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Original Article

Kirenol protects against oxidized low-density lipoprotein induced damages in endothelial cells

Kirenol protege contra danos induzidos por lipoproteínas de baixa densidade oxidadas em células endoteliais

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Abstract

Kirenol (KNL) has recently been reported to have anti-inflammatory properties. Yet, little is known about the potential mechanisms of its anti-inflammatory properties. In HUVECs, we elucidated the anti-inflammatory mechanisms of kirenol. RT-PCR was used to test mRNA of pro-inflammatory mediators produced by Ox-LDL. The viability of cells was measured using MTT. Western blots analyzed protein levels. On Ox-LDL-stimulated HUVECs, KNL significantly inhibited the production of pro-inflammatory mediators such as NO, IL-1β, iNOS, TNF-α and IL-6. p38, ROS and Nrf2 expression were inhibited by KNL. Inhibition of p38, ROS, and KNL caused nuclear accumulation of Nrf2. KNL attenuated Ox-LDL-induced phosphorylation of ERK1/2 and p38, too. Based on our results, KNL inhibits NF-κB and MAPK signaling in HUVECs by activating Nrf2 signaling. There's a possibility that KNL could be developed into an anti-inflammatory drug.

Keywords: kirenol, Ox-LDL, ROS, anti-inflammation, Nrf2.

Resumo

Kirenol (KNL) foi recentemente relatado como tendo propriedades anti-inflamatórias. No entanto, pouco se sabe sobre os potenciais mecanismos de suas propriedades anti-inflamatórias. Em HUVECs, elucidamos os mecanismos anti-inflamatórios do kirenol. RT-PCR foi usado para testar mRNA de mediadores pró-inflamatórios produzidos por Ox-LDL. A viabilidade das células foi medida usando MTT. Western blots analisaram os níveis de proteína. Em HUVECs estimuladas por Ox-LDL, o KNL inibiu significativamente a produção de mediadores pró-inflamatórios como NO, IL-1β, iNOS, TNF-α e IL-6. A expressão de p38, ROS e Nrf2 foi inibida por KNL. A inibição de p38, ROS e KNL causou acúmulo nuclear de Nrf2. O KNL também atenuou a fosforilação induzida por Ox-LDL de ERK1/2 e p38. Com base em nossos resultados, o KNL inibe a sinalização de NF-κB e MAPK em HUVECs, ativando a sinalização de Nrf2. Existe a possibilidade de que o KNL possa ser desenvolvido em um medicamento anti-inflamatório.

Palavras-chave: kirenol, Ox-LDL, ROS, anti-inflamação, Nrf2.

1. Introduction

Worldwide, cardiovascular disease (CVD) is the number one cause of death and premature death (Kivimäki and Steptoe, 2018; Kovacic et al., 2019; Silva et al., 2015; Singh et al., 2020; Yang et al., 2021). A chronic inflammatory lipid-depositing disease causes peripheral artery disease, coronary artery disease, and myocardial infarction (Gao and Liu 2017). A dysfunction of vessel endothelium can initiate plaques and lead to atherosclerosis (Dotta et al., 2015; Rajendran et al., 2013). Oxidative stress and inflammatory reactions are important risk factors for atherosclerosis progression (Steven et al., 2019). Macrophages smooth muscle cells, and endothelial cells also apoptosis (Rajendran et al., 2021b). Stresses like Ox-LDL activate several inflammatory pathways, including NF-κB and mMAPK. NF-κB is normally inactivated by endogenous inhibitor IκBα, which is present in the cytoplasm (Steven et al., 2019). LPS, however, phosphorylates IκBα, which leads to IκBα degradation. IκBα degradation breaks up the complex between IκBα and NF-κB, which leads to NF-κB being translocated into the nucleus, which contributes to the expression of mediators that cause inflammation, like NO,IL-1β and IL-6. (Haybar et al., 2019) MAPKs are involved in the inflammatory response through activating ERK1/2 and p38 and which lead to signal transduction from the surface of the cell to the nucleus for pro-inflammatory mediators to be expressed. Therefore, NF-κB and MAPKs signaling have been considered potential targets for anti-inflammatory drugs.

Atherosclerosis is treated with a variety of chemical drugs, but some of them cause serious side effects

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(Nakhlband et al., 2018). Therefore, understanding endothelial dysfunction is important for developing effective therapeutic strategies to deal with atherosclerosis. Herba Siegesbeckiae, a historical tropical plant, produces a natural diterpene called Kirenol (KNL) (Alzahrani et al., 2021). In China, especially, it's used for treating arthritis, malaria, hypertension, and snakebites (Ibrahim et al., 2021a). Aside from inhibiting pro-inflammatory cytokines, kirenol activates annexin-1, IL-2, BMP, and Wnt (Liu et al., 2020). Kirenol's administration lowered IFN-γ and IL-17A serum expressions, as well as the ratio of Th1 and Th17 cells in draining lymph nodes. In kirenol-treated EAE mice, lymphocyte priming was reduced and the apoptosis of myelin oligodendrocyte glycoprotein (MOG)-activated CD41T cells was increased. Based on the additional in vitro investigations by Xiao et al (Xiao et al., 2015). According to Rajendran et al.,, pretreatment with kirenol (25 µmoL) significantly improved the cell survival of human umbilical vein endothelial cells (HUVECs), whereas DNA damage and the formation of reactive oxygen species (ROS) caused by benzo(a)pyrene (B(a)P) was inhibited. Kirenol's potential as an antioxidant is directly associated with the increased expression of an antioxidant gene and the nuclear translocation activation of Nrf2, even in the absence of B(a) P, a ubiquitous environmental mutagen. Furthermore, this study established that Nrf2 translocation is mediated by the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signalling pathways as validated by the activation of It also stimulates LRP-5 and β-catenin mRNA expression, and inhibits glycogen synthase kinase 3 beta (GSK3β)-phosphorylation by β-catenin (Ibrahim et al., 2021a). This makes kirenol anti-inflammatory, antiadipogenic, immunoregulatory, antioxidant, and antiarthritic. According to a systematic literature review, KNL boosts inflammation while reducing oxidative stress and reducing the risk of heart disease. Additionally, kirenol potentially ameliorates lung injury. Overall, despite the fact that the anti-inflammatory mechanism and underlying targets are unclear, kirenol's favourable effects on LPS-induced inflammation make it an important lead molecule in future drug development and research (Nasir et al., 2022).

2. Materials and Methods

2.1. Reagents

Cell activity was assessed using a MTT assay kit (Promega, Madison, WI, USA). Kirenol (Cas#52659-56-0) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2′7′-dichlorodihydrofluorescein diacetate (DCFH2-DA; CAS 4091-99-0).Ox-LDL(L34357**)** pP65, P65, pP38, P38, Nrf2, and actin, derived from Invitrogen, Waltham, MA-based Thermo Fisher Scientific, Inc.

2.2. Cell culture

HUVECs were collected from the American Type Culture Collection (ATCC, Manassas, VA, USA). In Dulbecco's modified essential medium (DMEM, ThermoFisher Scientific, Waltham, MA, USA), we cultured HUVECs endothelium from the ATCC (VA, USA). HUVECs cells were treated with 15 µmol (EC50) of KNL co-treated with Ox-LDL was added. We collected the cell lysate after 24 hours.

2.3. MTT assay

HUVECs were cultured at 37°C with 10% FBS in DMEM. After 24 hours, HUVECs were treated with Ox-LDL at the indicated concentrations. The MTT assay (Sigma, MO USA) was used to measure the proliferation of the cells (Deng et al., 2021).

2.4. Nitric oxide formation

By Griess assay, we tested KNL's NO production inhibitory activity. HUVECs cells (1 × 105 cells) were pretreated with KNL for 6 h and co-treated with Ox-LDL (100 *g/ml) for 18 h. Then, we mixed the supernatants with Griess reagent and let it sit for 15 minutes at room temperature. Using a UV/Visible spectrophotometer, we measured the absorbance at 540 nm after the reaction (Yang et al., 2018).

2.5. ROS accumulation measurements

DCFH2-DA fluorescence dye was used to amount of intracellular ROS accumulation (Ismail et al., 2021). We seeded 1 X 10^7 cells/mL in a 6-well plate, then treated with SPN (for 2 h) followed by DOX treatment. Afterward, DCFH2-DA was supplementary to the culture medium for 30 min at 37°C. Using fluorescence microscopy, we examined the dichlorofluorescein (DCF) fluorescence intensity inside cells. By comparing the fluorescence intensity of treated cells and vehicle-treated cells, we measured ROS levels (Rajendran et al., 2021a).

2.6. Cytokine measurements

HUVECs were cultured in a 12-well plate with approximately 6.5 X 10^5 cells/well to measure cytokines, levels in the culture medium. We measured KNL protective effect on Ox-LDL-stimulated cells after pretreatment with KNL using ELISA (0-50 µmol, 2 h) and Ox-LDL treatment (100 µg/ml, 72 h). IL-6, IL-1β, TNF-α, were quantified using their respective ELISA kits (Yang et al., 2020).

2.7. Western blotting

Post-treatment, we harvested the cells and used cold PBS to wash them. We then prepared nuclear, cytoplasmic, and total extracts in the aforementioned manner. For detecting the status of the protein, we used a Bio-Rad protein assay in each sample, with bovine serum albumin (BSA) as the reference standard. To obtain protein (50 µg) in equal amounts, we used SDS-PAGE (8–15%) and transferred the proteins to nitrocellulose membranes overnight. We blocked the membranes using 5% skimmed milk at 3 °C for 30 min and then incubated them for 2 h with the indicated primary antibodies (1:1000 dilution). Subsequently, a horseradish-peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:5000 dilution) was incubated using the nitrocellulose membranes for 1 h. Samples were scanned with a LI-COR 3600-00-C-Digit Blot Scanner (AbuZahra et al., 2021).

2.8. Statistics

This study analyzed the data using an analysis of variance (one-way analysis of variance) and compared the controls using Tukey's post-hoc test. The results were considered significant at $p<0.05$.

3. Results

3.1. KNL inhibits Ox-LDL-induced cytotoxicity in HUVECs cells

MTT was used to test Ox-LDL and KNL for cytotoxicity. Figure 1A shows that treatment of HUVECs with Ox-LDL have cytotoxic effects. Figure 1B shows KNL didn't have any cytotoxic effects up to 20 µmol/mL. We used a KNL concentration of 15 µm/mL in the experiments. Figure 1C shows that OX-LDL (100 µg) significantly reduced cell viability up to 42.54%, ($p<0.05$) but KNL dose-dependently protected the OX-LDL-induced induction of cell death. It's clear from these results that KNL is protective against OX-LDL when exposed to HUVECs.

3.2. KNL suppresses intracellular ROS

DCFH2-DA fluorescence was used to measure intracellular ROS. KNL was pretreated then Ox-LDL was stimulated for 24 h. ROS accumulation in these cells was measured. KNL pretreatment attenuated Ox-LDL-induced ROS accumulation dose-dependently and significantly ($p<0.05$) (Figure 2A).

3.3. KNL affects PGE2 expression in Ox-LDL-induced HUVECs.

In Ox-LDL supplemented cells, we tested whether KNL inactivates the PGE2. According to spectrophotometer, cells pre-treated with KNL had significantly lower levels of PGE2 level than cells pre-treated with Ox-LDL alone (Figure 2B) ($p<0.05$) KNL seems to suppress PGE2 production that causes Ox-LDL-induced inflammation.

3.4. Pro-inflammatory cytokine suppression by KNL

Endothelial cells stimulated with Ox-LDL showed KNL anti-inflammatory effects. In this experiment, cells were pretreated with KNL for co-treated being stimulated with Ox-LDL for 72 h. Figure 3A-C shows that Ox-LDL stimulation alone significantly increased the production of TNF-α, IL-1β and IL-6. In contrast, KNL pre-treatment significantly ($p<0.05$) suppressed the release of pro-inflammatory cytokines, indicating a protective role for KNL in Ox-LDL-induced inflammation.

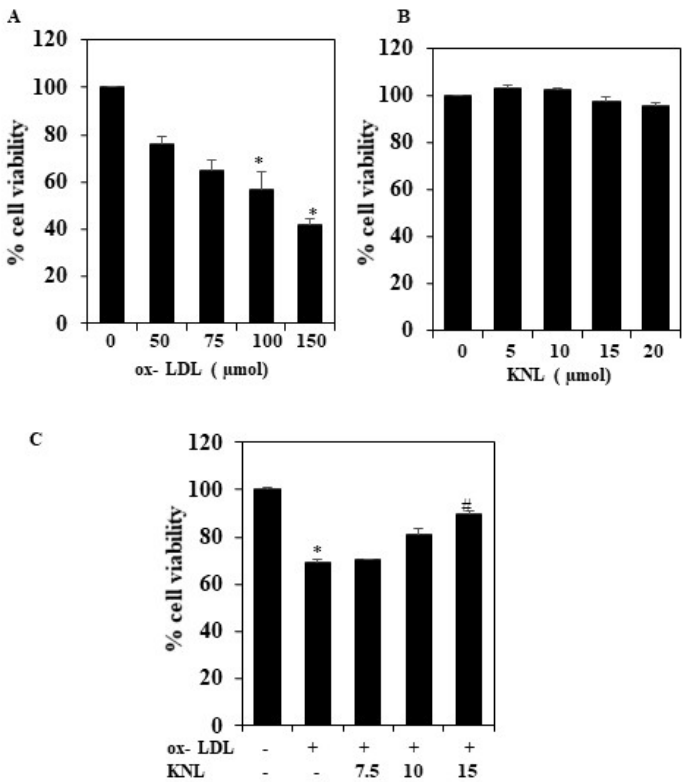

Figure 1. KNL inhibit viability of HUVECs on Ox-LDL induced toxicity. (A and B) Cell growth effect of Ox-LDL and KNL indicated Dose for 24 hr by MTT. (C) Cell growth effect of SPN on DOX induced H9c2 cells. The data using an analysis of variance (one-way analysis of variance) and compared the controls using Tukey's post-hoc test. Data are presented as the mean ± SD. *$p<0.05$ vs control, #$p<0.05$ vs KNL treatment.

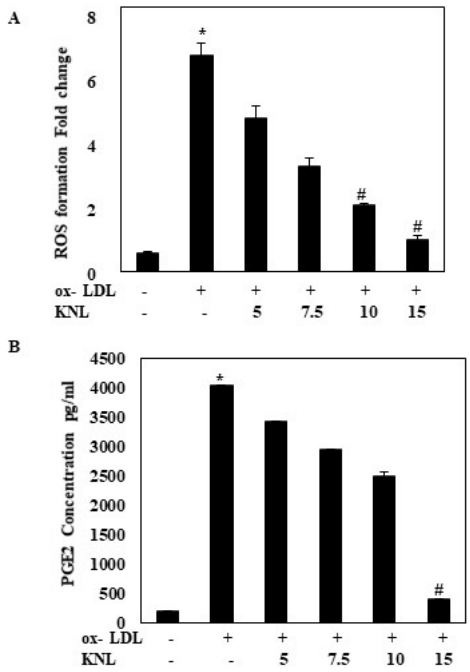

Figure 2. KNL inhibit the NO and PGE2 production in Ox-LDL induced endothelial cells cells. (A) Nitric oxide formation control treated cells. (B) Effect of PEG2 on Ox-LDL induced HUVECs cells. The data using an analysis of variance (one-way analysis of variance) and compared the controls using Tukey's post-hoc test. Data are presented as the mean ± SD. *p<0.05 vs control, #p<0.05 vs KNL treatment.

3.5. KNL affects IL-6, iNOS, COX-2 and IL-1β gene expression in Ox-LDL-induced HUVECs.

In Ox-LDL supplemented cells, we tested whether KNL inactivates the mRNA expression of IL-6, iNOS, COX-2 and IL-1β. According to qRT-PCR, cells pre-treated with KNL had significantly lower levels of mRNA than cells pre-treated with Ox-LDL alone (Figure 4 A-D) ($p<0.05$) KNL seems to suppress cytokine production that causes Ox-LDL-induced inflammation.

3.6. KNL inhibits the inflammatory reaction via Nfκ-B pathways in Ox-LDL-induced HUVECs.

KNL may alleviate OX-LDL-induced endothelial dysfunction by activating Nrf2 and its downstream target genes. Co-treating with KNL didn't reduce Nrf2 levels, but Ox-LDL did (Figure 5). Furthermore. This suggests that KNL enriched Nrf2 expression in nuclei. Further evaluate expression pP65, pP38 and PERK1/2 by western blot. In Figure 4 KNL could inhibits activation of this proteins where as KNL treatment significantly($p<0.05$) suppressed in Ox-LDL induced cells.

4. Discussion

Kirenol (Kr) is an ent-pimarane type diterpenoid that has been reported from Siegesbeckiaorientalis, S. pubescens, and S. glabrescens (family Asteraceae). These plants have been used traditionally for treating various ailments such as hypertension, neurasthenia, rheumatoid arthritis, asthma,

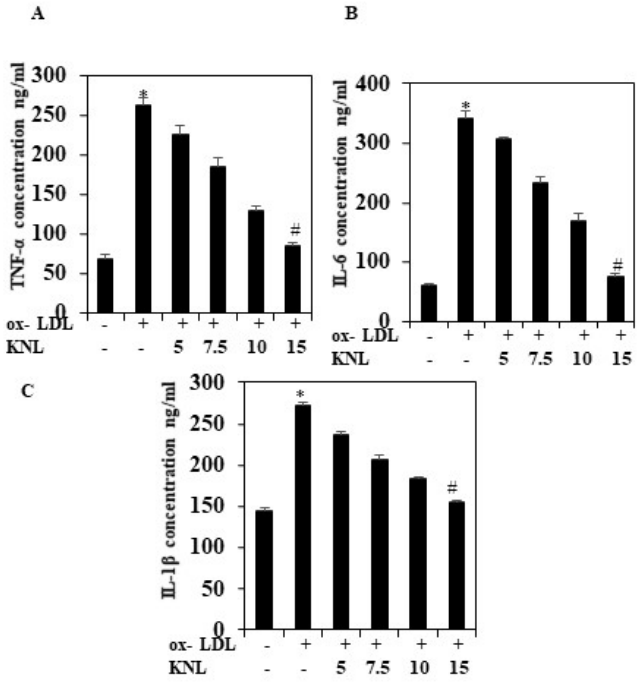

Figure 3. KNL on pro-inflammatory cytokine expression Ox-LDL induced endothelial cells. Expression of TNF-α (A), IL-6 (B) and IL-1β (C) by spectrophotometer. The data using an analysis of variance (one-way analysis of variance) and compared the controls using Tukey's post-hoc test. Data are presented as the mean ± SD. *p<0.05 vs control, #p<0.05 vs KNL treatment.

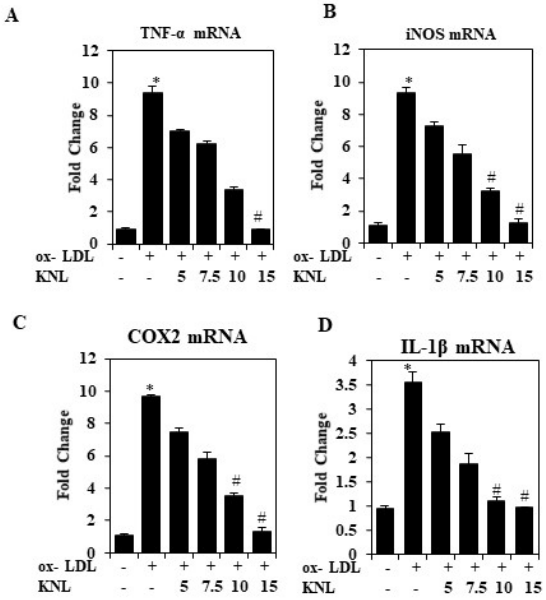

Figure 4. Efect of KNL on mRNA expression. (A) TNF-α., (B), iNOS (C) COX-2 and (D) Il-1β analyzed by RT-PCR the data using an analysis of variance (one-way analysis of variance) and compared the controls using Tukey's post-hoc test. Data are presented as the mean ± SD. *p<0.05 vs control, #p<0.05 vs KNL treatment.

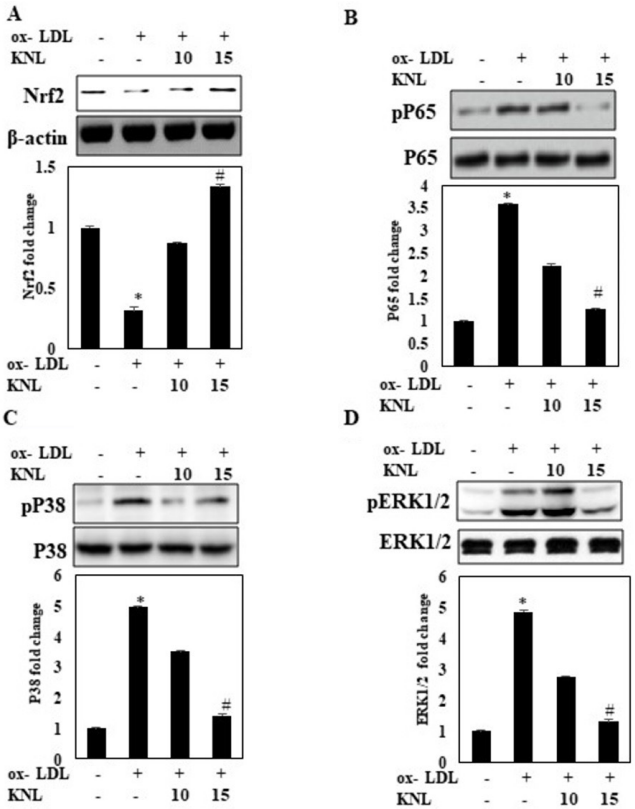

Figure 5. Effect of KNL on Ox-LDL induced inflammation. (A) Activation of Nrf2 by western blot experiment. (B) pP65 expression internal control total p65 (C) pP38 expression internal control p38. (D) PERK1/2 analyzed by WB internal control ERK1/2. the data using an analysis of variance (one-way analysis of variance) and compared the controls using Tukey's post-hoc test. Data are presented as the mean ± SD. *p<0.05 vs control, #p<0.05 vs KNL treatment.

snakebites, allergic disorders, paralysis, soreness, cutaneous disorders, rubella, menstrual disorders, numbness of limbs, dizziness, headache, and malaria. Importantly, in recent years, Kr has received great attention due to its diversified pharmacological activities (Ibrahim et al., 2021b). In this study, we tested the anti-inflammatory effects of ox-LDL on HUVECs. Furthermore, we analyzed how KNL affected ROS, PGE2, TNF-α, IL-1β, IL-6, the outflow of iNOS and COX-2, and NF-κB activation. In ox-LDL stimulated endothelial cells, KNL efficiently repressed the discharge of NO, PGE2, TNF-α, IL-1β, and IL-6 by barricading the NF-κB and MAPK pathways. The inhibitory impact of KNL was one of the instruments in charge of its calming effect and potential to be a good specialist for treating inflammatory diseases. Over the top inflammatory mediators and pro-inflammatory cytokines from macrophages act synergistically under obsessive conditions (Chatterjee, 2018). The influence of natural active compounds on NO or PGE2 creation might be advantageous for mitigating inflammatory illnesses and, for this reason, the inhibitory effects of natural active compounds on these preparations have been thoroughly studied to build up remedies (Chan et al., 2021; Kong et al., 2019).

In addition, excessive production of proinflammatory cytokines leads to intense inflammatory reactions and chronic inflammatory illnesses (Rajendran et al., 2018). More recent studies have shown that in vivo or in vitro medications of active compounds can decrease inflammation by preventing the release of inflammatory cytokines, which can aggravate inflammatory-related illnesses like atherosclerosis, cancer, and joint inflammation (Wolf and Ley, 2019). To control inflammation, the direction of those molecles is key. KNL restrains NO and PGE2 production in ox-LDL-animated HUVECs cells through iNOS and COX-2 reductase downregulation, which seems to be the primary contributing factor to transcriptional inhibition of iNOS and COX-2. NF-κB is a transcription figure that accepts iNOS and COX-2. Proinflammatory cytokines, like NO and PGE2 created by iNOS and COX-2, are also controlled by the NF-κB pathway and play a role in the establishment of the intrinsic resistance reaction to the acquired immune reaction (Khan and Khan, 2018). We've always known that dark tea concentrate restrains NF-κB activation by phosphorylating, ubiquitinating, and degrading IκB by way of the ubiquitin-proteosome pathway. Although its biochemical activities on NF-κB remain unclear, the present review indicates that KNL conceivably represses the NF-κB promoter-driven blot expression induced by ox-LDL in HUVECs cells. As a result, the KNL inhibits NF-κB initiation in HUVECS cells because of the ox-LDL flag. The initiation/deactivation of NF-κB pathway probably plays a big role in decreasing iNOS, COX-2, and proinflammatory cytokine expression in HUVECs. It looks like ox-LDL treatment is addressing the elements of KNL through the NF-κB pathway in this report. Different flagging kinases, including MAPKs (ERK and p38) and Akt, are likewise essential to NF-κB activation. NF-κB activates in ox-LDL-fortified immune cells when MAPKs are present. As a result, anti-inflammatory mechanisms are strongly linked to MAPK inhibition in enacted HUVECs. We found that KNL treatment restrained MAPK phosphorylation in light of ox-LDL. This study found that Nrf2, a downstream controller of ROS, was also restrained by KNL due to oxidized LDL in HUVECs.

5. Conclusion

In summary, we have identified KNL as a drug that inhibits endothelial inflammation via regulating NF-κB and Nrf2 signaling pathways. Our study supports the hypothesis that KNL may become an essential pharmacological agent by controlling key aspects of HUVECs homeostasis in physiologic variation or pathologic states. The anti-inflammatory effect of KNL has critical implications for its clinical application as a preventative or treatment option for rheumatoid arthritis, and cardiovascular disease, inflammatory diseases and cancer.

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