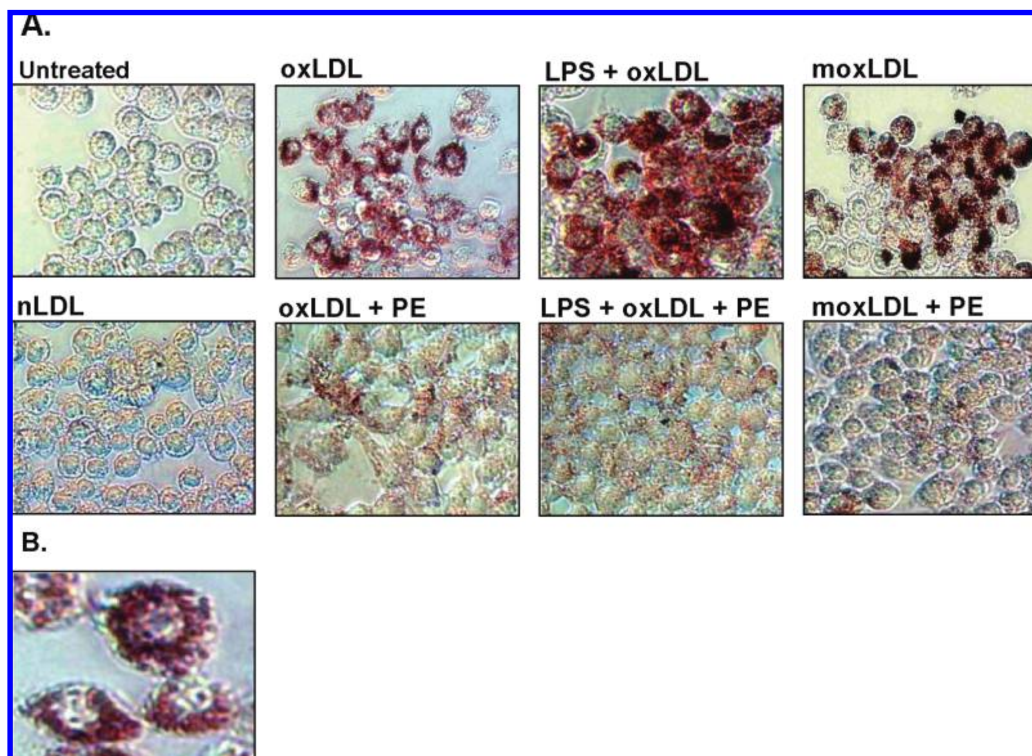

Figure 2. (**A**) Foam cell formation assayed by oil red O staining. RAW 264.7 macrophages were exposed to 25 μg/mL nLDL, oxLDL, LPS + oxLDL, or moxLDL for 24 h in the presence or absence of PE, fixed and stained with oil red O. Foam cell formation in the oxidized LDL-exposed cells is indicated by the punctate oil red O staining of lipid droplets. (**B**) Representative enlargement of RAW 264.7 macrophages oil red O staining.

expression was markedly modified as expected by LPS + oxLDL treatment, which is reflected in the increase of lipid peroxidation in this model. In addition, PE could reduce iNOS mRNA relative expression in all of the treatments performed (**Figure 3C**). Neither treatment with nLDL nor treatment with moxLDL modified the level of IκBα expression. In contrast, oxLDL and LPS + oxLDL increased the transcription of IκBα, and only in oxLDL treatment did PE reduce IκBα expression (**Figure 3D**).

We also determined COX-2 mRNA levels and showed that COX-2 expression was induced by only LPS + oxLDL treatment and that PE could not modulate this induction (LPS + oxLDL, 1.02 ± 0.02; LPS + oxLDL + PE, 0.938 ± 0.05).

CD36 Expression Positively Correlates with Total Cholesterol Accumulation. To test possible associations between mRNA levels of CD36, the total cholesterol accumulation and the effect of PE on these parameters, Spearman's rank correlation test was performed analyzing the data from the three independent experiments. We show a significant positive correlation between CD36 expression and total cholesterol levels ($\rho = 0.738$, $p < 0.05$).

DISCUSSION

Procyanidins are biochemically active compounds with anti-inflammatory and free radical-scavenging properties (*1*). In this study we assessed the corrective effect in vitro of a procyanidin extract (PE) on foam cell formation. For this reason we tested the PE effect in three foam cell models. Cells were treated with moderately oxidized lipoprotein (oxLDL) or minimally oxidized lipoprotein (moxLDL) or pretreated with endotoxin and incubated with oxLDL (LPS + oxLDL) to mimic the inflammatory state of atherosclerosis.

The bioavailability of procyanidins is a matter of intensive study. To better reflect the situation in vivo, we carefully studied the dosage used for the experiment. We used a dose of procyanidins of 45 μg/mL (~50 μM) that contains approximately 9.6 μg/mL of monomers (~26 μM), 7.8 μg/mL of dimers (~13.5 μM), 7.3 μg/mL of trimers (~8.4 μM), 6 μg/mL of tetramers (~11.5 μM), and 14.3 μg/mL of further polymerizated procyanidins (5−10 units). It is widely accepted that monomeric forms are absorbed in humans, reaching concentrations that range from 0.1 to 13 μM (*21, 22*). Further polymerized procyanidins have been detected in their native form in rat plasma (dimers to tetramers) and reached concentrations of ~3 μg/mL for dimers and of ~7.5 μg/mL for each trimeric and tetrameric form (*23*). Within these data, we selected a concentration that contained approximately the concentrations found in plasma of each component of the extract.

We first evaluated the PE effect on lipid accumulation in response to different degrees of lipoprotein oxidation. In agreement with the previously reported effects, after assessing the total cholesterol and triglyceride contents, we showed that native LDL had not caused any change in lipid accumulation in the cells because high cellular cholesterol levels down-regulate native LDL receptor gene transcription (*14, 20*). In contrast, total cholesterol increased because of incubation with the different oxidized lipoproteins. Free cholesterol is toxic for the cell and, as we show in our results, its levels must remain unchanged (*14*), whereas cholesterol esters were increased after the incubation with the oxLDLs. Once we confirmed the accumulation of lipids in the foam cells, we assessed the PE effect on the lipid content and observed that PE decreased the total cholesterol accumulation because of a decrease of approximately 30% in the content of cholesterol esters. PE also reduced TG accumulation in all treatments performed.

Experimental data suggest there is a role for oxLDL in the pathogenesis of atherosclerosis (*24*). Dietary antioxidants attenuate the cytotoxic effects of oxidatively modified LDL and restore endothelial function in patients with coronary artery

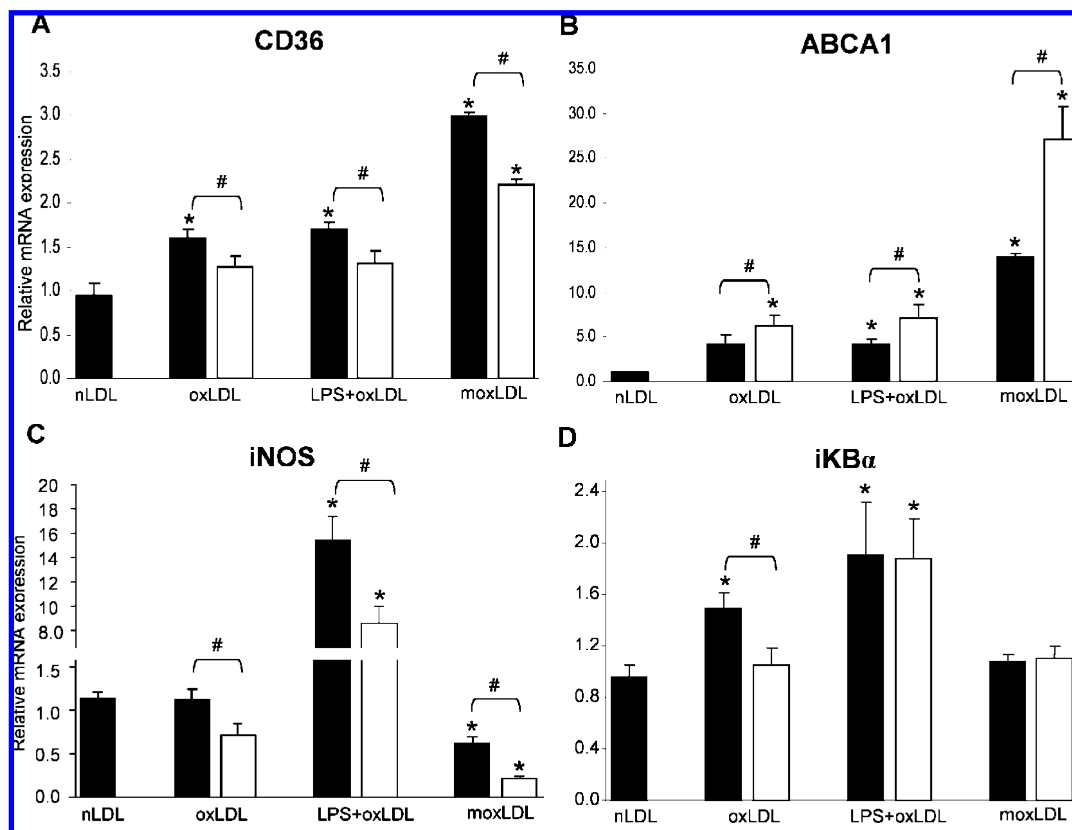

Figure 3. PE effect on CD36, ABCA1, iNOS, and iKBαa relative mRNA expression. Open bars indicates PE treatment. Cells were incubated for 24 h with native LDL (nLDL; 25 μg/mL), moderately oxidized LDL (oxLDL; 25 μg/mL), pretreated for 12 h with LPS (500 ng/mL), and then incubated with moderately oxidized LDL (LPS + oxLDL; 25 μg/mL) or incubated with minimally oxidized LDL (moxLDL; 25 μg/mL), in the absence or presence of the procyanidin extract (PE; 45 μg/mL). Values are means ± SEM of three independent experiments performed in triplicate. *, $P < 0.05$ compared to nLDL; #, $P < 0.05$ compared in each treatment in the absence or presence of PE.

Table 3. Effect of PE Treatment on the TC, CE, FC, and TG Contents[a]

treatment	TC	CE	FC	TG
none (μg/μg of protein)	0.54 ± 0.06	0.31 ± 0.05	0.23 ± 0.04	0.79 ± 0.14
none	100.0 ± 11.1	100.0 ± 16.9	100.0 ± 22.5	100.0 ± 17.6
nLDL	91.8 ± 20.6	81.2 ± 30.4	88.4 ± 14.5	122.3 ± 11.1
oxLDL	202.0 ± 10.2*	162.0 ± 22.0*	96.6 ± 12.0	102.4 ± 12.9
oxLDL + PE	147.8 ± 14.9*#	111.1 ± 13.2#	93.6 ± 13.9	60.9 ± 13.9*#
LPS + oxLDL	270.6 ± 12.4*	254.5 ± 33.0*	110.3 ± 5.1	178.3 ± 26.0*
LPS + oxLDL + PE	204.0 ± 24.2*	174.2 ± 46.8*#	117.7 ± 13.9	134.6 ± 17.8*#
moxLDL	137.7 ± 17.6	145.4 ± 13.0*	99.2 ± 13.0	141.2 ± 9.8*
moxLDL + PE	98.9 ± 11.1#	103.4 ± 14.8*#	102.6 ± 9.9	102.4 ± 5.1#

[a] Cells were incubated for 24 h with native LDL (nLDL; 25 μg/mL), moderately oxidized LDL (oxLDL; 25 μg/mL), pretreated for 12 h with LPS (500 ng/mL), and then incubated with oxLDL (LPS + oxLDL; 25 μg/mL) or incubated with minimally oxidized LDL (moxLDL; 25 μg/mL), in the absence or presence of the procyanidin extract (PE; 45 μg/mL). The total cholesterol (TC), cholesterol ester (CE), free cholesterol (FC), and triglyceride (TG) contents of the cells were determined. Lipid concentrations were normalized to the total cell protein content. Data are expressed as a percentage of the value found in the untreated macrophages and are the mean ± SEM from three separate experiments. *, $P < 0.05$ compared to untreated macrophages; #, $P < 0.05$ compared in each treatment in the absence or presence of PE.

disease (*25*). Some authors have described how flavonoids prevent and protect LDL from lipid peroxidation in vivo and in vitro (*2, 3, 5*). To further investigate the way PE works, we assessed the potential corrective effect of procyanidins after lipoprotein oxidation. We showed that the LPS + oxLDL model produced higher levels of lipid peroxidation than the oxLDL model. These results reveal that the LPS activation of the macrophage oxidases amplifies the lipid peroxidation of LDL. Many human trials have demonstrated a preventive role of natural antioxidants on LDL oxidation (*26, 27*), but with our results we should rule out a corrective effect of procyanidins because PE treatment could not return lipoproteins to their nonoxidized state at the concentration tested. These results indicate that PE is inhibiting different mechanisms, other than lipid peroxidation, that are able to reduce lipid accumulation in these foam cell models.

Because fatty streaks of atherosclerosis contain large numbers of macrophage derived foam cells that express class B scavenger receptor CD36 as well as other scavenger receptors, we investigated the PE effect on CD36 expression. OxLDL is not a single, well-defined entity, but has structural and physical properties that vary according to the degree of oxidation. The

extent of LDL lipid oxidation affects the induction of CD36 and influences its effect on cell function. As other authors have reported (*28*), in this work there appears to be greater induction of CD36 by moxLDL compared with oxLDL, suggesting that early products from moxLDL (such as lipid hydroperoxides) are involved in the transcriptional regulation of CD36 and that they are gradually degraded in oxLDL (*10*). Our results also show that PE modulates the expression of CD36. PE is able to reduce CD36 expression to basal levels in both oxLDL and LPS + oxLDL models and to inhibit CD36 expression significantly in the moxLDL model. As a result of this inhibiting effect, PE could reduce the cholesterol influx from oxidized lipoproteins.

Previously reported works showed a differential expression profile of ABCA1 gene in response to the treatment with different lipoproteins (*29, 30*). We show an enhanced expression of ABCA1 after the treatments with oxidized lipoproteins, where the highest induction resulted in moxLDL model. In this study, we observed for the first time how treating cholesterol-loaded macrophages with PE significantly induced ABCA1 expression in all of the treatments. PE then may exert antiatherogenic effects by facilitating the removal of cholesterol from macrophages via the cholesterol transporter protein ABCA1, but further studies must be performed to confirm such hypothesis.

Both clinical and experimental data suggest that atherosclerosis is a chronic inflammatory disorder associated with endothelial and cholesterol balance dysfunction (*17, 18*). Members of the NF-κB family coordinately regulate gene clusters that control inflammatory responses and have been involved in the development of atherosclerosis (*31*). The development of NF-κB inhibitors will be of interest because of their potential antiatherogenic properties (*32*). We have previously reported that PE inhibits the NF-κB pathway in LPS-activated macrophages (*1*). In the present study we investigated whether PE could inhibit the inflammatory response in a macrophage-derived foam cell model. For this reason we studied IκBα expression, which is an early target gene of NF-κB, the expression of which positively correlates with NF-κB activation (*33*). As expected, the LPS + oxLDL model produced a 2-fold induction of its expression. It is also important to appreciate that incubation with oxLDL also activated NF-κB, as other authors have reported (*31*), which could add to local inflammation when occurring in macrophages in atherosclerotic plaques. Interestingly, PE reduced IκBα expression when cells were treated with oxLDL, restoring its levels to the basal state.

Nitric oxide (NO) is a potent oxidant produced by both endothelial cells and macrophages. NO produced via the much higher capacity iNOS in macrophages can amplify the oxidative reactions in the foam cell. iNOS expression is mainly transcriptionally regulated through the activation of the NF-κB pathway. Evidence that inducible nitric oxide synthase contributes to LDL oxidation in vivo has recently been provided by studies demonstrating that apo E-deficient mice which lack iNOS develop less atherosclerosis and that inhibitors of iNOS decrease atherosclerosis in rabbits (*34*). We have shown that the extent of lipid peroxidation was higher in the LPS + oxLDL model, probably due to the induced activity of iNOS and other macrophage oxidases, as myeloperoxidase and NADPH, that contribute to LDL oxidation. In our study, iNOS expression was also induced in oxLDL treatment. Furthermore, in this work we have shown that iNOS expression is reduced by PE treatment, probably as a result of the inhibition of NF-κB activity. We also investigated the role of PE in the regulation of COX-2 expression, an inducible isoform responsible for high levels of prostaglandin production during inflammation and immune responses. As expected, we showed that COX-2 expression was induced by LPS + oxLDL treatment, although PE could not reduce this induction.

The current study reveals that foam cell formation was markedly induced after incubation with different oxidized lipoproteins. We have demonstrated that the endotoxin-induced model showed the highest lipid accumulation and peroxidation levels, probably because of the enhanced inflammatory response. However, the most important gene induction of CD36 and ABCA1 resulted in the moxLDL model. It has been suggested that there is a link between lipid metabolism and inflammatory pathways driven through the cross-talk between PPARs and NF-κB (*17*). CD36 and ABCA1 expressions are mostly regulated by PPARα and PPARγ nuclear receptors, which have been described as anti-inflamatory factors through inhibition of the NF-κB activation pathway. In this work we show that the lowest levels of iNOS and IκBα correspond to the highest induction of CD36 and ABCA1 in the moxLDL model, which can indicate a reduction in NF-κB activity driven by PE and the subsequent activation of PPARs.

In conclusion, we found that procyanidins have the ability to regulate the expression of genes causing the imbalance between lipid influx and efflux. These findings also show a down-regulation of the inflammatory response mediated by PE through the inhibition of iNOS and IκBα expression. These in vitro data help to interpret the beneficial effects of procyanidins in reducing the risk of atherosclerosis after the administration of flavonoid-rich foods or supplements. The differentially expressed genes modulated by procyanidins help to uncover novel targets and may help to target disease interactions in atherosclerosis in the future. Further studies utilizing atherosclerosis-prone animal models are needed to clarify our findings.

ABBREVIATIONS USED

ABCA1, ATP binding cassette A1; IκBα, alpha inhibitor of kappa beta; LPS, lipopolysaccharide; LDL, low-density lipoprotein; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; NF-κB, nuclear factor-kappaB; PE, procyanidin extract; PPARγ, peroxisome proliferator-activated receptor-gamma.

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