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RESEARCH ARTICLE

Dietary curcumin inhibits atherosclerosis by affecting the expression of genes involved in leukocyte adhesion and transendothelial migration

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Scope: The aim of the study was to examine the atheroprotective effect of dietary curcumin in a mouse model of atherosclerosis and to identify its cellular and molecular targets at the vascular level.

Methods and results: ApoE$^{-/-}$ mice were fed with curcumin at 0.2% (wt/wt) in diet for 4 months. This supplementation reduced the extent of atherosclerotic lesion by 26% and induced changes in expression of genes implicated in cell adhesion and transendothelial migration or cytoskeleton organization, as revealed by a transcriptomic analysis in the aorta. Expression profile of these genes suggests reduction in both leukocyte adhesion and transendothelial migration. In agreement with this hypothesis, we observed a reduction (–37%) in macrophage infiltration in the plaque, as measured by immunohistochemistry, and, in vitro, a lower adhesion of monocytes to TNF-α-stimulated endothelial cells (–32%) after exposure to a nutritionally achievable concentration of curcumin. These changes in gene expression could be related to the observed increased expression of IκB protein and decrease of TNF-α-induced NF-κB/DNA binding and NF-κB-transcriptional activity upon exposure to curcumin.

Conclusion: Our findings pointed out that the antiatherogenic effect of curcumin could be linked to its effect on gene networks and cell functions related to leukocyte adhesion and transendothelial migration via NF-κB-dependent pathways.

Keywords:
Atherosclerosis / Curcumin / Macrophage infiltration / Monocyte adhesion / Nutrigenomics

1. Introduction

Atherosclerosis represents an important chronic inflammatory process associated with several pathophysiological reactions in the vascular wall. It is correlated with high plasma cholesterol and oxidized LDL levels and oxidative stress [1] and is initiated by endothelial dysfunction triggered by various atherogenic risk factors. This leads to monocyte adhesion and migration across the endothelial layer into the subendothelial space. Monocytes differentiate into macrophages and take up oxidized lipoproteins, forming lipid-rich cells known as foam cells. The lesion formation is accompanied by smooth muscle cell proliferation and dedifferentiation as well as extracellular matrix formation [2].

A growing number of epidemiological, clinical, and experimental data suggest that some bioactive compounds present in plant foods, namely polyphenols, could contribute to the positive association between fruit and vegetable consumption and coronary heart disease risk [3]. Even if the putative health benefit of herbs and spices need further investigations,

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this issue could be considered since both of them constitute rich sources of common or particular polyphenols. Among these bioactives is curcumin, the principal curcuminoid in turmeric, a spice obtained from the rhizomes of *Curcuma longa* that is commonly consumed in South and southeast Asian countries. Curcumin has been shown to exhibit potent antioxidant, anti-inflammatory, immunomodulatory, proapoptotic, and antiangiogenic properties [4]. These effects have been observed to be mediated by the capacity of curcumin to modulate activity of signaling pathways, transcription factors, and in consequence gene expression [5, 6]. Epidemiological studies and clinical trials have shown an important chemoprotective effect of curcumin on colorectal and pancreatic cancers [7–9]. So far, no clinical trial had considered the cardiovascular protective effect of curcumin. Nevertheless, several animal studies have reported hypocholesterolemic [10–14], antiatherogenic [14–16], antiplatelet [17], and antioxidant activities [11, 16] of this compound. Furthermore, in vitro, curcumin has been shown to exhibit anti-inflammatory action on human vascular cells. Curcumin attenuates inflammatory response of TNF-α-stimulated human endothelial cells by interfering with NF-κB [18–20] and is capable of preventing platelet-derived growth factor (PDGF)-stimulated migration of vascular smooth muscle cells (VSMCs) by inhibiting MMP-9 activity and expression [21,22].

Based on the above considerations, the purpose of the present study was to establish the effect of a dietary curcumin supplementation on atherosclerosis development in apolipoprotein E deficient (apoE$^{-/-}$) mice and to identify, using nutrigenomic approach, the cellular and molecular targets of curcumin in the aorta. We demonstrated that curcumin exhibits antiatherogenic effects in apoE$^{-/-}$ mice without affecting lipemia. The nutrigenomic study revealed genes which expression is directly modulated by curcumin in aorta. These genes are involved in different processes such as cellular adhesion, cellular cytoskeleton, focal adhesion, or actin cytoskeleton. Expression profile of these genes suggests reduction in leukocyte adhesion and transendothelial migration, hypothesis in agreement with the observed reduction in macrophage number in the plaque, and the lower adhesion of monocytes to endothelial cells. As revealed from these in vivo and cell experiments, the protective effect of curcumin seems to be mediated by its capacity to modulate, at the vascular level, expression of genes involved in the early steps of atherosclerosis development.

2 Materials and methods

2.1 Animals and diets

Homozygous apoE-deficient mice were purchased from Jackson Laboratories (Charles River Laboratories, L'Arbresle, France) and interbred to obtain the males used for the present study. Mice were individually housed in wire-bottomed cages in a temperature-controlled room (22 ± 0.8°C) with a 12-h light-dark cycle and a relative humidity of 55 ± 10%. The mice had free access to food and water. All animals were maintained and handled according to the recommendations of the Institutional Ethics Committee of the INRA, in accordance with decree No 87–848. At 8 weeks of age, male mice were divided into two groups (15 mice per group) fed iso-energetic diet: a control diet or the same diet supplemented with 0.2% of curcumin (Sigma Aldrich, Saint-Quentin Fallavier, France) for 16 weeks (Supporting Information Table S1). The dose of curcumin used in this study is equivalent to 1 g curcumin consumption in humans, a level of consumption achievable in South-Asian countries [23]. No significant difference in weight gain was observed between the two groups at the end of the experimental period (data not shown). After 16 weeks, mice were anaesthetized (40 milligram pentobarbital per kilogram of body weight) and blood was collected from the abdominal aorta into heparinized tubes and plasma was stored at −80°C. After washing with sterilized PBS, aorta and heart samples were immediately frozen in liquid nitrogen and stored at −80°C.

2.2 Quantification of atherosclerotic lesions

Atherosclerotic lesions were assessed by measuring lipid deposit in the aortic sinus as previously described [24]. Briefly, five sections of 10 μm thickness were harvested per slide and 35 slides per mouse were prepared and stained with Oil red O (Merck, Darmstadt, Germany) and counterstained with hematoxylin (Diapath, Martinengo, Italy). Each section was evaluated for Oil red O staining area under microscope. Image analysis was carried out using the ImageJ free software (http://rsb.info.nih.gov/ij/) to quantify the cross-section surface areas of the lesions and the cross-section surface area of the aorta. The fractionation area, expressed as the percentage, of the lesion was calculated by dividing the surface of the lesion by the surface of the vessel. This approach allows correcting for errors caused by oblique sections that could lead to overestimation of the surface area occupied by a lesion.

2.3 Microarray analysis

The different steps of the microarray analysis, RNA extraction and labeling, hybridization, and image and data analyses were realized as previously described [25]. Briefly, aortae were placed in RNA*later* (Sigma, St. Louis, MO, USA) to remove the surrounding adventitial fat tissue. Two aortas were pooled for total RNA extraction to obtain eight pools: four pools of aortas from each diet group. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and RNA amplification was performed using Amino Allyl MessageAmp^{TM} II aRNA Kit (Ambion, Austin, TX, USA) according to the manufacturers' instructions. Hybridization was carried out on the Operon mouse microarray (OpArrays^{TM,} Operon Biotechnology, Cologne, Germany). In all, eight microarrays were used for a total of four independent comparisons. Hybridization

was carried out in a Ventana hybridization system (Ventana Medical Systems, S.A, Illkirch, France) at 42°C for 8 h and microarrays were scanned using the Agilent Micro Array Scanner G2505B (Agilent Technologies, Inc., Santa Clara, CA, USA) at 5 μm resolution. The signal and background intensity values for each spot in both channels were obtained using ImaGene 6.0 software (Biodiscovery, Inc, Proteigene, Saint Marcel, France), base-2 logarithm transformed and corrected for systemic dye bias using Lowess normalization. The log-ratio between curcumin-supplemented and control samples was analyzed with Student's *t*-test to detect differentially expressed genes and probability values were adjusted using a Bonferroni correction for multiple testing at 1% to eliminate false positives. Microarray data have been deposited in the public ArrayExpress Archive database of microarray gene expression data at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under accession number: E-MEXP-3185.

To validate data of microarray study, real-time quantitative PCR was performed on the same RNA for a subset of genes identified as differentially expressed (*Acaca, Pla2g6, Acox-1, TNF-α, Ndufb-9, Tnxb, Itgb-1*). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe RNA to cDNA. The primers were identified using Primer Express software (Applied Biosystems) (Supporting Information Table S2). The qRT-PCR was carried out on Mastercycler epgradient S Realplex (Eppendorf, Hambourg, Germany) using the Power SYBR®Green PCR Master Mix kit (Applied Biosystems). After initial denaturation of 10 min at 95°C, a two-step cycling conditions were: 15 s denaturation at 95°C and annealing/extension at 60°C for 30 s, cycled 40 times. The expression levels were calculated using the ΔΔCt method.

Gene ontology (GO) annotations of biological processes for differentially expressed genes were conducted using GOstat (http://gostat.wehi.edu.au) [26]. To extract maximum biological information of differentially expressed genes, together with GO, genes were also classified according to their role(s) in cellular or metabolic pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/pathway.html) database.

2.4 Macrophage quantification by immunohistochemistry

Immunohistochemistry analysis was performed on consecutive aortic sections cut between 500 and 700 μm from the cusp origin as previously described [27]. Macrophages were identified with a purified rat anti-mouse Mac-3 monoclonal antibody (BD Biosciences, Le Pont de Claix, France, dilution 1:30). The staining was revealed by using a fluorescent (Alexa Fluor 488) goat anti-rat IgG (H+L) secondary antibody (Life Technologies SAS, Saint Aubin, France, dilution 1:200). Fluorescence detection and image acquisition were performed as detailed in [27]. Macrophages were counted on five digitally captured nonoverlapping 40× magnification fields in the infiltrated area, and reported as average of macrophage number in the lesion.

2.5 Protein extraction and Western blotting

Total aorta samples were lysed in 100 μL RIPA buffer containing protease and phosphatase inhibitors (aprotinin, PEFAblock, β-glycerol phosphate, leupeptine). Samples were rotated for 2 h at 4°C and centrifuged for 10 min at 8000 *g*. Ten microliters of SDS sample buffer and 2.5 μL DTT were added to 40 μL of supernatant of each lysate. The total lysates were sonicated for 1 min and heated at 95°C for 10 min before loading on a polyacrylamide gel. Separated proteins were transferred onto nitrocellulose membranes and analyzed by western blotting for the presence of Iκ-Bα (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p65 (Santa Cruz Biotechnology). All detected proteins were normalized with tubulin (Sigma Aldrich, Bornem, Belgium). Detection was performed using fluorescent secondary antibodies (Rockland, Gilbertsville, PA, USA) via the Odyssey Imaging System (Licor, Lincoln, NA, USA).

2.6 Quantification of cell adhesion on human umbilical vein endothelial cells (HUVECs) monolayer

Primary HUVECs (Lonza, Walkersville, MD, USA) were used at passage 5 and were cultured in EBM®-Phenol Red Free medium (Lonza) supplemented with EGM®-2 BulletKit® (Lonza). A human monocytic cell line U937 (ATCC, Manassas, VA, USA) was cultured in RPMI medium (Pan Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (Sigma, St Quentin Fallavier, France).

HUVECs were seeded in 24-well plates to proliferate until 60–70% confluence is achieved. The medium was replaced with fresh one containing either vehicle (DMSO 0.1%, control wells) or curcumin (both from Sigma Aldrich) (0.1, 0.5, or 1 μM). After 3 h, the medium was replaced by basal EBM®-Phenol Red Free for further 15–18 h. After this period, the confluent monolayer was stimulated with TNF-α (0.1 ng/mL) (R&D Systems, Lille, France) for 4 h and 50 μL of a 5×10^6 U937 cells/mL suspension was added to the wells. The cells were co-incubated at 37°C for 1 h and rinsed with PBS before fixing with crystal violet (Sigma Aldrich). In each well, the number of attached U937 cells was counted in three random microscopic fields defined by an eyepiece. Experiments were performed three times in triplicate.

2.7 Quantification of gene expression by qRT-PCR in endothelial cells in vitro

The cell culture was realized as described in section 2.6. After 5-h exposition to TNF-α (0.1 ng/mL), wells were

washed two times with PBS and were stored at −80°C. The RNA were extracted using the RNeasy Plus Micro Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions and RNA quantities were determined on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to reverse transcribe 1 μg RNA.

Impact of curcumin on expression of 96 different genes was analyzed using TaqMan Low Density Array (TLDA) (Applied Biosystems). qRT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and 2 μL of the obtained cDNA solution applied into sample-loading ports of TLDA. qRT-PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system (Applied Biosystems) with the following thermal cycler conditions: 2 min at 50°C and 10 min at 94.5°C, followed by 40 cycles of 30 s at 97°C and 30 s at 59.7°C. Raw data were analyzed using Sequence Detection System Software v2.4 (Applied Biosystems).

2.8 Electrophoretic mobility shift assay (EMSA) and reporter gene studies

The human endothelial cell line EAhy926 was obtained from ATCC and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics [28]. EAhy926 were seeded in six-well plates at 3×10^5 cells/well and treated for 2 h with the compound of interest (20 μM) and 4 h with or without TNF-α (0.1 ng/mL). After treatment, cells were washed with ice-cold PBS and pelleted in 1 mL PBS by centrifugation for 10 min at 2600 rpm (4°C). Preparation of nuclear extracts has been described previously [29]. For EMSA, equal amounts of protein were incubated for 30 min at room temperature with an NF-κB-specific ^{32}P-labeled oligonucleotide and binding mix as described previously [30]. Labeling of the oligonucleotides was performed with [α-^{32}P]-dCTP by using Klenow enzyme (Boehringer Mannheim Bruxelles, Belgium). The NFκB oligonucleotide comprises the sequence: 5′-AGCTATGTGGGTTTTCCCATGAGC-3′, in which the single IL6 promoter-derived NF-κB motif is bold and italicized. Samples were loaded on a 6% polyacrylamide gel run in 0.5× TBE buffer (pH 8) and complexes formed were analyzed using Phosphor Imager Technology. Extensive characterization of NF-κB binding complexes by supershift analysis and competition analysis has been published elsewhere [31]. NF-κB luciferase reporter gene cells have been described previously [29].

2.9 Statistical analyses

Data were analyzed with a Student's *t*-test or by one-way analysis of variance (ANOVA) coupled with the Student–Newman–Keuls multiple comparison test (InStat3) except for the lesion areas and macrophage quantification. These latter analyses were done with a two-way ANOVA (mouse groups and aortic sections or macrophage number) (REGWG test; Statview; SAS Institute Inc., Cary, NC, USA). Differences were considered significant when the p value was ≤ 0.05.

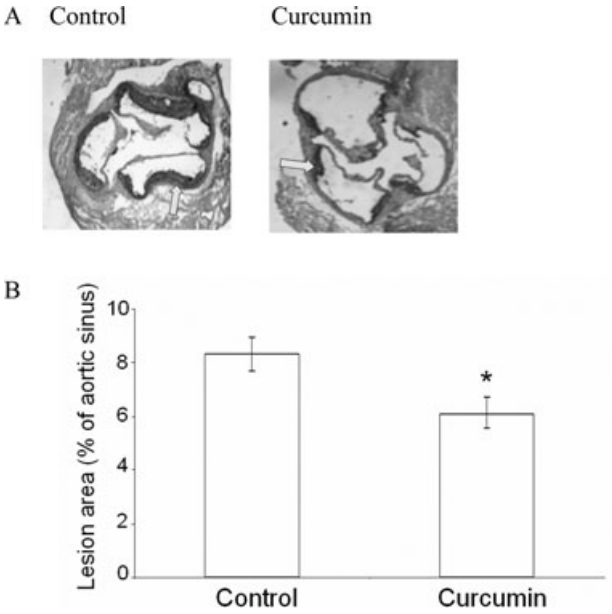

Figure 1. Quantification of atherosclerotic lesions. (A) Sections of aortic sinus (at 500 μm from the cusps) stained with oil red O in apoE-deficient mice fed with a control or curcumin-supplemented diet for 16 weeks. The arrows indicate lipid deposit. (B) Atherosclerotic lesion severity is expressed as percent changes of lesion area in total cross-sectional area. Data represent means ± SEM for 15 mice per group; significance: $^{*}p < 0.05$

3 Results

3.1 Curcumin significantly reduces atherosclerotic lesion size without any change in lipemia

The lesion area, expressed in percent of total aortic sinus area, in control and curcumin mice after 16 weeks on diet was 8.33 ± 0.64% and 6.10 ± 0.53%, respectively (Fig. 1A and B). These data showed that curcumin supplementation led to a 26% reduction of the lesion extent compared to controls ($p < 0.02$). By contrast, curcumin consumption did not modify the antioxidant capacity of plasma (FRAP) nor the plasma lipid concentrations (Supporting Information Table S3).

3.2 Curcumin modulates the expression of genes in aorta

To obtain insight of the molecular mechanism underlying the observed antiatherogenic effect of curcumin, we used microarray analysis for gene expression profiling in aortas.

Using this approach, 1670 genes were revealed as differentially expressed in aortas of curcumin-fed mice compared to aortas of mice on the control diet (Supporting Information Table S4). Among those 1670 genes, 1022 were upregulated and 648 were downregulated with magnitude fold-change ranging from 1.27 to 3.47 and from −1.25 to −4.1, respectively. These fold-changes are consistent with previously reported nutrigenomic studies [25, 32]. Real-time quantitative PCR was performed using same RNA samples on a subset of genes identified as differentially expressed in aorta and for the majority of genes studied, the expression values were concordant with data from microarray analysis (Supporting Information Fig. S1).

To decipher biological processes affected by curcumin, differentially expressed genes were subjected to gene-annotation enrichment analysis using GOstat bioinformatics resources (Supporting Information Table S5). This analysis revealed that differentially expressed genes are implicated in different processes, such as metabolic processes, signaling cascade, or cell cycle process. Among the highly represented GO identified, some processes could be related to the development of atherosclerosis such as cell communication, cell adhesion, cell morphogenesis, or cell mobility. To further refine biological function in which differentially expressed genes are implicated, we placed the genes according to their role(s) in cellular or metabolic pathways using KEGG database. Interestingly, we identified pathways potentially implicated in the initial process of atherosclerosis development (Supporting Information Table S6). Numerous genes were identified as involved in cytoskeleton regulation and focal adhesion as well as coding for adhesion molecules entailed in leukocyte recruitment and transmigration through the endothelium.

3.3 Curcumin decreases macrophage number in atherosclerotic lesions

Immunofluorescence analyses have been performed using Mac-3-specific antibody on aortic root sections from the same aortas used to evaluate atherosclerotic lesions and transcriptome analysis. Quantification of macrophages in the atherosclerotic lesions indicated a significant ($p < 0.05$) 37% reduction in macrophage number in the curcumin-fed mice (Fig. 2A and B) compared to control mice.

3.4 Curcumin increases aortic expression of IκB protein

Using MetaCore software (http://www.genego.com/metacore.php), transcription factors potentially involved in the genomic regulation of curcumin supplementation have been identified. Among different transcription factors, NF-κB and its pathway seem to regulate the expression of numerous genes (Supporting Information Fig. S2). To examine the impact of curcumin on the protein level of

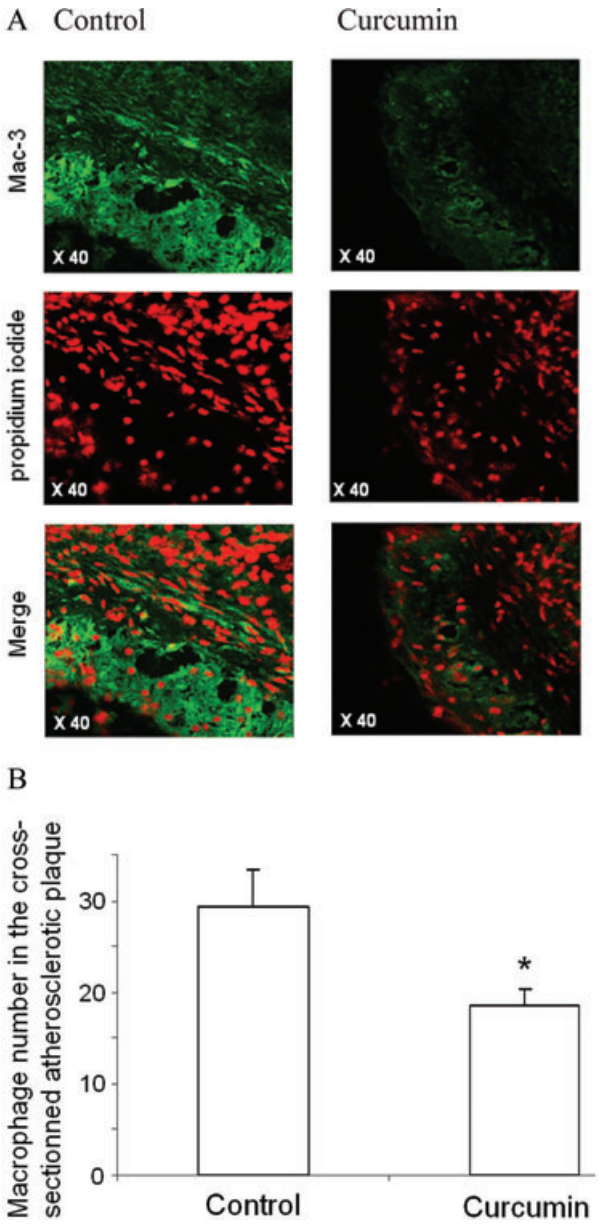

Figure 2. Immunohistochemical staining of macrophages in the plaque. (A) Ten micrometer thick cryostat sections of plaques were stained with M3/84 mAb (1:30 dilution) which reacts with the Mac-3 antigen and visualized with a goat anti-rat IgG antibody labeled with Alexa Fluor® 488 (1:200 dilution). The nuclei of cells were stained with propidium iodide (1:500 dilution). (B) Nuclei surrounded with green fluorescence are identified to be macrophages in atherosclerotic plaque. Their number in the plaque is determined with the Leica Qwin® software. Data represent means ± SEM for eight mice per group; significance: $^{*}p < 0.05$

NF-κB in vivo in aorta, we quantified by Western blot the protein levels of the p65 subunit of NF-κB as well as of IκB, an inhibitor of NF-κB. This analysis revealed a higher level of IκB in curcumin group, while the amount of p65 did not differ between the two groups (Fig. 3A). Additional proof for

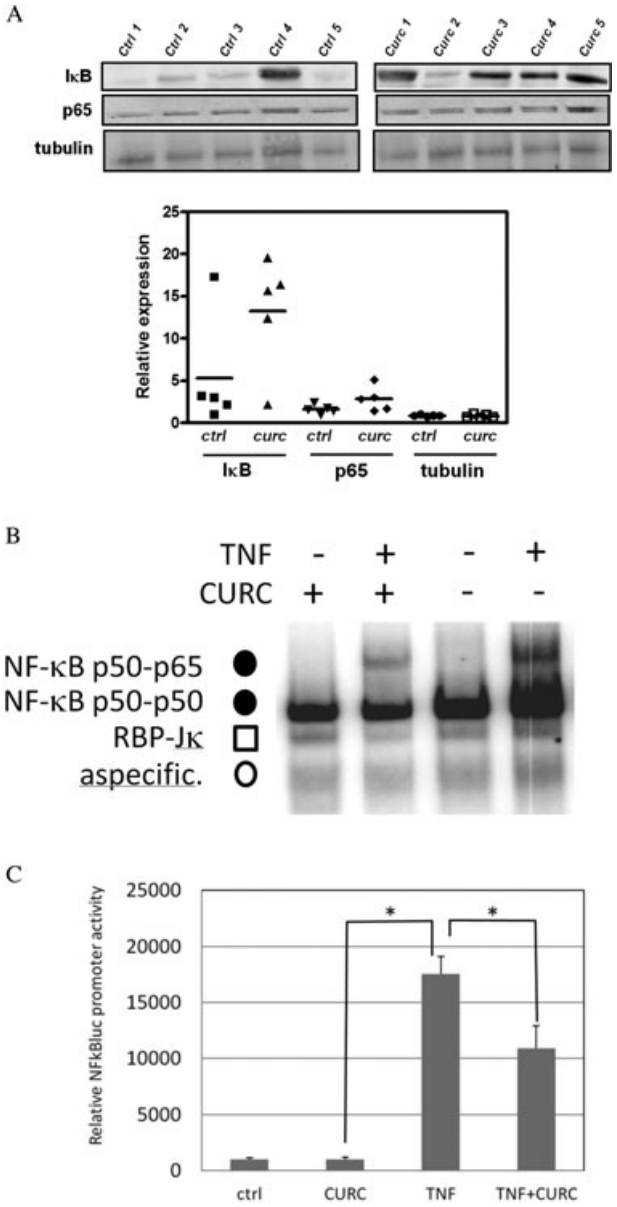

Figure 3. Curcumin attenuates NF-κB-dependent gene activation. (A) Protein lysates of aorta isolated from mice fed a control or curcumin-supplemented diet for 16 weeks were analyzed by western blotting against the p65 subunit of NF-κB and IκB. Intensity of western signals was quantified via the Odyssey Imaging System. Relative expression levels of IκB, p65, and tubulin proteins are presented as a scatterplot of control or curcumin-treated mice. Data represent relative expression for five mice per group. (B) NF-kB/DNA binding activity was determined by electrophoretic mobility assay in the human endothelial cell line EAhy926, exposed for 4 h to TNF (2500 IU/mL) alone, or upon cotreatment with curcumin (1 μM). (C) Curcumin effects on TNF induced NF-κB-dependent transcriptional activity were measured in stable transfected NF-κB luciferase reporter gene cells [21] exposed for 4 h to TNF (2500 IU/mL) alone, or upon cotreatment with curcumin (1 μM).

a role of NF-κB in the antiatherogenic effects of curcumin was obtained in electrophoretic mobility shift assays and reporter gene studies, which reveal decreased levels of TNF-α-induced NF-κB/DNA binding and NF-κB-dependent reporter gene expression upon exposure to curcumin (Fig. 3B and C).

3.5 Curcumin inhibits monocyte adhesion to TNF-α-stimulated endothelial cells in vitro

In order to assess the hypothesis raised from microarray analysis that curcumin could affect monocyte adhesion process to endothelial cells by modulating expression of genes implicated in the initial steps of atherosclerosis, we performed in vitro experiments using primary endothelial cells HUVEC. As expected, TNF-α stimulation of HUVECs significantly increased ($p < 0.01$) monocyte adhesion when compared to non-stimulated cells (from 31% to 113%). Interestingly, a 3-h pre-exposure of HUVECs to different concentrations of curcumin (0.1, 0.5, and 1 μM) resulted in a significant ($p < 0.05$) reduction of monocyte adhesion to HUVECs (Fig. 4). The reduction levels observed were 32%, 42%, and 51%, respectively.

3.6 Curcumin modulates expression of genes in endothelial cells in vitro

TLDAs were used to study the impact of curcumin, in vitro, on the expression of 95 genes in HUVECs and 18S was considered as housekeeping gene. Some of these genes were selected based on the literature for their role in the early stages of atherosclerosis development and others have been identified by microarray analysis. Among these 95 genes, 16 were not expressed in any studied condition. Nonsupervised hierarchical cluster of expression profiles (Supporting Information Fig. S3) suggests that the expression profile in HUVECs stimulated by TNF-α is different from those of stimulated HUVECs exposed to curcumin. In vitro, TNF-α alone induced expression of 48 genes and reduced expression of 29 genes. Among the most upregulated genes are ICAM-1, VCAM-1, SELE, NF-κB1, or CCL2. Interestingly, the expression of some of the genes upregulated in response to TNF-α were identified as downregulated by curcumin, such as VCAM-1, NF-κB1, RDX, CDC42, ACTR2/3 at concentrations of 0.5 μM and 1 μM. These genes are implicated in the processes of adhesion, actin cytoskeleton, or docking structure formation. The expression of other genes such as ICAM-1 or SELE was only downregulated by curcumin at the dose of 1 μM.

4 Discussion

In this study, we demonstrated the antiatherogenic effect of dietary supplementation with curcumin in apoE-deficient mice and identified potential molecular mechanisms

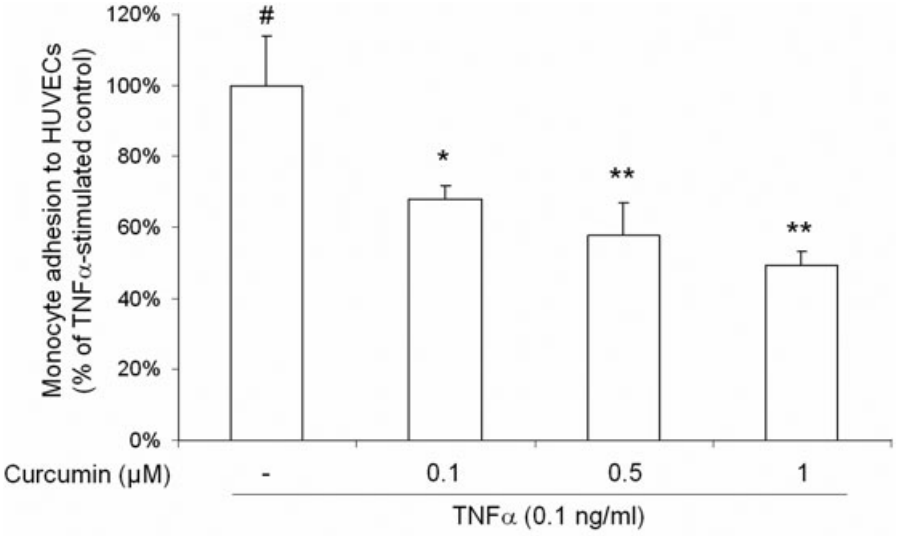

Figure 4. Monocyte adhesion to HUVECs. The values of monocyte adhesion to HUVECs, 16 h after a 3-h incubation with curcumin (0.1–1 μM), are represented in comparison to monocyte adhesion on TNF-α-stimulated HUVECs without curcumin. Values are means ± SEM from three independent experiments. Significances: $^{*}p < 0.05$; $^{**}p < 0.01$. $^{\#}$The TNF-α-stimulated positive control is normalized at 100%.

underlying the observed atheroprotective effect. Our results point out on the preventive effect of curcumin by acting on early events of atherogenesis, i.e. monocyte adhesion to the endothelium and migration into the vascular wall.

The present study showed that consumption of curcumin at 0.2% in the diet for 4 months is sufficient to reduce atherosclerosis progression in apoE-deficient mice. Recently, an antiatherogenic effect of curcumin has been reported in $LDLR^{-/-}$ mice fed high-cholesterol diet [14]. In this previous study, the atheroprotective effect of curcumin was associated with the lower plasma cholesterol and triglycerides levels, compared to untreated mice, via hepatic regulation of lipoprotein cholesterol metabolism. Several other studies using high-fat/high-cholesterol diets have already reported a hypocholesterolemic effect of dietary curcumin [10–13]. A high level of supplementation of fat and cholesterol in diets appears necessary to observe such effect. Indeed, other studies using standard diets or diets supplemented with moderate level of cholesterol failed to observe, as we did, any plasma cholesterol lowering effect [33, 34], even when an antiatherogenic effect was described [15]. Therefore, these observations suggest that the antiatherogenic effect of curcumin may result from a direct action at the vascular level. One of the means by which curcumin may impact biological processes in the arterial wall may involve genomic effects. A previous study reported the ability of curcumin to regulate expression of hepatic genes related to inflammation and lipoprotein metabolism in mice [14]. Nonetheless, the molecular targets of curcumin at the vascular level remain unknown.

To decipher potential molecular mechanisms involved in the curcumin-mediated inhibition of atherosclerosis, we performed a global transcriptomic analysis on aorta from apoE-deficient mice fed for 4 months a curcumin-supplemented diet. The microarray analysis showed that the expression of 1670 genes has been affected by curcumin in aorta. It has been previously reported, using transcriptome approach, that curcumin can modulate expression of genes in different cell lines, such as Y79 retinoblastoma cells [35], microglial cells [36], breast cancer cell [37], or human colon cancer cells [38], as well as in-vivo, in rat heart and liver [39, 40] or mice colon [41]. To our knowledge, our study is the first to report impact of curcumin on expression of genes in aorta using genome-wide approach in relation to the antiatherogenic effect of this compound. GO classification of the differentially expressed genes into functional categories according to their biological function, suggests that curcumin presents multitarget mode of action. Genes involved in different cellular functions have been identified, such as cellular metabolic process (GO:0044237), signal transduction (GO:0007135), cell death (GO:0008219), protein processing (GO:0016485), cell adhesion (GO:0007155), or apoptosis (GO:0006915) (Supporting Information Table S5). Some of these cellular functions have already been described as modulated at the genomic level by curcumin in different cell lines [35, 37, 38]. Interestingly, in our study, classification of identified differentially expressed genes using both GO and KEGG databases, revealed that over 100 genes could be involved in cytoskeleton organization, cell junction, cell communication, or cell adhesion. These processes are crucial in initial steps of atherogenesis by controlling adhesion and recruitment of circulating monocytes into the subendothelial space [42]. The recruitment of monocytes is mediated by different chemokines [43], some of which have been identified as downregulated by curcumin, such as Cxcl5 and Ccr7. It has been previously shown that the inactivation of the chemokine receptor Ccr7 preserved the lesion size and foam cell content in apoE-deficient mice [44]. This chemotaxis step is followed by the firm adhesion of monocytes to endothelium mediated by different cell surface adhesion molecules, such as ICAM-1, VCAM-1, but also interleukins, particularly IL17, IL2r, and IL1 [45]. Transcriptome analysis also revealed that curcumin downregulated the expression of over 60 genes coding for adhesion molecules

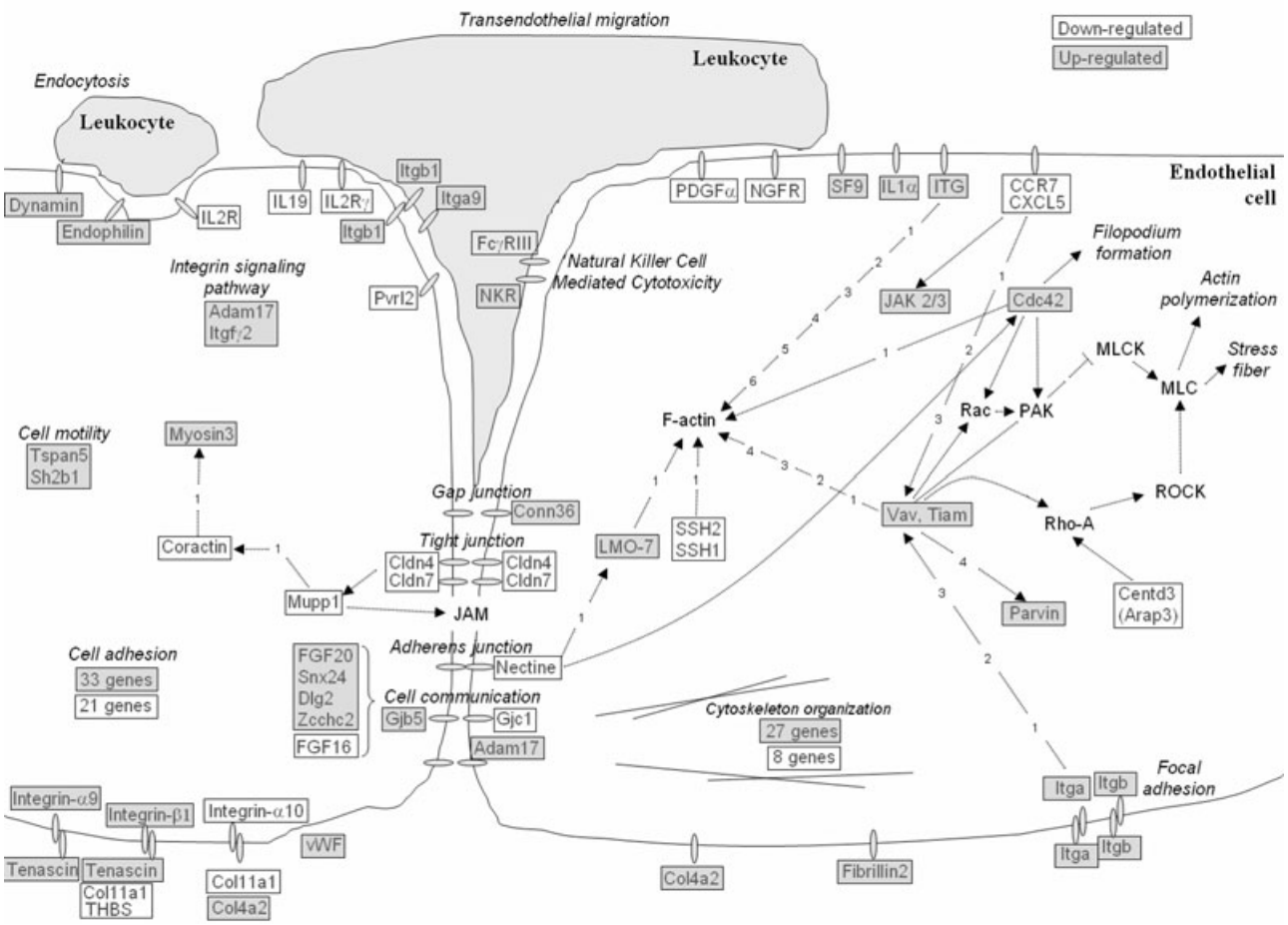

Figure 5. Differentially expressed genes in aorta potentially involved in transendothelial migration of leukocytes. Genes which expressions have been identified as modulated by curcumin in aortas of apoE$^{-/-}$ mice and potentially implicated in different processes of transendothelial migration as identified from Gene Ontology and Kegg pathway analyses are presented. Downregulated genes are presented in empty squares and upregulated in grayed squares.

and interleukins, such as L1CAM, ITGA10, IL19, or IL2r. Regarding these observations, downregulation of genes coding for chemokines and adhesion molecules could be, at least in part, responsible for the decreased monocyte infiltration. Monocyte adhesion to endothelial cells induces formation of "docking structures" that are actin-rich membrane extensions surrounding the adherent cells. In our study, curcumin was shown to reduce the expression of the arp2/3 complex gene (ARP3), which is involved in actin filament polymerization. This modulation of expression of ARP3 could lead to reduce docking structure formation and consequently lower monocyte adhesion and infiltration. Monocyte adhesion is followed by diapedesis, a process during which circulating cells migrate between or through endothelial cells. Diapedesis is characterized by endothelial cell contraction that increases intercellular space allowing the migration of monocytes. This process, as well as docking structure formation, is regulated through actin cytoskeleton and focal adhesion pathways. As presented in the Table S6, numerous differentially expressed genes are implicated in these two pathways, suggesting that curcumin could regulate transendothelial migration. Furthermore, junctions between endothelial cells are tightly dependent on homophilic cell adhesion and cell communication proteins that form a barrier between blood cells and the underlying tissues. In our study, genes coding for homophilic cell adhesion, cell communication, and cell–cell junction proteins have been identified as upregulated by curcumin. Lmo7, Cdh15, Dsg1b, and Gjb5 are associated with intercellular interaction. Therefore, the observed upregulation of these genes by curcumin could result in a lower endothelial permeability and hence in a reduction of monocyte infiltration. Taken together, genes whose expression has been identified as modulated by curcumin and which are potentially involved in the initial steps of atherogenesis are presented schematically in Fig. 5. Overall, the expression profile of these genes could be related to a possible beneficial effect of curcumin toward atherosclerosis initiation and progression by decreasing monocyte chemotaxis, adhesion, and strengthening

endothelial barrier, therefore lowering macrophage accumulation in the intima of aorta.

The results obtained from our transcriptomic analysis in aorta strongly suggest that curcumin could impair monocyte adhesion to endothelial cells by modulating expression of genes involved in cell adhesion. To assess this hypothesis, an additional study has been performed in vitro on endothelial cells. The selection of concentrations of curcumin as well as the conditions of exposure to endothelial cells was based on literature data on the bioavailability of curcumin in humans [46]. Indeed, as described for other polyphenolic compounds [47], upon its oral administration curcurmin is conjugated in the small intestine and later in the liver. In human plasma, curcumin and its metabolites are detectable at nanomolar to micromolar concentrations following the consumption of several grams of curcumin [48–50]. The peak of plasmatic concentration of curcumin is reached between 1 and 2 h after ingestion [48]. From bioavailability studies performed in mice, the consumption of curcumin, in a range from 2.5 to 30 mg/day, led to plasma curcumin concentrations of 0.095–0.6 μM, respectively [51,52]. Therefore, regarding these published data, concentration of curcumin chosen to be used in in vitro experiments were 0.1, 0.5, and 1 μM, respectively, concentrations that could be considered as physiologically relevant. We showed that curcumin at 0.1μM decreased monocyte adhesion to HUVECs for as much as 32%. Furthermore, as observed in vivo, curcumin also displays the capacity to modulate the expression of genes in endothelial cells. Gene expression analysis revealed that curcumin counteracted the TNF-α-induced expression of pro-atherogenic genes; for example, curcumin downregulated the expression of genes coding for adhesion molecules, such as VCAM1, ICAM1, or SELE as well as genes coding for chemokines, such as CCL2. The observed downregulation of expression of these genes, known to be involved in monocyte adhesion, presents molecular mechanism underlying the reduction in monocyte adhesion. In vitro, curcumin also modulated the expression of genes, such as RDX, CDC42, or ACTR2/3, implicated in cellular actin cytoskeleton organization and docking structure formation. Interestingly, our microarray analysis in aorta also revealed genes implicated in the same cellular processes. Finally, changes in gene expression observed in vivo (aorta) and in vitro (endothelial cells) in response to curcumin are all going in the direction of a reduction in endothelial permeability that would result in a lesser transendothelial migration of monocytes. This hypothesis is entirely consistent with the results of the quantification of macrophages in the lesion showing a lower macrophage number in the lesions of aortas of apoE mice that received curcumin in the diet. Overall, the results from both in vivo and in vitro experiments revealed that curcumin can decrease monocyte adhesion to endothelial cells, as well as monocyte infiltration into intima via transendothelial migration, by modulating expression of related genes, revealing potential cellular and molecular mechanisms underlying the antiatherogenic activity of curcumin.

Bioinformatic analysis of transcriptome data revealed transcription factors potentially modulated by curcumin. Among these transcription factors is NF-κB which could regulate expression of genes involved in atherogenesis, such as chemokines (Ccl-2 or Ccr-7), interleukins or inflammatory genes (IL-2R, IFN-αR, or HSP-27), or cell adhesion molecules (Adam-17 or Vav-1) (Supporting Information Fig. S2). NF-κB is a dimeric transcription factor formed by the hetero- or homodimerization of proteins of the Rel family, including p50 and p65. In its inactive form, NF-κB is bound to inhibitor of κB (IκB) in the cytoplasm. The phosphorylation of IκB is followed by its ubiquitination, leading to its degradation and NF-κB activation. In our study, the amount of p65 subunit of NF-κB is similar in control and in curcumin group, whereas IκB protein level is increased with curcumin. Therefore, it could be hypothesized that curcumin consumption prevents IκB degradation by inhibiting IKK2 kinase activity involved in IκB Ser32–36 phosphorylation, which attenuates its ubiquitination and proteasomal degradation and therefore, reduces levels of NF-κB activation and decreases target gene expression involved in inflammation, adhesion, or cell migration. It is also possible that the enhanced total amounts of IκB by curcumin are due to the induction of the expression of IκB or impact of curcumin on expression of miRNA implicated in the regulation of mRNA stability or protein synthesis of IκB. Regarding the last hypothesis, it has been observed that curcumin in apoE$^{-/-}$ mice modulated expression of miRNA [53]. Bioinformatics analysis using miRBase and TargetScan database (http:////www.mirbase.org; http:////www.targetscan.org) revealed, by homology of sequences, that miR-199 could bind to the IκB mRNA. Interestingly, we observed that in apoE$^{-/-}$ mice, curcumin significantly decreased the expression of this miRNA. Therefore by decreasing the expression of these miRNA, mRNA and protein levels of IκB could increase, hypothesis that could be related to the observed increase in protein level of this gene. The increased IκB protein level that we observed in response to curcumin is in agreement with previous studies that have reported an inhibitory effect of curcumin on phospho-p65/NF-κB or phospho-IκB protein expression in mice and on the DNA-binding activity of NF-κB in hepatic or tumor cells [54–56]. Also, this observation is in line with previous in vitro studies which demonstrated inhibitory action of curcumin on IκB phosphorylation via inhibition of IκB kinase complex (IKK) [57, 58] and thus increasing free IκB [59]. Gareus and co-workers showed in apoE$^{-/-}$ mice that the inhibition of NF-κB abrogated adhesion molecule induction in endothelial cells, impaired macrophage recruitment to atherosclerotic plaques and reduced expression of cytokines and chemokines in the aorta [60]. Thus, the modulation of NF-κB activity could be one of the mechanisms involved in the biological effects of curcumin that could be related to its antiatherogenic action [61]. Of particular interest, in favor of our hypothesis, IκB deficiency was found to promote atherogenesis by promoting leukocyte recruitment to plaques [62]. Furthermore, the expression of atherogenic genes, such as

genes coding for adhesion molecules and NOS3, was studied in the aorta of mice fed experimental diets only for 4 weeks. The obtained results showed that curcumin induced significant changes in the expression of these genes without affecting the size of the early lesion (Supporting Information Fig. S4A and B). This observation suggests an early and direct genomic effect of dietary curcumin at the vascular level during atherosclerosis progression.

In conclusion, in the present study, we have demonstrated that curcumin is able to attenuate atherosclerosis progression in apoE-deficient mice. Data accumulated from in vivo (aorta) and endothelial cells experiments strongly suggest that the atheroprotective effect of dietary curcumin could be related to its action on the early steps of atherogenesis by acting at the genomic level. Curcumin appears particularly efficient to affect the expression of genes that control leukocyte adhesion and transendothelial migration as supported by the numerous modulated genes involved in these processes (see Fig. 5). These results point out new molecular targets of curcumin that could be responsible for the observed reduction of macrophage infiltration in vivo and monocyte adhesion in vitro and they highlight the anti-inflammatory properties of curcumin in mediating its antiatherogenic effect. Based on our findings, by acting on the early stages of atherogenesis curcumin may appear as an attractive candidate for nutritional prevention of this disease.

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