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［原著］

Human neutrophil elastase alters the chemokine profile of human visceral adipocytes through induction of nuclear factor kappa B repressing factor

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Abstract

Human neutrophil elastase (HNE) induces NF-κB repressing factor (NKRF), which inhibits chemokine synthesis. Obesity is a state of low-grade chronic inflammation and chemokine expression in inflamed adipose tissue is mainly mediated by nuclear factor kappaB (NF-κB). We hypothesized that chemokine expression would differ between human visceral adipocytes cultured with or without HNE due to the influence of NKRF.

NKRF was detected in adipocytes from subjects with a body mass index (BMI) >30 kg/m^2 by western blotting and was upregulated by incubation with HNE, whereas NKRF was not detected in adipocytes from subjects with a BMI <30 kg/m^2. Adipocytes from both groups of subjects expressed mRNA for monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), epithelial cell-derived neutrophil-activating peptide-78 (ENA-78), and interleukin-8 (IL-8). However, only adipocytes from subjects with a BMI >30 kg/m^2 expressed mRNA for lymphotactin-β and fractalkine. Incubation with HNE downregulated the expression of MCP-1 and RANTES mRNA and inhibited MCP-1 protein production by adipocytes from subjects with a BMI >30 kg/m^2. Pretreatment of adipocytes with U73122 (a phospholipase C inhibitor) further reduced MCP-1 mRNA expression and MCP-1 protein levels after incubation with HNE. While PAR-2 agonists (LIGRLO and A264613) did not reduce MCP-1 mRNA expression, silencing of PAR-2 blunted this response to HNE. In conclusion, HNE may modulate interactions between innate and adaptive immunity by altering the chemokine profile of visceral adipocytes.

Keywords: body mass index, chemokine, monocyte chemotactic protein-1, neutrophil elastase, nuclear factor-kappaB repressing factor, visceral adipocyte

Ⅰ. Introduction

Neutrophils are the first line of defense of the innate immune system against infections. Recent studies have suggested that neutrophils also play additional roles in the immune response by producing chemokines that attract other immune cells to infectious foci and cytokines that modulate both innate and adaptive responses[1)]. Moreover, there is evidence that some cytokines produced by neutrophils may have a direct regulatory effect on the subsequent adaptive immune response[2)]. Thus, neutrophils play an integrated role in activation and regulation of the innate and

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adaptive immune systems[3, 4].

Morbid obesity is associated with activation of the innate immune response. Neutrophils transiently infiltrate intra-abdominal fat early in the course of feeding with a high-fat diet. This infiltration of neutrophils into adipose tissue precedes macrophage infiltration and is mediated by neutrophil CD11b and adipocyte ICAM-1[5]. Neutrophils express a functional leptin receptor and leptin stimulates neutrophil chemotaxis and oxidative bursts[6]. In addition, apoptotic pathways are inhibited in neutrophils by activation of the leptin receptor[7]. Infiltration of neutrophils into white adipose tissue occurs early in the development of dietary obesity and is persistent[5, 8]. Insulin resistance associated with obesity is a major risk factor for type 2 diabetes and cardiovascular disease, and chronic inflammation characterized by T cell and macrophage infiltration of visceral adipose tissue is a hallmark of obesity-associated insulin resistance and glucose intolerance. Moreover, insulin resistance in obese persons is associated with a unique IgG autoantibody profile, suggesting the importance of B cells and adaptive immunity in the mechanisms underlying resistance to insulin[9]. Interestingly, cells of the adaptive immune system, specifically B and T lymphocytes, have emerged as unexpected promoters and regulators of insulin resistance[10]. This finding suggests the existence of a link between innate and adaptive immunity through cell-mediated and humoral immune responses. Thus, chemokines are key players in linking innate immunity with adaptive immunity[11, 12], while neutrophils are involved in the activation, regulation, and effector functions of innate and adaptive immune cells. Neutrophil effector molecules include a broad array of cytokines and extracellular traps. It has also been reported that neutrophils mediate insulin resistance in mice fed a high-fat diet through secretion of elastase[8]. Human neutrophil elastase (HNE) represses the synthesis of IL-8 by human airway smooth muscle cells through induction of NF-κB repressing factor (NKRF)[13]. NKRF is a transcriptional silencing protein that specifically counteracts the basal activity of several NF-κB-dependent promoters by binding directly to specific neighboring DNA sequences[14]. HNE induces NKRF, which inhibits chemokine synthesis by peripheral blood mononuclear cells and alveolar macrophages[15]. Thus, HNE may modulate chemokines produced by the target genes of NF-kB. The present study was performed to investigate the influence of NKRF induced by HNE on the chemokine profile of human visceral adipocytes.

Ⅱ. Materials and Methods

1. Ethics Statement

Human cryopreserved preadiocytes were obtained from Zen-Bio, Inc. (Research Triangle Park, NC). The study utilizing human preadipocytes was approved by the Kumamoto Health Science University Institutional Review Board.

2. Chemicals and reagents

HNE with an activity of 0.2 U/mL was purchased from SERVA Electrophoresis (Heidelberg, Germany). U73122 (Merck Millipore, Bedford, MA) and two protease-activated receptor (PAR)-2 agonists (AC- 264613 [2-oxo-4-phenylpyrrolidine-3-carboxylic acid [1- (3-bromophenyl)-(E/Z)-ethylidene]-hydrazide], Tocris Bioscience, Bristol, UK and 2-furoyl-LIGRLO-amide, Tocris Bioscience) were purchased to study the intracellular signal transduction pathways involved in PAR-2 stimulation. All reagents and chemicals were negative for endotoxin, as determined by an Endospecy test [16].

3. Human visceral adipocytes

Human visceral preadipocytes were supplied by Zen-Bio, Inc. (Research Triangle Park, NC), cultured and differentiated into adipocytes according to the company's instructions. Briefly, these fibroblast-like precursor cells were

cryopreserved at the end of primary culture and thus could be propagated for two passages prior to differentiation into human adipocytes. In brief, human visceral preadipocytes (2.5×10^4 cells/well) were cultured in 12-well plates containing preadipocyte medium (DS Pharma Biomedical Co., Ltd., Suita, Japan) in a humidified incubator at 37℃ under 5% CO_2. At 24 h after plating, cells were checked for confluence and differentiation was induced with the differentiation medium according to the manufacturer's instructions. At 2 weeks after the initiation of differentiation, cells were rounded with large lipid droplets in the cytoplasm. These cells were considered to be mature adipocytes.

4. Extraction of RNA and reverse transcription polymerase chain reaction (RT-PCR).

Visceral adipocytes (2.5×10^4 cells) were extracted with 1 ml of ISOGEN RNA kit (NIPPON GENE, Toyama, Japan). Total RNA was isolated and precipitated according to the manufacturer's instructions, after which 200 ng of total RNA was reverse-transcribed using Origo dT primer with PrimeScript™ RT reagent Kit (TAKARA BIO, Shiga, Japan). Then reverse-transcribed RNA was amplified by PCR using an TaKaRa PCR thermal cycler (TAKARA BIO). The chemokine production profile of visceral adipocytes was determined by RT-PCR. In addition, visceral adipocytes were incubated with HNE (0 or 50 μM) for 6 hours, after which chemokine mRNA expression was analysed by RT-PCR. The primer sequences used were as follows: adiponectin forward,
5'-AGACTGGGCCTCCTGAATTT-3', and reverse,
5'-TGTTCAGAATTTAAAACTGTAAGCAAA-3'; forward,
5'-AGAAGCCTTATTGGTAAGGTT-3' and reverse
5'-AACATCATGACAGGTGGTGAT-3' (PAR-2) ; forward,
5'-CAAACTGAAGCTCGCACTC-3' and reverse
5'-CATTTCCACAATAATATTTTAG-3' (MCP-1) ; forward,
5'-CTACTCGGGAGGCTAAGGCAGGAA-3' and reverse
5'-GAGGGGTTGAGACGGCGGAAGC-3' (RANTES) ; forward
5'-ATCTCCGCTCCTCCACCCAGT-3' and reverse
5'-TTCTTGTCTTCCCTGGGTTCAGA-3' (ENA-78) ; forward
5'-GCTTTCTGATGGAAGAGAGC-3' and reverse
5'-GGCACAGTGGAACAAGGACT-3 (IL-8) ; forward
5'-TCTGCTCTCTCACTGCATAC-3' and reverse
5'-CAGCTGTATTGGTCGATTGC-3' (Lymphotactin-β) ; forward
5'-AGGAGAATGCTCCGTCTGAA-3' and reverse
5'-AGAAGAGGAGGCCAAGGAAG-3 (Fractalkine) ; forward
5'-CTTGTGTAGAGATATAGCCAGG-3' and reverse
5'- GGGAACAAAAGTGAATGTGACC-3' (Tissue factor (TF)) ; forward
5'-GTGGGGCGCCCCAGGCACCA-3' and reverse
5'-CTCCTTAATGTCACGCACGATTTC-3' (beta-Actin). The PCR conditions were as follows: for adiponectin, 40 cycles (94℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds), PAR-2, 45 cycles (94℃ for 60 seconds, 60℃ for 60 seconds, 72℃ for 60 seconds), MCP-1, 35 cycles (94℃ for 60 seconds, 55℃ for 60 seconds, 72℃ for 60 seconds), RANTES, 45 cycles (94℃ for 60 seconds, 60℃ for 60 seconds, 72℃ for 60 seconds), ENA-78, 45cycles (94℃ for 60 seconds, 60℃ for 60 seconds, 72℃ for 60 seconds), IL-8, 35 cycles (94℃ for 60 seconds, 55℃ for 60 seconds, 72℃ for 60 seconds), Lymphotactin-β, 45 cycles (94℃ for 60 seconds, 60℃ for 60 seconds, 72℃ for 60 seconds), Fractalkine, 45 cycle (94℃ for 60 seconds, 60℃ for 60 seconds, 72℃ for 60 seconds), TF, 35 cycles (94℃ for 30

seconds, 55 ℃ for 30 seconds, 72 ℃
for 30 seconds), beta-Actin, 40 cycles (94 ℃ for 30 seconds, 60 ℃ for 30 seconds,
72 ℃ for 30 seconds) were used. The PCR products were analyzed on agarose gels.

5. Protease-activated receptor (PAR)-2 expression by visceral adipocytes

G protein-coupled receptor signaling regulates obesity[17] and modulates macrophage-dependent inflammation of adipose tissue[18]. PAR-2 signals via G protein for activation. PAR-2 mRNA levels were investigated by RT-PCR to explore whether exposure to HNE (50 μM) influenced PAR-2 mRNA expression by visceral adipocytes.

6. Chemokine profile of visceral adipocytes

Expression of mRNA for monocyte chemoattractant protein-1 (MCP-1/CCL2), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), epithelial cell-derived neutrophil-activating peptide-78 (ENA-78/CXCL5), IL-8 (CXCL8), lymphotactin-β (XCL2), and fractalkine (CX3CL1) was investigated in visceral adipocytes from subjects with a BMI <30 kg/m^2 or >30 kg/m^2 to assess differences of the chemokine profile.

7. Western blotting for NF-κB repressing factor (NKRF)

Adipocytes from subjects with a BMI <30 kg/m^2 or >30 kg/m^2 were treated with HNE (0 or 50 μM) for 6 hours and then NKRF was detected by Western blotting. Equivalent amounts of whole-cell lysates from adipocytes were subjected to electrophoresis and the products were transferred to polyvinylidene difluoride membranes. Then the membranes were incubated with 1.0×10^3 μg/L rabbit anti-human NF-κB repressing factor IgG (Abcam Inc., Cambridge, MA), washed, and incubated with alkaline phosphatase-conjugated guinea pig anti-rabbit IgG (Abcam Inc.) diluted to 1:4000. Next, the membranes were incubated with a chemiluminescence enhancer (Immun-Star, Bio-Rad, Hercules, California) and exposed to XAR film (Kodak, Rochester, NY). After the film was developed, bands were quantified with a densitometer and ImageQuant software (Molecular Dynamics, Sunnydale, CA).

8. Effect of HNE on MCP-1 mRNA expression by visceral adipocytes

Visceral adipocytes obtained from subjects with a BMI <30 kg/m^2 or >30 kg/m^2 were incubated with or without HNE (50 μM) for 6 h and then the level of MCP-1 mRNA was determined by reverse transcription and polymerase chain reaction (RT-PCR). Differential effect of HNE on MCP-1 and tissue factor expression by visceral adipocytes.

Visceral adipocytes from subjects with a BMI >30 kg/m^2 were incubated with or without HNE (50 μM) for 6 h, after which MCP-1 and tissue factor (TF) mRNA levels were determined by RT-PCR.

9. Effect of PAR-2 agonists on MCP-1 mRNA expression by visceral adipocytes

A native peptide agonist (neutrophil elastase), a synthetic peptide agonist (2-furoyl-LIGRLO-amide), or a non-peptide (AC-264613) agonist were employed for this experiment. Visceral adipocytes from subjects with a BMI >30 kg/m^2 were incubated with or without HNE (50 μM) for 6 hr. Adipocytes pretreated with U73122 (10 mM) were also incubated with HNE (50 μM). LIGRLO (1 mM) and AC- 264613 (5 mM) were utilized to investigate the influence of PAR-2 on chemokine production.

10. Enzyme-linked immunosorbent assay (ELISA) for MCP-1

Visceral adipocytes from subjects with a BMI >30 kg/m^2 were incubated with or without HNE (50 μM) for 6 h. Then the level of MCP-1 protein in whole cell lysates was measured by ELISA (Abcam, Inc., Cambridge, MA) with an anti-MCP-1 monoclonal antibody.

11. Effect of silencing PAR-2 on MCP-1 production by visceral adipocytes after stimulation with HNE

siRNA for PAR-2 was purchased from Santa Cruz Biotechnology. Transfection of mature visceral adipocytes from subjects with a BMI >30 kg/m^2 was performed using siRNA for PAR-2 (50 nM) and Lipofectamine™ RNAiMAX (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The transfected adipocytes (2.5×10^4 cells) were incubated with or without HNE (50 μM) for 6 hr and MCP-1 protein levels in whole-cell lysates were determined by ELISA with an anti-MCP-1 monoclonal antibody (Abcam).

12. Statistical analysis

Results are expressed as the mean ± SE. Analysis of variance and the *t*-test of independent means were used to assess differences between multiple group and differences between two groups, respectively. When the F ratio was significant, mean values were compared by using a post hoc Bonferroni test. A P value <0.05 was considered to indicate a significant difference in all analyses.

Ⅲ. Results

Western blotting revealed NKRF expression in visceral adipocytes from subjects with a BMI >30 kg/m^2, whereas it was not found in adipocytes from subjects with a BMI <30kg/m^2. Interestingly, incubation with HNE upregulated NKRF expression by adipocytes from subjects with a BMI >30 kg/m^2 (Fig. 1). Visceral adipocytes from subjects with a BMI >30 kg/m^2 expressed mRNA for monocyte chemoattractant protein-1 (MCP-1/CCL2), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), epithelial cell-derived neutrophil-activating peptide-78 (ENA-78/CXCL5), interleukin-8 (IL-8/CXCL8), lymphotactin-β (XCL2), and fractalkine (CX3CL1) . Expression of MCP-1 and RANTES mRNA repressed significantly after exposure of adipocytes to HNE (50 μM), whereas beta-Actin was not affected. PAR-2 mRNA expression was not affected by exposure to HNE (Fig.2). Although visceral adipocytes from subjects with a BMI <30 kg/m^2 also showed expression of MCP-1, RANTES, ENA-78, and IL-8 mRNA, neither lymphotactin-beta nor fraktalkine mRNA was detected. Incubation of these cells with HNE did not repress the MCP-1 or RNTES mRNA level (Fig. 3). Next, we investigated the effects of HNE on expression of chemokines, MCP-1, and the prothrombotic molecule tissue factor (TF) by adipocytes from subjects with a BMI >30 kg/m^2. TF expression was detected in unstimulated adipocytes. Exposure of these cells to HNE caused a repression of MCP-1 mRNA expression, whereas TF mRNA was upregulated. Protein level of MCP-1 was significantly decreased in the lysates of adipocytes (2.5×10^4 cells) incubated with HNE (50 μM) compared to the lysates of adipocytes incubated without HNE (Fig.4). Pretreatment of visceral adipocytes from subjects with a BMI >30 kg/m^2 by a phospholipase C inhibitor (U73122) led to a further decrease of MCP-1 mRNA after exposure of the cells to HNE. In contrast, PAR-2 agonists (2-furoyl-LIGRLO-amide and AC-264613) did not reduce MCP-1 expression (Fig. 5). Finally, silencing of PAR-2 did not blunt MCP-1 production by visceral adipocytes stimulated with HNE (Fig. 6).

Ⅳ. Discussion

Expression of NKRF was detected by western blotting in visceral adipocytes from subjects with a BMI >30 kg/m^2, whereas it was not found in adipocytes from subjects with a BMI <30 kg/m^2. Incubation with HNE upregulated NKRF expression in visceral adipocytes from subjects with a BMI >30 kg/m^2. Interestingly, HNE (50 μM) significantly decreased expression of MCP-1 and RANTES mRNA by visceral adipocytes from subjects with a BMI >30 kg/m^2 and both MCP-1

and RANTES are direct targets of NF-κB [19, 20]. In contrast, neither MCP-1 nor RANTES mRNA was downregulated in visceral adipocytes from subjects with a BMI <30 kg/m^2, which is why NKRF was not detected in these visceral adipocytes by western blotting.

Serine proteases have various important physiological roles that are mediated via G protein-coupled protease-activated receptors (PARs) and PAR-2 is a trypsin-activated member of the G-protein-coupled PAR family[21]. Neutrophil elastase may influence cell signaling by targeting PAR-2[22]. Next, we investigated the influence of PAR-2 agonists on MCP-1 mRNA expression using 2-furoyl-LIGRLO-NH_2, a PAR-2-activating peptide with modification of the N-terminal furoyl group that increases intracellular calcium more potently than SLIGRL-NH_2[23] and has been shown to be a selective agonist of PAR-2 over PAR-1 [24]. We also used AC 264613 [2-oxo-4-phenylpyrrolidine-3-carboxylic acid [1-(3-bromo-phenyl)-(E/Z)-ethylidene]-hydrazide], a low molecular weight PAR-2 agonist, stimulates hydrolysis of phosphatidylinositol and Ca^{2+} mobilization, and does not act on other PAR receptor subtypes[25]. We found that PAR-2 agonists (2-furoyl-LIGRLO-NH_2 and AC-264613) did not reduce MCP-1 mRNA expression in visceral adipocytes from subjects with a BMI >30 kg/m^2. It has been reported that U73122 (a phospholipase C inhibitor) suppresses phosphorylation of p65 and its translocation into the nucleus. Thus, activation of NF-κB is inhibited by U73122[26], which explains why we found that U73122 caused a further decrease of MCP-1 mRNA expression in cells exposed to HNE.

It has been reported that expression of IL-8 [27], ENA-78[28], lymphotactin-β [29], and fractalkine[30] is associated with increased activity of NF-κB. However, none of these mRNAs showed downregulation by HNE through induction of NKRF. HNE induces the production of MUC5AC mucin via generation of reactive oxygen species (ROS) and activation of transforming growth factor (TGF)-alpha-dependent epidermal growth factor receptor (EGFR). Tumor necrosis factor (TNF)-alpha-converting enzyme (TACE) cleaves pro-TGF-alpha to release soluble TGF-alpha and is activated by ROS[31]. TACE (also known as ADAM metallopeptidase domain 17: ADAM17) is a member of the ADAM (a disintegrin and metalloprotease domain) family[32]. TACE/ADAM17 has been reported to promote transactivation of EGFR in response to HNE. The TACE/TGF-α /EGFR axis regulates IL-8/CXCL8[33] and ADAM17 regulates ENA-78/CXCL5[34]. The membrane-bound form of fractalkine is generated by ADAM17 together with ADAM10 and[35, 36]. Fractalkine was originally identified on the basis of its sequence homology to lymphotactin (XCL1), a C-type chemokine. It is a transmembrane protein in which the chemokine domain sits on a mucin-like stalk, and the characteristic cysteines are separated by 3 amino acids[37]. The extracellular domain is shed by proteolysis with ADAM10 to produce a soluble form, while shedding induced by phorbol-12-myristate-13-acetate (PMA) is mediated by TACE/ADAM17[35, 37, 38]. Thus, expression of IL-8, ENA-78, lymphotactin-beta, and fractalkine mRNA was upregulated by TACE/ADAM17 in response to incubation of cells with HNE.

In this study, we found that HNE reduced MCP-1 expression. Tumor necrosis factor (TNF)-α has been implicated as a causative factor in obesity-associated insulin resistance and the pathogenesis of type 2 diabetes[39]. Interleukin (IL-4) or IL-10 is a potent inhibitor of TNF- α expression[40, 41]. MCP-1 stimulates IL-4 and IL-10 production[41]. Indeed, MCP-1 is involved in Th2 polarization by IL-4 or IL-10[42]. Interestingly, it was reported that MCP-1-/- mice injected with lipolysaccharide (LPS) showed higher levels of TNF- α compared to LPS-treated MCP-1+/+ mice[43]. Taken together, it is possible that MCP-1 is associated with the Th1/Th2 imbalance which occur in obesity.

V. conclusion

HNE induced NF-kB repressing factor and altered the chemokine profile of human visceral adipocytes, suggesting that it may modulate the relation between innate and adaptive immunity in obesity-related diseases.

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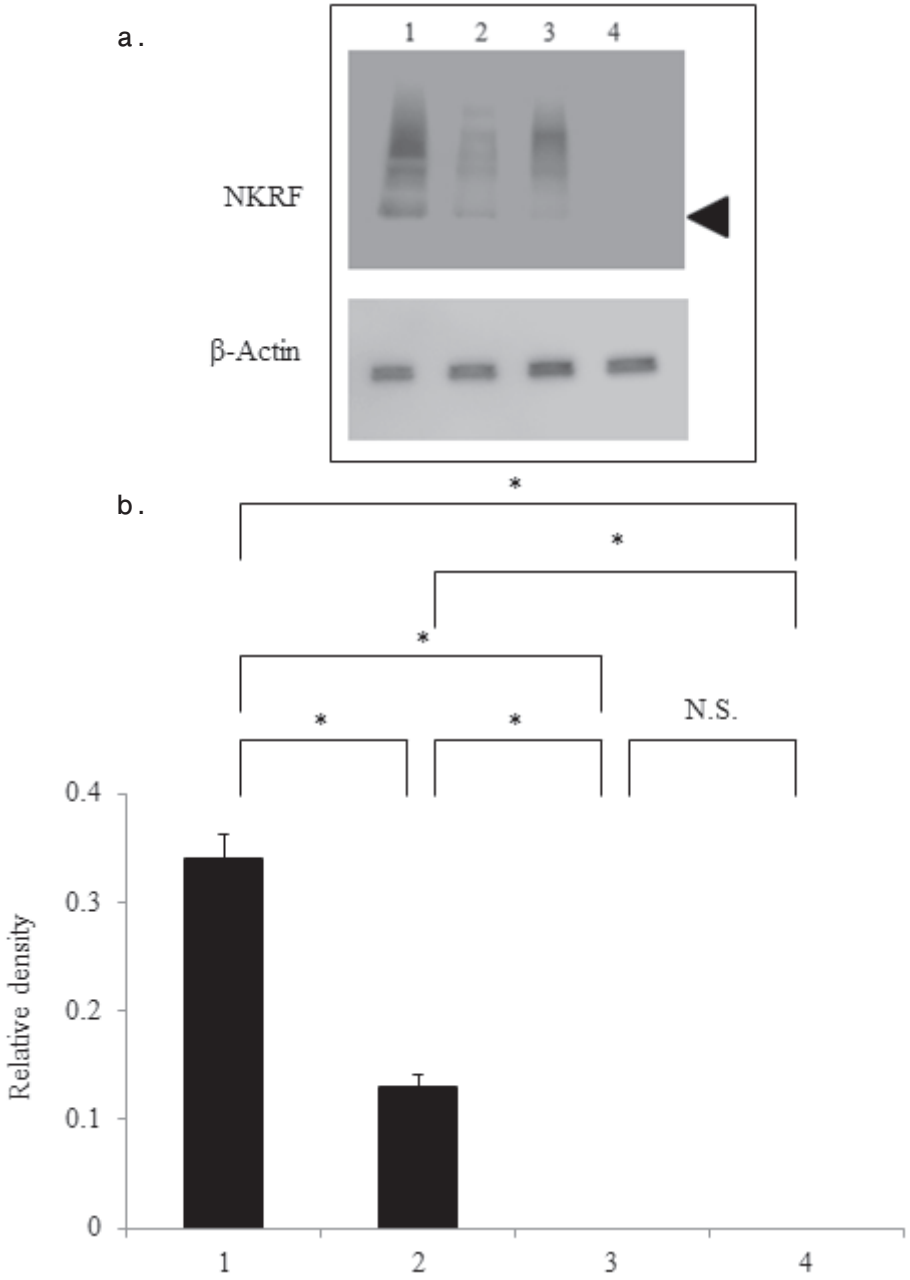


Figure 1. Western blotting for NF- κB repressing factor (NKRF)

After adipocytes from subjects with a BMI >30 kg/m^2 or <30 kg/m^2 were incubated with HNE (50 μM) or without HNE for 6 hours, NKRF was detected by western blotting. Beta-actin was also detected in adipocytes with or without HNE stimulation. The density of each NKRF band was normalized to that of beta-actin.HNE: human neutrophil elastase. Samples were run in triplicate and three separate experiments were performed. (a) Representative western blot. (b) Densitometry data. Data were obtained using cells from three donors in each experiment (mean + SE). *P< .01; N.S., not significant

1. Visceral adipocytes from subjects with a BMI >30 kg/m^2 + HNE (50 μM)
2. Visceral adipocytes from subjects with a BMI >30 kg/m^2 (no HNE)
3. Visceral adipocytes from subjects with a BMI <30 kg/m^2 + HNE (50 μM)
4. Visceral adipocytes from subjects with a BMI <30 kg/m^2 (no HNE)

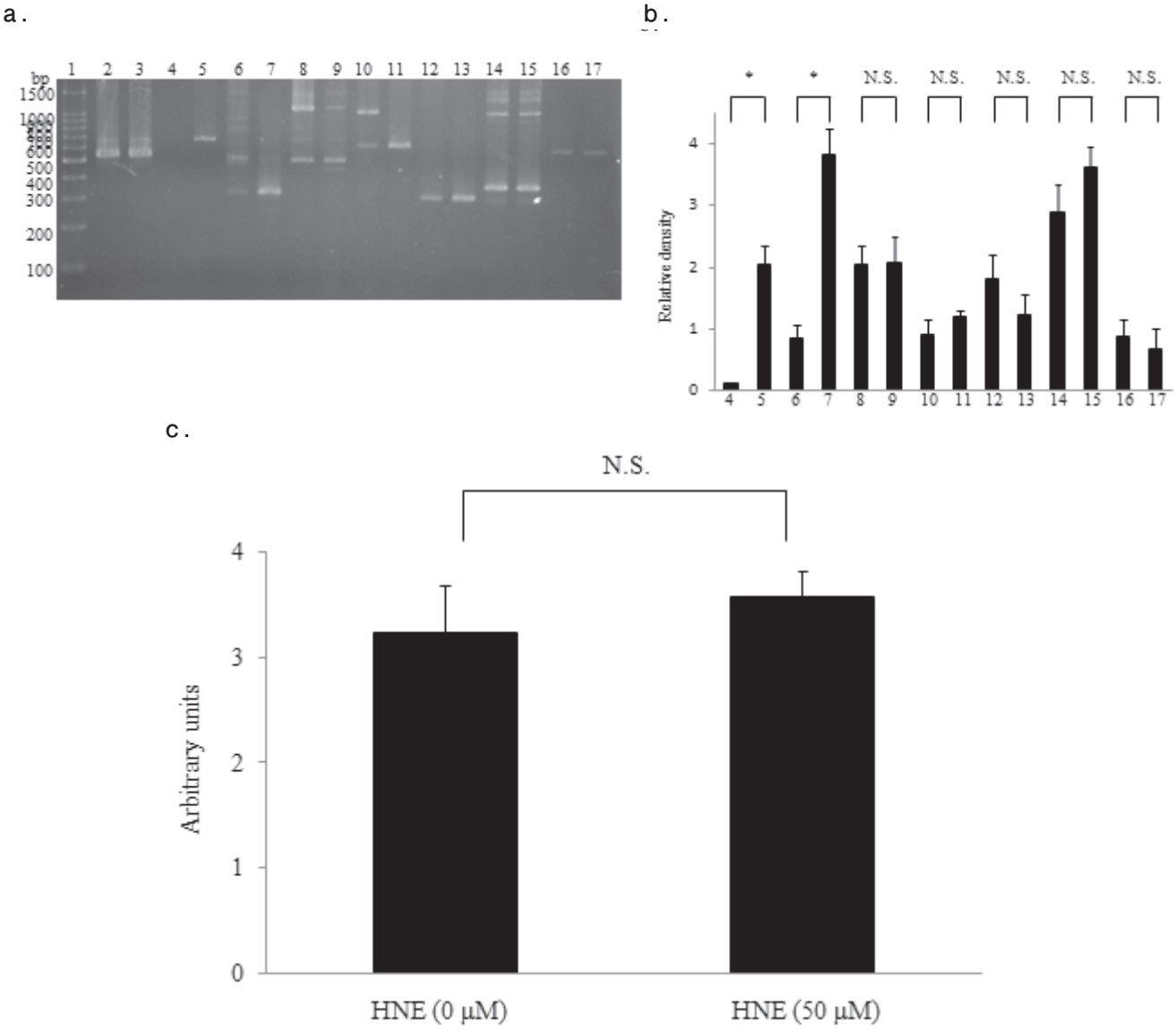


Figure 2. Chemokine profile of visceral adipocytes from subjects with a BMI >30 kg/m^2 after incubation with or without HNE stimulation

a. RT-PCR identified expression of MCP-1, RANTES, ENA-78, IL-8, lymphotactin-beta, and fractalkine mRNA. Expression of MCP-1 and RANTES mRNA was significantly repressed by exposure to HNE (50 μM). HNE had no inhibitory effect on PAR-2 mRNA expression. b. The density of each band was normalized to that of beta-actin. Data were obtained from three donors in each group and represent the mean + SE. *P< .01; N.S., not significant

1. 100 bp ladder
2. β -Actin: HNE (50 μM)
3. β -Actin: HNE (0 μM)
4. MCP-1: HNE (50 μM)
5. MCP-1: HNE (0 μM)
6. RANTES: HNE (50 μM)
7. RANTES: HNE (0 μM)
8. ENA-78: HNE (50 μM)
9. ENA-78: HNE (0 μM)
10. IL-8: HNE (50 μM)
11. IL-8: HNE (0 μM)
12. Lymphotactin-β : HNE (50 μM)
13. Lymphotactin- β : HNE (0 μM)
14. Fractalkine: HNE (50 μM)
15. Fractalkine: HNE (0 μM)
16. PAR-2: HNE (50 μM)
17. PAR-2: HNE (0 μM

c. HNE (50 μM) did not repress beta-Actin mRNA expression. The density of each band was normalized to that of beta-actin. HNE; human neutrophil elastase. Data were obtained from three donors in each group and represent the mean + SE. *P< .01; N.S., not significant

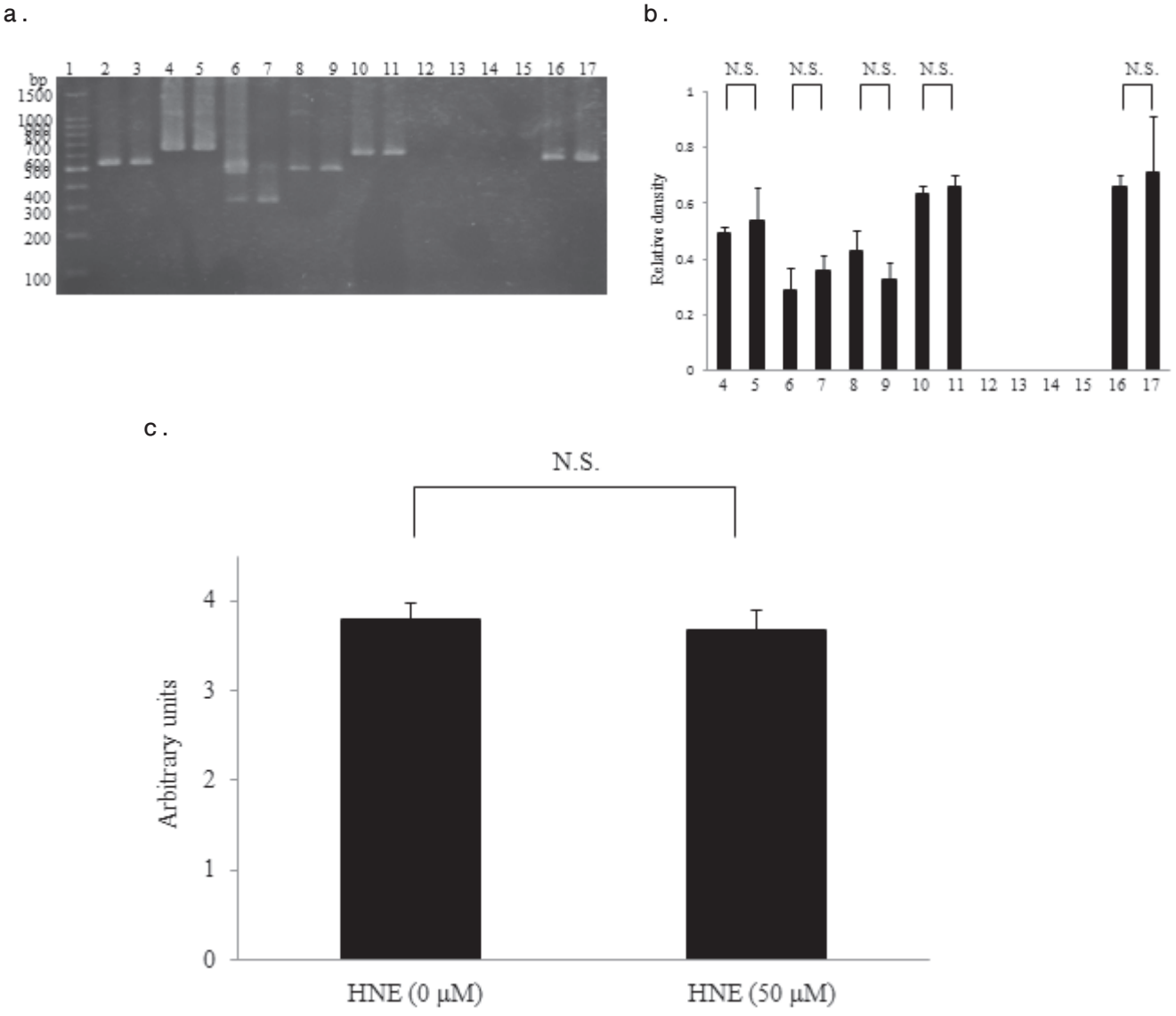


Figure 3. Chemokine profile of visceral adipocytes from subjects with a BMI <30 kg/m² after incubation with or without HNE

a. RT-PCR detected the expression of MCP-1, RANTES, ENA-78, and IL-8 mRNA, but neither lymphotactin-beta nor fractalkine mRNA was detected. MCP-1 mRNA was not repressed by HNE (50 μM).
b. The density of each band was normalized to that of beta-actin. Data were obtained from three donors in each group and represent the mean + SE. N.S., not significant

1. 100 bp ladder
2. β -Actin: HNE (50 μM)
3. β -Actin: HNE (0 μM
4. MCP-1: HNE (50 μM)
5. MCP-1: HNE (0 μM)
6. RANTES: HNE (50 μM)
7. RANTES: HNE (0 μM)
8. ENA-78: HNE (50 μM)
9. ENA-78: HNE (0 μM)
10. IL-8: HNE (50 μM)
11. IL-8: HNE (0 μM)
12. Lymphotactin- β : HNE (50 μM)
13. Lymphotactin- β : HNE (0 μM)
14. Fractalkine: HNE (50 μM)
15. Fractalkine: HNE (0 μM)
16. PAR-2: HNE (50 μM)
17. PAR-2: HNE (0 μM)

b. HNE (50 μM) did not reduce beta-actin mRNA expression. The density of each band was normalized to that of beta-actin. HNE; human neutrophil elastase. Data were obtained from three individuals in each group and represent the mean ± SE. N.S., not significant

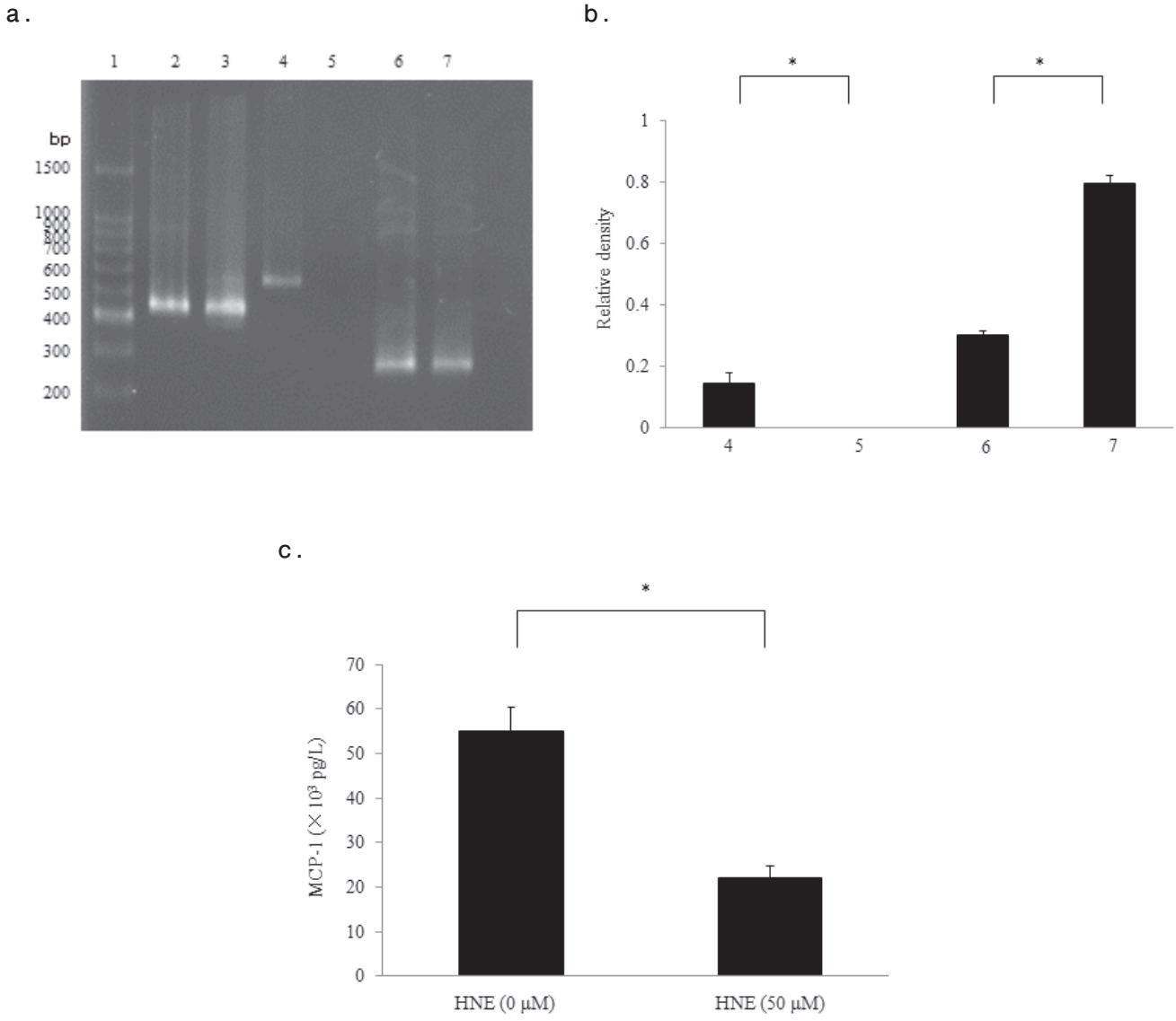


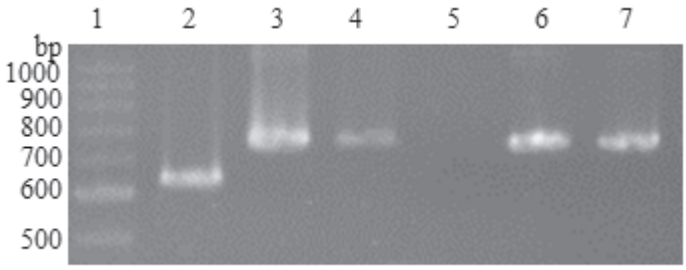
Figure 4. Effect of HNE on MCP-1 and TF mRNA expression by adipocytes from subjects with a BMI >30 kg/m²

a.TF mRNA was detected in adipocytes derived from cryopreserved preadipocytes. HNE (50 μM) repressed MCP-1 mRNA expression, although TF mRNA was upregulated. The density of each band was normalized to that of beta-actin (b). Data were obtained from three donors in each group and represent the mean ± SE. *P< .01; N.S., not significant

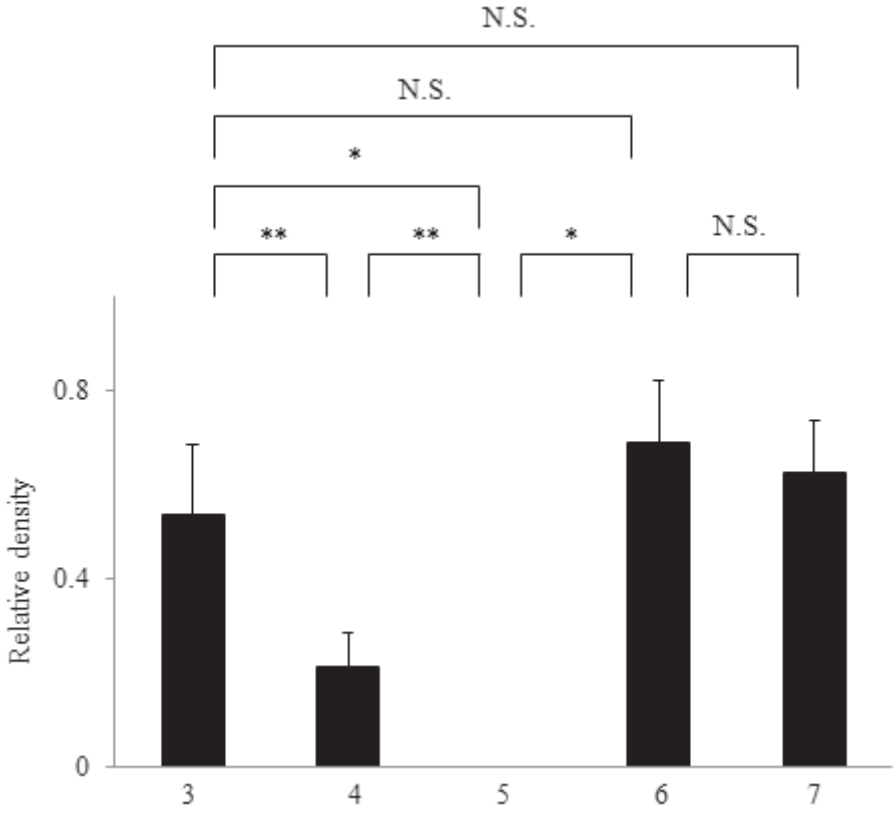
1. 100 bp ladder
2. β -Actin:HNE (0 μM)
3. β -Actin:HNE (50 μM)
4. MCP-1: HNE (0 μM)
5. MCP-1: HNE (50 μM)
6. TF: HNE (0 μM)
7. TF: HNE (50 μM)

c. MCP-1 was significantly decreased in lysates of adipocytes (2.5×10^4 cells) stimulated with HNE (50 μM) compared to lysates of cells cultured without HNE. HNE; human neutrophil elastase. Data were obtained from three individuals in each group and represent the mean ± SE. *P< .01; N.S., not significant

a.


1
2
3
4
5
6
7
bp
1000
900
800
700
600
500

b.


N.S.
N.S.
*
**
**
*
N.S.
Relative density
0.8
0.4
0
3
4
5
6
7

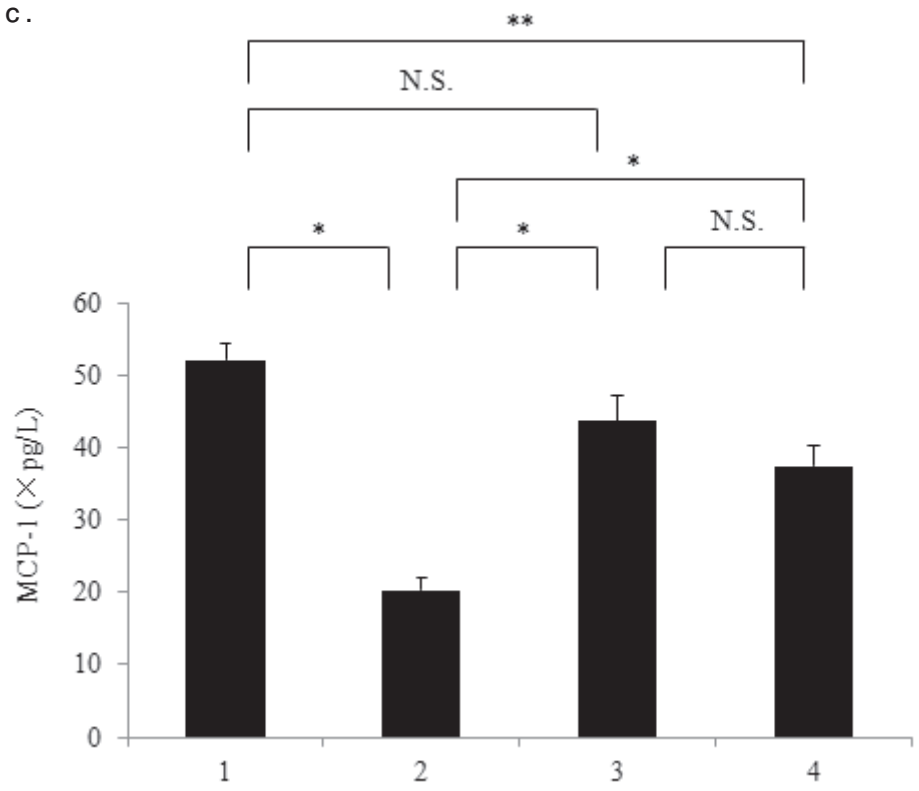


Figure 5. Effect of PAR-2 agonists on MCP-1 mRNA expression by adipocytes from subjects with a BMI >30 kg/m²

Treatment of visceral adipocytes with HNE (50 μM) partially inhibited MCP-1 mRNA expression. Pretreatment of adipocytes with U73122 (10×10^3 mM/L) blunted the decrease of MCP-1 after exposure to HNE. PAR-2 agonists (2-Furoyl-LIGRLO-amide and A264613) did not decrease MCP-1. HNE; human neutrophil elastase. Data were obtained from three donors in each group and represent the mean ±SE. *P< .01; **P< .05; N.S., not significant

a. b.

1. 100 bp ladder
2. β -Actin: HNE (0 μM)
3. MCP-1: HNE (0 μM)
4. MCP-1: HNE (50 μM)
5. MCP-1: U73122 (10mM) + HNE (50 μM)
6. MCP-1: 2-Furoyl-LIGRLO-amide (1 mM)
7. MCP-1: AC 264613 (5 mM)

b. Visceral adipocytes were treated with HNE (50M), 2-Furoyl-LIGRLO-amide (1 mM), or AC 264613 (5 mM) for 6 hr and then protein levels of MCP-1 were determined by ELISA. Data were obtained from three donors in each group and represent the mean ± SE. *P< .01; **P< .05; N.S., not significant

1. no treatment
2. HNE (50 μM)
3. 2-Furoyl-LIGRLO-amide (1 mM)
4. AC 264613 (5 mM)

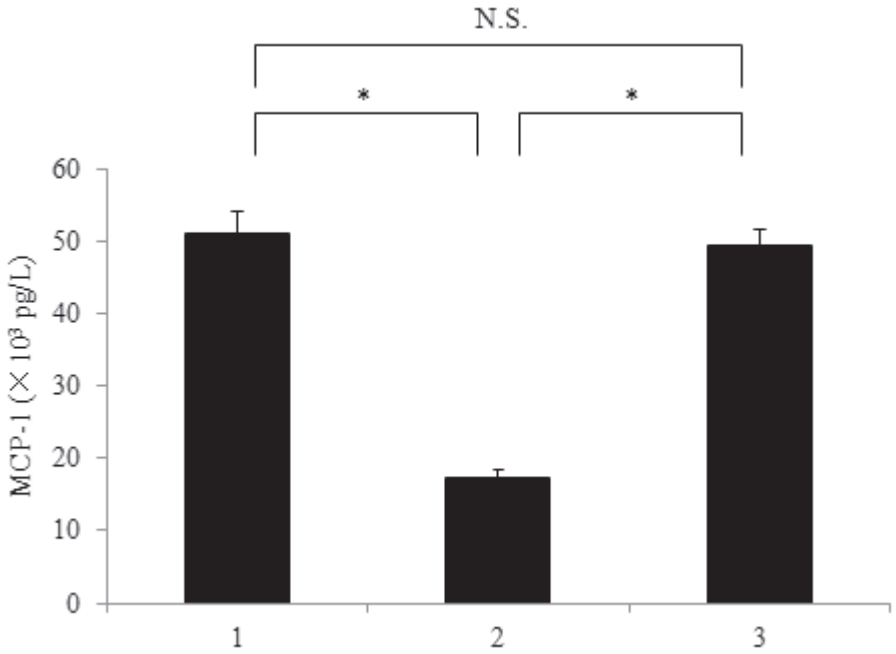


Figure 6. Effect of PAR-2 siRNA on MCP-1 production by visceral adipocytes stimulated with HNE.

Visceral adipocytes were transfected with PAR-2 siRNA and incubated with or without HNE (50 μM) for 6 hr. MCP-1 protein levels in whole-cell lysates were measured by ELISA. HNE: human neutrophil elastase, MCP-1: monocyte chemotactic protein-1, PAR-2: protease-activated receptor 2, siRNA: small interfering RNA. Data were obtained from cells of three donors in each group and represent the mean ± SE. *P< .01; N.S., not significant

1. no treatment
2. HNE (50 μM)
3. PAR-2 siRNA (50 nM) + HNE (50 μM)