Deficiency of G-protein coupled receptor 40, a lipid-activated receptor, heightens in vitro- and in vivo-induced murine osteoarthritis

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Original article

Curcumin modulates endothelial permeability and monocyte transendothelial migration by affecting endothelial cell dynamics

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ABSTRACT

Curcumin is a phenolic compound that exhibits beneficial properties for cardiometabolic health. We previously showed that curcumin reduced the infiltration of immune cells into the vascular wall and prevented atherosclerosis development in mice. This study aimed to investigate the effect of curcumin on monocyte adhesion and transendothelial migration (TEM) and to decipher the underlying mechanisms of these actions.

Human umbilical vein endothelial cells (HUVECs) were exposed to curcumin (0.5–1 μM) for 3 h prior to their activation by Tumor Necrosis Factor alpha (TNF-α). Endothelial permeability, monocyte adhesion and transendothelial migration assays were conducted under static condition and shear stress that mimics blood flow. We further investigated the impact of curcumin on signaling pathways and on the expression of genes using macroarrays.

Pre-exposure of endothelial cells to curcumin reduced monocyte adhesion and their transendothelial migration in both static and shear stress conditions. Curcumin also prevented changes in both endothelial permeability and the area of HUVECs when induced by TNF-α. We showed that curcumin modulated the expression of 15 genes involved in the control of cytoskeleton and endothelial junction dynamic. Finally, we showed that curcumin inhibited NF-κB signaling likely through an antagonist interplay with several kinases as suggested by molecular docking analysis.

Our findings demonstrate the ability of curcumin to reduce monocyte TEM through a multimodal regulation of the endothelial cell dynamics with a potential benefit on the vascular endothelial function barrier.

1. Introduction

Impairment of the endothelial cell function is a major factor in the development of vascular damage. Endothelial cell dysfunction is characterized by the abnormal expression of genes including those coding for adhesion molecules, which leads to the firm adhesion of leukocytes to the endothelium and their subsequent infiltration into the vascular wall, which is one of the initial events in atherosclerosis onset [1].

Curcumin is a polyphenolic compound of turmeric derived from the rhizome of *Curcuma longa*. This compound is commonly used as a dietary spice and food coloring agent. Curcumin exhibits potent antioxidant and anti-inflammatory properties that could be beneficial for vascular health [2]. Some clinical data showed that daily curcumin intake, from 450 mg/day to 8 g/day for 1–4 months, can reduce some cardiovascular risk factors such as systolic blood pressure [3], serum triglycerides and low density lipoprotein (LDL) [4], and can improve endothelial function [5]. Moreover, curcumin has been shown to reduce the development of atheromatous lesions in different animal models of atherosclerosis [6–11]. Coban et al. revealed a decrease in macrophages in the atherosclerotic lesions of $ApoE^{-/-}$ mice that were supplemented with dietary curcumin [6]. In the same study, the authors identified that most of the differentially expressed genes in response to curcumin supplementation were entailed in leukocyte recruitment and transmigration. Um et al. showed a lower expression of endothelial adhesion molecules (VCAM-1, ICAM-1) in the aortas of rabbits fed with a high cholesterol diet supplemented with curcumin [9].

In vitro studies have demonstrated that curcumin reduces monocyte adhesion to human endothelial cells activated by the pro-inflammatory

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tumor necrosis factor alpha (TNF-α) by modulating the expression of adhesion molecules [6,12–15]. Down-regulation of such molecules has been related to an inhibitory effect of curcumin on several cell-signaling molecules, of which NF-κB is one of the major transcription factors activated by TNF-α [16,17].

Following their firm adhesion to the endothelium, monocytes can migrate through the endothelium. This process, also known as diapedesis, transendothelial migration (TEM) or extravasation, is a dynamic process entailing endothelial cytoskeleton remodeling and disruption of endothelial cell junctions that cause an increase in vascular permeability [18,19]. These cellular modifications can be induced *in vitro* by the stimulation of endothelial cells with TNF-α [20,21]. So far, little is known about the effect of curcumin on the regulation of cytoskeleton and junctional dynamics in the endothelium. It has been reported that oral supplementation of rats with curcumin may reduce coronary artery permeability to a fluorescent macromolecule [22]. To our knowledge, no studies have investigated the impact of curcumin on leukocyte transmigration through the endothelium.

Based on the above considerations, this study aimed to investigate the effect of curcumin on monocyte transendothelial migration and on the expression of genes involved in this process. Special attention was paid to reproduce the physiological environment of vascular endothelial cells as closely as possible. For that, the cells were exposed to low concentrations of curcumin for a 3-h period, in respect to pharmacokinetics data of curcumin [23]. Moreover, effects of curcumin were also investigated in endothelial cells exposed to physiological shear stress that mimicked blood flow.

2. Materials and methods

2.1. Chemicals

Curcumin was purchased from Extrasynthese (France). Reagents for cell culture (RPMI 1640, penicillin/streptomycin, Trypsin/EDTA, Fetal Bovine Serum) were purchased from PAN biotech (Germany). Gelatin, paraformaldehyde (PFA), FITC-dextran, dimethylsulfoxide (DMSO), anti-protease cocktail, anti-phosphatase cocktail, Nonidet P40 and DAPI were obtained from Sigma-Aldrich (France). Recombinant-human-TNF-α and MCP-1 were obtained from R & D systems and Miltenyi Biotec SAS (Paris, France) respectively.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) (Lonza, Amboise, France) were cultured in a phenol-red-free endothelial growth medium supplemented with 2% fetal bovine serum, 0.4% human fibroblast growth factor, 0.1% human epidermal growth factor, 0.1% vascular endothelial growth factor, 0.1% insulin-like growth factor, 0.1% ascorbic acid, 0.1% heparin, 0.1% gentamicin/amphotericin-B and 0.04% hydrocortisone (all from Lonza, France). A monocyte THP1 cell line obtained from ACC (Manassas, VA) was cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Both cultures were maintained at 37 °C in humidified atmosphere with 5% CO_2.

2.3. Cell adhesion assay

HUVECs were seeded into 0.2% gelatin-coated 24-well plates at a density of 50,000 cells/well. At 60–70% confluence, cells were made quiescent for 24 h in medium with 0.2% FBS, and then were exposed to 0.5 μM, 1 μM curcumin or a DMSO vehicle (0.02%) for 3 h. Following pre-incubation with curcumin, inflammatory stress was induced by 1 ng/mL of TNF-α for 4 h. After TNF-α stimulation, 5×10^5 THP1 cells were added to each well, and cells were co-incubated for 15 min. Non-adhering THP1 cells were rinsed away by washing with 1X DPBS. The remaining attached cells were subsequently detached using trypsin/EDTA (0.05%/0.02%), fixed with 1% PFA and stored at 4 °C prior to immunostaining and analysis by flow cytometry.

2.4. Flow cytometry

Cell immunostaining was performed according to the manufacturer instructions (Miltenyi Biotec SAS, Paris, France). Briefly, fixed cells from the adhesion assay were pelleted by centrifugation at 300 g for 10 min and treated with FcR blocking reagent for 10 min. Subsequently, they were stained with CD45 antibody conjugated to FITC for 30 min in the dark. IgG2a isotype antibodies conjugated to FITC were used as a negative control. The number of CD45-positive cells (THP1) and unstained cells (HUVECs) was measured by BD FACSCalibur (BD Bioscience, San Jose, CA), set at 20,000 events, and analyzed using CellQuest Pro software, version 5.1.1 (BD Bioscience, San Jose, CA) for Macintosh. Relative monocyte adhesion to endothelial cells was expressed as the ratio of monocytes to HUVECs.

2.5. Monocyte transendothelial migration assay

HUVECs were seeded at 15,000 cells/well onto Corning Transwell filters (5 μm pore, 6.5 mm diameter) (Corning, USA) previously coated with 0.2% gelatin and grown to confluence. Cells were made quiescent for 24 h in medium with 0.2% FBS and were then exposed to 0.5 μM and 1 μM curcumin or vehicle, respectively (DMSO, 0.02%), for 3 h. Following this incubation, 100 ng/mL of MCP-1 were added to the lower chamber, and 5×10^5 THP1 above HUVECs. Cells were incubated for 3 h at 37 °C and 5% CO_2. THP1 cells that had transmigrated to the lower chamber were harvested and counted using a Beckman cell coulter (Beckman Coulter, USA). The results are expressed as a percentage of transmigrated monocytes relative to the condition 'vehicle + TNF-α'.

2.6. Endothelium permeability assay

As for monocyte transendothelial migration assay, confluent cultured HUVECs loaded onto Coning Transwell filters were made quiescent for 24 h in medium with 0.2% FBS before being exposed to 0.5 μM or 1 μM curcumin or vehicle (0.02% DMSO) for 3 h, followed by stimulation with 1 ng/mL of TNF-α for 4 h. Permeability of the endothelium was evaluated by passage of FITC-dextran at 40 kDa through endothelial monolayer. One hundred microliters of FITC-dextran at 40 kDa (2 mg/mL) was added to the upper chamber and allowed to equilibrate for 20 min, after which FITC fluorescence (excitation 488 nm; emission 520 nm) in the lower chamber was measured using a Biotek synergy 2 plate reader (BioTek, France). Four independent experiments were performed.

2.7. Endothelial cell area measurement

HUVECs were seeded on 0.2% gelatin-coated glass coverslips (13 mm diameter). At 60–70% of confluence, HUVECs were treated with TNF-α as previously described for the monocyte adhesion assay. After 15 min of THP1-HUVECs co-incubation, cells were rinsed to remove non-adherent cells and then fixed with 1% PFA for 15 min at 4 °C. Fixed cells were permeabilized by a 1% Triton ×100/1X PBS/3% BSA solution, and the actin cytoskeleton was stained with rhodamine-phalloidin (Invitrogen). Nuclei were counterstained with DAPI (100 nM). Fluorescence was detected and imaged using a Zeiss AxioPlan E microscope (Karl Zeiss, Jena, Germany) and a ZeissAxioCam MRM (Carl Zeiss Vision, Hallbergmoos, Germany). The mean endothelial cell area was measured in 10 fields of view per condition from 2 independent experiments using "ImageJ" image analysis software (http://www.imagej.nih.gov/).

2.8. Chemoattractant capacity of TNF-α activated HUVECs

Chemoattractant potency of HUVECs was evaluated using a transwell assay and conditioned media harvested from HUVECs exposed 3 h to 1 μM curcumin or DMSO vehicle, with or without stimulation by 1 ng/mL TNF-α for 4 h. Conditioned media were added to the lower chamber and 5×10^5 THP1 was added above the filter. After incubation for 3 h at 37 °C with 5% CO_2, transmigrated THP1 cells were harvested and counted using a Beckman cell counter (Beckman Coulter, USA). The results were expressed as a percentage of transmigrated monocytes compared to that measured in response to the supplementation of basal conditioned medium with 100 ng/mL MCP-1.

2.9. Monocyte adhesion and transendothelial migration assessment under shear stress

HUVECs were seeded at 15,000 cells/cm^2 in gelatin-coated microchannels of the Vena8 Endothelial Biochip (Cellix, Ireland). After adhesion (2 h), biochips were connected to the Kima pump (Cellix), and cells were grown under a 1 Hz-pulsatile aortic shear stress of 7 dyne/cm^2 for 72 h. Cells were exposed to low concentrations of curcumin (0.5–1 μM) or a vehicle (DMSO 0.02%) for 3 h prior to their stimulation with 1 ng/mL of TNF-α for 4 h under shear stress. Following TNF-α stimulation, a suspension of 2×10^6 THP1/mL was perfused through the microchannels at 0.5 dyne/cm^2 for 10 min. Interactions between monocytes and HUVECs were visualized with videomicroscopy using phase contrast optics (Diaphot-TMD microscope and DS-Fi 1 CCD camera, Nikon, USA). Each experimental condition was recorded on a single field for the 10 min minutes. Additionally, images of ten fields were captured for each condition and distributed along the channel. Firmly adherent monocytes to HUVECs accumulated throughout the experiment, and those that had transmigrated were quantified at the end of the experiment. Adherent and transmigrated monocytes were identified based on their phase white and round appearance and on their phase dark and flattened shape, respectively [24]. Quantifications were performed on pictures obtained from 2 to 3 independent experiments using “ImageJ” image analysis software (www.imagej.nih.gov/).

2.10. Quantitative real-time PCR analysis

Total RNAs were extracted using Trizol Reagent (Thermo Fisher Scientific, France) following manufacturer conditions. RNA concentration was measured using a spectrophotometer NanoDrap ND-1000 (LabTech, Ringmer, UK) and their quality checked by 1% agarose gel electrophoresis. 1 μg total RNA extract was used as template for single-strand cDNA synthesis using High Capacity cDNA RT kit (Applied Biosystems, USA) in a total volume of 20 μl containing 1 X RT buffer, 4 mM dNTP mix, 1 X random primers, 50 U reverse transcriptase and 20 U RNase inhibitor. The reverse transcription reactions were ran under the following conditions: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 s. PCR was carried out in the final volume of 20 μl containing 10 μl Power SYBR Green PCR Master Mix kit (Applied Biosystems, USA), 0.4 μl of each primer at 10 pmol/μl and 2 μl of the cDNA solution. The primers used were: h-MCP-1-F: TCA TAG CAG CCA CCT TCA TTC C, h-MCP-1-R: GGA CAC TTG CTG CTG GTG ATT C; GAPDH-F: CTG GGC TAC ACT GAG CAC C, GAPDH-R: AAG TGG TCG TTG AGG GCA ATG. RT-PCR amplification was performed using an Applied Biosystems Prism 7900HT (Applied Biosystems, France) system 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 (SDS) Software v2.4 (Applied Biosystems, Courtaboeuf, France) and compared by the ΔΔCt method. Results are expressed relative to the housekeeping gene transcript quantity and normalized to untreated cells.

Impact of curcumin on the expression of 93 other genes in HUVECs involved in endothelial cell function was performed using TaqMan Low Density Array (TLDA) (Applied Biosystem). Genes were chosen according to their role in atherosclerosis development from published articles (Supplemental Table S1). Two hundreds ng (2 μl) cDNA of each sample was combined with, 98 μl of nuclease-free water and 100 μl 2X TaqMan Universal PCR Master Mix (Applied Biosystems, USA) for the quantitative real-time PCR (qPCR) measurements. This mixture was divided equally over two sample-loading ports of the TLDA. The arrays were centrifuged once (1 min, 1300 rpm at room temperature) to equally distribute the sample over the wells. Subsequently, the card was sealed to prevent an exchange between wells. qPCR amplification was performed using an Applied Biosystems Prism 7900HT system (Applied Biosystem, USA) 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 (SDS) Software v2.4 (Applied Biosystems). Analyses were performed in triplicates. Bioinformatics analyzes with these genes were performed using the miRWalk database (link) to predict the validated miRNAs modulated by curcumin.

2.11. Western blot analyses

HUVECs pre-exposed to curcumin or vehicle were harvested at the end of a 4-h-TNF-α activation period and washed twice with ice-cold PBS. Total proteins were extracted using lysis buffer containing 50 mmol/L Tris pH 7.8, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP40, anti-protease and anti-phosphatase. Protein concentration was determined using BCA protein assay reagent Kit (Interchem). Lysates were loaded onto a 10% SDS-polyacrylamide gel for electrophoresis and then transferred onto immobilon-P membrane (GE Healthcare). The membrane was incubated in 5% (wt/vol) dried milk protein in TBS containing 0.05% Tween-20 for 1 h, and then further reacted with primary antibodies: rabbit anti-iCAM (Santa Cruz, 1:1000), rabbit anti-vCAM-1 (1:1000, GTX), rabbit anti-NFκB total (1:1000, Cell Signaling), rabbit anti-NFκB phosphor-ser536 (1:1000, Cell Signaling), rabbit anti-IκBα total (1:1000, Cell Signaling) and rabbit anti-IκBα phosphor-ser32 (1:1000, Cell Signaling). After extensive washes, membrane was incubated with anti-rabbit IgG antibody conjugated to HRP (1:5000, Santa Cruz, USA). Protein bands were visualized using ECL detection kit (Millipore, USA) and then analyzed using Image J software (www.imagej.nih.gov/). Graphs represent protein level expressed as the mean +/- standard deviation of 4 independent experiments.

2.12. Molecular docking analysis

Prior to virtual screening (docking), an academic license of *MarvinSketch* was used for drawing, displaying of 2D structure and 3D optimization of curcumin and generation of the required input file for docking (Tripos MOL2 file format), MarvinSketch 16.10.24.0, 2016, ChemAxon (https://www.chemaxon.com). Docking also requires the 3D structure of the target and it was necessary to cross-reference 2 online databases in order to identify the possible targets and their high-resolution complete 3D structures – a resolution better than 2.0 Å being recommended for docking [25]: The Universal Protein Resource – UniProt (http://www.uniprot.org) and RCSB Protein Data Bank – PDB (http://www.rcsb.org). Since our search criteria did not had positive feed-back, were constructed homologues models with the help of SWISS-MODEL [26]. Virtual screening of curcumin against the selected targets was conducted with *PyRx* – Python Prescription 0.9.2 using AutoDock Vina [27] as docking software. AutoDock Vina uses a scoring function inspired by X-score and tuned with PDBbind dataset [28] to predict the noncovalent binding of macromolecules and automatically calculates the grid maps and clusters the results. Data extraction and rendering of docking poses were performed with Molegro Molecular Viewer 2.5 (MMV – Molegro, A CLC bio company, Aarhus N, Denmark). The "backface culling" method was used for simulation of the molecular

surface of proteins. This method allowed us to determine whether a polygon of a graphical object is visible (*e.g.,* primary structure, secondary structure, or inner part of binding side shown as a molecular surface); when it was not visible, the polygon was "culled" from rendering process, which increases efficiency by reducing the number of polygons the hardware has to draw [29]. All docking runs were conducted in an extended search space, set to cover the entire target, with a volume bigger than 27.0 $Å^3$ and the exhaustiveness was manually increased to 80in order to improve the accuracy of predictions.

2.13. Statistical analyses

Prism software, version 6.0.c (GraphPad, La Jolla, CA) for Macintosh, was used for the statistical analysis of data. Data were analyzed using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test. For the analysis of gene expression data, two-way ANOVA was performed, followed by Dunnett's multiple comparison test with a single pooled variance. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Curcumin decreases both monocyte adhesion to endothelial cells and their transendothelial migration (TEM)

As expected, activation of HUVECs by TNF-α stimulated the firm adhesion of monocytes to the endothelial surface compared to those incubated with the non-stimulated HUVECs (3-fold increase; $p < 0.001$) (Fig. 1A). This adhesion was significantly reduced by 25% ($p < 0.001$) in HUVECs pre-exposed to curcumin at 0.5 μM and 1 μM compared to vehicle-treated endothelial cells.

Regarding TEM, Fig. 1B shows that the chemoattractant protein MCP-1 induced a 4.5-fold higher monocyte migration in a transwell assay compared to the cells incubated without MCP-1 ($p < 0.001$). The magnitude of this MCP-1-induced migration was significantly reduced when HUVECs were pre-treated with 1 μM curcumin (−24%, $p < 0.05$).

3.2. Curcumin preserves endothelium permeability and cell surface area during inflammation

Cell permeability assays, performed using Transwell system, revealed that stimulation of HUVECs by TNF-α resulted in a significant 4-fold increase of endothelial permeability to FITC-dextran (Fig. 2A). This increase in permeability was inhibited by 34% with a pre-incubation of endothelial cells with 1 μM curcumin ($p = 0.028$). In addition, the endothelial cell surface within the endothelium cell monolayer was modified in response to TNF-α induced monocyte adhesion to HUVECs (Fig. 2B). A 50% decrease of the mean cell area of HUVECs was observed after 1 h of monocyte/TNF-α−activated endothelial cells interaction ($p < 0.01$). In TNF-α activated HUVECs pre-exposed to 1 μM curcumin, the surface of HUVECs decreased by only 25%. Thus, exposure of endothelial cells to 1 μM curcumin reduced by 50% the TNF-α-induced contraction of HUVECs ($p < 0.05$).

3.3. Curcumin affects neither chemoattractive capacity of TNF-α-activated endothelial cells nor the expression of MCP-1

Fig. 3A shows that monocyte chemotaxis induced by the culture medium from TNF-α-activated endothelial cells was similar to the chemotaxis induced by the chemokine MCP-1. In addition, we observed that the treatment of endothelial cells with 1 μM curcumin did not affect the endothelial mediated monocyte chemotaxis. The expression of the gene coding for MCP-1 in endothelial cells was quantified by RT-qPCR (Fig. 3B). A 4-h treatment of HUVECs with TNF-α highly increased the expression of this gene ($p < 0.01$). Pre-exposure of endothelial cells to curcumin before their activation by TNF-α had no significant effect on the TNF-α-induced expression of the MCP-1 gene.

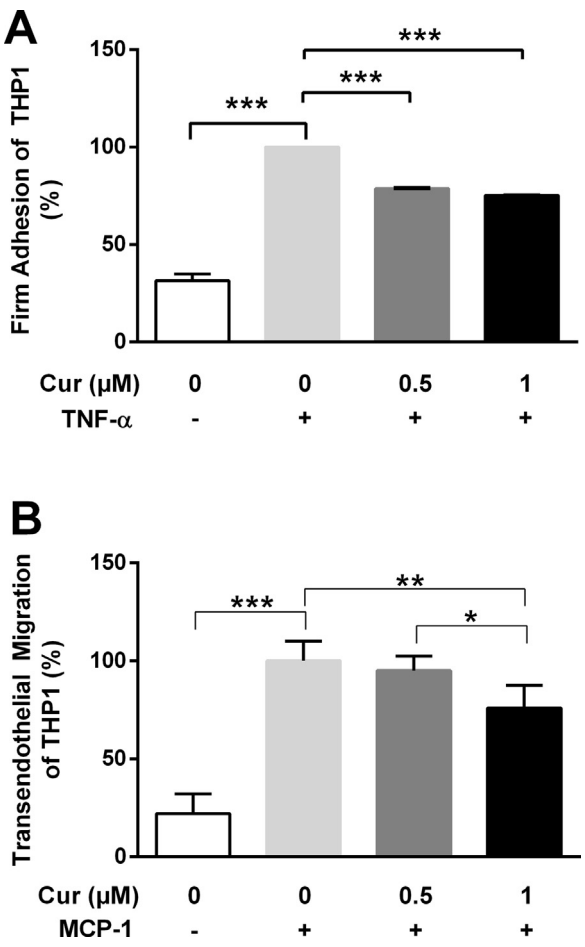

Fig. 1. Effect of curcumin on monocyte adhesion to endothelial cells and their transendothelial migration (TEM) in the static condition of culture. (A) Monocyte adhesion to TNF-α activated HUVECs after a 3-h exposure of endothelial cells to curcumin. In the absence of curcumin, HUVECs were exposed to vehicle (0.2% DMSO). The results are expressed as a percentage of monocyte adhesion to 'vehicle + TNF-α' treated HUVECs that is normalized at 100%. (B) TEM of monocytes in response to MCP-1 across HUVEC monolayer exposed to curcumin or vehicle for 3 h. The results are expressed as the percentage of migration measured in the vehicle + MCP-1 condition that is normalized at 100%. Bars represent the mean +/- SEM of 3–4 independent experiments. Significances: *p ≤ 0.05, **p ≤ 0.01 ***p ≤ 0.001.

3.4. Curcumin decreases monocyte-endothelial interaction under shear stress

Under physiological shear stress (~7 dyne/cm^2), TNF-α significantly increased the tight adhesion of monocytes to endothelial cells by 20-fold (Fig. 4A and suppl. Movie). The pretreatment of HUVECs by 1 μM curcumin significantly reduced monocyte adhesion by 36% ($p = 0.017$). A tendency for a decrease was observed with 0.5 μM of curcumin but did not reach significance.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.freeradbiomed.2017.07.019.

Time-lapse microscopy assessing monocyte transendothelial migration under shear stress showed that the pretreatment of HUVECs with 0.5 μM or 1 μM curcumin tended to decrease the number of migrated monocytes in comparison to migration observed for the vehicle-treated cells under shear stress (Fig. 4B and suppl. Movie).

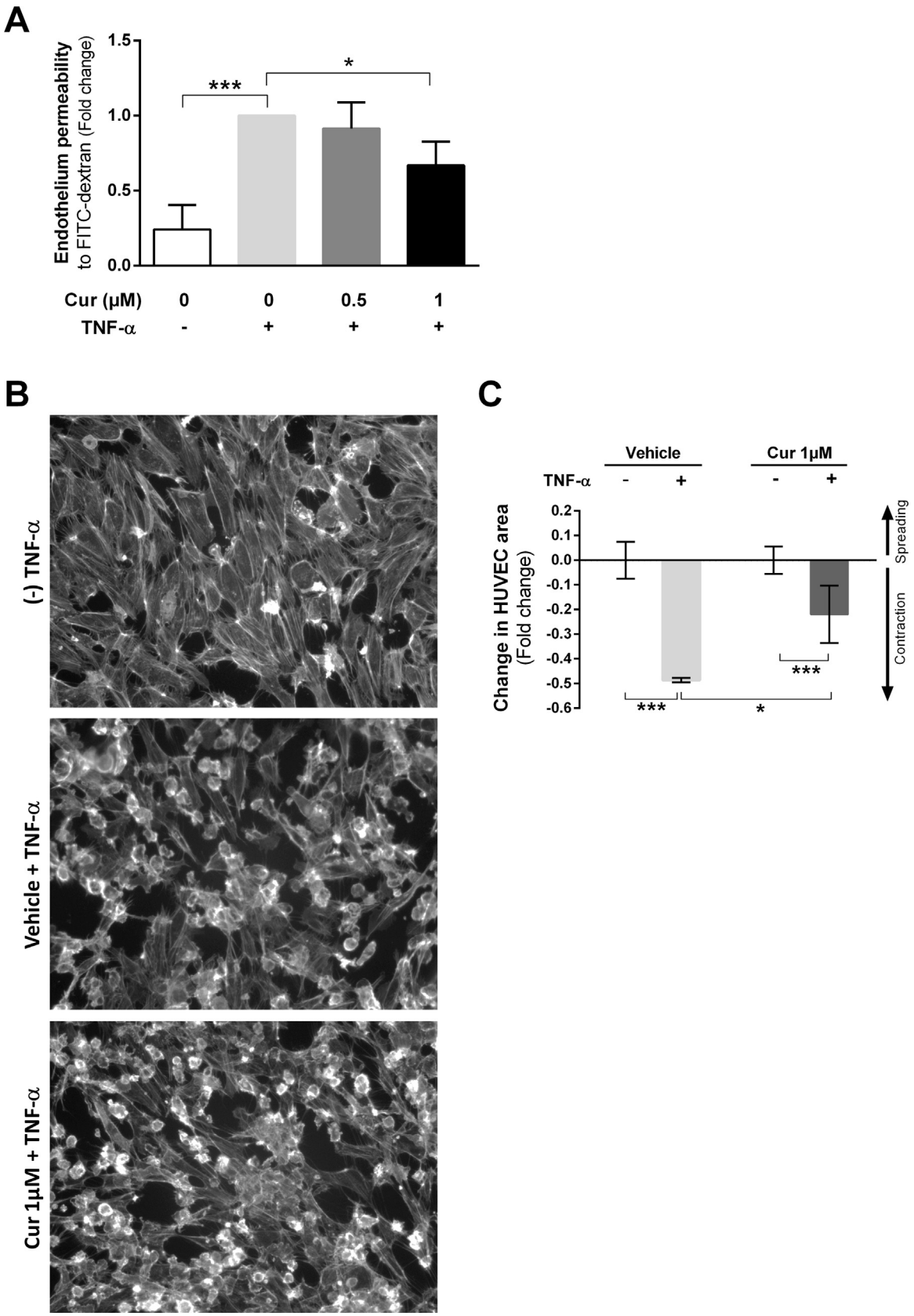

Fig. 2. *Effect of curcumin on endothelium integrity.* (A) Permeability of the HUVEC monolayer to FITC-dextran (40 kDa) after 3-h exposure to vehicle or curcumin (0.5 and 1 μM) followed by 4-h TNF-α activation. The results are expressed as a percentage of permeability measured in 'vehicle + TNF-α' treated HUVECs that is normalized at 100% (n = 4). (B, C) Impact of curcumin exposition on mean HUVEC surface area measured after monocyte/TNF-α − activated endothelial cells interaction. (B) Representative immunostainings of the actin cytoskeleton of HUVECs after 3-h exposure to vehicle or curcumin (1 μM) followed by 4-h TNF-α activation and co-incubation with monocytes (Magnitude: 20x). (C) Quantification of the mean HUVEC surface area. The results are expressed in comparison to the mean area measured at steady sate (n = 3). Significances: *$p \leq 0.05$, ***$p \leq 0.001$.

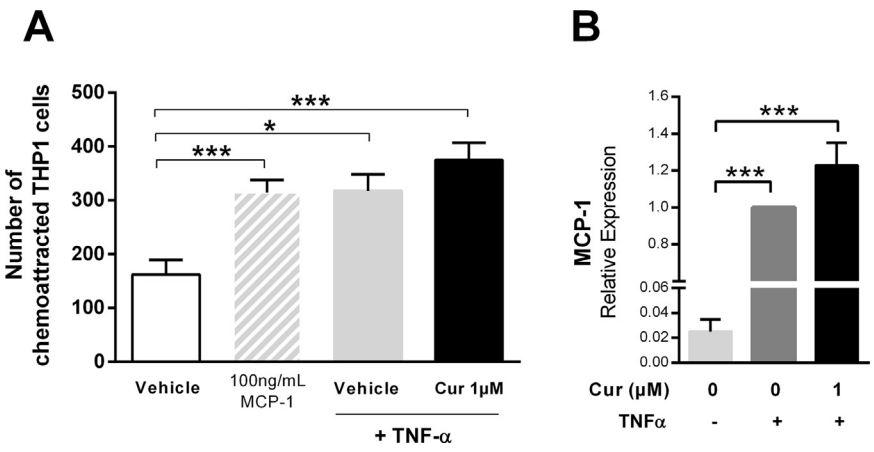

Fig. 3. *Chemoattractive potency of TNF-α-activated endothelial cells in response to curcumin.* (A) Transmigrated monocytes stimulated by the conditioned media harvested from vehicle/-TNF-α-, vehicle/+TNF-α- and Cur 1 µM/+TNF-α-HUVECs were assessed using a transwell assay. Bars represent means +/- SEM (n=4). (B) Relative expression of gene coding the chemoattractive protein MCP-1 normalized first to the expression of the housekeeping gene GAPDH and then to the relative expression detected in vehicle/+TNF-α-treated HUVECs. Values indicate fold-changes expressed as the mean +/- SEM (n=4). Significances: *p≤0.05, **p≤0.01 ***p≤0.001.

3.5. Curcumin modulates expression of genes in HUVECs

Of 93 genes for which the expression of TEM was evaluated, seventeen were identified as not expressed in our condition (Fig. 5A and Suppl. Table 1). Our nutrigenomic study revealed that the exposure of endothelial cells to curcumin prior their activation by TNF-α significantly modulated the expression of sixteen genes. Bioinformatic analyses showed that these differentially expressed genes are involved in different cellular processes, such as antioxidant defense (SOD-1), metabolism (IGFR1), cell signaling (CAV1, CASK, PDPK1), focal adhesion (PXN, VCL, ADAM10), intercellular junction (TJP2) and cytoskeleton organization (CDC42 subunits, ARPC2, PAK4, ACTN1, VIM). For three genes, tendencies in the modulation of their expression were observed; these genes encode two small Rho GTPases involved in the regulation of the cytoskeleton (RHOC and RAC1) and for the CALD1 gene which encodes caldesmon 1.

Additionally, bioinformatic analysis, performed using the miRWalk database, identified 459 putative miRNAs potentially modulated by curcumin and involved in the regulation of the aforementioned genes differentially expressed (data not shown).

Moreover, in TNF-α non-stimulated endothelial cells, a low basal expression of cell adhesion proteins (VCAM-1 and ICAM-1) was observed (Fig. 6A-C). A 4-h treatment of endothelial cells with TNF-α highly increased the expression of these two proteins. The expression of these proteins was not affected by the exposure of HUVECs to curcumin prior to TNF-α-activation.

3.6. Curcumin modulates phosphorylation of NF-κB cell-signaling proteins

We observed that a 3-h exposure to 0.5 µM or 1 µM of curcumin in TNF-α non-stimulated endothelial cells did not modify the basal phosphorylation level of NF-κB p65 and IκBα (Fig. 6). Following stimulation with TNF-α, levels of phosphorylated NF-κB p65 (ser536) and phosphorylated IκBα (ser32) were increased, and the total amount of IκBα was reduced compared to those observed in the non-stimulated endothelial cells. As a result, the phosphorylated/total ratio of NF-κB p65 and IκBα were higher in TNF-α-activated cells compared to the non-stimulated cells. Pre-exposure of HUVECs to 0.5 µM curcumin prior to their stimulation to TNF-α did not modify the phosphorylated/total ratio of NF-κB p65 and IκBα. In contrast, the exposure to 1 µM curcumin significantly reduced the level of phosphorylation of NF-κB p65 (Fig. 6A and D) without affecting that of IκBα (Fig. 6A and E).

3.7. Curcumin reveals favorable binding properties to cell-signaling proteins

The analysis of protein binding between curcumin and cell-signaling proteins of the NF-κB signaling pathway revealed that of the 13 proteins evaluated (Table 1 and Suppl. Table 2), 2 protein structures were not available, and 7 computational predictions had a low degree of confidence due to low resolution of the 3D structure of the target (resolution > 2.0 Å is recommended for docking) [25]. *In silico* predictions demonstrated that curcumin could interact directly with TAK1 and PDK1 to form a covalent bond within the active site of these proteins with a binding affinity of −7.8 Kcal/mol and −6.5 Kcal/mol, respectively (Table 1). Curcumin can also interplay with the kinases AKT 1 and AKT 2 through a non-covalent interaction of −7.3 kCal/mol and −6.3 kCal/mol, respectively. These interactions can be seen in Fig. 7.

4. Discussion

In this study, we set out to identify the cellular effects and the molecular mechanisms responsible for the ability of curcumin to prevent impairments in endothelial cell function. A special focus was on the impact of curcumin on endothelial cell processes involved in the interactions between vascular endothelium and circulating immune cells.

The bioavailability of pure curcumin is known to be very low in humans, with plasma concentrations most often not detectable for

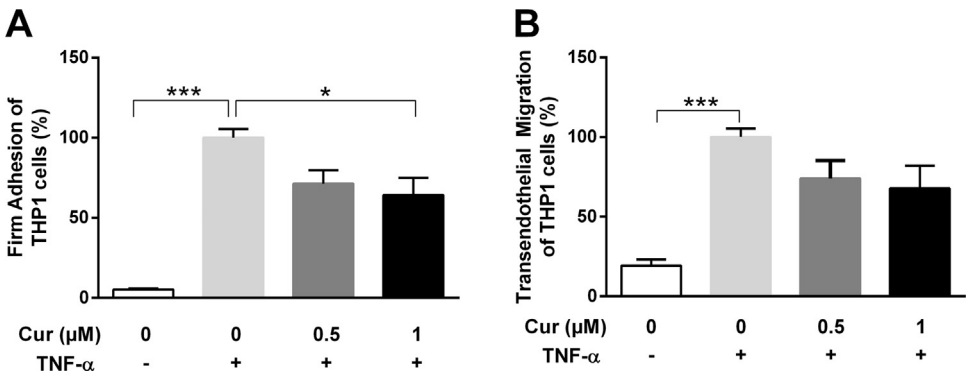

Fig. 4. *Effect of curcumin on monocyte adhesion to endothelial cells and their transendothelial migration under shear stress.* (A) Monocyte adhesion to TNF-α activated HUVECs after a 3-h exposure of endothelial cells to curcumin under 1 Hz pulsatile and 7 dyne/cm^2 shear stress. The results are expressed as a percentage of monocyte adhesion to 'vehicle/+TNF-α' treated HUVECs that normalized at 100%. (B) Transmigrated monocytes accumulated throughout the entire 10-min duration of the adhesion assay. The results are expressed as a percentage of transmigrated monocytes to 'vehicle/+TNF-α' treated HUVECs that is normalized at 100%. Significances: *p≤0.05, **p≤0.01 ***p≤0.001.

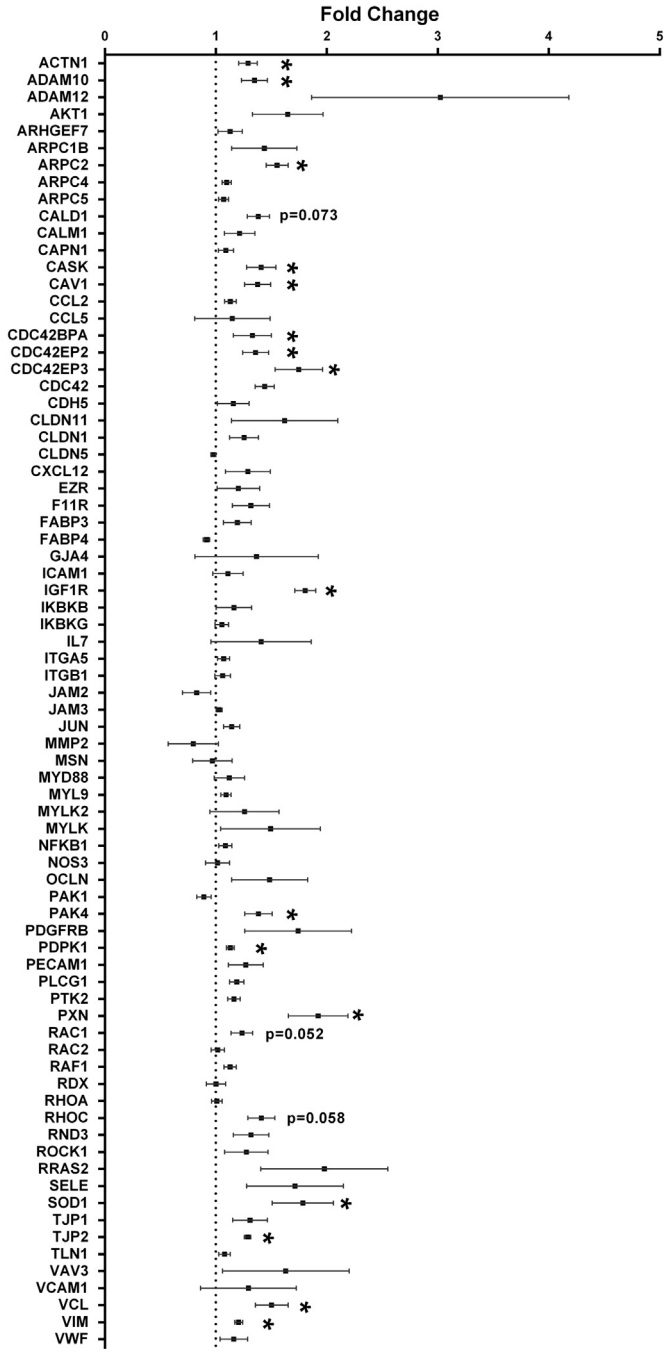

Fig. 5. *Gene expression in curcumin-exposed HUVECs.* Expression of 93 genes coding proteins involving TEM quantified by TLDA. Values (mean +/- SEM, n = 4) indicate gene expression fold change measured in 1 μM Cur/+TNF-α HUVECs compared to Vehicle/+TNF-α-exposed HUVECs. *p ≤ 0.05 in comparison to vehicle/-TNF-α group.

administered dose below 4 g and peaking at 1.77 μM after the intake of 8 g of curcumin [30–33]. However, in recent years many strategies have been developed to markedly increase the bioavailability of oral curcumin, including the use of natural enhancers or of encapsulation systems to improve its delivery [34], with a positive impact on the bioefficacy of curcumin in clinical studies [35]. With such approaches, the concentrations of curcumin tested in the present study (0.5 and 1 μM) could be easily reached in the plasma of human subjects consuming a reasonable dose of curcumin. Our study showed a reduction of monocyte adhesion to endothelial cells exposed to curcumin (0.5 μM and 1 μM) for 3 h that corresponds to the resident time of curcumin within blood circulation after curcumin intake [23]. This effect did not seem to result from an altered monocyte chemotaxis as revealed by the lack of effect of curcumin on chemoattractive potency of activated endothelial cells. This result is not consistent with previous studies reporting that curcumin can alter the expression of the gene coding for chemoattractant chemokine (MCP-1) in several cell lines [36]. However, these studies used curcumin at much higher concentrations than those used in the present study.

To mimic the endothelial dysfunction observed in vascular diseases [21], endothelial cells were activated by the inflammatory cytokine TNF-α. This activation induces the expression of cell adhesion molecules such as ICAM and VCAM, which are involved in the firm adhesion of monocytes [20]. In our *in vitro* conditions, curcumin at 1 μM significantly reduced monocyte adhesion, but it did not modify the expression of adhesion molecules induced by TNF-α. This observation is not in line with results from other studies showing a reduction of monocyte adhesion to endothelial cells along with a decrease in the expression of adhesion molecules. This discrepancy could result from differences in the experimental conditions used, for example, in previous studies, endothelial cells were treated with higher concentrations of curcumin (≥ 5 μM) for 1 h prior to TNF-α activation [12–14]. Nevertheless, the chronic consumption of curcumin has been associated *in vivo* with a decrease in the expression of genes coding for ICAM-1 and VCAM-1 in the aorta of atherosclerotic rabbits [9].

Tethering of monocytes with endothelial cells activates ICAM-1 and VCAM-1 clustering in lipid raft domain of the endothelial membrane [37–39] resulting in the firm adhesion of monocytes to the endothelial cells prior to their extravasation [39–41]. Wojciak-Stothard et al. [42] have demonstrated that inhibition of adhesion molecule clustering reduced the adhesion of monocytes to activated endothelial cells. Moreover, it has been reported that curcumin, at 5–10 μM, may affect delocalization of integrin and the formation of a signaling complex at a lipid raft, both of which are required for cell mobility [43,44]. Taken together, these results suggest that the decrease in monocyte adhesion to endothelial cells could result from the capacity of curcumin to affect the subcellular movement of adhesion molecules required for monocyte adhesion and diapedesis.

Following the firm adhesion to endothelial cells, monocytes undergo diapedesis. For the first time, we showed that curcumin can also reduce monocyte transendothelial migration. Extravasation of monocytes across the endothelium requires regulation of endothelial junctional permeability [18]. In addition, we demonstrated that 1 μM curcumin reduced the TNF-α-induced increase in permeability of the confluent monolayer of HUVECs. In agreement with these results, Li et al. previously reported that oral supplementation with curcumin protects against the alteration in permeability of the coronary artery as measured in a rat model of coronary disease [22]. Moreover, in western-diet induced atherosclerotic mice, curcumin has been previously shown to reduce intestinal epithelium permeability by acting on tight junction proteins involved in the selective barrier function such ZO-1 and claudin 1 [10,18]. Endothelial permeability is controlled not only by the regulation of cell-cell junctions but also by cytoskeleton dynamics [45]. Indeed, re-organization of actin into contractile stress fibers or microtubule disassembly increases endothelial contraction, resulting in interendothelial gap formation and consequently an increase in permeability. Interestingly, we observed that endothelial cells pre-exposed to curcumin were less sensitive to TNF-α induced modification of the cell surface, which is dependent on the actin cytoskeleton. All together, these results suggest that curcumin may have an impact on endothelial permeability by acting on the regulation of junction proteins and on the dynamics of the actin cytoskeleton. This hypothesis is in agreement with the lower number of monocytes within the atheromatous lesion observed in curcumin-supplemented ApoE$^{-/-}$ mice [6].

It is worth noting that endothelial cells in vessels are exposed to the luminal surface of blood flow that induces a physical strain called shear stress [46,47]. This physiological shear stress is known to activate endothelial mechanoreceptors resulting in the induction of the expression

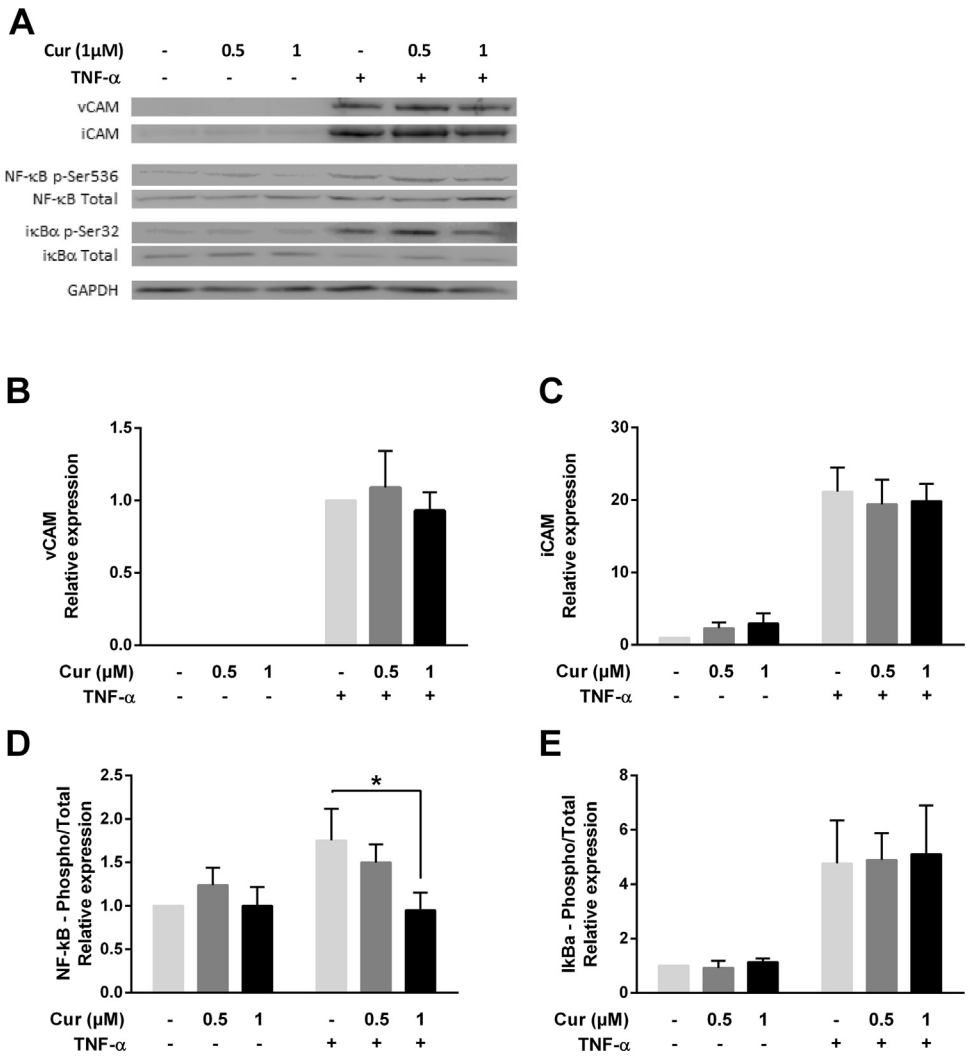

Fig. 6. *Impact of curcumin on endothelial adhesion molecules and on NF-κB signaling pathway proteins in HUVECs.* (A) Representative images of a western blot showing the detection of adhesion molecules, total NF-κB and phosphor-ser536, total IκBα and phosphor-ser532, and GAPDH in HUVECs exposed to curcumin (0.5 µM and 1 µM) or vehicle and stimulated by 1 ng/mL TNF-α for 4 h (vCAM and ICAM) or 15 min (NF-κB.and IκBα)⊡ (B, C) Quantification of the expression of endothelial adhesion molecules (vCAM and ICAM respectively). (D, E) Ratios of phosphorylated/total NF-κB.and IκBα respectively. Graphs represent means +/- SEM (n = 3). *p ≤ 0.05.

Table 1
Binding score of curcumin interplay with cell-signaling proteins. (N/A: none adapted).

Target	Binding Affinity of curcumin *(Kcal/mol)*	Number of H-Bonds	Location of H-Bonds
PDK1	−6,5	1	Tyr126 of the active site
AKT1	−7.3	0	N/A
AKT2	−6.3	0	N/A
TAK1	−7.8	1	Lys227 (coil) of the protein kinase domain, blocking the access at the ATP binding site and interaction with MAPK8IP1

of transcription factors such as Krüppel-like factor 2 (KLP2) and nuclear factor (erythroid-derived 2)-like 2 (NRF2), among others [48]. The activation of these factors contributes to the preservation of endothelial function by inducing vasodilatory, antioxidant, antithrombotic, anti-adherent and antiangiogenic responses. Here, we demonstrated for the first time that the exposure of endothelial cells to low concentrations of curcumin under physiological shear stress could reduce monocyte adhesion and extravasation. To the best of our knowledge, only one other study, that of Chacko et al., demonstrated a similar observation under flow in response to 1 µM of isoflavone, another subfamily of polyphenols [49,50]. In addition, we observed that the magnitude of the effect of curcumin on adhesion and TEM in the condition of shear stress was markedly higher (+44% and +37%, respectively) than those observed in the static condition. Hence, one can speculate that curcumin may potentiate the beneficial effect of physiological shear stress on endothelial function.

To decipher the underlying mechanisms of action of curcumin, we also screened for the expression of 93 genes coding for adhesion and permeability proteins in response to curcumin exposure in endothelial cells for the first time. Among the genes significantly modulated by the exposure to curcumin, our results revealed the overexpression of PXN, VIM and VCL, coding for paxilin, vimentin and vinculin, respectively, which are three proteins involved in the focal adhesion of endothelial cells to extracellular matrix [19]. Down-regulation of such proteins was associated to an increase in endothelial barrier permeability [48,51]. Similarly, the expression of genes TJP2 and CASK, coding the tight junction protein 2 (also called ZO-2) and the Ca^{2+}/Calmodulin dependent serine protein kinase (CASK), both involved in endothelial cell-cell junction [45], were shown to increase in response to curcumin. Such change in the expression of these genes has been described to be associated to a reduction in endothelial permeability [52]. Our gene expression assay also showed that curcumin affects the expression of caveolin-1 (CAV1), which plays an important role in the organization of protein interactions between cells and the extracellular matrix that contributes to control endothelial permeability [53]. Moreover, we observed that curcumin modulated the expression of two Rho GTPases (ARPC2 and CDC42), which are known to control microtubule dynamics and actin-rich surface projections involved in TEM process [54], and the expression of the PDPK1 kinase, which regulates cell migration [55]. In addition, we observed the modulation of PAK4 expression by

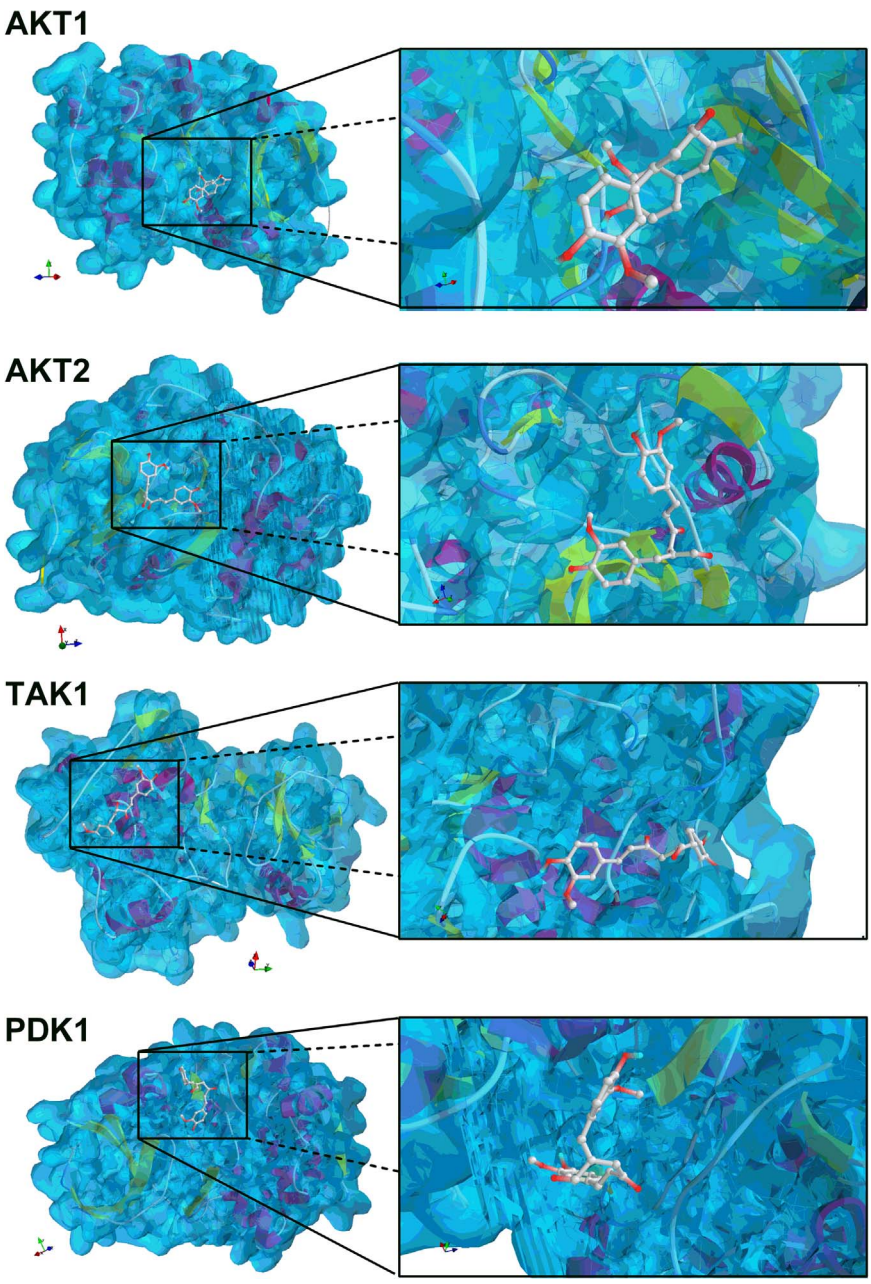

Fig. 7. 3D structures from *in silico* docking of curcumin to cell proteins of the NF-κB signaling pathway.

curcumin. PAK4 has been described to activate the downstream target RHOA involved in the regulation of the assembly of focal adhesions and actin stress fibers [19]. The expression of caldesmon (CALD1), a binding protein that plays a role in the stabilization of actin filament structure [56], was also significantly upregulated in curcumin-exposed HUVECs. Taken together, the changes in gene expression that we observed suggest that curcumin could maintain endothelium integrity by reinforcing cell-cell and cell-matrix interactions, and by stabilizing endothelial cell cytoskeleton through nutrigenomic effect. This hypothesis fits with the observed impact of curcumin on endothelial cell function and surface area.

The molecular mechanisms of curcumin involve a large range of targets including signaling pathways and transcription factors [16]. In a previous animal study, we demonstrated that a dietary supplementation with 0.2% curcumin inhibits atherosclerosis by affecting NF-κB-transcriptional activity [6]. In the present *in vitro* study, we have stimulated endothelial cells with TNF-α, an activator of the NF-κB signaling pathway [57], and we showed that 1 μM of curcumin significantly reduced the phosphorylation of NF-κB p65 (serine 536). Others have previously described such an inhibition with curcumin in a similar range of concentrations [12,13,21]. In contrast to previous observations [12–14], in our experimental conditions, inhibition of NF-κB by curcumin did not result in a lowering of the expression of NF-κB target genes such as ICAM and VCAM. However, Cobb et al. [58] reported that induction of VCAM1 can be NF-κB-independent and that proteasome can regulate VCAM-1 and ICAM-1 expression in endothelial cells without affecting NF-κB activation. Recently, Hasima et al. [59] suggested that curcumin can modulate proteasomal pathways in a dose-dependent manner. Thus, it can be speculated that curcumin may regulate the expression of adhesion molecules in a different way than the NF-κB pathway, potentially by acting on proteasomal activity.

The inhibition of the NF-κB pathway observed in response to exposure of endothelial cells to curcumin is also supported by our results obtained *in silico* that revealed favorable binding of curcumin to some kinases, namely, TAK1, PDK1 and AKT. TAK1 and PDK1 have been reported to activate the NF-κB canonical pathway by phosphorylating

the IκB Kinase β (IKKβ) [60]. AKT is known to be activated by TNF-α stimulation [61] and to regulate the activity of NF-κB by inducing phosphorylation and subsequent degradation of inhibitor of NF-κB (IκB) [62]. Regarding the binding mode of curcumin in the active domain of these kinases, the occupancy and the interactions we observed mimic those of a heterocyclic core of known inhibitors of kinases that have been shown to attenuate NF-κB activation [63–65]. Similarly, Gupta et al. reported that curcumin might interact directly with a large range of targets including signaling proteins and protein kinases, leading in their inhibition [66]. In addition, *in silico* structural evaluation highlighted that quercetin, another polyphenol, may interact and therefore inhibit serine/threonine kinases such as AKT [67]. Collectively, our *in silico* analysis suggests that curcumin can bind to cell-signaling proteins and affect their activity, which consequently results in modulation of the expression of genes as we observed.

Another possible mechanism of action by which curcumin could modulate adhesion and monocyte transendothelial migration in endothelial cells may involve the modulation of miRNAs. miRNA are noncoding single-strand RNAs of 22 nucleotides that constitute a class of gene regulators at the posttranscriptional level [68]. Among the miRNAs potentially modulated by curcumin and involved in the regulation of the 15 aforementioned genes shown as differentially expressed in response to curcumin in the present study, 4 were previously detected as modulated by dietary curcumin in murine ApoE$^{-/-}$ liver (miR-137, miR-188-5p, miR-324-5p and miR-335-5p) [69]. MiR-137 has been described as a regulator of Cdc42 expression and a Rho GTPase [70], and miR-335-5p is involved in cytoskeleton dynamics [71]. In addition, Howell et al. have recently described the ability of curcumin to modulate 29 miRNAs in epithelial cell line ARPE-19 [72]. Nine of these miRNAs were reported to regulate the expression of cell-cell junction proteins and adhesion molecules (miR181a, miR18a, miR150 miR101, miR126, miR181b, miR27a, miR155, and miR20A) [73]. These findings were supported by bioinformatic analyses suggesting that the miRNAs detected as modulated by curcumin in murine ApoE$^{-/-}$ liver [69] and in murine melanoma [74] may regulate several pathways influencing cell communication and focal adhesion. Therefore, further investigation is needed to determine the impact of curcumin on miRNAs as post-transcriptional mode of regulation of monocyte TEM in endothelial cells.

In conclusion, this *in vitro* study provides new mechanistic insights into the ability of dietary curcumin to preserve vascular health during inflammation, as summarized in Fig. 8. Our findings clearly support that, in physiologically relevant conditions, exposure of endothelial cells to curcumin can reduce endothelial permeability and monocyte adhesion and transmigration, ultimately promoting vascular integrity. This effect seems to be related to a complex and multimodal action of curcumin on the intracellular dynamics of endothelial cells. However, these mechanisms warrant further investigation to be fully understood.

4.1. Statement of authorship

The authors' responsibilities were as follows: LEM designed methods and experiments, carried out the experiments, analyzed the data and wrote the paper; SM carried out cell experiments and flow cytometry analysis, and DB performed the gene expression assay; RT performed molecular docking analysis; NBC participated in discussion and revision of the article; DM & CM carried out study design, data interpretation and manuscript preparation. All authors have contributed to read and approved the manuscript.

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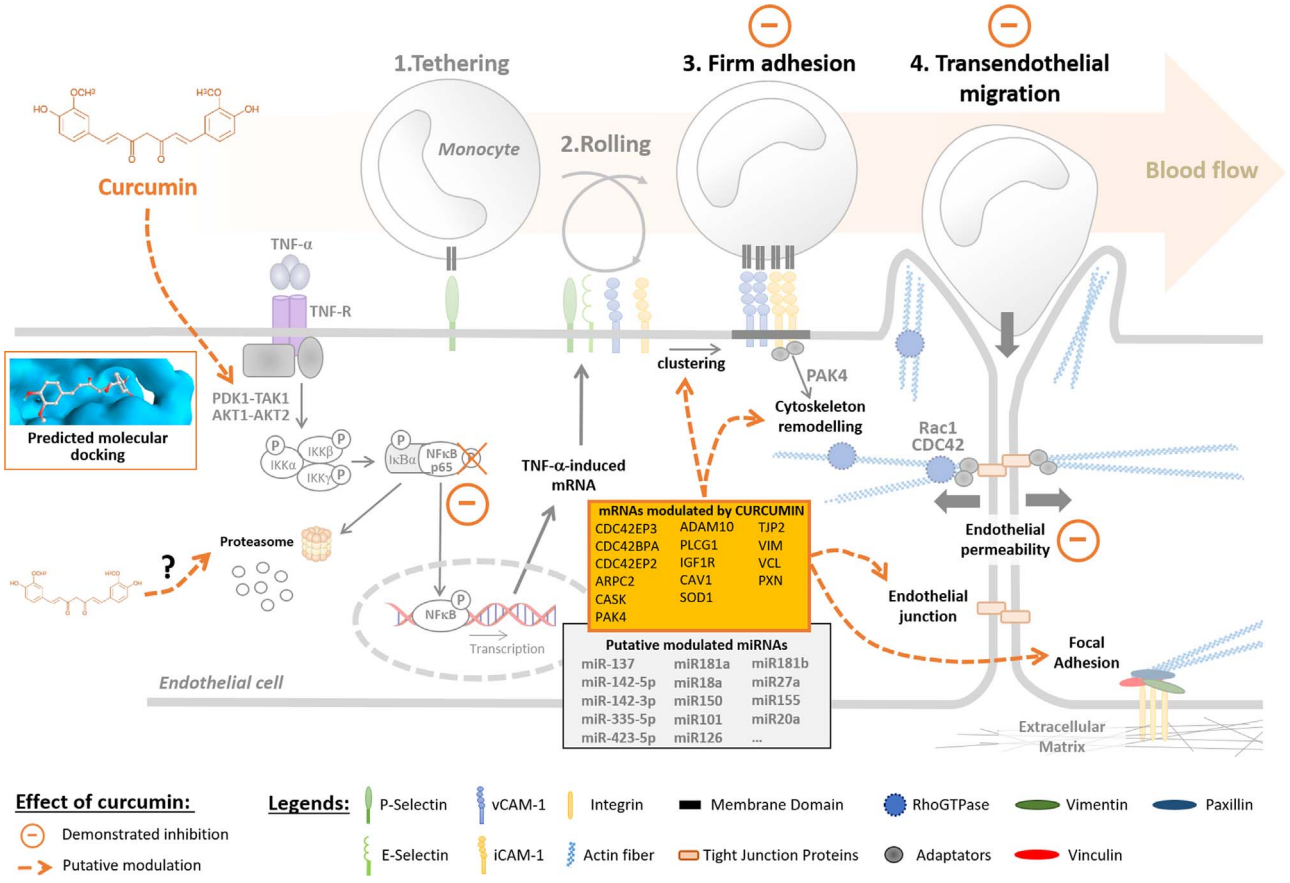

Fig. 8. Overview of the demonstrated and putative mechanisms by which curcumin can modulate endothelial cell activation.

plant food bioactives and determinants involved).

Conflict of interest

None of the authors has any conflict of interest to declare.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2017.07.019.

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