Avicenna Journal of Phytomedicine (AJP)

Original Research Article

Nordihydroguaiaretic acid attenuates TNFα-dependent intercellular adhesion molecule-1 expression in cultured human umbilical vein endothelial cells via targeting TNFα-PI3K-NF-κB-ICAM1 pathway

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Article history:
Received: Aug08, 2024
Received in revised form: Feb02, 2025
Accepted:Mar12, 2025
Epub ahead of print

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Keywords:
Human umbilical vein endothelial cells (HUVECs)
TNFα
Intercellular adhesion molecule-1 (ICAM1)
NDGA
PI3K
NF-κB

Abstract

Objective: During inflammation and oxidative stress, vascular endothelial cell surfaces express high levels of adhesion molecules such as intercellular adhesion molecule 1 (ICAM1) which bind the circulatory leukocytes (e.g. macrophages), through counter receptors LFA/Mac1. The bound leukocytes on sub-endothelial translocation accumulate oxidized lipids and proteins, developing atherosclerotic plaques by foam cell and fatty streak formations. Herewith, nordihydroguaiaretic acid (NDGA) prevails as a polyphenol in the *Larrea tridentata* plant, with potent antioxidant and anti-inflammatory traits. This study for the first time elucidates that NDGA attenuates TNFα-dependent ICAM1 expression in the cultured human umbilical vein endothelial cells (HUVECs), by targeting the TNFα-PI3K-NF-κB-ICAM1 pathway.

Materials and Methods: Cultured HUVECs were treated with pro-inflammatory and pro-oxidative cytokine TNFα, to induce ICAM1 mRNA level and protein expression on HUVECs cell surface as documented by northern and western blot, respectively. The effect of varying NDGA concentrations was examined on TNFα-stimulated ICAM1 expression and monocyte attachment assay.

Results: Pre-TNFα-NDGA treatment of HUVECs moderated TNFα-dependent ICAM1 expression and monocyte attachment on vascular endothelium by inhibiting the PI3K-NF-κB-ICAM1 signaling pathway.

Conclusion: In this study, the NDGA anti-inflammatory and anti-adhesion essence is elucidated *via* impaired cytoplasm to nucleus translocation of pro-oxidative and pro-inflammatory transcription factor NF-κB, moderating the ICAM1 expression and monocyte attachment.

Please cite this paper as:
Mukherjee TK, Malik P, Singh R, Haribhai Patel V, Kumar R. Nordihydroguaiaretic acid attenuates TNFα-dependent intercellular adhesion molecule-1 expression in cultured human umbilical vein endothelial cells via targeting TNFα-PI3K-NF-κB-ICAM1 pathway. Avicenna J Phytomed, 2025. Epub ahead of print.

Introduction

Adhesion of leukocytes to the damaged vascular endothelium is one of the early critical events associated with fatty streak formation which subsequently manifests as atherosclerosis plaque. In the early events of fatty streak formation and subsequent atherosclerotic plaque development, vascular endothelial cell surface adhesion molecules such as intercellular adhesion molecule 1 (ICAM1) serve as critical regulators by binding to the surface receptors (LFA/Mac1) of the circulating leukocytes (e.g. macrophages). The bound leukocytes are translocated to the sub-endothelial region *via* chemotactic actions, initiating a series of pro-inflammatory and pro-oxidative cellular events to aggravate inflammation, and oxidative stress and intensify the atherosclerotic plaque development (Navab et al. 1995; Libby 2002)

Various pro-inflammatory (e.g. tumor necrosis factor alpha (TNFα) and pro-oxidative (e.g. H_2O_2) molecules have been demonstrated to increase the expression of cell surface adhesion molecules (Mukherjee et al. 2002a, 2003a, 2007a). On the other hand, antioxidants (Toledo-Ibelles and Mas-Oliva 2018) and anti-inflammatory molecules ((Charo and Taub 2011; Poznyak et al. 2021) including female steroids estrogens ((Mukherjee et al. 2003a) mitigate the expression of adhesion molecules, exhibiting pertinent promise as potential anti-atherosclerosis agents.

Towards this end, plant-derived natural compounds have been reported in the *in vitro* and *in vivo* experiments (Siasos et al. 2013). Nordihydroguaiaretic acid (NDGA, IUPAC: 4-[4-(3,4-dihydroxyphenyl)-2,3-dimethylbutyl] benzene-1,2-diol) is a creosote bush-polyphenol, structurally distinguished by o-dihydroxy (catechol) moiety (Figure 1a)(Hyder et al. 2002). Familiar as masoprocol, NDGA is harvested *via* five plant species from the genus, *Larrea*(Peralta et al. 2018). Structurally, NDGA comprises two catechol rings, harboring 4 symmetrical –OH groups for its cytoprotective and cytotoxic abilities (Figure 1b). The cytoprotective effect manifests from the scavenging of manifold reactive oxygen species (ROS), oxidizing NDGA to catechol-quinone, further to ortho-quinone and superoxide spontaneously or *via* peroxidases and cytochrome p450 (Floriano-Sánchez et al. 2006; Billinsky and Krol 2008) (Figure 1b). Back conversion of ortho-quinone to semi-quinone *via* NADPH-dependent reductase evokes superoxide-oxidative stress, cautioning for optimal NDGA intake (Chichirau et al. 2005). Thus, the net physiological effect of NDGA depends on the balance of its protective (ROS scavenging and electrophilic actions) and toxic (excess superoxide formation and reduced glutathione (GSH) depletion) fates. Cautionary surveillance *vis-à-vis* redox potential and geometric distribution of atoms render moderate NDGA concentrations feasible for cysteine interactions, averting significant GSH depletion.

From a functional consideration, NDGA hydrophobicity (Dissociation Factor: Log p: 4.48) is attributed to its therapeutic essence, with 14.7 mg/ml as the highest plasma extent for a 50 mg/kg intravenous intake, in rats (Lambert et al. 2001). Studies reckon ~99.8% plasma-NDGA as protein bound with a 30, 135 min $t_{1/2}$ distribution and terminal $t_{1/2}$ besides 201.9 mL/min/kg renal clearance, inferring an abysmal *in vivo* bioavailability (Lambert et al. 2004). Prominent therapeutic mechanisms of NDGA are (i) moderated pro-oxidant inflammation, (ii) suppressed lipoxygenases (LOX)-mediated reduction of lipid hydroxyl peroxides, fueling the oxidative stress *via* NADPH oxidases triggered decomposition to free radicals (Mashima and Okuyama 2015; Nagahora et al. 2017) and (iii) activated NRF2-regulated endogenous antioxidant fates.

NDGA biochemical actions are characterized by its pleiotropic essence (Chen et al. 2017; Manda et al. 2020).Despite recalling NDGA anti-inflammatory abilities, no mechanism was proposed for impaired ICAM1, and VCAM-1 endothelial cell surface expression.

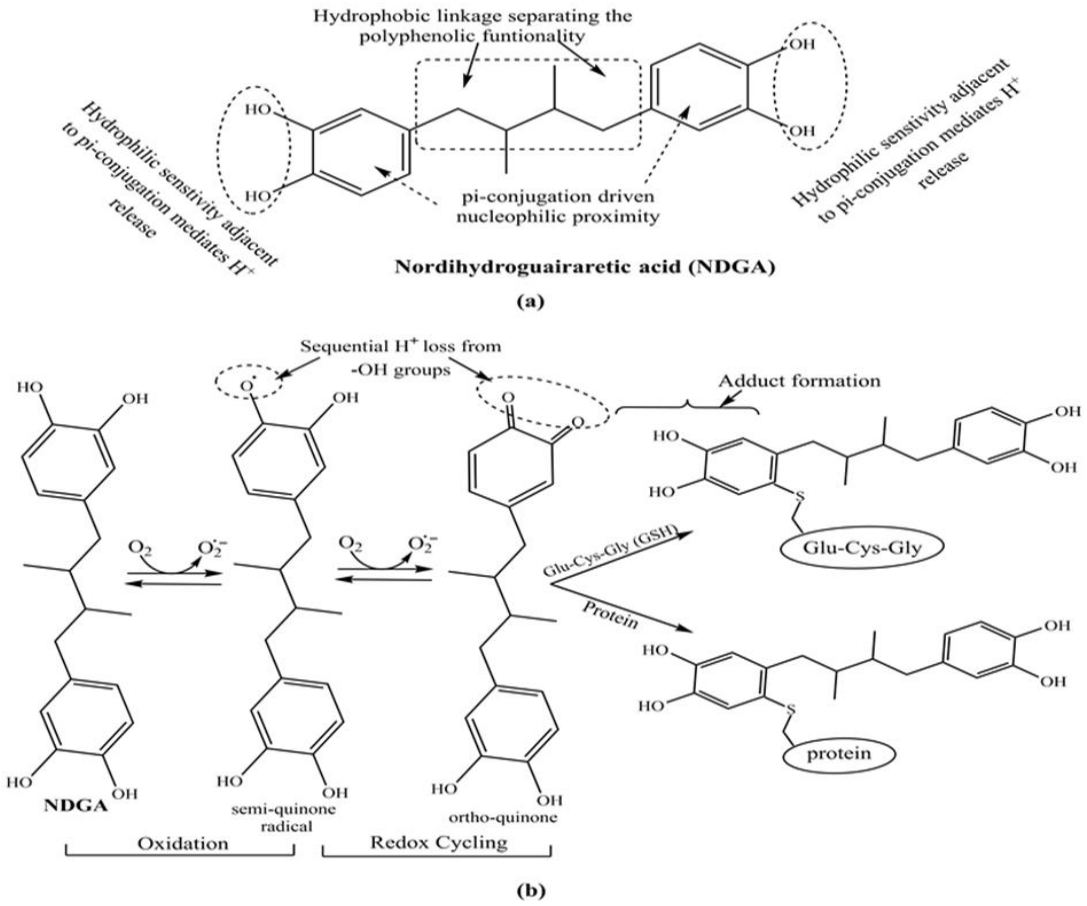

Figure 1(a) Chemical structure of nordihydroguairaretic acid (NDGA), distinguishing terminal -OH substituted phenyl rings sandwiched by a 4C-hydrophobic linkage, (b) ROS scavenging fates of NDGA, forming semi-quinone and ortho-quinone on sequential oxidation. Further adduct formation could alter normal physiology via engaging proteins or intracellular glutathione

In the present study, primary cultured HUVECs were treated with pro-inflammatory cytokine TNFα to induce cell surface ICAM1 expression by activating the PI3K-NF-κB-ICAM1 signaling pathway to screen the increased monocyte attachment on HUVEC surfaces. Further, the present study assesses the NDGA role in attenuating TNFα-dependent ICAM1 expression in the HUVECs. Pre-TNFα treatment of HUVECs with varied NDGA concentrations attenuated cytoplasm to nucleus translocation of pro-inflammatory transcription factor NF-κB and decreased TNFα-dependent ICAM1 expression. Another observation was that the NDGA pretreatment attenuated the attachment of monocytes on the endothelial cell surfaces. Thus, NDGA may be experimentally used as a potential anti-atherosclerosis agent *via* varied intake.

Materials and Methods

Materials

TNFα and NDGA were procured from Sigma Aldrich, while PI3K inhibitor LY-294002 and antibodies against NF-kB-p65, ICAM1, PI3K-p85, FAK were obtained from Cell Signaling Technology, Thermo-Fischer and Abcam. The U937 monocyte cell line was collected from ATCC (USA) sub-cultured using standard ATCC protocol. Meanwhile, local reagents were sourced from Sigma Aldrich.

Methods

Isolation, seeding, and *in vitro* culture of HUVECs

Freshly collected umbilical vein was utilized for isolation, seeding, and primary culture of HUVECs in complete M199 medium containing 5% Fetal Bovine Serum (FBS), 100 U/ml penicillin-G sodium,100

µg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B and 5 µg/ml vascular endothelial growth factor (VEGF) (Mukherjee et al. 2003b).

Treatment of *in vitro* cultured HUVECs with the drugs of interest

Here, 70-80% confluent HUVECs were washed with phosphate-buffered saline (PBS) and incubated overnight (O/N) with phenol red-free M199 medium with 2% charcoal dextran treated FBS, but without any VEGF, antibiotic, and anti-mycotic. Following O/N incubation, HUVECs were treated with TNFα, NDGA, phosphoinositide 3-kinase (PI3K) inhibitor, and LY294002 for varied durations as per the experimental standards (Mukherjee et al. 2003b).

Isolation of total RNA and northern blot analysis

The TNFα-treated HUVECs were utilized as the source of total RNA, whose concentration was ascertained spectrophotometrically. The isolated RNA was used in the Northern blot to screen the extent of ICAM1 mRNA changes after TNFα treatment. For northern blot analysis, 10 µg total RNA loaded in a 1% agarose-formaldehyde gel was electrophoresed with 3-(N-morpholino) propanesulfonic acid (MOPS) buffer, before Hybond membrane transfer. The Northern blot was completed as described previously(Mukherjee et al. 2002b).

Isolation of total cell lysate and western blot analysis

Here, 70-80% confluent HUVECs were washed and incubated with an experimental medium for O/N and treated either with TNFα (10 ng/ml for 4 hrs), or NDGA (1-100) µM extents till O/N or LY294002 (1-2 µM for 1 hr). The western blot was used to ascertain the level of ICAM1, PI3K-p85, and focal adhesion kinases (FAK) protein changes (Mukherjee et al. 2003b).

Isolation of cytosolic and nuclear extracts and detection of NF-κB transcription factor translocation

Following treatment with various drugs of interest, HUVECs were harvested. Thereafter, cytosolic and nuclear extracts were prepared, and the protein titer was screened spectrophotometrically. The extract was subjected to western blot analysis as described earlier against NF-κB-p65 antibody (Mukherjee et al. 2007b).

Monocyte attachment assay

The monocyte adhesion/attachment assay was performed as described previously (Shih et al. 1999) with modifications (Mukherjee et al. 2007b). Briefly, HUVECs were cultured in a 6-well plate and grown till complete confluency. The cultured HUVECs were treated with various agents as illustrated in the cell culture and treatment section. Following treatment, the HUVECs were washed twice with the cell culture medium. Freshly grown human peripheral blood monocytes (U937 cell line) were added to the culture plate containing HUVECs. In the next step, the culture plates were incubated in a CO_2 incubator for 1 hr at 37°C to enable monocyte binding on the HUVECs surface. The unattached monocytes were washed with PBS, and the culture plates containing monocytes attached/adhered to HUVECs were fixed with 1% glutaraldehyde in PBS. An inverted microscope with an eyepiece grid was used to count the HUVEC-bound monocytes. For each well, an average of five fields was computed.

Data analysis

Values are expressed as means±SE obtained from at least three different experiments in each group. Differences between groups were assessed by one-way ANOVA and Newman-Keuls multiple comparison tests, validated using appropriate using Origin Pro Software. p values of 0.05 were considered significant.

Results

In this study, HUVECs were isolated from freshly collected and primary cultured umbilical cord veins as we described previously (Mukherjee et al. 2002b, 2003b, 2007b). The freshly cultured HUVECs were treated with varying concentrations of pro-oxidative and pro-inflammatory cytokine, TNFα to attain the threshold ICAM1 expression, a cell surface immunoglobulin class of molecule. The HUVECs were also treated with varying NDGA concentrations to attenuate TNFα-dependent-ICAM1 expression. Further, the role of signaling pathways affected by NDGA in the TNFα-dependent-ICAM1 expression including the involvement of various upstream cell signaling molecules such as PI3K, FAK, and the transcription factor, NF-κB, was ascertained.

Thus, for experimental purposes, 70-80% confluent HUVECs incubated till O/N along with experimental medium were treated with varied TNFα concentrations the following day for 4 hr. Results of this experiment revealed that TNFα-dose-dependently enhanced both ICAM1 mRNA and protein expression with the maximum propensity at 10 ng/ml for 4 hr (Figures 2 and 3). The TNFα-mediated ICAM1 expression was dose-dependently attenuated by HUVECs pretreatment with NDGA before TNFα treatment (Figure 4). Analysis revealed enhanced ICAM1-mRNA and protein extents on 10 ng/ml TNFα administration to HUVECs for 4 hr, exhibiting dosage-driven moderation on NDGA pretreatment (Figures 2-4).

Subsequently, we probed the mechanism through which TNFα enhanced ICAM1 mRNA and protein concentrations. Towards this end, we screened the NF-κB effect on TNFα-dependent-ICAM1 expression in the HUVECs. Note that NF-κB is a pro-inflammatory transcription factor and can bind with the promoter region of the ICAM1 gene and regulate its transcription. The p65 subunit of NF-κB, i.e. NF-κB-p65 is its imperative active component. Contrary to the cytoplasmic prevalence of NF-κB in resting cells, the TNFα activated cells mandate its cytoplasm to nucleus translocation for binding to ICAM1 promoter and subsequent transcription of responsible genes. Thus, cytosolic and nuclear extracts were prepared on treatment with various drugs including pro-inflammatory cytokine TNFα and anti-inflammatory agent NDGA. Inspection using Western Blotting revealed TNFα-dependent enhancedNF-κB-p65 cytoplasm to nucleus translocation is moderated with O/N NDGA pretreatment (Figure 5). This result confirms NF-κB involvement in TNFα-dependent-ICAM1 expression in cultured HUVECs besides NDGA attenuated TNFα-dependent NF-κB-translocation and ICAM1 expression.

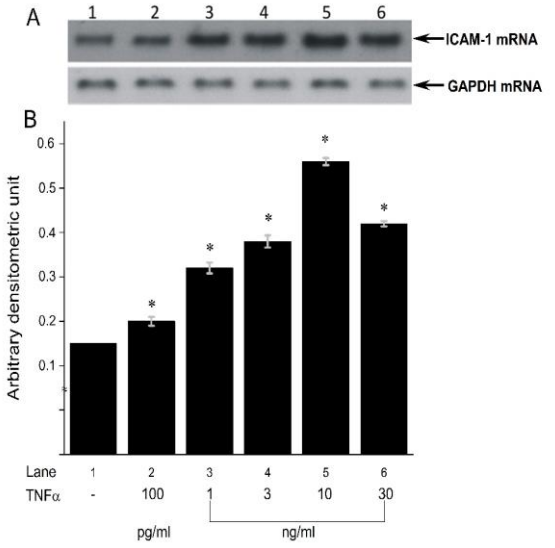

Figure 2. A. Representative autograph of a Northern blotting experiment showing the effects of varied TNFα concentrations on the ICAM1 mRNA level in the HUVECs, keeping GAPDH mRNA as internal control. TNFα-dose dependently induces the ICAM1 mRNA level with a maximum effect at 10 ng/ml without affecting the standard internal control, i.e., GAPDH mRNA level. B. Arbitrary densitometric unit of the above experiment, representing the ICAM1 mRNA enhancement on varied TNFα treatment of HUVECs for 4 hrs. Values were mean±SEM of the three independent experiments in triplicate. *p<0.05 compared to non TNFα HUVECs.

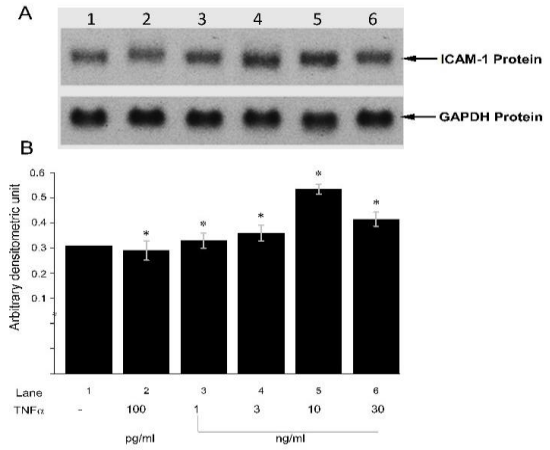

Figure 3. A. Representative autograph of a western blotting experiment, depicting the effects of varied TNFα extents on ICAM1 expression in the in vitro cultured HUVECs with GAPDH protein as the internal control. TNFα-dose dependently induces the ICAM1 expression with a maximum effect at 10 ng/ml without affecting the standard internal control, i.e. GAPDH protein expression. B. Arbitrary densitometric unit of the above experiment representing the ICAM1 enhancement on HUVECs with varying TNFα extents till 4 hr. Values were mean±SEM of the three independent experiments in triplicate. *p<0.05 compared to non TNFα HUVECs.

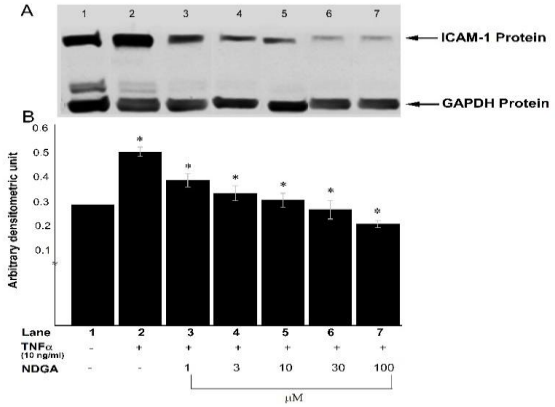

Figure 4. A. Representative autograph of a western blotting experiment portraying the effects of various NDGA concentrations on TNFα-dependent ICAM1 expression in the in vitro cultured HUVECs with GAPDH protein as the internal control. HUVECs were treated overnight with 1, 3, 10, 30 and 100 μM NDGA before treating with 10 ng/ml TNFα till 4 hr. B. Arbitrary densitometric unit of above experiment representing attenuation of TNFα-dependent ICAM1 protein expression by the HUVECs pretreatment with varying NDGA concentrations.Values were mean±SEM of the three independent experiments in triplicate. *p<0.05 compared to non (TNFα+NDGA) human umbilical vein endothelial cells.

In the subsequent experiments, we checked the upstream cell signaling molecules involved in TNFα-dependent ICAM1 expression in the cultured HUVECs. Previous studies decipher PI3Kas the prominent upstream cell signaling molecule mediated in TNFα-dependent activation of the downstream transcription factor, NF-κB, and subsequent ICAM1 activation in the endothelial cells. FAK is another molecule that could be activated *via* TNFα treatment(Tseng et al. 2010; Lu et al. 2014). Thus, to identify the upstream cell signaling molecules involved in TNFα-stimulated NF-κB-p65 cytoplasm to the nucleus translocation, HUVECs were treated with PI3K inhibitor LY294002 and the relative PI3K-p85 protein level was monitored by western blot. The PI3K inhibitor, LY294002 exhibited a dosage-driven moderation of TNFα-dependent PI3K-p85expression (Figure 6). Like LY294002, NDGA treatment also reduced the TNFα-dependent PI3K-p85expression (Figure 7), indicating NDGA alleviated PI3K to regulate the ICAM1 expression. Further, LY294002 also moderated FAK phosphorylation (data not shown).This result infers PI3K-FAK interaction since PI3K inhibitor LY294002 attenuated functional pFAK extent. The PI3K-FAK interaction was further revealed *via* FAK immunoprecipitation followed by PI3K-p85 western blot (data not shown).

Finally, a monocyte attachment assay was conducted to observe the consequences of increased ICAM1 expression on the HUVEC surface. For this assay, a freshly cultured U937 monocyte cell line was added to the freshly cultured, confluent

HUVECs and kept in a CO_2 incubator at 37ºC for 1 hr. Following incubation, the HUVECs were extensively washed and observed under an inverted microscope, and the attached monocytes were counted. Analysis revealed that while TNFα exhibits ~29% enhancement in the monocyte attachment on the surface of HUVECs, NDGA treatment attenuated the TNFα-dependent monocyte attachment (Figure 8).

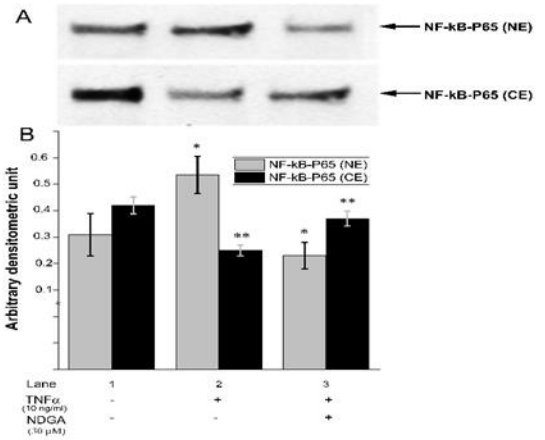

Figure 5. A. Representative autograph of a western blot analysis illustrating the effect of NDGA on the TNFα-dependent NF-kB-p65 cytoplasm to nucleus translocation in the cultured HUVECs. Following TNFα and NDGA treatment, the cytosolic and nuclear extracts (CE, NE) were prepared and subjected to a western blot experiment. The cytoplasm to the nucleus translocation of transcription factor, NF-kB was monitored by screening the NF-kB-p65 protein level in the CE and NE. B. Arbitrary densitometric unit representing 30 μM NDGA effects on the TNFα-dependent NF-kB-p65 cytoplasm to nucleus translocation. Values were mean±SEM of the three independent experiments in triplicate. *p<0.05 (NF-kB-p65(NE)), **p<0.05 (NF-kB-p65(CE)) compared to non (TNFα+NDGA) HUVECs.

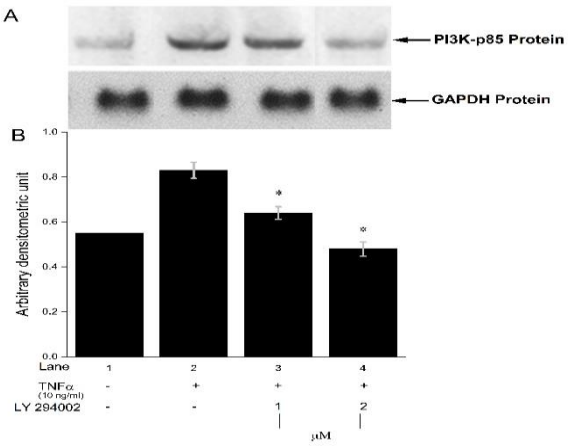

Figure 6. A. Representative autograph of a western blot, elucidating the effects of varying LY294002 (a PI3K inhibitor) extents on the TNFα-dependent PI3K-p85 protein expression in the cultured HUVECs with GAPDH protein as the internal control. B. Arbitrary densitometric unit of the above experiment representing the effects of varied LY294002 concentrations on the TNFα-dependent PI3K-p85 protein expression. Values were mean±SEM of the three independent experiments in triplicate. *p<0.05 compared to non LY294002 (PI3K inhibitor) human umbilical vein endothelial cell (HUVECs).

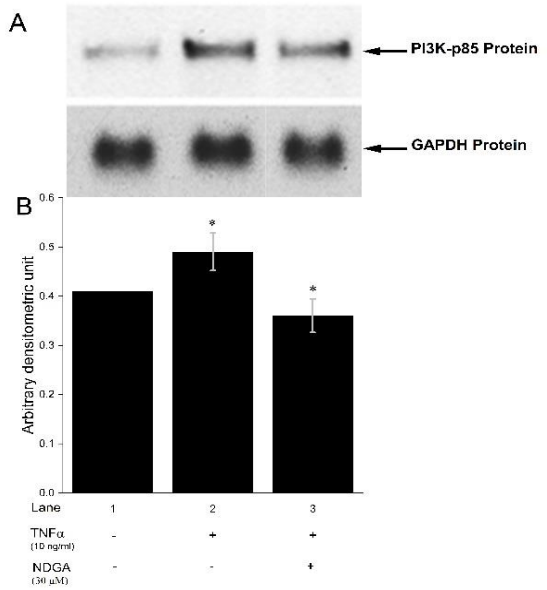

Figure 7. Representative autograph of a western blot, signifying the effects of NDGA (30 μM) on the 10 ng/ml TNFα-dependent PI3K-p85 protein expression in the cultured HUVECs. B. Arbitrary densitometric unit representing the effects of 30 μM NDGA on the TNFα-dependent PI3K-p85 protein expression. Values were mean±SEM of the three independent experiments in triplicate.*p<0.05 compared to non (TNFα+NDGA) HUVECs.

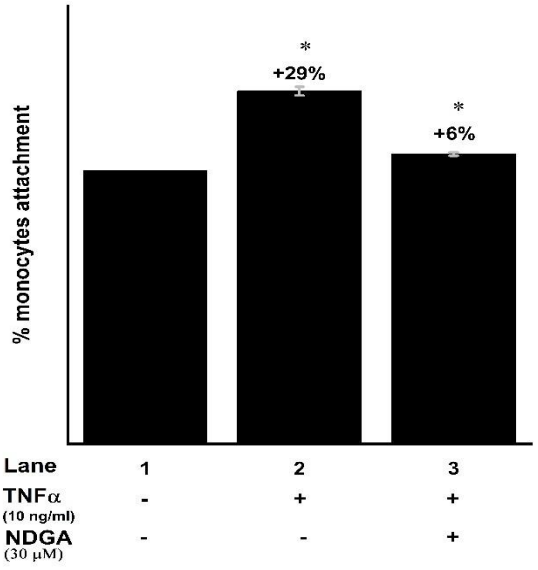

Figure 8. Representative view of monocyte attachment assay, demonstrating the attenuated effects of NDGA (30 μM) on the 10 ng/ml TNFα-dependent monocyte attachment on HUVEC surfaces. Analysis revealed that TNFα exhibits ~29% enhancement in monocyte attachment on HUVECs surface and NDGA treatment attenuated TNFα-dependent monocyte attachment. Values were mean±SEM of the three independent experiments in triplicate. *p<0.05 compared to non (TNFα+NDGA) HUVECs.

Discussion

Leukocyte adhesion (e.g. monocytes/macrophages) on vascular endothelium is one of the critical steps in atherosclerotic complications. The endothelial cells express ICAM1asaprominent cell-surface adhesion molecule, furthered *via* interacting with counter receptors such as macrophage antigen 1 (Mac-1; CD11b/CD18), β_2-integrins and lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18). Of note, Mac-1 and LFA-1 are known to bind ICAM1, Ig1, and Ig3 domains respectively, and therefore act as counter receptors of ICAM1(Miller et al. 1995; Frick et al. 2005).

ICAM1 is regarded as a cell surface immunoglobulin class of molecules. The pro-inflammatory and pro-oxidative conditions including vascular injury induce the expression of ICAM1 on the vascular endothelium surface. Once expressed ICAM1 further induces inflammatory conditions by aggravating the adhesion of various leukocytes such as macrophages on the vascular endothelium via binding with the conjugated receptor proteins LFA1 and Mac1(Lawson and Wolf 2009). Thus, ICAM1 is regarded as a master regulator of cellular responses to inflammation by facilitating the release of inflammatory mediators (e.g. cytokines, chemokines, etc.) by macrophages and other immunological cells (Bui et al. 2020). Vascular endothelium-attached leukocytes are translocated from the vascular luminal cell surface to the sub-endothelial space *via* chemotaxis. Thereafter, oxidized lipids and proteins build up in sub-endothelial macrophages, causing fatty streak formation. Consequently, increased inflammatory and oxidative stresses culminate in pathophysiological changes characterizing atherosclerosis(Bentzon et al. 2014).

Thus, ICAM1 is an important adhesion molecule involved in monocyte/macrophage attachment on endothelial cell surface, fatty streak formation, and eventual atherosclerotic plaque development (Bourdillon et al. 2000). Previous studies established a correlation between soluble ICAM1, atherosclerosis, and cardiovascular severity, deciphering ICAM1 inhibition as a feasible atherosclerosis treatment strategy (Kitagawa et al. 2002). Thereby, leukocyte localization to inflammatory sites can progress early atherosclerosis onset, predominantly initiated by adhesion molecules including ICAM1. The therapeutic intervention that attenuates ICAM1 expression on the endothelial cell surface may protect from atherosclerotic plaque development.

The role of TNFα as a potential pro-inflammatory agent *vis-à-vis* adhesion molecule expression is well established (Lukacs et al. 1994; Kim et al. 2008). It is also known that the ICAM1 promoter harbors the NF-κB binding site, with TNFα treated endothelial cells aggravating its cytoplasm to nucleus translocation (Amrani et al. 1999; Mukherjee et al. 2003b). Once NF-κB binds ICAM1 gene promoter, it induces ICAM1 expression(Chen et al. 2017; Zhang et al. 2020). The present study re-established these cellular events whereby inducing ICAM1 expression in TNF-α treated HUVECs was attenuated by NDGA pretreatment (Figures 2 and 3).

Previous studies established transcription factor NF-κB as an intermediate molecule in the TNFα-dependent ICAM1 expression in the endothelial cells by binding to the promoter of the ICAM1 gene(Amrani et al. 1999; Min et al. 2005). NF-κB serves as a pivotal mediator of immune and inflammatory responses by regulating multiple aspects of innate and adaptive immune responses including encoding the expression of cytokines and chemokines and participating in inflammasome regulation(Tak and Firestein 2001; Liu et al. 2017). Several earlier studies including our studies (Mukherjee et al. 2003b) have shown the pro-inflammatory cytokine TNFα mediated-NF-κB cytoplasm to nucleus

translocation in the *in vitro* cultured HUVEC. The p65 subunit of NF-κB, i.e. NF-κB-p65 is the most active component of NF-κB. Once translocated to the nucleus, NF-κB binds to the specific promoter site of pro-inflammatory molecule ICAM1, stimulating its transcription (Liu et al. 2017). Therefore, in our study, NF-κB-p65 cytoplasm to nucleus translocation was examined and as predicted, the analysis revealed that TNF-α treated HUVECs enhanced the NF-κB-p65 cytoplasm to the nucleus translocation (Figure 5). The nuclear translocated NF-kB-p65 binds with the ICAM1 gene promoter to activate its transcription.

The present study also identified upstream cell signaling molecules involved in TNFα-dependent-ICAM1 expression on HUVECs surface, supporting the demonstrated PI3K activity as an upstream cell signaling molecule involved in TNF-α-dependent-ICAM1 expression in endothelial cells(Tsoyi et al. 2010; Lu et al. 2014).Considerable FAK-PI3K crosstalk prevails amidst TNFα-dependent IL6 production in myofibroblasts(Tseng et al. 2010). Of note, FAK is an upstream PI3K/Akt regulator for transduced β_1 integrin signaling in collagen matrices (Xia et al. 2004). Based on these studies, the PI3K-p85 subunit was examined in presence of LY294002 (a PI3K inhibitor) and TNFα, revealing activation (with TNFα) and inhibition (with LY294002) (Figure 6). LY294002 also assuaged TNFα-dependent FAK phosphorylation (data not shown). This study also elucidated for the first time that like LY294002, NDGA attenuated TNFα-dependent-PI3K-p85 expression (Figure 7) and FAK phosphorylation (data not shown).

Previously, we have shown that TNFα-dependent high-level ICAM1 expression aggravates the monocyte attachments in the human pulmonary aortic endothelial cells (HPAECs) (Mukherjee et al. 2007b). Therefore, the enhanced endothelial cell surface ICAM1 expression in HUVECs was screened *via* monocyte attachment, aggravation of which inferred TNFα involvement in early fatty streak formation (Figure 8). Further, the attenuation of TNFα-dependent PI3K-p85 expression by NDGA pre-treated TNFα-treated cells inferred NDGA assuaged HUVECs surface-monocyte attachment (Figure 8). In this study, attenuation of TNFα-dependent-ICAM1 expression in cultured HUVECs by NDGA (creosote bush metabolite) has been elucidated.

The study results conclude that NDGA, a plant-derived lignan, attenuates the TNFα-dependent ICAM1 expression and monocyte attachment on the HUVEC surface by attenuating the PI3K-NF-κB-ICAM1 pro-inflammatory signaling pathway. The results of this study clarify the basis through which NDGA counteracts inflammation-associated diseases, namely atherosclerosis.

Acknowledgment

Authors are thankful to Amity University, Kolkata, Central University of Gujarat, Gandhinagar, Maharishi Markandeshwar University, Mullana, Haryana for infrastructural and round-the-clock internet facility, ensuring steady communication amongst the authors.

Conflicts of interest

The authors declare no conflicts from academic or financial viewpoints. For isolation of endothelial cells freshly collected discarded umbilical cord was used by fulfilling the ethical norms of the University Ethical Committee (UEC). No animal or human subjects were used for this purpose.

Funding

No funding was used to complete this work.

Ethical Considerations

All the experiments were conducted on isolated endothelial cells, without the use of any human/animal tissues, blood, etc. The university recommends conducting

experimental work with no ethical concerns.

Code of Ethics

No specific code of ethics was used during experiments. The level 2 Biosafety cabinet (Laminar flow hood) was used for mammalian cell culture, and standard ethical and biosafety guidelines were used.

Authors' Contributions

TKM planned the experiments, TKM and PM wrote the initial version of the manuscript, which was revised and appraised by RS and RK. RS discussed the outcome feasibility and reliability with TKM. VHP contributed to analyzing the data and contributed significantly to the preparation of the figures in adequate resolution.

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