

Neutrophil functions in morbidly obese subjects

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Introduction

Obesity is increasingly accepted as a condition characterized by low-grade chronic inflammation [1]. Systemically, this is evidenced by elevated levels of various inflammatory markers, including C-reactive protein, tumour necrosis factor (TNF)- α , interleukin (IL)-6 and IL-8 [2]. Obesity has been identified as a risk factor for infection and poor wound healing following surgical procedures and burns [3,4]. Morbidly obese individuals coincided with low neutrophil bactericidal capacity [5,6]. Adipose tissue in obesity has been shown to be infiltrated by macrophages, at least some of which are of bone marrow origin [7,8]. The close relationship between adipocyte size and the abundance of macrophages in adipose tissue suggests that the influence of adipocyte size on adipocyte function may be conveyed through paracrine pathway involving adipose tissue macrophages [9]. Currently, most

Summary

The present study aimed to determine different peripheral blood neutrophil functions in 18 morbidly obese subjects with body mass index (BMI) ranging between 35 and 69 kg/m² in parallel with age- and gender-matched lean controls. Peripheral blood neutrophil functions of obese subjects and matched lean controls were determined. Neutrophils of obese subjects showed significant elevation of the release of basal superoxides ($P < 0.0001$), formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated superoxides ($P < 0.0001$) and opsonized zymosan (OZ)-stimulated superoxides ($P < 0.045$) compared with lean controls. Interestingly, there were no differences in phorbol myristate acetate (PMA)-stimulated superoxide production by neutrophils of the obese subjects and controls. There was also a significant elevation of chemotactic ($P < 0.0003$) and random ($P < 0.0001$) migration of neutrophils from obese subjects compared with lean controls. Phagocytosis, CD11b surface expression and adherence of neutrophils from obese subjects were not significantly different from those of the lean controls. The elevated superoxide production and chemotactic activity, together with the normal phagocytosis and adherence, suggest that neutrophils from obese subjects are primed and have the capability to combat infections. However, neutrophils in the priming state may participate in the pathogenesis of obesity-related diseases.

Keywords: cell activation, chemotaxis, neutrophils, phagocytosis

myeloid cell types have been implicated in the process, including B cells, various T cell classes and even eosinophils and mast cells [10,11]. In our previous studies [12,13] we demonstrated a significant body weight gain and infiltration of neutrophils to the parenchyma of intra-abdominal adipose tissue early (3 and 7 days) after initiating high-fat feeding of C57BL/6J mice. This early appearance of neutrophils in adipose tissue, which was recently confirmed [14], suggests that adipose tissue inflammation in obesity largely follows the classical inflammation paradigms of acute *versus* chronic inflammatory cell infiltrates. Neutrophils are the body's first line of defence against microorganisms and a critical effector cell in both innate and humoral immunity [15]. There are conflicting reports about their activation state in obese subjects [5,6,16]. Given their importance for host defence and the potential for increased susceptibility of obese subjects to infections, the present study aimed to

determine different neutrophil functions in highly obese subjects.

Methods and procedures

Subjects

Eighteen morbidly obese subjects [body mass index (BMI) 35–68 kg/m²] scheduled to elective laparoscopic surgery (gastric banding) and age- and gender-matched lean healthy controls were enrolled into the study. In some cases, two patients were studied in parallel to one matched lean control. All the subjects were healthy. Diabetic patients were excluded from the study, as glucose has been shown to activate superoxide production by neutrophils [17]. Eight obese people were receiving statin treatment and were analysed in comparison to statin-treated lean controls. The study was approved by the Soroka University Medical Center ethical committee. All subjects signed an informed consent form. Heparinized blood was drawn from the subjects at the time blood tests were performed, as well as from healthy donor lean controls, and were transferred at room temperature for evaluation within 1 h.

Materials

Ferricytochrome c, formyl-methionyl-leucyl-phenylalanine (fMLP), Ficoll-Hypaque and zymosan were purchased from Sigma (St Louis, MO, USA). Percoll was obtained from Pharmacia (Uppsala, Sweden). Sodium dodecyl sulphate (SDS) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Cell-culture media and sera were purchased from Biological Industries (Beit Haemek, Israel).

Preparation of granulocytes

Granulocytes at 95% purity were obtained by Ficoll-Hypaque centrifugation, dextran sedimentation and hypotonic lysis of erythrocytes, as described [18], within 2 h of blood drawn. Cells were counted and their viability was determined by trypan blue exclusion.

Superoxide generation

The production of the superoxide anion by intact monocytes or granulocytes was measured as the superoxide dismutase inhibitable reduction of ferricytochrome c by the microtitre plate technique, as described previously [19]. Cells (2.5×10^5 /well) were suspended in 100 μ l Hanks's balanced salts solution (HBSS) containing ferricytochrome c (150 mM). Superoxide production by the cells was stimulated with 1 mg/ml opsonized zymosan (OZ), 5×10^{-7} M formyl-methionyl-leucyl-phenylalanine (fMLP) or 50 ng/ml phorbol 12-myristate 13-acetate (PMA). The reduction of acetyl ferricytochrome c was followed by the

change of absorbance at 550 nm at 2-min intervals on a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA). The maximal rates of superoxide generation were determined and expressed as nmol O₂⁻/10⁶ cells/min using the extinction coefficient $E_{550} = 21 \text{ mM/cm}^1$.

Chemotaxis

Cell migration was assessed as described earlier [20]. Agarose was dissolved in sterile, distilled boiling water for 10 min. After cooling to 48°C in a water bath, the agarose was mixed with an equal volume of prewarmed $\times 2$ minimal essential medium (MEM) with 10% heat-inactivated fetal calf serum (FCS) and 7.5% (w/v) sodium bicarbonate. Five ml of the agarose medium was delivered to 60 \times 15 mm tissue culture dishes and allowed to harden. Six series of three wells, 2.4 mm in diameter and spaced 2.4 mm apart, were plucked out. The centre well of each three-well series received a 10 μ l aliquot cell suspension containing 2.5×10^5 purified cells in MEM. The outer well received 10 μ l of fMLP (10^{-8} M) and the inner well received 10 μ l of MEM. The dishes were incubated subsequently at 37°C in a humidified atmosphere containing 5% CO₂ in air. Dishes containing granulocytes were incubated for 2 h. The plates were fixed by addition of 3 ml methanol at 4°C overnight or for 30 min at room temperature. After the methanol was poured off, the plates were placed in glutaraldehyde (2.5%) for 30 min at room temperature. The agarose gel was removed intact after fixation and the plates stained by Giemsa and air-dried. Chemotaxis was defined as the ratio between the linear migration towards fMLP and the random migration towards control medium (MEM).

Phagocytosis

Cells (5×10^5) were suspended in RPMI-1640 containing 10% heat-inactivated FCS and incubated at 37°C for 1 h with 5 μ l zymosan (1 mg/ml), opsonized by pooled human serum. Subsequently, the cells were smeared and stained with differential Wright-Giemsa. Phagocytosis was determined under the microscope in at least 100 cells, and defined as the percentage of cells containing more than two phagocytized particles of OZ.

Surface expression of CD11b

Resting or stimulated neutrophils were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD11b antibody or with a control FITC-conjugated IgG2b antibody for 40 min in 4°C in HBSS. After two washes with the same buffer the cells were fixed with 2.5% formaldehyde, and surface CD11b was detected by fluorescence activated cell sorter (FACS) analysis (Becton Dickinson, Mountain View, CA, USA).

Table 1. Characteristics of the subjects enrolled into the study

	Lean	Obese > 35
Gender F/M	8/6	13/5
Age (years)		
Mean	40.2 ± 2.3	39.7 ± 1.7
Range	(24–49)	(21–50)
BMI (kg/m ²)		
Mean	22.1 ± 0.5	45.7 ± 2*
Range	(19–24)	(38–69)
WBC (*10 ³ /μl)		
Mean	6.1 ± 0.5	6.9 ± 0.3
range	(4.1–9.1)	(4.5–8.7)
Neutrophils (*10 ³ /μl)		
Mean	4.1 ± 0.3	4.5 ± 2.4
Range	(2.9–6.7)	(3.1–6.7)
Cholesterol (mg/dl)		
Mean	184.6 ± 11.3	208.4 ± 7.3
Range	(125–247)	(157–288)
Triglycerides (mg/ml)		
Mean	96.2 ± 10.9	178.6 ± 16.9†
Range	(46–179)	(65–659)

P < 0.001 compared to the lean controls; †*P* < 0.01 compared to the lean controls. WBC = white blood cells; BMI = body mass index; F/M = female/male.

Adherence of neutrophils to endothelial cells

Neutrophils were labelled with 1 mCi Cr⁵¹/10⁶ cells at 37°C during 1 h of gentle shaking [21]. Following two washes with cold phosphate-buffered saline (PBS) the neutrophils were resuspended in HBSS²⁺ or HBSS²⁻ at 7.5 × 10⁵ cells/ml. Cr⁵¹ radiolabelled neutrophils were pretreated for 3 min with 50 ng/ml PMA or 5 × 10⁻⁷M fMLP added onto 12-well plates of confluent ECV304 cell line and allowed to adhere for 30 min in a 5% CO₂ incubator (37°C). Following the adherence period, cells were washed twice thoroughly with PBS, and the remaining adhered prelabelled phagocytic cells, together with the ECV304 cells, were lysed in 500 μl lysis buffer containing 1% Triton-X 100, 50 mM Tris-HCl (pH 7.5), 1 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM ethylene glycol tetraacetic acid (EGTA). Radioactivity was then measured in the lysates by γ counter (Diagnostic Products Corporation, Biermann, Germany), along with a sample of prelabelled phagocytic cells to calculate the 100% count, based on which the proportional radioactivity in the adherence assay was calculated as the relative percentage.

Statistics

Data are presented as the mean ± standard error of the mean (s.e.m.). Statistical significance for between-group comparisons was determined using Student's paired two-tailed *t*-test or analysis of variance (ANOVA) followed by

Bonferroni correction. Correlations were analysed using Pearson's χ^2 tests.

Results

Obese subjects

The characteristics of morbidly obese subjects enrolled into the study are presented in Table 1. There was a significant (*P* < 0.001) elevation in BMI (kg/m²) and of serum triglycerides (*P* < 0.01) in the obese group compared with the lean control group. All other parameters, including age, white blood cell (WBC) count, neutrophil count and cholesterol were similar in the obese and lean subjects.

Neutrophil functions of obese versus lean donors

Neutrophil functions were studied in obese subjects in parallel with matched lean control subjects. Figure 1 presents superoxide production from unstimulated and stimulated neutrophils with two physiological agonists (OZ or fMLP) and a non-physiological agonist, PMA. As shown in Fig. 1, the levels of basal superoxide production by unstimulated neutrophils from all obese subjects were higher than that of the matched controls with no overlap between the groups. The mean ± s.e.m. was significantly higher (*P* < 0.0001) in the obese individuals than in the controls, 15.33 ± 0.34 and 6.72 ± 0.72 nmol/10⁶ cells/min, respectively. Moreover, there was a correlation (*r* = 0.78) between basal superoxide production and BMI. Similarly, a significant (*P* < 0.0001) elevated superoxide release was obtained in neutrophils of obese people stimulated with fMLP (mean ± s.e.m. of 32.06 ± 2.10 nmol/10⁶ cells/min compared with 1732 ± 094 nmol/10⁶ cells/min in the controls). Stimulation of neutrophils with OZ also resulted in increased superoxide production by neutrophils of the obese subjects compared with the controls, but with lower significance (*P* < 0.045). The mean ± s.e.m. was 22.92 ± 1.50 and 18.22 ± 1.25 nmol/10⁶ cells/min in the obese and control subjects, respectively. In contrast, there was no significant difference (*P* = n.s.) in the release of superoxides between both groups when neutrophils were stimulated with PMA; 35.17 ± 3.78 and 31.66 ± 2.54 nmol/10⁶ cells/min in the obese and control subjects, respectively. A lower concentration of PMA (5 ng/ml) studied did not show a difference between both groups (not shown).

Chemotactic migration and random migration of neutrophils from obese subjects were also elevated. As shown in Fig. 2, the obese subjects showed elevated neutrophil chemotactic migration and random migration compared to the lean controls. The mean ± s.e.m. of the chemotactic migration was 35.54 ± 1.48 and 24.93 ± 1.45 nm (*P* < 0.0003) of the random migration was 27.52 ± 0.83 and

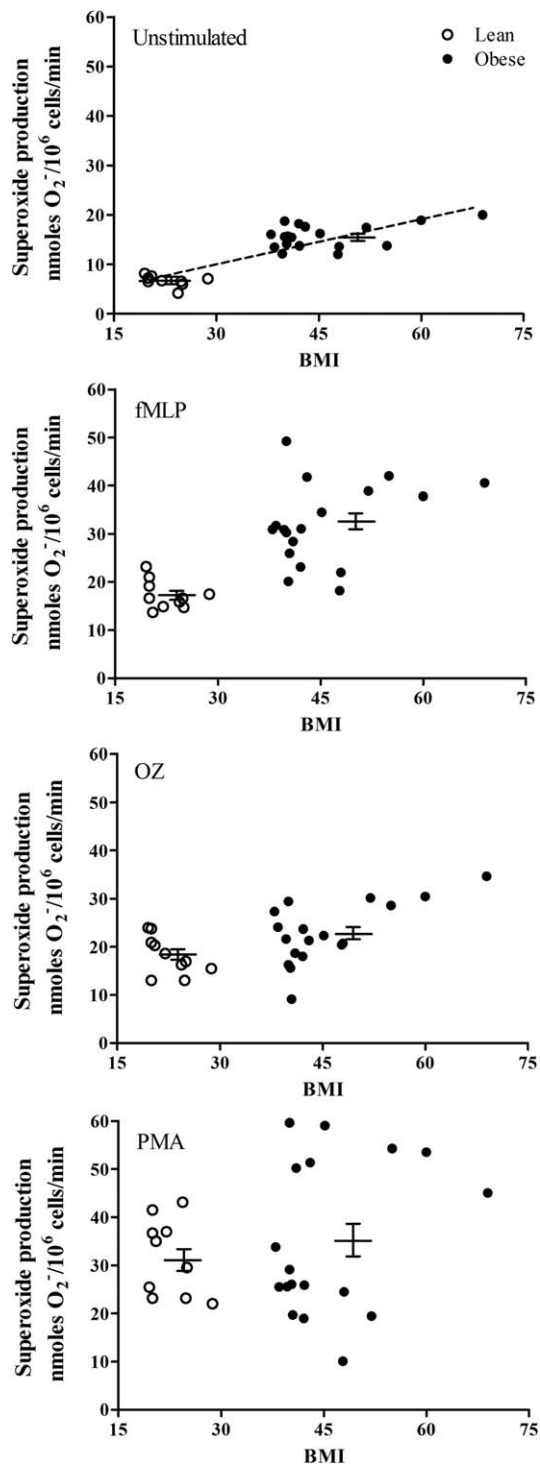


Fig. 1. Superoxide production was measured in neutrophils from obese subjects or from healthy donors unstimulated or stimulated with either 5×10^{-7} M formyl-methionyl-leucyl-phenylalanine (fMLP), 1 mg/ml opsonized zymosan (OZ) or 50 ng/ml phorbol myristate acetate (PMA). Each value is a mean of two independent tests from each subject. The mean \pm standard error of the mean are presented by the horizontal lines. There is a correlation ($r = 0.78$) between basal superoxide production secreted from unstimulated neutrophils and the body mass index.

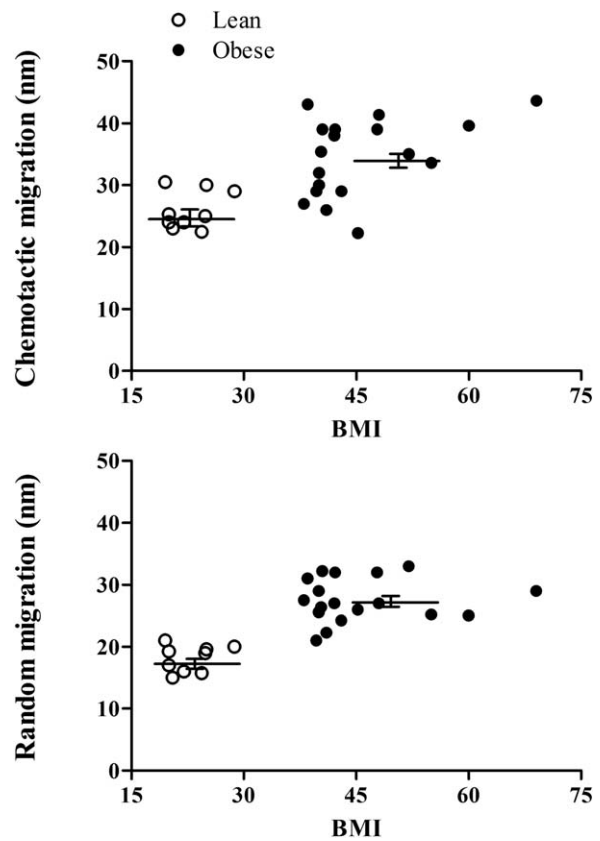


Fig. 2. Chemotactic and random migration of neutrophils towards formyl-methionyl-leucyl-phenylalanine (fMLP). Chemotactic migration towards 10^{-8} M fMLP and random migration were studied in neutrophils from obese patients and healthy controls. Each value is a mean of two independent tests from each subject. The mean \pm standard error of the mean are presented by the horizontal lines.

17.36 ± 1.03 nm ($P < 0.0001$) for obese subjects and lean controls, respectively.

Phagocytosis activity was similar in neutrophils of the obese and lean subjects, with mean \pm s.e.m. of $80.4 \pm 1.5\%$ and $72.2 \pm 1.8\%$, respectively (Fig. 3).

Neutrophil adherence

CD11b surface expression determined by FACS analysis was similar to unstimulated and stimulated neutrophils of the obese patients and the matched controls (Table 2). Adherence of unstimulated and stimulated neutrophils to endothelial cells was assayed. As shown in Fig. 4, the adherence of unstimulated neutrophils or stimulated neutrophils with either fMLP or PMA was similar in obese and lean subjects.

Discussion

The present study shows that superoxide production by neutrophils of the obese people with BMI ranging

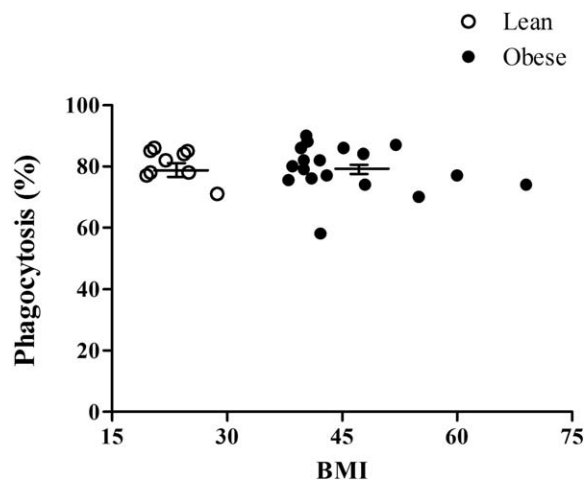


Fig. 3. Phagocytosis of opsonized zymosan (OZ) by neutrophils of obese subjects. Each value is a mean of two independent tests from each subject. The mean \pm standard error of the mean are presented by the horizontal lines.

between 38 and 69 kg/m² was elevated significantly. The major alteration was the elevated basal superoxide production by unstimulated neutrophils of obese people that was more than twofold higher than that of lean controls, suggesting that the neutrophils were at a primed state. Moreover, there was a correlation between basal superoxide production and BMI. Stimulation with the physiological stimuli fMLP or OZ also caused a significant increase in superoxide production, further supporting their primed state. Stimulation with the non-physiological stimuli PMA [that does not bind to specific receptor on the plasma membranes, but activates protein kinase C (PKC) directly] caused a non-significant elevation of superoxide production. The phagocytosis process of OZ particles, number of circulating neutrophils, surface expression of CD11b and adhesion of neutrophils to endothelial cells were not affected by obesity and were similar to those of the lean controls. All functions were not altered by age and gender (not shown), in accordance with an earlier study [22] of neutrophil function in obese people. Similar to our results, that study [22] showed an increased release of basal superoxides from unstimulated

Table 2. Neutrophils' CD11b surface expression

	Lean (median)	Obese > 35 (median)
Unstimulated	76 \pm 4.5	78 \pm 6.1
Stimulated with PMA	265 \pm 5.8	278 \pm 9.1
Stimulated with fMLP	158 \pm 3.1	150 \pm 6.2

Surface CD11b on unstimulated neutrophils and neutrophils stimulated with 50 ng/ml phorbol myristate acetate (PMA) or 5×10^{-7} M formyl-methionyl-leucyl-phenylalanine (fMLP). The means \pm standard error of the mean of median fluorescence of 10 000 cells of all subjects in each group are presented.

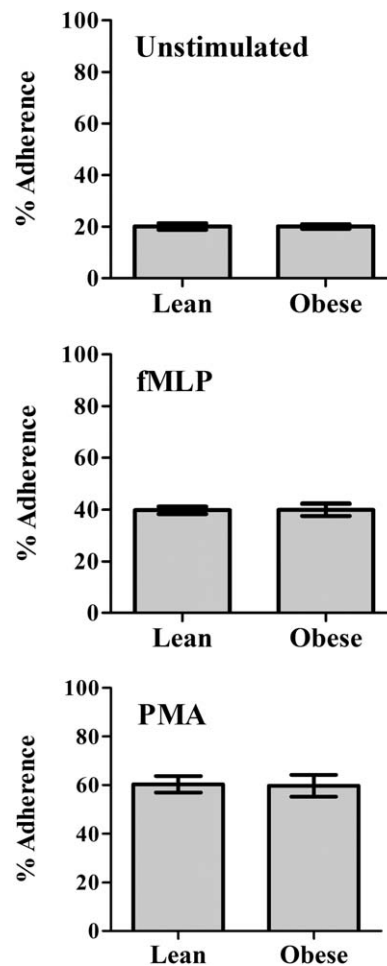


Fig. 4. Neutrophil adherence to endothelial cells was determined in unstimulated neutrophils or stimulated neutrophils by 5×10^{-7} M formyl-methionyl-leucyl-phenylalanine (fMLP) or 50 ng/ml phorbol myristate acetate (PMA). Results are mean \pm standard error of the mean of % adhered neutrophils of the subject studies.

neutrophils, although the elevation was much lower, only 59.6%. They have reported a low reduction in total superoxide production by neutrophils of obese people stimulated by 0.1 μ mol/l PMA and a low reduction in the rate of superoxide production stimulated with 0.01 μ mol/ml. They did not analyse superoxide production stimulated by physiological stimuli. Similar to our results, they [22] reported elevated migration and normal phagocytosis. The normal surface expression of CD11b in neutrophils of obese people, consistent with others' results [23], may explain the normal adherence to endothelial cells, as this integrin is the major one facilitating the adherence process [12]. Although obesity is a well-known risk factor for several morbid conditions and infections were reported to be more common in obese people than in those with normal weight [24], our results suggest the neutrophils' capability to combat infections in normal individuals, as none of the neutrophil functions studied

was shown to be defective. Moreover, stimulated superoxide production by physiological stimuli and chemotactic activity were significantly higher in neutrophils of obese subjects in comparison to those of lean controls. Thus, the increased risk of infections in obese subject is probably not attributing to neutrophil dysfunction.

The elevated basal superoxide production from unstimulated neutrophils of obese subjects is in line with studies demonstrating that obesity is associated with oxidative stress [25–30]. The basal elevated superoxide release from neutrophils indicates a low grade of inflammation, in accordance with the proinflammatory state of circulating mononuclear cells of obese subjects [31]. The elevated basal superoxide production may be a result of neutrophil activation by high levels of triglycerides in their blood (Table 1), as an immediate activation of circulating neutrophils, release of superoxides and increased expression of nicotinamide adenine dinucleotide phosphate oxidase (NOX2-NADPH) were demonstrated in healthy human subjects exposed to a high-fat diet [32–37]. It has been reported that long-chain saturated free fatty acids induced insulin resistance in cell cultures and *in vivo* by affecting several steps in signal transduction, such as insulin receptor substrate 2 (IRS-2) tyrosine phosphorylation, IRS-2-associated phosphoinositide (PI) 3-kinase activity and phosphorylation of protein kinase B (Akt), p70 S6 kinase, glycogen synthase kinase 3 (GSK-3) and forkhead box protein O1A (FOXO1A) [38–40]. Although neutrophils are usually considered to be a part of the innate immune system and the first line of defence against infection, accumulating evidence indicates a role for neutrophils in the pathogenesis of non-infectious inflammatory diseases. For example, there was a significant increased infiltration of neutrophils into systemic vasculature of pre-eclamptic women that was associated with inflammatory markers [41]. A number of studies have reported that women with pre-eclampsia are at an increased risk of developing cardiovascular disease late in life [42,43]. Similarly, obese women have significant vasculature infiltration of neutrophils, which may put them at risk of cardiovascular disease [44]. Our earlier study, as well as others [13,45,46], demonstrated that non-stimulated peripheral blood neutrophils from mice on a 3-day high-fat diet (3dHFD) with elevated body weight released significantly higher levels of basal superoxides, and their infiltration to adipose tissues was responsible for the development of insulin resistance in the liver. It was reported recently that overproduction of oxidants from NADPH oxidase and mitochondrial sources caused subsequent enhanced engagement of stress-activated kinases, associated with diminished insulin-stimulated insulin signalling and reduced glucose transport activity in obese subjects [47–49], as well as in mutant mouse models [50].

In conclusion, our results show that superoxide production from unstimulated or stimulated neutrophils

with the physiological stimuli and neutrophil migration were elevated in obese subjects while all other functions were normal, indicating the capability of neutrophils to combat infections. The high basal superoxide production from unstimulated neutrophils is consistent with oxidative stress associated with obesity.

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Disclosure

The authors declare that they have no financial and commercial conflicts of interest.

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