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Investigation the role of nitric oxide pathway in TNF-α induced HUVEC cells

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ABSTRACT: Nitric oxide (NO) is a highly reactive molecule involved in a variety of physiological and pathophysiological processes, such as inflammation. eNOS-produced NO has anti-inflammatory properties and play a role in vascular homeostasis. Through a variety of mechanisms, tumor necrosis factor alpha (TNF-α) has been shown to induce inflammation in HUVECs. The purpose of this study was to investigate the role of NO in HUVEC cells under inflammatory conditions induced by TNF-α. To identify TNF-α induction mechanisms, the phosphorylation of ERK, Akt, and eNOS was investigated. Sildenafil and L-NAME used to examine the role of NO in this process. In TNF-α-induced HUVEC cells, cell viability, nitrite/nitrate production, phosphorylated and total ERK, Akt, and eNOS levels were measured with or without sildenafil and L-NAME. At 20 and 40 ng/ml concentrations, TNF-α increased nitrite/nitrate and decreased cell viability ($p<0.05$). Sildenafil and L-NAME had no effect on cell viability and they both decreased nitrite/ nitrate in HUVEC that had been stimulated by TNF-α. TNF-α had no effect on the phosphorylation of ERK, Akt, and eNOS, but it did increase total eNOS levels. The phosphorylation of ERK, Akt, and eNOS was unaffected by sildenafil and L-NAME; however, L-NAME decreased total eNOS. According to our findings, there is no known direct relationship between TNF-α, sildenafil, or L-NAME and protein phosphorylation.

KEYWORDS: HUVEC; TNF-α; nitric oxide; sildenafil; L-NAME

1. INTRODUCTION

Nitric oxide (NO) is a highly reactive molecule involved in numerous physiological and pathophysiological processes, including inflammation. NO is generated by nitric oxide synthases (NOS), which include endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [1].

NO has been shown to regulate a variety of inflammatory processes, including leukocyte adhesion, vascular permeability and angiogenesis. eNOS is expressed in the endothelial cells lining the blood vessels and is responsible for the synthesis of NO under physiological conditions. NO produced by eNOS has anti-inflammatory properties and plays a role in maintaining vascular homeostasis. NO has been shown to inhibit leukocyte adhesion to the endothelium, a crucial step in the inflammatory response. iNOS is induced in response to inflammatory stimuli, such as bacterial infection or tissue injury. NO produced by iNOS has pro-inflammatory properties and plays a role in the host defense against infectious agents [1]. Inhuman umbilical vein endothelial cells (HUVECs), tumor necrosis factor alpha (TNF-α) has been shown to induce inflammation through a variety of mechanisms [2].

One signaling pathway that TNF-α can activate is ERK (extracellular signal-regulated kinase) pathway. ERK is a member of protein kinase family that they are activated by extracellular signals, such as growth factors and stress signals. Upon activation, ERK phosphorylates a number of target proteins, including transcription factors, cytoskeletal proteins, and enzymes, leading to changes in cell behavior [3].

Another signaling pathway that TNF-α can regulate is Akt (also known as protein kinase B) pathway. Akt is a serine/threonine protein kinase that is activated by various stimuli, including growth factors and hormones. Akt phosphorylates a number of target proteins, including transcription factors, cytoskeletal proteins, and enzymes, leading to play a key role in various cellular processes, including cell growth, survival, and metabolism. TNF-α has been shown to decrease the phosphorylation of Akt in HUVECs [4]. Furthermore, TNF-α has been shown to stimulate the phosphorylation of eNOS, which leads to the activation of eNOS and the increased production of NO, while some others studies demonstrated no effect of TNF-α on eNOS phosphorylation [5–7].

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The purpose of the current study was to investigate the role of NO in TNF-α-induced inflammation of HUVECs. The phosphorylation of Akt, ERK, and eNOS under inflammatory conditions were studied. To investigate the role of NO in this process, sildenafil and N-nitro-L-arginine methyl ester (L-NAME) were utilized.

2. RESULTS

In order to induce inflammation in HUVEC cells, TNF-α concentration were optimized using viability and nitrite/nitrate assays. A non-toxic (viability >85%) concentration that was sufficient to induce nitrite and nitrate production was chosen. **Figure 1A** shows that 20 ng/ml and 40 ng/ml TNF-α decreased viability of HUVEC cells. However, at 40 ng/ml, viability reduction was more severe. **Figure 1B** is showing TNF-α induced nitrite/nitrate production. 20 ng/ml and 40 ng/ml increased the production of nitrite/nitrate. Experiments were continued with non-toxic 20 ng/ml concentration of TNF-α.

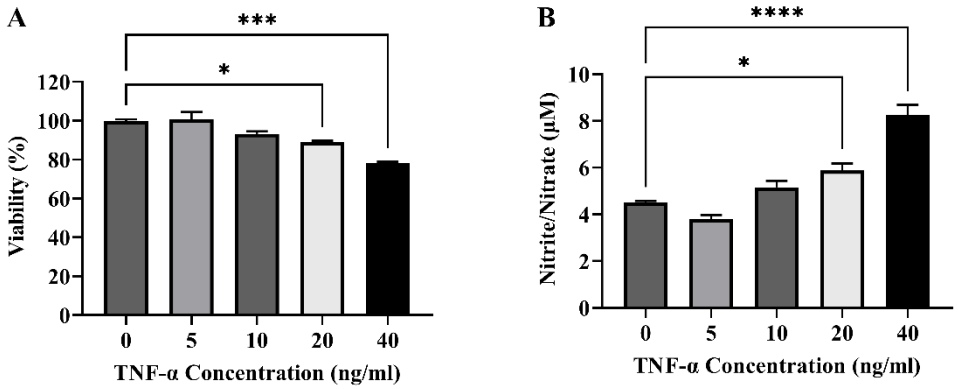

Figure 1. Optimization of TNF-α concentration. **A.** Viability (%) of HUVEC cells treated with 0-40 ng/ml TNF-α. **B.** TNF-α (0-40 ng/ml) induced nitrite/nitrate (µM) production (n=3).

HUVEC cells were incubated with sildenafil (10^{-6} M), and L-NAME (10^{-4} M) to determine their effects on cell viability. Under the inflammatory conditions, no agents affected cell viability **(Figure 2A)**. From the cell culture supernatants nitrite/nitrate was measured to evaluate nitric oxide production. TNF-α induced nitrite/nitrate production in HUVEC cells at 20 ng/ml concentration ($p<0.05$). Sildenafil and L-NAME decreased TNF-α induced nitrite/nitrate production ($p<0.01$) **(Figure 2B).**

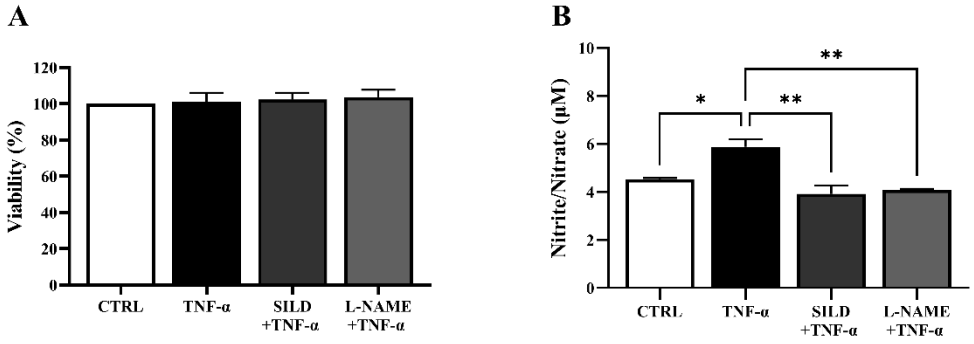

Figure 2. Effects of Sildenafil (10^{-6} M), and L-NAME (10^{-4} M) on cell viability (%) **(A)** and nitrite/nitrate production **(B)** in TNF-α(20 ng/ml) induced HUVEC cells under inflammatory conditions (n=3). CTRL: control, SILD: sildenafil.

We have evaluated protein levels of p-eNOS, eNOS, p-Akt, Akt, p-ERK, ERK in HUVEC cells under inflammatory conditions. TNF-α increased only total eNOS in HUVEC cells ($p=0.02$) **(Figure 3B).** p-eNOS, p-Akt, Akt, p-ERK, ERK did not changed compared to control **(Figure 3A-G)**. Similarly, sildenafil ($p=0.06$)

insignificantly and L-NAME (p=0.03) significantly decreased TNF-α induced eNOS expression **(Figure 3B)**, however did not affect p-eNOS, p-Akt, Akt, p-ERK, ERK expression **(Figure 3A-G).**

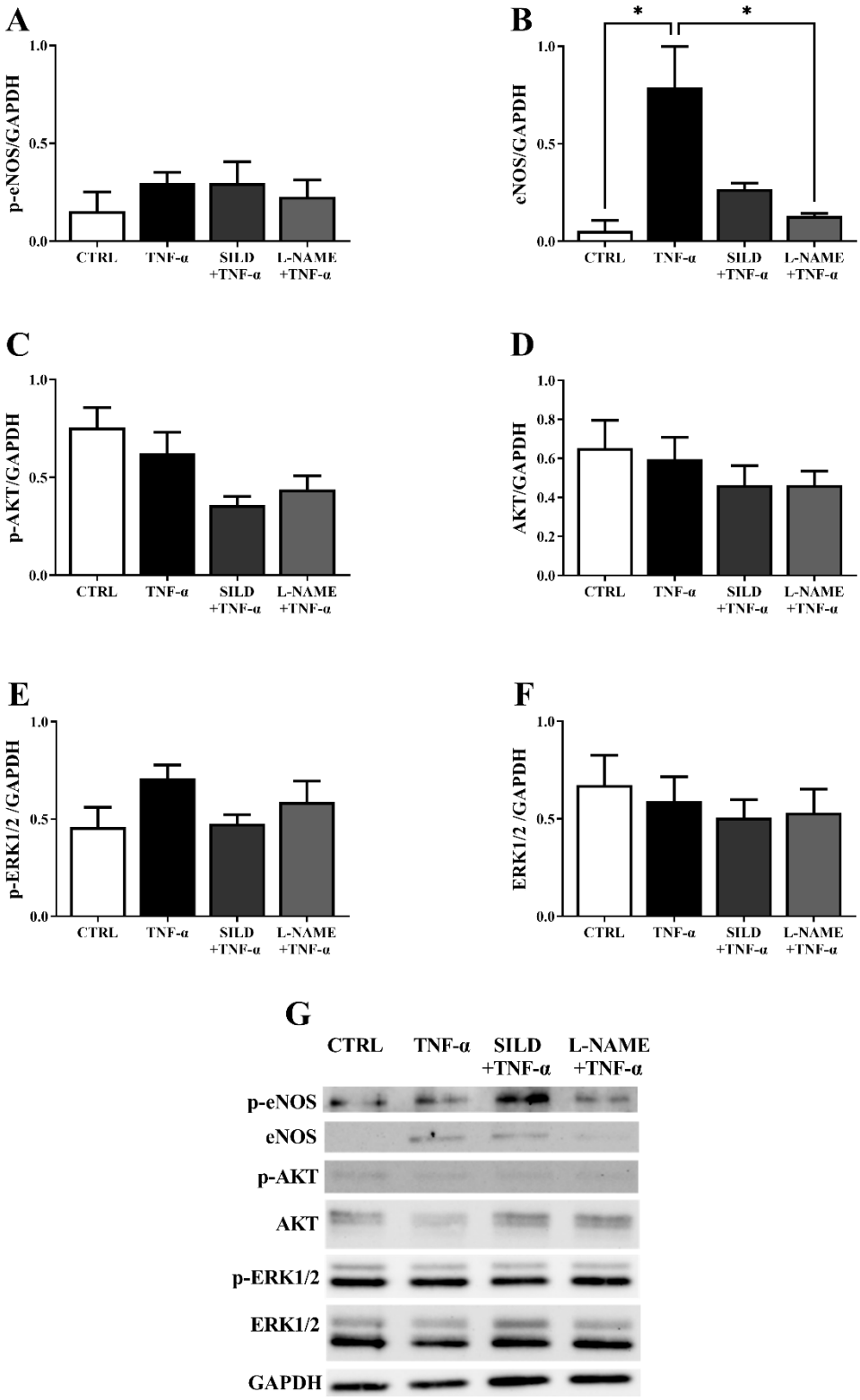

Figure 3. Effects of Sildenafil (10^{-6} M), and L-NAME (10^{-4} M) on expression of p-eNOS **(A)**, eNOS **(B)**, p-AKT **(C)**, AKT **(D)**, p-ERK 1/2 **(E)**, and ERK1/2 **(F)** proteins relative to GAPDH in TNF-α induced HUVEC cells under inflammatory conditions. **(G)** shows representative images of the bands (n=3-6).CTRL: control, SILD: sildenafil, p-: phospho.

3. DISCUSSION

TNF-α is a cytokine that plays a key role in inflammation. When TNF-α is produced and released by immune cells, it can stimulate inflammation in various tissues and organs of the body. TNF-α can stimulate the production of adhesion molecules and chemokines, which attract immune cells to the site of inflammation. In addition to these effects, TNF-α has been shown to modulate the production of other inflammatory mediators, such as interleukin-1 beta (IL-1β) and interleukin-6 (IL-6), and to stimulate the expression of enzymes that contribute to the breakdown of extracellular matrix proteins. Overall, TNF-α plays a central role in the initiation and maintenance of inflammation [8].

TNF-α has been shown to play a role in the regulation of nitrite levels in HUVECs. In the present study, to induce inflammation in HUVEC cells, TNF-α concentrations were optimized using viability and nitrite/nitrate assays. 20 ng/ml and 40 ng/ml concentrations of TNF-α decreased the viability of HUVEC cells while increasing the production of nitrite/nitrate. 20 ng/ml TNF-α is a non-toxic (viability >85%) and however enough to induce nitrite/nitrate production concentration, so we have continued our experiments with 20 ng/ml concentration of TNF-α. Similarly, another study also demonstrated that cell viability was significantly decreased by 30-40 ng/ml concentration of TNF-α, while 20 ng/ml concentration of TNF-α had little effect on HUVECs viability [9].

Several studies demonstrated that TNF-α treatment of HUVECs resulted in a decrease of NO levels with downregulation of eNOS [7,9–12]. On the other hand, others demonstrated TNF-α has no significant effect on NO levels in HUVECs [13,14] while some others showed TNF-α treatment of HUVECs resulted in an increase in NO levels, which was mediated through the activation of iNOS [15]. Contradictory results could be due to the different incubation period and concentration of TNF-α used. Depending on the concentration of TNF-α, the interaction with NOS and/or scavenging by NO from radicals induced by oxidative stress could be changed and this could result in different levels of NO.

In order to investigate the contribution of NO in TNF-α-induced inflammation, we have used sildenafil and L-NAME. NO activates an enzyme called guanylate cyclase, which converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). Sildenafil enhances this process by inhibiting the breakdown of cGMP by phosphodiesterase-5 enzyme (PDE-5), resulting in even higher levels of cGMP. L-NAME is a NOS inhibitor that blocks the production of NO. Our results demonstrated that both sildenafil and L-NAME did not affect HUVEC viability in the presence of TNF-α. As expected, L-NAME reversed nitrite/nitrate levels induced by TNF-α. In addition, sildenafil significantly reduced nitrite/nitrate levels induced by TNF-α. In the absence of TNF-α, another study demonstrated that sildenafil increased eNOS expression in HUVECs [16].

Phosphorylation is a chemical process in which a protein is modified by the addition of a phosphate group, and it can be regulated by various signaling pathways. In the next step of study, we investigate the role of TNF-α on Akt, ERK and eNOS phosphorylation and the contribution of NO in these effects. We did not find any significant effect of TNF-α on the levels of p-ERK, p-Akt and p-eNOS as well as the total levels of these proteins except total eNOS. Several studies showed that TNF-α did not change the levels of p-eNOS/t-eNOS in HUVECs [6,7]. On the other hand, some other studies indicated a decrease or increase in the phosphorylation of ERK or Akt by TNF-α in HUVECs [3,4]. Contradictory results could be due to the different incubation period and concentration of TNF-α used.

The role of sildenafil and L-NAME were also studied on the phosphorylation of ERK,Akt or eNOS in the presence of TNF-α. Our results demonstrated that both sildenafil and L-NAME did not modulate the phosphorylation of these proteins, however L-NAME decreased total eNOS. One study showed that chronic treatment with sildenafil enhanced eNOS but not Akt phosphorylation in HUVECs [16]. On the other hand, another study indicated that sildenafil treatment in pulmonary hypertensive rats reduced ERK1/2 and p38 MAPK activation while enhancing activation of the cytoprotective Akt pathway [17]. In the present study, we have investigated the in vitro effects of sildenafil under inflammatory conditions. These differences in experimental techniques could lead to different regulation of phosphorylation of these proteins.

4. CONCLUSION

Overall, our results demonstrated that in vitro incubation with TNF-α increased nitrite/nitrate levels but did not modify the phosphorylation of ERK, Akt, and eNOS, however increased total eNOS. L-NAME and sildenafil decreased nitrite/nitrate levels, which were induced by TNF-α. Both sildenafil and L-NAME did not regulate the phosphorylation of ERK, Akt, and eNOS, however L-NAME decreased total eNOS. Our results suggested there is no known direct relationship between the TNF-α and sildenafil/L-NAME in terms of their

effects on protein phosphorylation. The phosphorylation of these proteins is complex and multifaceted, and further research is needed to fully understand the mechanisms underlying this process.

5. MATERIALS AND METHODS

5.1. Cell culture

HUVEC (ATCC; CRL-1730™), is a vascular endothelial cell line derived from human umbilical cord, was used as an in vitro model for this study. Cells were cultured by using DMEM/F12 (1:1) (1X) (Gibco, MA, USA) (with 1% Penicillin-streptomycin, 10% FBS) medium at 37°C with humidified atmosphere incubator containing 5% CO_2 (cell culture conditions). The passaging procedure was performed every 2-3 days. Whole experiments were performed between passages 12 – 18.

5.2. Drugs and treatment procedure

Sildenafil (SML3033, Sigma, USA), a phosphodiesterase-5 enzyme inhibitor, and L-NAME (N5751, Sigma, USA), nitric oxide synthase inhibitor, were dissolved in PBS to prepare 10^{-2} M concentrations for both. TNF-α (300-01A, Peprotech, USA) was dissolved in PBS to prepare concentration of 10 µg/ml.

5.3. Optimization of TNF-α concentration

TNF-α concentration was optimized via MTT assay in 24 well plates for further studies. HUVEC cells were seeded as $1x10^5$/ well in basal medium. TNF-α concentrations of 5, 10, 20, and 40 ng/ml were applied in triplicate to 24 well plates. After 24 hours of incubation at cell culture conditions, medium was collected into 1.5 ml centrifuge tubes, MTT was added (final concentration 0.5 µg/ml) and plates were incubated for an additional 4 hours. After dissolving the formazan crystals with DMSO, absorbance was read at 570 nm in the Biotek H1 plate reader. Nitrate/Nitrite Colorimetric Assay Kit (Cay780001-192 Cayman, USA) was used to measure nitrite/nitrate production from collected cell culture supernatants. Nitrite/nitrate (µM) were normalized with respect to viability [9].

5.4. Cell viability

HUVEC cells were seeded as $1x10^5$/well in basal medium in 24 well plates and incubated for overnight at cell culture conditions. 4 hours prior to the experiment, serum-free medium was added to starve the cells. Sildenafil (10^{-6} M) [16] and L-NAME (10^{-4} M) [18] were added as triplicate. 30 min later 20 ng/ml TNF-α was applied all the wells except controls. After 24 hours of incubation at cell culture conditions, MTT was added (final concentration 0.5 µg/ml) and plates were incubated for an additional 4 hours. After dissolving the formazan crystals with DMSO, absorbance was measured at 570 nm in Biotek Synergy H1 plate reader. Viability (%) is calculated using negative controls (untreated cells) [16].

5.5. Nitrate/Nitrite Colorimetric Assay

HUVEC cells were seeded as $1x10^5$/well in basal medium in 24 well plates and incubated for overnight at cell culture conditions. 4 hours prior to the experiment, serum-free medium was added to starve the cells. Sildenafil (10^{-6} M), and L-NAME (10^{-4} M) were added as triplicate. 30 min later 20 ng/ml TNF-α was applied all the wells except controls. After 24 hours of incubation at cell culture conditions, medium was collected into 1.5 ml centrifuge tubes for all samples and kept at -20°C until the experiment. Nitrate/Nitrite Colorimetric Assay Kit (Cay780001-192 Cayman, USA) was used to measure NO in cell culture supernatants as instructed in the kits. Absorbance was measured in Biotek Synergy H1 plate reader [6].

5.6. Western blot

After supernatant collection for colorimetric assay, cells were trypsinized, collected into 1.5 ml centrifuge tubes, and centrifuged at 2500 rpm for 5 min. The supernatant was removed, and the cell pellet was lysed in Ripa Lysis Buffer (Santa Cruz, USA). After several vortexing, tubes were centrifuged at 14000 rpm for 20 min at 4°C and clear supernatants were collected in clean tubes. Total protein concentrations were measured using the Qubit Protein Assay Kit and Qubit Fluorometer 2.0 (Thermo Fisher, USA). Protein samples were mixed with 2X Laemmli Buffer (Bio-Rad, USA) and heated at 95°C for 5 min. 50 µg total protein were loaded into the wells of the 4-10% SDS gel and run at 120 V for 2 hours. Gels were transferred to PVDF membranes (Millipore, USA) in tank overnight at 0.1 A. Membranes were blocked with 5% non-fat milk at room temperature (RT) for 1 h. Primary antibody incubations were made at RT for overnight. Phospho-eNOS-(S1177) pAB (AP0421, Abclonal, USA), eNOSpAB (A1548, Abclonal, USA), Phospho-Akt (Ser473) mAB (4060S,

CST, USA), Akt mAB (4691S, CST, USA), Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) mAB (9101S, CST, USA), p44/42 MAPK (ERK1/2) mAB (4695S. CST, USA), GAPDH mAb (AC002, Abclonal, USA) primary antibodies were used. Membranes were incubated with anti-rabbit or anti-mouse secondary antibody (CST, USA) at RT for 1 h. Imaging was made using ECL (Bio-Rad, USA) in Bio-Rad Chemidoc Imaging System. Band intensities were analyzed in ImageLab 6.0 (Bio-Rad, USA), and normalization was made using GAPDH as housekeepingprotein [16].

5.7. Statistical analysis

Results were given as mean ± standart mean of error (SEM). Sample size (n) was 3 to 6 according to different experiments. One-way ANOVA was used to compare statistical differences between groups. $p<0.05$ was considered significant. Statistical analysis and graph production were made using Graphpad Software 9.0.

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