Assessment of NF-κB Inhibitor (SN50) Effect on Adipose Tumor Necrosis Factor-Alpha and Angiotensinogen Secretion and Expression †

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Abstract: Central adiposity is one of the significant determinants of obesity-related hypertension risk, which may arise due to the abdominal fat depot’s pathogenic inflammatory nature. Pro-inflammatory cytokines and adipokines up-regulation through nuclear factor-kappa B (NF-κB) activation in adipose tissue has been considered an essential function in the pathogenesis of obesity-related hypertension. This study aimed to ascertain the NF-κB inhibitor (SN50) effect on TNF-α and angiotensinogen (AGT) secretion and expression in mediating the anti-inflammatory effect through its impact on NF-κB activity in humans adipose tissue. Primary human adipocytes were isolated from 20 subjects among 10 overweight and 10 obese with and without hypertension and treated with 10ng/ml LPS in the presence and absence of NF-κB inhibitor, SN50 (50μg/ml). TNF-α secretion and NF-κB p65 activity were detected in supernatants extracted from cultured cells treated and untreated with LPS (10ng/ml) and SN50 (50μg/ml) using enzyme-linked immunosorbent assay (ELISA). The western blot technique detected the protein of NF-κB p65 and AGT. Gene expression of TNF-α and AGT was detected in cells and performed using quantitative real-time polymerase chain reaction (RT-PCR). Treatment of AbdSc adipocytes with LPS (10ng/ml) caused a significant increase in NF-κB p65 among overweight and obese subjects with and without hypertension (P=0.001) at 24 hours incubation. In contrast, SN50-NF-κB inhibitor causes a reduction of NF-κB p65 in overweight (P ≤0.001) and obese subjects with and without hypertension (P=0.001) at 24 hours incubation. Treatment of AbdSc adipocytes with 10ng/ml LPS caused a significant increase in TNF-α secretion in overweight and obese subjects at all-time points (P ≤0.001), whereas SN50 leads to a decrease in TNF-α secretion at 3 and 12 hours incubation. Treatment of AbdSc adipocytes with LPS (10ng/ml) caused increased TNF-α and AGT gene expression twofold compared with untreated cells, whereas, in the presence of SN50, it reduces mRNA AGT levels in both groups. Taken together, these adipokines with NF-κB activation may represent essential biomarkers to evaluate hypertension risk and to provide insight into the pathogenesis of obesity-related hypertension.

Keywords: abdominal subcutaneous adipocytes; angiotensinogen; nuclear factor-kappa B; lipopolysaccharide; tumor necrosis factor-alpha
1. Introduction

Abdominal adipose tissue is considered to be particularly pathogenic in nature with increasing obesity [1]. Adipose tissue is an endocrine organ that secretes numerous bioactive peptides, often referred to as adipocytokines, which have been proposed to participate in the development of hypertension [2]. From a basic physiological perspective, adipose tissue growth is tightly coupled to angiogenesis and microcirculation development. Tumor necrosis factor-alpha (TNF-α) is known to regulate angiotensinogen (AGT) in hepatocytes, as the AGT promoter contains a cytokine-inducible enhancer known as the acute phase response element [3].

Pro-inflammatory adipocytokine secretion from adipose tissue is coordinated through the activation of the nuclear factor-kappa B (NF-κB) transcription factor [4], which regulates the transcription of genes involved in inflammatory responses, cell growth control, and apoptosis. Thus, additional pathways in the development of hypertension may emanate from NF-κB activation. In resting cells, NF-κB is bound to IκB (IκB) inhibitors, which prevent NF-κB from entering the nucleus.

Extracellular stimuli such as pro-inflammatory cytokines, chemokines, and lipopolysaccharides (LPS) activate a set of proteins termed IκB kinases (IKKs), which phosphorylate IκB, leading to an altered conformation of IκB, which releases NF-κB to enter the nucleus and activates transcription of target genes [5]. Lipopolysaccharides increase the secretion of cytokines such as TNF-α via NF-κB activation [6]. TNF-α also induces the transcription of AGT via NF-κB [7]. Moreover, there is accumulating evidence that indicates angiotensin II (ANG II) is capable of inducing an inflammatory response in the cardiac tissue through the activation of NF-κB [8].

However, the study of NF-κB has, to some extent, been hampered by the lack of selective and specific inhibitory compounds. Our study, therefore, investigated the role of a cell-permeable peptide, SN50, as an NF-κB inhibitor. This peptide consists of the nuclear localization sequence of NF-κB subunit (p50) fused with the hydrophobic region of the signal sequence of Kaposi’s fibroblast growth factor. Lin et al. [9] demonstrated that SN50 is capable of inhibiting the nuclear import of NF-κB in human monocytic cells and murine endothelial cells stimulated with LPS and TNF-α.

We hypothesized that the activation of NF-κB and the subsequent secretion and expression of TNF-α and AGT from human adipose tissue might play an important role in the development of arterial hypertension in obese subjects. The results may enable us to understand the role of various pro-inflammatory agents upregulated by activated NF-κB in the development of arterial hypertension. Our study was aimed to ascertain the effect of the NF-κB inhibitor, SN50, on reducing TNF-α and AGT secretion and expression in mediating the anti-inflammatory effect through its effects on the NF-κB inflammatory pathway in humans adipose tissue.

2. Materials and Methods

2.1. Subjects and Adipose Tissue

Abdominal subcutaneous adipose tissue (AbdScAT) samples were obtained from 10 overweight (Mean age: 30.71±9.69; and a mean BMI: 27.92±0.66), and 10 obese with and without hypertension (Mean age: 41.6±12.62; and a mean BMI: 36.2±6.95). They were undergoing abdominal adipose tissue liposuction for cosmetic reasons. Subjects with chronic debilitating diseases were excluded from the study. Personal information and anthropometric measurements were taken from each participant. All participants fasted overnight before surgery, and only normal saline was administered intravenously until
the tissue liposuction was taken. Informed consent was taken from all participants. The study was carried out at the Chair for Biomarkers of Chronic Diseases and Obesity Research Center, King Saud University, Riyadh, KSA. Ethical approval was granted by the King Khalid University Hospital, College of Medicine, King Saud University, Riyadh, KSA.

2.2. Isolation and Culture of Mature Adipocytes

Adipose tissue liposuction samples were digested for 30 minutes at 37 °C in Hank’s balanced salt solution (HBSS) containing 2 mg/ml collagenase under intermittent shaking as described by [10]. In brief, the mixture was centrifuged at 2000 rpm for 5 minutes at room temperature. After centrifugation, the tubes were tilted, allowing the densely packed adipocytes layer (cake of fat cell) to flow gently into a clean falcon tube. Mature fat cells were then washed by pouring in 10-15 ml of Dulbecco’s modified eagle medium (DMEM F-12) phenol-red free (1 ml containing 15 mmol/l glucose, supplemented with 100 U/ml of penicillin and 100 μg/ml streptomycin. Fat cells were centrifuged at 1000 rpm for 1 min. Once the adipocytes have been washed, the cells were collected into a new falcon tube. Following isolation of these cells, cells (0.2 ml, 100,000 adipocytes) were plated in 6-well tissue culture plates with culture medium (1 ml, containing 15 mmol/l glucose, supplemented with 100 U/ml of penicillin, 100 μg/ml streptomycin, and 100 μg/ml transferrin). The cells were preincubated with SN50 and SN50M peptides (50 μg/ml) before the initiation of stimulation to enable efficient membrane translocation at 37 °C in 95% air and 5% CO2 for 2 hours. After preincubation, the cells were treated with ten ng/ml LPS for 03, 12, 24, and 48 hours before the experiments. To optimize the experiments, we used different concentrations of NF-κB inhibitor (1, 10, 50, and 100 μg/ml SN50) and LPS (1, 10, and 100 ng/ml) without causing cellular death; this was deemed as 50 μg/ml SN50, and ten ng/ml LPS. Following treatment, conditioned media and adipocytes were centrifuged for 1 minute at 1000 rpm. The infranatant then separated and stored at -80°C until adipokines measurement. In contrast, fat cells were used for protein expression (NF-κB p65 activity assay and western blot) and gene expression of TNF-α and ANG.

2.3. NF-κB p65 Activity Assay

NF-κB p65 activity was assessed using the transcription factor assay kit according to the manufacturer’s instructions (TransAM NF-κB p65 kit, Active Motif, Rixenart, Belgium; Cat. No. 40096), as described by [11]. In brief, 20 μg of total protein was used to detect NF-κB p65 activity. Jurkat cell nuclear extract (2.5 μg) was used as a positive control for NF-κB p65 activation. Wild-type consensus oligonucleotides were used in the assay as a competitor for NF-κB binding. 20 pmol/well of oligonucleotides used were enough to prevent NF-κB binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotides were used as an uncompetitor for NF-κB binding.

2.4. Isolation and Protein Quantification

Equal amounts of protein from each sample (20 μg) and pertained markers were heated for 5 min at 95 °C in a loading sample buffer, loaded, and separated by 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) running gel and 4% SDS-PAGE stacking gel. In brief, equal amounts of protein from each sample (20 μg) and prestained markers were heated for 5 min at 95 °C in loading sample buffer and then separated by 12% Sodium Dodecyl Sulphate-Polyacrylamide (SDS-PAGE) running gel, and 4% SDS-PAGE stacking gel. Gels were run for 2 hours at 120 volts and then blotted and transferred onto polyvinylidene difluoride (PVDF) membrane for 25 minutes at 15 volts using a liquid transfer buffer. After transfer, the membrane was washed with 1X tris buffered saline (TBS) for 5 minutes and then blocked in a blocking buffer (TBS with 0.1% Tween20, 5% nonfat dried milk, and 1% bovine serum albumin (BSA) for one hour at room temperature. After
blocking, the membrane was then incubated with gentle agitation overnight at 40°C with antibodies against the following: polyclonal rabbit IgG NF-kB p65 (1:1000) (Cell Signaling Technology), polyclonal rabbit IgG angiotensinogen (1:200) (Phoenix Pharmaceuticals Industries, USA), polyclonal goat IgG TNF-α (1:1000) (R&D Systems, USA). After incubation, the membrane was then washed three times with TBS/T for 5 minutes each wash and then incubated with the appropriate HRP-conjugated secondary antibody for one hour. After incubation, the membrane was washed five times with TBS/T and then incubated with chemiluminescent development (luminol and peroxide substrate) with gentle agitation for 1 minute in a dark room. The membrane was drained, wrapped in plastic wrap, and exposed on the x-ray image.

2.5. Isolation and Purification of Total RNA

Cells from 6 wells were extracted with 200 μl of RNA later (for RNA stabilization). Total RNA was isolated using the RNeasy mini kit (Qiagen, GmbH, Hilden; Germany). RNA concentration and purity were performed using the Nanodrop ND-1000 spectrophotometer, Thermo Fisher Scientific, Wilmington, DE 19810 USA.

2.6. cDNA Synthesis

The reverse transcription step was conducted on 200ng RNA using the quantitect reverse transcription kit (Qiagen, USA) described by Heller et al. [13]. In brief, purified RNA samples (200 ng/μl) were incubated with 2 μl of genomic DNA wipeout buffer at 42°C for 2 minutes to remove contaminating genomic DNA effectively. After genomic DNA elimination, the RNA samples were reverse transcribed using a master mix prepared from quantiscript reverse transcriptase (1 μl), RT primer mix (1 μl), quantiscript RT buffer (4 μl) at 42°C for 15 minutes.

2.7. Quantitative Real-Time PCR

All the experiments were performed in 96 well plates with CFX 96- Real-Time PCR detection system (Bio-Rad, USA) using a premade TaqMan probe for (AGT: Hs01586213_m1 and TNF-α Hs00174128_m1). Real-time relative expression experiments were performed according to the manufacturer’s instructions. In brief, 1 μl cDNA was used in a final PCR volume of 20 μl, containing 10 μl of the TaqMan Master Mix (AB Applied Biosystems, Warrington, UK), 8μl RNA-free water, and 1 μl TaqMan probe. Polymerase chain reaction cycles were as follows: 10 minutes at 42°C, followed by 40 cycles for 15 seconds at 95°C, and then 1 minute at 60°C. All reactions were multiplexed with the housekeeping gene human 18S ribosomal RNA (AB Applied Biosystems, Warrington, UK) and were used as a reference, enabling data to be expressed as delta cycle threshold (ΔCT) values (where ΔCT=CT 18s- CT gene of interest). Quantification of target mRNA was carried out by comparing the number of cycles required to reach the reference and target threshold values (ΔΔCT method). All reactions were performed in triplicate. All statistical analyses were performed at the ΔCT stage to exclude potential bias due to the averaging of data transformed through a 2-ΔΔCT equation.

2.8. Statistical Analysis

Data were analyzed using the SPSS Windows Statistical Package for Social Sciences (Version 16.0 SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard error (S.E.). An independent sample t-test was done for delta C.T. (ΔCT) among untreated and various treatment groups. P<0.05 was considered as a significant value.
3. Results

3.1. Inhibitory Effect of SN50 on NF-κB Translocation and Activity in Cultured Human AbdSc Adipocytes

Figure 1 shows the effect of SN50 on NF-κB translocation and activity in untreated and LPS-treated cells taken from overweight, and obese with and without hypertension at different time points. Treatment of adipocytes with 10 ng/ml LPS resulted in a significant increase in NF-κB activity in obese with and without hypertension than overweight subjects at 3 and 12 hours respectively (Controls: 0.32±0.06 vs. LPS: 0.54±0.06; \( P=0.005 \); 0.34±0.02 vs. 0.57±0.04; \( P=0.001 \)), and a significant increase in both groups at 24 hours (overweight: controls: 0.21±0.07 vs. LPS: 0.46±0.02; \( P=0.002 \); obese subjects with or without hypertension: controls: 0.33±0.04 vs. 0.54±0.04; \( P=0.005 \)). Coincubation with SN50 (50 μg/ml) effectively abrogated LPS-stimulated NF-κB activity in the obese subjects with or without hypertension at 3, 12, and 24 hours (3 h, LPS: 0.54 ± 0.06 vs. SN50+LPS: 0.35 ± 0.06; \( P=0.01 \); 12 h, LPS: 0.57 ± 0.04 vs. SN50+LPS: 0.31 ± 0.05; \( P \leq 0.001 \); 24 h, LPS: 0.54 ± 0.05 vs. SN50+LPS: 0.41 ± 0.11; \( P \leq 0.001 \)) respectively, and in overweight subjects only at 24 hour (LPS: 0.46 ± 0.02 vs. SN50+LPS: 0.24 ± 0.03; \( P \leq 0.001 \)). In contrast, SN50M (Inactive peptide) did not affect the ability of LPS to activate and translocate NF-κB. Incubation with SN50 alone did not influence NF-κB activity in both groups (overweight: 3 hr.; controls: 0.38 ± 0.04 vs. SN50: 0.36 ± 0.04; \( P=NS \); obese with or without hypertension: 3 hr.; control: 0.32 ± 0.06 vs. SN50: 0.41 ± 0.06; \( P=NS \); A 12 hr.; controls: 0.33 ± 0.07 vs. 0.29 ± 0.1; \( P=NS \); B 12 hr.; control: 0.34 ± 0.02 vs. SN50: 0.29 ± 0.04; \( P=NS \); overweight, 24 hr.; controls: 0.21 ± 0.07 vs. SN50: 0.14 ± 0.09; \( P=NS \); obese with or without hypertension, 24 hr.; controls: 0.33 ± 0.04 vs. SN50: 0.32 ± 0.08; \( P=NS \)). Values are expressed as mean ± SE using the sample student t-test.

(A)
Figure 1. Inhibitory effect of the NF-κB inhibitor, SN50, on NF-κB p65 activity in cultured human abdominal subcutaneous (AbdSc) adipocytes. (a) Adipocytes treated with lipopolysaccharides (LPS) (10 ng/mL) in the presence and absence of SN50 (50 μg/mL) of the overweight and the obese ± hypertension group at 3 h of incubation. Values are expressed as means ± SEMs using the student t-test. Values of p < 0.05 were considered significant versus untreated and treated cells. * P-value <0.05, ** p-value = 0.001, *** p-value ≤0.001. (b) Adipocytes treated with LPS (10 ng/mL) in the presence and absence of SN50 (50 μg/mL) of the overweight and the obese ± hypertension group at 12 h of incubation. Values are expressed as means ± SEMs using the student t-test. Values of p < 0.05 were considered significant versus untreated and treated cells. * P-value <0.05, ** p-value = 0.001, *** p-value ≤0.001. (c) Adipocytes treated with LPS (10 ng/mL) in the presence and absence of SN50 (50 μg/mL) of the overweight and the obese ± hypertension group at 24 h of incubation. Values are expressed as means ± SEMs using the student t-test. Values of p < 0.05 were considered significant versus untreated and treated cells. * P-value <0.05, ** p-value = 0.001, *** p-value ≤0.001.
3.2. SN50 Reduces NF-κB p65 Protein in LPS-Stimulated Adipocytes

To explore whether inhibition of NF-κB p65 protein reduces LPS-stimulated adipocytes, we isolated protein from adipocytes treated with 10 ng/ml LPS for 3, 12, and 24 hours with or without SN50 (50 μg/ml). LPS alone increased NF-κB protein levels compared with those in control cells (figure 2). In contrast, co-incubation with LPS and SN50 resulted in a marked reduction of NF-κB p65 protein levels at 3 and 12 hours, but did not affect at 24 hours.

3.3. LPS-Induced TNF-α Secretion is Linked to the Activation of NF-κB Pathway in Cultured Human AbdSc Adipocytes

The role of the NF-κB pathway in the gene expression and secretion of TNF-α has been determined through the use of a specific inhibitor to this pathway; cell permeable inhibitor peptide, SN50. Figure 3 shows that the use of SN50 (50 μg/ml) causes a dose dependent reduction in the LPS (10 ng/ml) induced activation of TNF-α secretion in both groups at different time points (overweight 3 h; LPS: 31.1 ± 3.4 vs. SN50+LPS: 12.6 ± 2.6
pg/ml; P=0.008; obese ± hypertension: LPS: 82.2 ± 4 vs. SN50+LPS: 54.4 ± 3.1 pg/ml; P ≤0.001; overweight 12 h; LPS: 140 ± 7 vs. SN50+LPS: 124.7 ± 5.1 pg/ml; P =0.09; obese ± hypertension: LPS: 177.3 ± 6.7 vs. SN50+LPS: 93.3 ± 5.3 pg/ml; P ≤0.001; overweight 24 hr.; LPS: 80.3 ± 4.3 vs. SN50+LPS: 47.3 ± 5.9 pg/ml; P=0.02; obese ± hypertension: LPS: 123.2 ± 6.2 vs. SN50+LPS: 59 ± 5.2 pg/ml; P ≤0.001). The maximum inhibition of TNF-α secretion (around 50 %) is obtained with a concentration of 50 μg/ml. Whereas, TNF-α secretion was increased in LPS-treated compared with untreated cells (controls) in both groups (overweight 3 h; controls: 7.1 ± 1.5 vs. LPS: 31.1 ± 3.4 pg/ml; P<0.001; obese ± hypertension: controls: 5.7 ± 1.3 vs. LPS: 82.2 ± 4 pg/ml; P ≤0.001; overweight 12 hr.; controls: 2.0 ± 0.62 vs. LPS: 140 ± 7 pg/mL; P ≤0.001; obese ± hypertension: controls: 8.1 ± 1.2 vs. LPS: 177.3 ± 6.7 pg/ml; P ≤0.001; overweight 24 hr.; controls: 32.2 ± 2.3 vs. LPS: 80.3 ± 4.3 pg/ml; P<0.001; obese ± hypertension: controls: 5.9 ± 2.1 vs. LPS: 123.2 ± 6.2 pg/ml; P ≤0.001). Moreover, the maximum effect of LPS was shown at 12 hours and gradually significantly decreased after 24 hours. TNF-α secretion was also significantly higher in obese with and without hypertension than overweight group. In contrast, there was no change observed in TNF-α secretion in the presence of SN50 alone compared with untreated cells in both groups (Figure 3), [(overweight 3 h; controls: 7.1 ± 1.5 vs. SN50: 4.3 ± 1.2 pg/ml; P=0.16; 12 hr.; controls: 2.0 ± 0.62 vs. SN50: 9.5 ± 1.2 pg/ml; P=NS; 24 hr.; controls: 32.2 ± 3.2 vs. SN50: 3.2 ± 1.1 pg/ml; P ≤0.001), (obese ± hypertension 3 hr.; controls: 5.7 ± 1.3 vs. SN50: 6.0 ± 1.1 pg/ml; P=0.86; 12 hr.; controls: 8.1 ± 1.2 vs. SN50: 7.6 ± 1.1 pg/ml; P=0.76; 24 hr.; controls: 5.9 ± 2.1 vs. SN50: 5.4 ± 1.5 pg/ml; P=0.92)]. Values are expressed as mean ± SE.
**Figure 3.** Secretion of TNF-α (pg/mL) in cultured human AbdSc adipocytes. Adipocytes treated with LPS (10 ng/mL) in the presence and absence of SN50 (50 μg/mL) among overweight subjects and obese ± hypertension subjects at (a) 3, (b) 12, and (c) 24 h incubation periods. Values are expressed as means ± standard errors using the student t-test. Levels of \( p < 0.05 \) were expressed as significant versus untreated cells. * \( p \)-value \(<0.05\), ** \( p \)-value \(=0.001\), *** \( p \)-value \(\leq0.001\).
3.4. mRNA Expression of TNF-α in Cultured Human AbdSc Adipocytes

Figure 4 shows the mRNA TNF-α level in cultured human adipocytes treated with LPS and SN50 for 12 hours taken from overweight (n=10) and obese with and without hypertension (n=10). Treatment of AbdSc adipocytes with LPS (10 ng/ml) increased the expression of the TNF-α gene twofold compared with untreated cells (controls) in both groups (Controls: 1 vs. LPS: 1.87; P=0.005; Controls: 1 vs. LPS: 2; P=0.02) respectively. In contrast, the treatment of AbdSc adipocytes with LPS in the presence of SN50 (50 μg/ml) reduced the expression of the TNF-α gene compared with treated cells in the overweight group (LPS: 1.87 vs. SN50+LPS: 1.15; P= 0.02), whereas, no significant change was observed in the expression of TNF-α mRNA level in obese ± hypertension group (LPS: 2 vs. SN50+LPS: 1.48; P= 0.14). Moreover, there was no effect for SN50 alone on TNF-α mRNA level in both groups (controls: 1 vs. SN50: 0.88; P= 0.51; controls: 1 vs. SN50: 1.39; P= 0.11) respectively. In contrast, expression of TNF-α gene was increased twofold in LPS-treated cell compared with controls in the combined groups studied (Figure 5), (controls: 1 vs. LPS: 1.96; P= 0.001), and significantly reduced in the presence of SN50 (LPS: 1.96 vs. SN50+LPS: 1.20; P= 0.02).
Figure 4. mRNA Expression of TNF-α level among overweight subjects and obese with and without hypertension subjects in cultured human AbdSc adipocytes. Total RNA was isolated from AbdSc adipocytes from (a) overweight subjects (n = 10) and (b) obese ± hypertension subjects (n = 10) and treated with LPS (10 ng/mL) in the presence and absence of SN50 (50 μg/mL) at 12 h of incubation. Quantitative RT-PCR was performed using a premade TaqMan probe for TNF-α. The quantitative fold changes in mRNA expression were determined as relative to 18S mRNA levels in each corresponding group and calculated using the 2-ΔΔCT method. Statistical analysis was undertaken using the independent sample t-test. \( P < 0.05 \) was considered as significant versus untreated cells. * \( P\text{-value} = 0.02 \), ** \( P\text{-value} = 0.005 \) (the overweight group), ** \( P\text{-value} = 0.002 \) (the obese ± hypertension group).

Figure 5. mRNA expression of TNF-α level in cultured human AbdSc adipocytes in the studied combined group. Total RNA was isolated from AbdSc adipocytes treated with LPS (10 ng/mL) in the presence and absence of SN50 (50 μg/mL) at 12 h of incubation. Quantitative RT-PCR was performed using a premade TaqMan probe for TNF-α. The quantitative fold changes in mRNA expression were determined as relative to 18S mRNA levels in each corresponding group and...
calculated using the 2-ΔΔCT method. Statistical analysis was undertaken using the independent sample t-test. *p < 0.05 was considered as significant versus untreated cells. *P-value < 0.02, **p-value = 0.001.

3.5. mRNA Expression of AGT in Cultured Human AbdSc Adipocytes

Figure 6 shows mRNA AGT level in human adipocytes treated with LPS and SN50 for 12 hours taken from overweight (n=10) and obese patients with and without hypertension (n=10). Treatment of AbdSc adipocytes with LPS (10 ng/ml) causes an increase in the expression of AGT gene twofold compared with untreated cells (controls) in both groups (controls: 1 vs. LPS: 2; P=0.002; controls: 1 vs. LPS: 2.23; P ≤0.001) respectively. In contrast, the treatment of AbdSc adipocytes with LPS in the presence of SN50 (50 μg/ml) reduced the expression of the AGT gene compared with treated cell in both groups (LPS: 2 vs. SN50+LPS: 1.23; P=0.02; LPS: 2.23 vs. SN50+LPS: 0.87; P ≤0.001) respectively. Moreover, there was a significant change observed in AGT mRNA level in the presence of SN50 alone in overweight and not in obese ± hypertension group (controls: 1 vs. SN50: 0.5; P ≤0.001; controls: 1 vs. SN50: 0.81; P=0.32) respectively. On the other hand, the expression of the AGT gene was increased twofold in LPS-treated cells and reduced in the presence of SN50 alone compared with untreated cells in the combined groups studied (Figure 7), (controls: 1 vs. LPS: 2.10, SN50: 0.65; P ≤0.001, P=0.04) respectively, and significantly reduced two-fold in the presence of SN50 compared with treated cells (LPS: 2.10 vs. SN50+LPS: 1.20; P=0.02).
Figure 6. mRNA expression of angiotensinogen (AGT) levels among overweight subjects and obese subjects with and without hypertension in cultured human AbdSc adipocytes. Total RNA was isolated from AbdSc adipocytes from (a) overweight subjects (n = 10) and (b) obese ± hypertension subjects (n = 10) treated with LPS (10 ng/mL) in the presence and absence of SN50 (50 μg/mL) at 12 h of incubation. Quantitative RT-PCR was performed using a premade TaqMan probe for AGT. The quantitative fold changes in mRNA expression were determined as relative to 18S mRNA levels in each corresponding group and calculated using the 2-ΔΔCT method. Statistical analysis was undertaken using the independent sample t-test. * p < 0.05 was considered as significant versus untreated cells. * P-value = 0.02, ** p-value = 0.002 (overweight), *** p-value ≤ 0.001 (obese ± hypertension).

Figure 7. mRNA expression of AGT levels in cultured human AbdSc adipocytes in the studied combined group. Total RNA was isolated from AbdSc adipocytes treated with LPS (10 ng/mL) in the presence and absence of SN50 (50 μg/mL) at 12 h of incubation. Quantitative RT-PCR was performed using a premade TaqMan probe for AGT. The quantitative fold changes in mRNA expression were determined as relative to 18S mRNA levels in each corresponding group and calculated using the 2-ΔΔCT method. Statistical analysis was undertaken using the independent sample t-test. * p < 0.05 was considered as significant versus untreated cells. * P-value = 0.04, *** p-value ≤ 0.001.
3.6. SN50 Reduces ANG Protein in LPS-Stimulated Adipocytes

LPS alone slightly increases AGT protein levels compared with those in control cells. Coincubation with LPS and SN50 resulted in a slight reduction of AGT protein levels 24 hours (Figure 8).

![Figure 8. Angiotensinogen antibody by western blot. Adipocyte cells were treated with LPS at 10 ng/mL in the presence and absence of SN50 (50 μg/mL) at 24 h of incubation. Proteins (20 μg per lane) were separated by 12% SDS-PAGE and analyzed by western blotting using the anti-AGT antibody. The loading equality was controlled using the antibody against the β-actin protein.](image)

4. Discussion

Despite the high prevalence of obesity and hypertension in Saudi Arabia, no study has examined the relationships between pro-inflammatory adipokines and blood pressure in the obese, hypertensive phenotype and the role of signaling pathways in developing hypertension in human adipose tissue. As obesity and hypertension are increasingly considered to produce subclinical chronic inflammation, the pro-inflammatory adipokines' position becomes increasingly essential to understand [14]. Our study focused on the NF-κB pathway as one signaling pathway contributing to the upregulation of pro-inflammatory adipokines such as TNF-α and RAS components like AGT in human adipose tissue, whose secretions in adipose tissue are coordinated through NF-κB activation. Harvested and isolated abdominal subcutaneous adipose tissue was used for this study.

Adipose tissue plays an essential role in the secretion of certain pro-inflammatory adipokines, and activation of these adipokines is coordinated through the NF-κB-dependent pathway [4, 15–17]. The present study examined, in vitro, the effect of an NF-κB blocker (SN50) on TNF-α secretion as well as mRNA, TNF-α protein, and AGT expression in primary human adipocytes, which were isolated from overweight subjects and obese subjects with and without hypertension and treated with 10 ng/mL LPS as a potent stimulant pathogen at different concentrations and time points.

Our findings demonstrated that LPS significantly stimulates NF-κB activation in a concentration higher or equivalent to Jurkat cells (positive control cells) in both groups. Lipopolysaccharide also increases NF-κB p65 protein levels compared with those in control cells at 3 and 12 h and did not affect 24 h. In contrast, coincubation with LPS and SN50 resulted in a marked reduction in NF-κB p65 levels than those treated only with LPS. However, SN50 alone did not affect LPS through NF-κB activation and expression. Our findings suggest that SN50 at least in part suppresses NF-κB-mediated inflammatory pathways in adipose tissue. Another interesting finding is the relationship between NF-κB activation and the degree of adiposity. Our study observed a strong relationship between body mass index (BMI) and NF-κB p65, which remained significant independent of age.

Liposaccharide is a well-preserved component of the external part of the Gram-negative bacterial cell wall [18]. The innate immune system recognizes this molecule via Toll-like receptors (TLRs), a class of proteins that play a crucial role in the natural immune
system, recognizing antigens, including LPS, on monocyte/macrophage activation. Activation of TLRs (particularly TLR-4) leads to translocation of NF-κB into the nucleus to initiate gene expression of cytokines like IL-1, IL-6, and TNF-α [18–20].

Numerous studies have shown that the endotoxin LPS has a potent inflammatory stimulant on cytokine secretion through NF-κB activation [4, 6]. Hence, our study addressed the principal activation regulatory pathways of the secretion of TNF-α by LPS via NF-κB activation. Treatment of adipocytes with 10 ng/mL LPS caused a significant increase in TNF-α secretion. In contrast, a significant decrease occurred in response to the presence of SN50 as compared with those cells treated with LPS at 3, 12, and 24 h. The maximum inhibitory action of SN50 on NF-κB activation was obtained with a concentration of 50μg/mL without causing cell death, which was observed at 12 h after treatment. This effect gradually decreased in both groups. In contrast, SN50 alone did not affect TNF-α secretion compared with control cells. Moreover, we observed that TNF-α secretion was significantly higher in the obese hypertensive group than in the overweight group.

The secretion of pro-inflammatory adipokines from adipose tissue has been studied by previous authors [4,21,22]. Adipocytes are known to secrete large quantities of IL-6 and non-negligible amounts of TNF-α compared to macrophages [21, 22]. There is increasing evidence supposing that adipocytes are highly implicated in the inflammatory phenomenon associated with obesity-related hypertension. However, Hoareau et al. [18] have shown that macrophages are more sensitive to LPS than adipocytes, responding to 5 ng/mL of LPS more than adipocytes, which are sensitive to 50 ng/mL LPS. The number of TLR4 on the cells’ surface could explain these differences in response between adipocytes and macrophages [18]. The production of TNF-α by adipocytes may be of particular importance because up to one-third of circulating TNF-α is secreted by adipose tissue [23]. An increase in central (visceral) adiposity confers a higher metabolic risk. This increased metabolic risk is associated with subclinical inflammation.

Numerous studies have noted this mechanism, demonstrating that LPS can stimulate the release of pro-inflammatory cytokines such as TNF-α via NF-κB activation [18,6]. They observed that TNF-α production in human adipocytes is dependent on the NF-κB pathway. Lehrke et al. [6] showed that LPS increases resistin production by inducing secretion of TNF-α. This increase in resistin production can be blocked by aspirin and rosiglitazone drugs with a dual anti-inflammatory and insulin-sensitizing action. They have been shown to antagonize NF-κB activity. Indeed, loss of NF-κB function abolishes LPS induction of resistin [6]. In a study of mice’s adipocytes, it was found that resistin caused insulin resistance and glucose intolerance [24], and the mice, who were lacking resistin, had low blood glucose levels [25]. The ability of resistin to modulate glucose metabolism is associated with the activation of SOCS3, an inhibitor of insulin signaling in adipocytes [24]. Regarding its effect on glucose metabolism, an increase in serum resistin also predicted the risk for increased systolic and diastolic BP in patients with type 2 diabetes mellitus (T2DM) independently of age, gender, BMI, fasting, blood glucose, and HDL-cholesterol [26].

Zhang et al. [27] showed that resistin can predict the risk of future hypertension among non-diabetic women aged ≥55 years, even after adjustment for inflammatory and endothelial markers, and can promote endothelial cell activation through the release of ET-1 and up-regulation of VCAM-1 and ICAM-1 [28]. Resistin also increases TNF-α and IL-6 expression in white adipose tissue [29].

NF-κB plays a role in regulating gene transcription, and the present study reported this role in the mRNA TNF-α and AGT levels of overweight patients and obese patients with and without hypertension at 12 h. Treatment of AbdSc adipocytes with LPS increased the mRNA TNF-α level twofold compared to those in untreated cells in both groups. In contrast, the treatment of AbdSc adipocytes with LPS in the presence of SN50 caused a decrease in the mRNA TNF-α level compared with those LPS-treated overweight subjects. In contrast, no significant change was observed in mRNA TNF-α level in obese
subjects without hypertension. Moreover, SN50 alone did not affect TNF-α mRNA levels in both of the experimental groups.

In contrast, our study demonstrated that the mRNA TNF-α level increased twofold in LPS-treated subjects compared with control cells in the combined group and significantly decreased in the presence of SN50. In addition to its effect on gene expression, LPS also slightly increased the TNF-α protein levels compared with those in control cells. In contrast, coincubation with LPS and SN50 resulted in a reduction in TNF-α protein levels at 24 h.

Similarly, treatment of AbdSc adipocytes with LPS caused a twofold increase in the mRNA AGT level than those in control cells in both groups. In contrast, the treatment of AbdSc adipocytes with LPS in the presence of SN50 caused a decrease in the mRNA AGT level. Moreover, the mRNA AGT was increased twofold in LPS-treated subjects and reduced in SN50 compared with LPS-treatment in the combined group. LPS alone also slightly increased the AGT protein levels compared with those in control cells. Coincubation with LPS and SN50 resulted in a slight reduction in AGT protein levels at 24 h. Thus, LPS can cause an inflammatory status in adipocytes, and the provocative quality led to increased TNF-α via NF-κB and increased AGT and ANG II via RAS. The blocking of NF-κB activation by SN50 led to a decrease in the inflammatory status, responsible for obesity and co-morbidities such as hypertension.

5. Conclusions

Our study clearly demonstrated that the LPS-induced activation pathway may be an integral part of the inflammatory process in white adipocytes linked to obesity and obesity-related complications. This stimulatory action seems to be mediated via the NF-κB activation. Thus, the NF-κB inflammatory pathway may represent a regulator of the inflammatory processes in obesity-related hypertension. Taken together, these adipokines with NF-κB activation may represent important biomarkers to evaluate hypertension risk and may provide mechanistic insight into the pathogenesis of obesity-related hypertension.

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Data Availability Statement: All requests for data access should be addressed to the corresponding author. Proposals requesting data access will have to specify how they plan to use the data.

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