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Original Research Article

The protective effect of curcumin on thrombin-induced hyper-permeability

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Abstract

Objective: Thrombin is a proinflammatory and pro-coagulant agent which is upregulated in several human diseases. Thrombin has a critical role in promoting cell proliferation and microvascular leakage in malignant cells, resulting in cancer growth and progression. Here, we explored the potential therapeutic value of curcumin on permeability induced by thrombin in mice.

Materials and Methods: To assess the activity of curcumin on thrombin-induced vascular permeability mice model, C57BL / 6 mice were randomly divided into four groups: (1) control (2) Thrombin (3) Thrombin + Curcumin and (4) Thrombin + Metformin. Thirty minutes after treatment, Evans blue was injected intravenously through the tail vein to mice. Then, animals were sacrificed and the dye was extracted from the skin tissue by incubation with formamide. Heatmap and correlation map were generated and protein-protein interaction network of the hub genes was drawn by Cytoscape software.

Results: Hub DEG expression rate showed that Heat shock protein a1 (Hspa1) family (comprised of HSPa1a, b, and HSPa5), caspase 3, and minichromosome maintenance complex component 2 were overexpressed after treatment with curcumin. Functional modules of curcumin enriched through Enrich gene biological process and revealed positive association of gene expression of apoptosis process with the therapy. Curcumin was also found to reduce leucocyte migration in murine tissues. Additionally, treatment with curcumin resulted in downregulation of heat shock proteins and proinflammatory cytokines such as monocyte chemotactic protein 1, interleukin-6 and chemokine (C-X-C motif) ligand 3.

Conclusion: Curcumin inhibited the proinflammatory cytokines and inflammatory HSPs in endothelial cells and reduced thrombin-induced barrier destabilization *in vivo*.

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Introduction

Thrombin is a trypsin-like serine protease formed by proteolytic cleavage of its zymogen, prothrombin, during blood coagulation (Norris, 2003). Thrombin converts fibrinogen into insoluble fibrin and activates factor XIII to form cross-linked fibrin clots (Crawley et al., 2007; Narayanan, 1999). Besides its procoagulant and anticoagulant properties, it acts as a potent inflammatory and mitogenic factor (Carney et al., 1984; Cirino et al., 1996; Vesey et al., 2005). Thrombin through proteolytic cleavage of N-terminal extracellular domains of the protease-activated receptors (PARs) can initiate several proliferative and inflammatory responses (Coughlin, 2000).

Thrombin activates proliferative signaling pathway Wnt and mammalian target of rapamycin (mTOR) (Parrales et al., 2013; Zhong et al., 2013), stimulates mast cell degranulation (Pervin et al., 1985), increases vascular permeability (Malik and Fenton, 1992), platelet aggregation, and overexpression of cell adhesion molecules such as VCAM-I,I-CAM and E-selectin, and induces the secretion of proinflammatory cytokines and chemokines (Rahman et al., 1999; Sugama et al., 1992). Moreover, it has been shown that thrombin affects Heat shock proteins (HSPs) which are critical for the regulation of inflammatory processes (Madamanchi et al., 2001). Thrombin as a potent proliferative and proinflammatory mediator is involved in pathogenesis of several diseases such as coronary thrombosis (Fitzgerald and FitzGerald, 1989), pulmonary emboli, atherogenesis (Strukova, 2001), neurodegenerative disorders (Ebrahimi et al., 2017) and carcinogenesis (Rickles, 2003). Thus, targeting thrombin represents a potentially novel therapeutic strategy for thrombin-associated disorders.

Curcumin is a polyphenolic compound (1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6- heptadiene-3,5-dione) isolated from the rhizomes of turmeric (*Curcuma longa* L.). It has been shown that curcumin plays critical roles in the modulation of oxidative stress (Motterlini et al., 2000), inflammation (Maithilikarpagaselvi et al., 2016) and multiple signaling pathways including AMP-activated protein kinase (AMPK), NF-KB, in several tumor types (Basha et al., 2016; Zhang et al., 2017).

In addition to being a powerful AMPK activator, metformin is initially classified as an antibiotic biguanide (Lee et al., 2017). Vascular endothelial cells have been shown to be activated by AMPK when exposed to metformin to inhibit the expression of pro-inflammatory cytokines and cell adhesion molecules (Park et al., 2012). As a prophylactic measure, metformin can also inhibit eosinophilic inflammation and reduce intestinal reactive oxygen species (Park et al., 2012). Previous studies have demonstrated various pharmacological effects of *C. longa* and its constituents, especially curcumin. Curcumin, inhibits vascular hyperpermeability following hemorrhagic shock (Tharakan et al., 2010). The antioxidant properties of curcumin also protect cells from oxidative stress because it induces heme oxygenase-1 (HO-1). HSP is one of the proteins that is stimulated by stress (Miller et al., 1993). In traumatic and ischemic injuries, vascular hyper-permeability occurs and blood vessels leak excessively (Oakley and Tharakan, 2014). In many cases, this hyper-permeability results in multiple organ failure and patient death as a result of vasogenic tissue edema (Oakley and Tharakan, 2014). Therefore, due to the importance of vascular hyper-permeability, its treatment can be useful. However, the effects of curcumin on thrombin- mediated signaling responses have not been fully investigated. Here, we investigated the modulatory effect of curcumin on thrombin-mediated signaling responses both *in vitro* and *in vivo*.

Materials and Methods

Chemical compound

Curcumin and metformin were obtained from Mashhad University of Medical Sciences, both dissolved in ethanol or sterile water. Human enzyme thrombin alpha was purchased from Haematologic Technologies, Inc. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Gaithersburg, MD).

Animal

C57/B6 mice were purchased from the Pasteur institute of Iran. The mice were maintained under a 12 hr light/dark cycle, in a temperature-controlled room (22–25°C) and they had access to food and water *ad libitum*. Animal experiments were carried out under the guidelines approved by the animal care committee of MUMS (IR. MUMS. MEDICAL. REC. 940938).

Induction of vascular permeability model

To investigate the regulatory effects of curcumin on thrombin-induced vascular permeability, the Evans-blue assay was performed as described previously (Hernández et al., 2018; Liu et al., 2020). Twenty-four C57BL/6 mice were randomly divided into four groups (n=6 for each group): (1) control group; (2) Thrombin group; (3) Thrombin + Curcumin group; and (4) Thrombin + Metformin group. The Thrombin group was given Thrombin (10 unit, intraperitoneal (ip)); the Thrombin + Curcumin group mice were injected thrombin (10-unit, ip) and then curcumin (50 mg/kg, ip); and the Thrombin + Metformin group mice received thrombin and then metformin (200 mg/kg, ip). For Induction of vascular permeability model, mice were injected with Evans blue 1% (100 µl) intravenously via tail vein, and then, perfused subcutaneously with thrombin, curcumin, or thrombin plus curcumin or metformin. Next, 30 min after the treatment, we collected a biopsy of mice's skin. After that, the mice were sacrificed and the dye was extracted from the skin tissue by incubation with formamide (56ºC for 24 hr) and the concentration of Evans blue was determined by spectrophotometry (620 nm). The results are expressed as microgram (µg) dye per gram tissue.

Leukocyte migration

Tissue samples were fixed in formalin and embedded in paraffin. The paraffin blocks were cut into 5-µm thick sections and dyed with hematoxylin-eosin stain. Histological investigation was performed by light microscopy to assess leukocyte migration (Farkas et al., 2006).

Gene expression microarray dataset

The gene expression profile of mice genome array was obtained from NCBI Gene Expression Omnibus (GEO) database. It comprises a platform: GPL18615 Affymetrix mouse genome 430 2.0 array [CDF: Mouse4302_Mm_ENTREZG.cdf Brainarray version 17.0.0] (Accession number: GSE72081).

Differentially expression genes

GEO2R was used to normalize the data. Differentially expressed genes between mouse groups that were treated with Curcumin vs. PBS (as a control sample) were identified by GEO database expression genes (DEGs). In the same part, fold changes (FC) were calculated, so upregulated and downregulated genes were identified via positive or negative results of FC.

Hub genes identification

Centrality parameters like degree, closeness, and betweenness were calculated by version 3.7.1 of Cytoscape software. Hub DEGs are types of genes that have the highest amount of these centrality parameters.

After identification of hub DEGs, heatmap and correlation map were drawn through R software version 3.2.5. Hub DEG expression rate is shown in the heat map.

The red and green colors in this plot demonstrate high and low expression, respectively. The protein-protein interaction network (PPTN) of the hub genes was drawn by Cytoscape software (Figure 1). A positive correlation was seen between node size and degree count indicating that increased numbers of degrees led to increased size of the node.

In the last step, enrichment of the identified hub DEGs and identification of GO biological process of hub DEGs (Figure 1B) were done using EnrichR website.

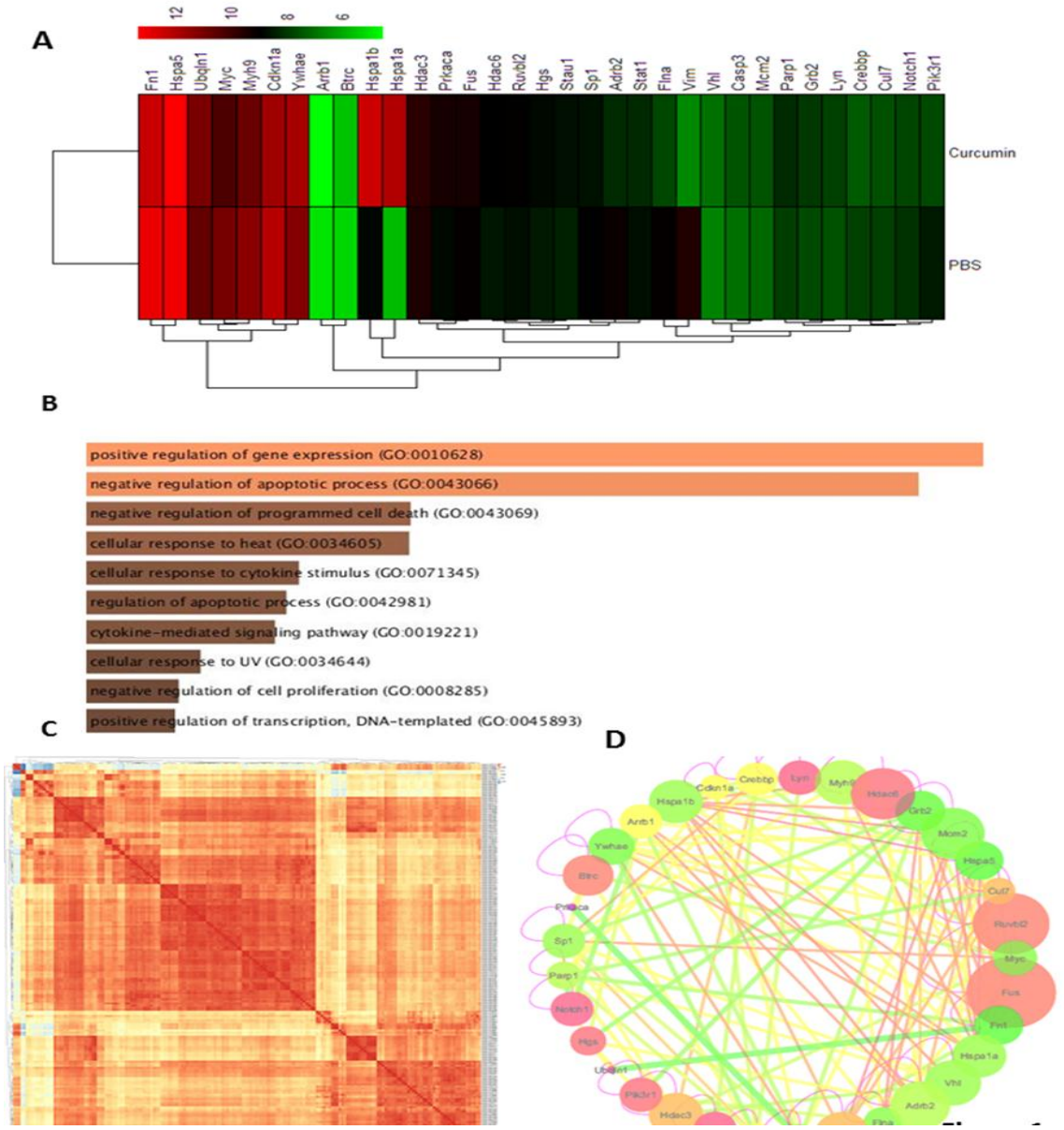

Figure 1. Expression analysis of all the hub genes. (A) A hierarchical clustering heat-map of the hub genes from control and curcumin-treated cells. The red and green colors in this plot demonstrate high and low expression, respectively. (B) The biological processes significantly enriched for proteins involved in cell proliferation, apoptosis, and inflammation. An adjusted p-value<0.05 was considered significant. (C) The distance between samples is shown by the correlation map which compares the samples based on the data expression profile. Red and blue colors indicate the highest and the lowest correlation, respectively. (D) The protein-protein interaction network of the hub genes is visualized by Cytoscape software.

Cell culture

HUVEC cell line was purchased from Pasteur Institute, Tehran, Iran. The cells were grown in DMEM containing 10% FBS and 1% penicillin and kept in 37°C in 5% CO_2 atmosphere.

Cell viability assay

The MTT assay was used to determine the cytotoxic effects of curcumin on HUVEC cells. Cells were treated with different concentrations of curcumin (0-1000 μM) for 24 hr. Data from three independent experiments was assessed and IC_{50} was determined as previously described (Avan et al., 2013).

Quantitative reverse-transcriptase polymerase-chain-reaction (qRT-PCR)

Real-Time PCR was performed as described previously (Rahmani et al., 2019). Briefly, RNAs were extracted from HUVEC cells and complementary DNAs (cDNA) were prepared according to the manufacturer's protocol (TaKaRa Bio, Shiga). Quantitative RT-PCR was performed using specific primers for *HSP10/60/70/90*, *CyclinD1*, *MCP1*, *IL-6*, and *CXCL3* genes (Macrogene Co. Seoul) (Table 1) in ABI-PRISM StepOne instrument (Applied Biosystems, Foster City, CA). The expression levels of target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the control gene.

Statistical analysis

Results were obtained from three independent experiments. All data are reported as mean±standard deviation (SD), and were analyzed by Student's t-test or ANOVA followed by Tukey's multiple comparisons test. Differences were considered to be statistically significant when $p<0.05$.

Table 1. qPCR primer sequences.

Gene	Source	Primer	Sequence
GAPDH	Mouse	Forward	CAACGACCCCTTCATTGACC
		Reverse	CTTCCCATTCTCGGCCTTGA
IL-6	Mouse	Forward	TCTATACCACTTCACAAGTCGGA
		Reverse	GAATTGCCATTGCACAACTCTTT
CXCL-3	Mouse	Forward	GATTTTGAGACCATCCAGAGCT
		Reverse	GGCAAACTTCTTGACCATCCT
MCP-1	Mouse	Forward	GTGAAGTTGACCCGTAAATCTGA
		Reverse	ACTAGTTCACTGTCACACTGGT
ICAM-1	Mouse	Forward	GGGCTGGCATTGTTCTCTAATGTC
		Reverse	TGGGATGGTAGCTGGAAGATCG
HSP 10	Mouse	Forward	ATGGCTGGACAAGCTTTT
		Reverse	GCTTCATGTGACACCTTTCAA
HSP 60	Mouse	Forward	AAAATTTGGTGCGGACGCT
		Reverse	AAAGCCCTCCTTGGCAATAGAT
HSP 70	Mouse	Forward	GGCAAGGCCAACAAGATCA
		Reverse	AGATGACCTCCTGGCACTTGTC
HSP 90	Mouse	Forward	GACGCTCTGGATAAAATCCGTT
		Reverse	TGGGAATGAGATTGATGTGCAG

Results

Curcumin affects various pathways involved in apoptosis and inflammation

Our results showed that most of statistically significant modules obtained from Enrichr gene biological process were associated with positive regulation of gene expression and negative regulation of the apoptosis process (Figure 1 A). HSPa1 family (comprised of HSPa1a, b, and HSPa5), caspase 3, and minichromosome maintenance complex component 2 (MCM2) were overexpressed after treatment with curcumin.

The distance of sample vs. sample is shown by the correlation map which compares the samples based on the data expression profile (Figure 1C).

Cytotoxic effects of curcumin in endothelial cells

As shown in Figure 2A, we demonstrated that curcumin significantly inhibited the growth of HUVEC cells in a dose-dependent manner with an IC_{50} of 10 μM.

Anti-inflammatory effects of curcumin in HUVEC cells

Our findings indicate that thrombin significantly increases the expression of inflammatory cytokines while curcumin suppresses the expression of these inflammatory mediators in HUVEC cells (Figure 2B). We next assessed the effects of curcumin on the expression of HSPs 10, 60, 70, and 90 in thrombin-treated HUVEC cells. As shown in Figure 3, curcumin suppressed the promotive effects of thrombin on the expression of HSPs 60, 70, and 90 while increased the expression of HSP 10.

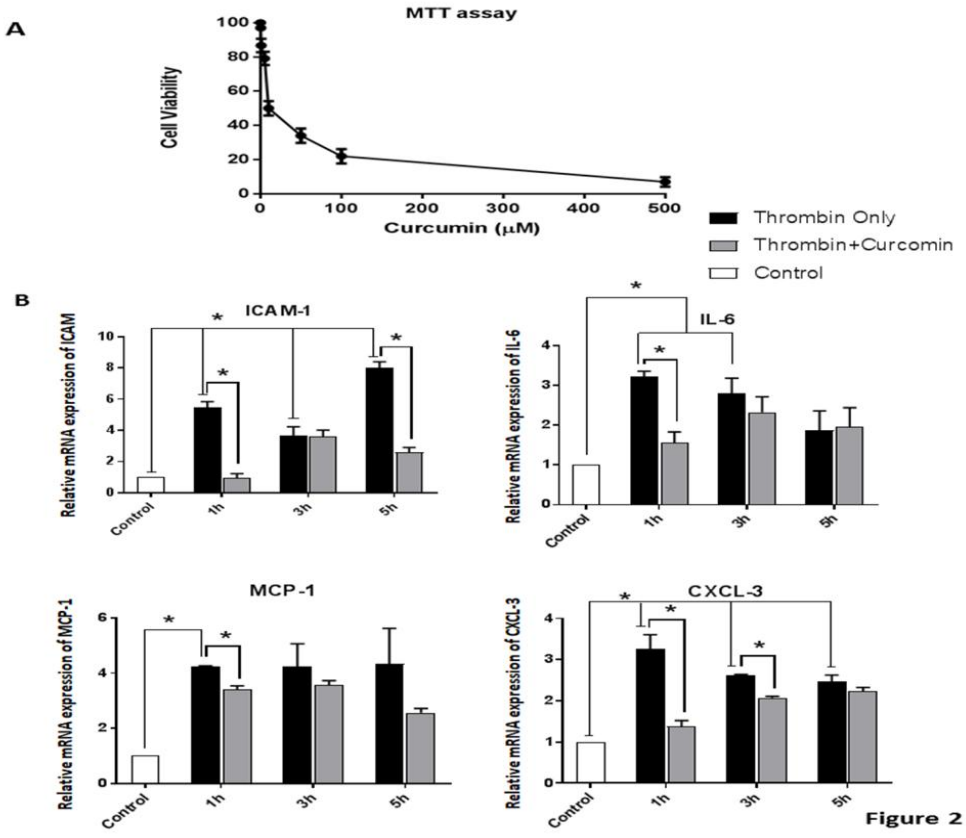

Figure 2. The effect of curcumin (10μM) on HUVEC cell viability and expression of inflammatory mediators. (A) HUVEC cells were treated with curcumin for 24 hr, and the cell viability was determined by MTT assay. (B) The result for time-dependent treatments on IL-6, MCP-1, CXCL-3, and ICAM-1. All data are reported as mean±standard deviation (SD), and were analyzed by Student's t-test or ANOVA followed by Tukey's multiple comparisons test.*$p<0.05$

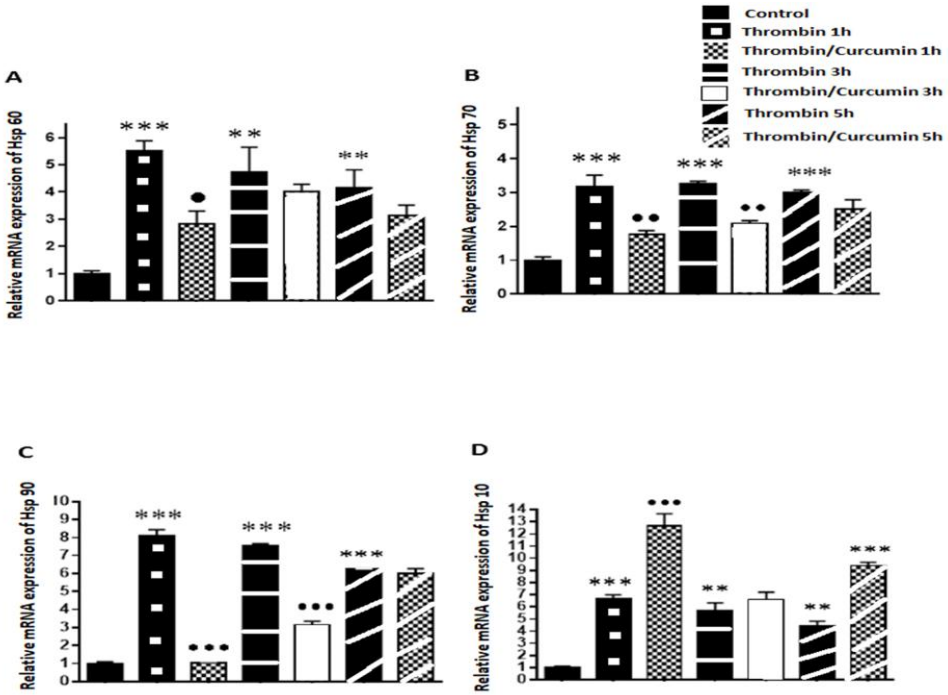

Figure 3. Curcumin treatment regulates expression of HSPs expression in endothelial cells. (A-D) Expression of HSP 60/70/90/10 in the presence and absence of curcumin treatment in a time dependent manner. *p<0.05; **p<0.01; ***p<0.001

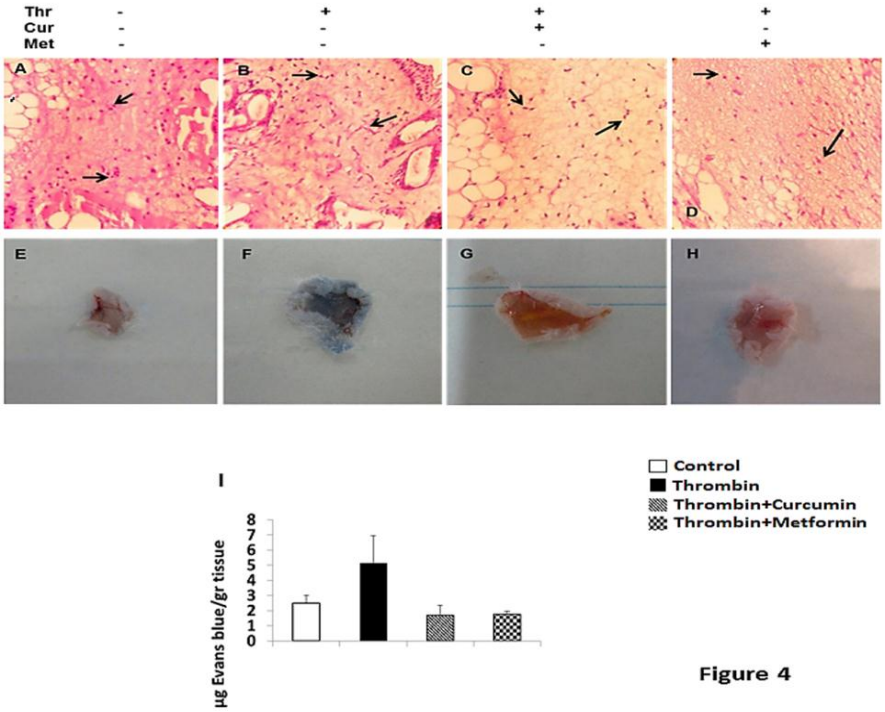

Figure 4. Curcumin inhibits thrombin-induced hyper-permeability and thrombin-induced microvascular leakage in endothelial cells. Mice were injected with Evans blue via tail vein, and then perfused subcutaneously with thrombin, curcumin, or thrombin plus curcumin or metformin. Next, 30 min after treatment, we collected a biopsy of mice skin. (A-D) Microscopic view of skin tissue shows leukocyte extravasation (black arrows). (E-H) Macroscopic view of skin tissue. (I) Evans blue was extracted from the skin biopsies and quantified by spectrophotometry. Statistical analysis was done as described in the Materials and Methods section; data are shown as mean±SD. All data were analyzed by Student's t-test or ANOVA followed by Tukey's multiple comparisons test. *p<0.05; **p<0.01; ***p<0.001

Inhibitory role of curcumin on thrombin-induced permeability in mice model

We evaluated the regulatory role of curcumin on leucocyte migration. As shown in Figure 4 (A-D), the H&E results revealed that curcumin decreases infiltration of leucocytes to thrombin-injected site. To further confirm the inhibitory role of curcumin on microvascular permeability, we used the Evans blue assay. Our results showed that thrombin significantly increased Evans blue extravasation in comparison with the control group whereas curcumin and metformin significantly reduced this elevation (Figure 4E-I).

Discussion

We have investigated the anti-inflammatory mechanisms of curcumin in controlling thrombin-induced microvascular permeability and leucocyte migration *in vitro* and *in vivo*. Our findings indicate that curcumin elicited its protective effects by down-regulation of pro-inflammatory cytokines including CXCL3, MCP-1, IL-6, and regulation of HSPs in thrombin-treated endothelial cells. Thrombin is a proinflammatory and pro-coagulant agent which is upregulated in many diseases and induces cell proliferation and microvascular leakage in malignant cells resulted in tumor progression and metastasis (Ebrahimi et al., 2017).

HSPs are molecular chaperones which can be up-regulated during various pathologic conditions including oxidative stress, ischemia, infection, and inflammation (Madamanchi et al., 2001; Fitzgerald et al., 1989). It has been shown that HSP60, HSP70 and HSP90 play functional roles in pathogenesis of several inflammatory diseases including arteriosclerosis (Strukova et al., 2001; Ebrahimi et al., 2017; Rickles et al., 2003). Madamanchi et al. showed the increased expression of HSP70 and HSP90 via thrombin through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway in vascular smooth muscle cells (VSMC). Thrombin-stimulated HSPs contributed to the VSMC proliferation which is considered a mechanism involved in the pathogenesis of atherosclerosis (Madamanchi et al., 2001). Consistently, our results demonstrated that thrombin (40 nM) increases the expression level of inflammatory HSPs 60/70/90, while reduces the expression of anti-inflammatory HSP10 in HUVEC cells, suggesting a potential proinflammatory role for thrombin (Figure 3D).

Moreover, the regulatory effects of curcumin on proinflammatory function of thrombin were reported in several studies (Motterlini et al., 2000; Maithilikarpagaselvi et al., 2016; Basha et al., 2016). It is interesting to note that curcumin as an AMPK activator significantly reverses the thrombin-modified HSPs expression in a time-dependent manner (Figure 3). The greatest effects were observed in the first hours in thrombin-exposed endothelial cells.

Excessive proinflammatory cytokines production such as interleukin (IL)-6 can lead to spread of local and systemic inflammation, severe pathophysiologic disorders, and even death (Zhang et al., 2017; Avan et al., 2013; Rahmani et al., 2019). Cho et al. revealed that curcumin administration inhibits the expression levels of IL-1 and IL-6 via suppression of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways in tumor necrosis factor-alpha (TNF-α)-treated HaCaT cells (Hernández et al., 2018). Furthermore, oral consumption of curcumin reduces the neutrophils-induced inflammation in aortic tissues of abdominal aortic aneurysm mouse model which was correlated with low concentrations of inflammatory cytokines such as IL-1, IL-6, and monocyte chemoattractant protein-1 (MCP-1) (Liu et al., 2020). Also, the regulatory effects of curcumin on the expression of chemokine

(C-X-C motif) ligand (CXCL)-1 and -2 were reported in breast cancer cells (Farkas et al., 2006). Similarly, our findings showed that curcumin can suppress the thrombin-promoted inflammatory mediators MCP-1, IL-6 and CXCL3 mRNA levels in HUVEC cell line (Figure 2B).

Cellular adhesion molecules are complex carbohydrate and protein molecules which are found on the cell surface, contributing in cell-cell and cell-extra cellular matrix (ECM) interactions (Ebrahimi et al., 2017). It has been shown that these molecules such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), or endothelial leukocyte cell adhesion molecule-1 (ELAM-1) have pivotal roles in inflammatory and neoplastic disorders (Loeffen et al., 2015). Kumar et al. showed that curcumin has the ability to significantly reduce the TNF-α-induced cell adhesion molecules in HUVECs, blocking the interaction of these cells to monocytes (Arsenault et al., 2012). To further investigate the anti-inflammatory effect of this compound, we measured the expression of ICAM-1 in thrombin-treated endothelial cells in the absence and presence of curcumin. Consistent with previous results, curcumin decreased the thrombin-induced ICAM-1 expression in endothelial cells (Figure 2B).

Recent studies indicate that thrombin has promotive effects on pro-inflammatory and proliferative signaling contributed in the pathogenesis of several diseases including neurodegenerative, cardiovascular, and cancer (Ebrahimi et al., 2017; Loeffen et al., 2015). From this point of view, modulation of thrombin may present a viable strategy in attenuating inflammatory and proliferative responses. Several drugs targeting thrombin have been designed and their therapeutic benefits have been documented in various *in vitro* and *in vivo* studies (Arsenault et al., 2012; Capodanno et al., 2020). However, clinical investigations of thrombin inhibitors are limited due to their toxicity, unfavorable pharmaceutical activities and risk of fatal bleeding (Nathan et al., 2017). Unlike synthetic thrombin inhibitors, natural inhibitors like curcumin have fewer toxic effects and can be used as an alternative to synthetic drugs (Akaishi et al., 2020; Hashemzehi et al., 2018).

Several studies reported that induction of AMP-activated protein kinase (AMPK) signaling pathway by metformin has a prominent role in modulating vascular permeability (Jian et al., 2013; Liu et al., 2014). Consistently, our results showed that metformin and curcumin as AMPK activators, have an inhibitory function in regulating leucocyte migration and barrier destabilization. To further support the curcumin regulatory mechanism, we evaluated the expression level of intercellular adhesion molecule type 1 (ICAM-1) in thrombin-treated HUVEC cells. Recent findings indicate that the ICAM signaling has a critical role in vascular permeability (Frank and Lisanti, 2008; Sumagin et al., 2008). In agreement with these data, we observed that ICAM-1 is significantly downregulated in curcumin-treated cells, suggesting that the protective effects of curcumin are at least partially mediated by suppressing expression of adhesion molecules in endothelial cells.

Taken together, we have shown that curcumin attenuates thrombin-induced microvascular permeability and leucocyte migration through suppressing inflammatory cytokines and adhesion molecules (Figure 4). Moreover, treatment with metformin as another AMPK activator, resulted in reduced microvascular permeability suggesting that targeting AMPK signaling represents a potentially new therapeutic strategy for thrombin-associated disorders (Hashemzehi et al., 2018). Obviously, further studies are required to examine the efficacy and safety of curcumin formulation and its structural analogues. It is recommended that further investigations be performed in order to determine the synergistic therapeutic effects of curcumin in combination with thrombin inhibitors on thrombin signaling.

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Conflicts of interest

The authors have declared that there is no conflict of interest.

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