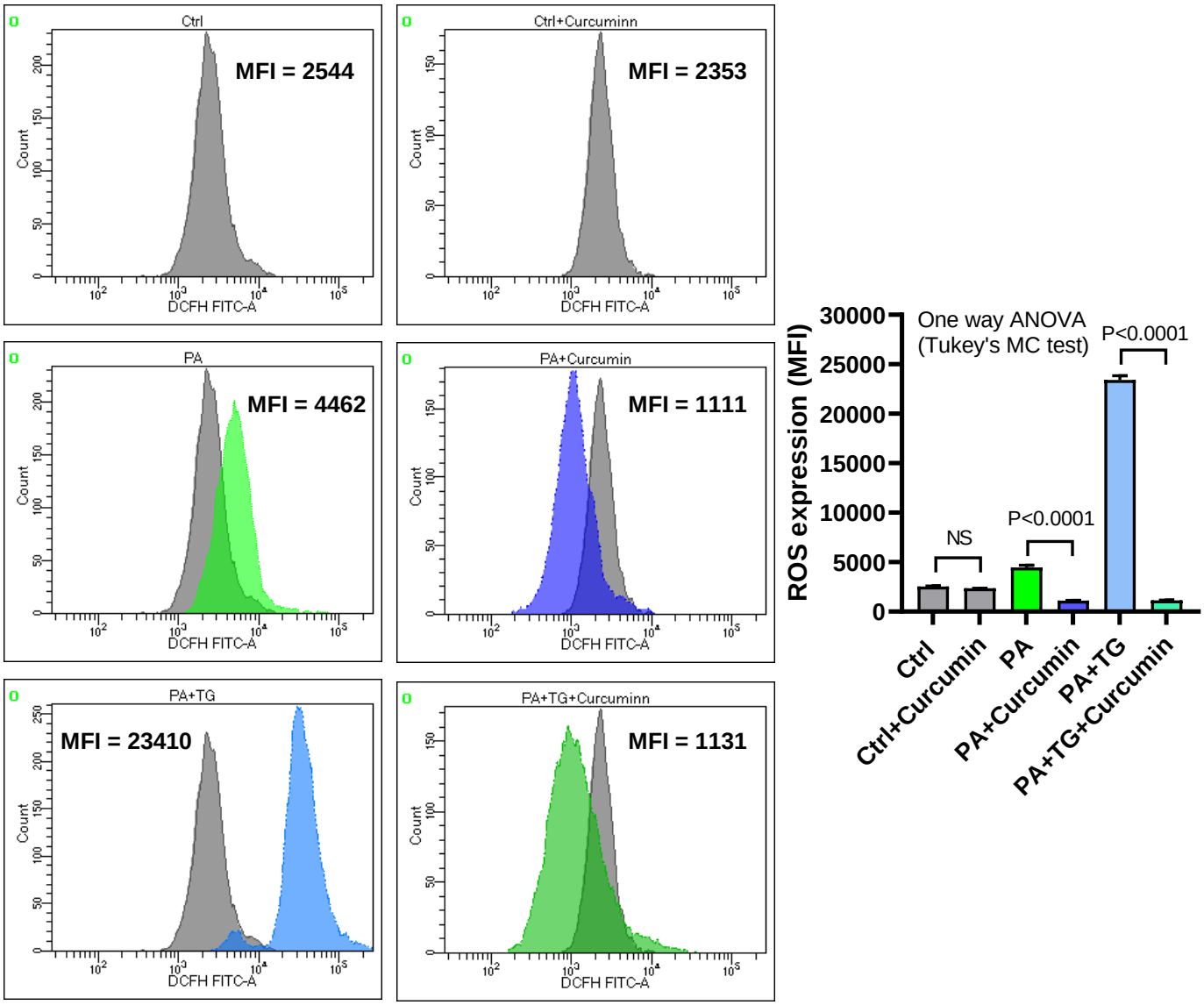
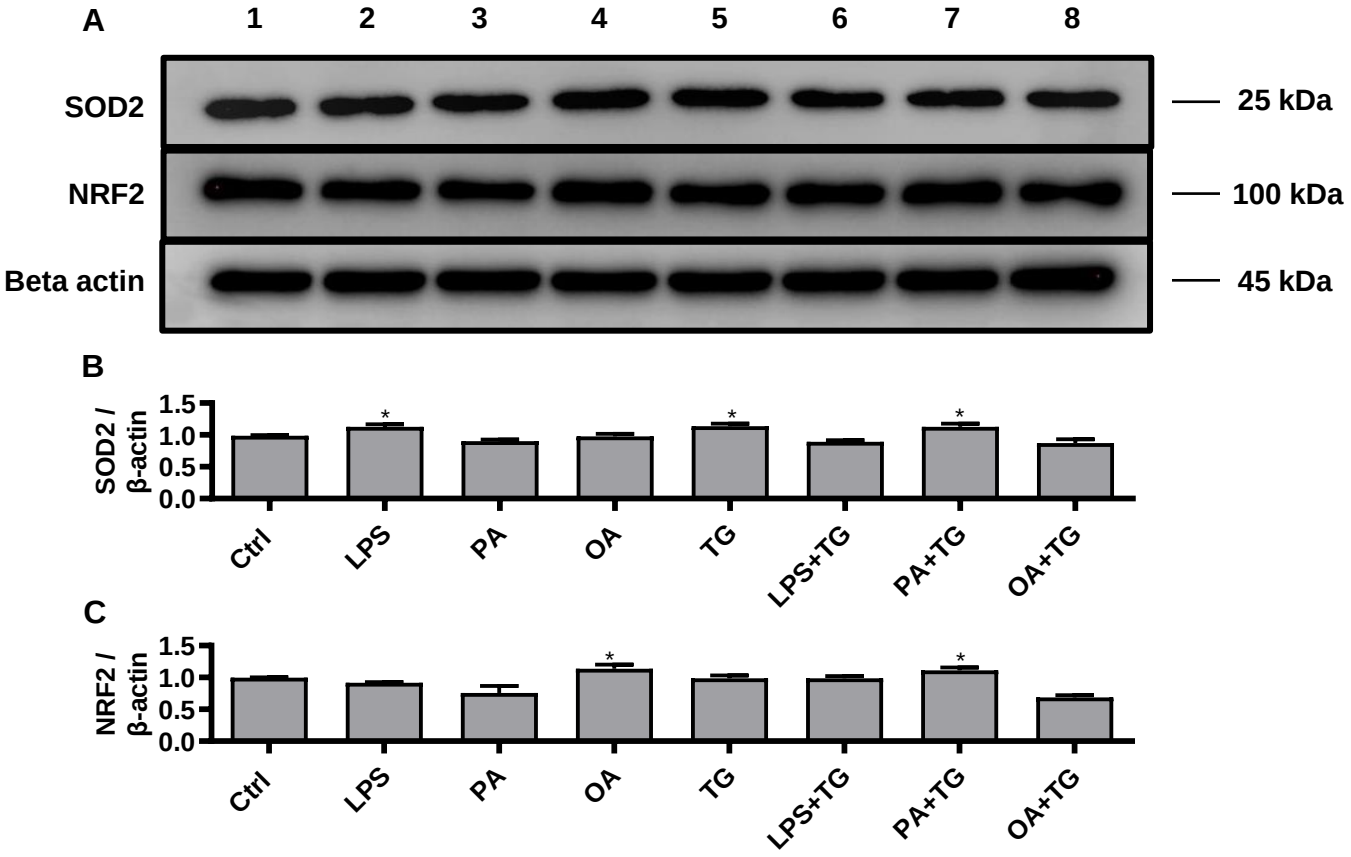
Supplementary Figures: S1 to S4

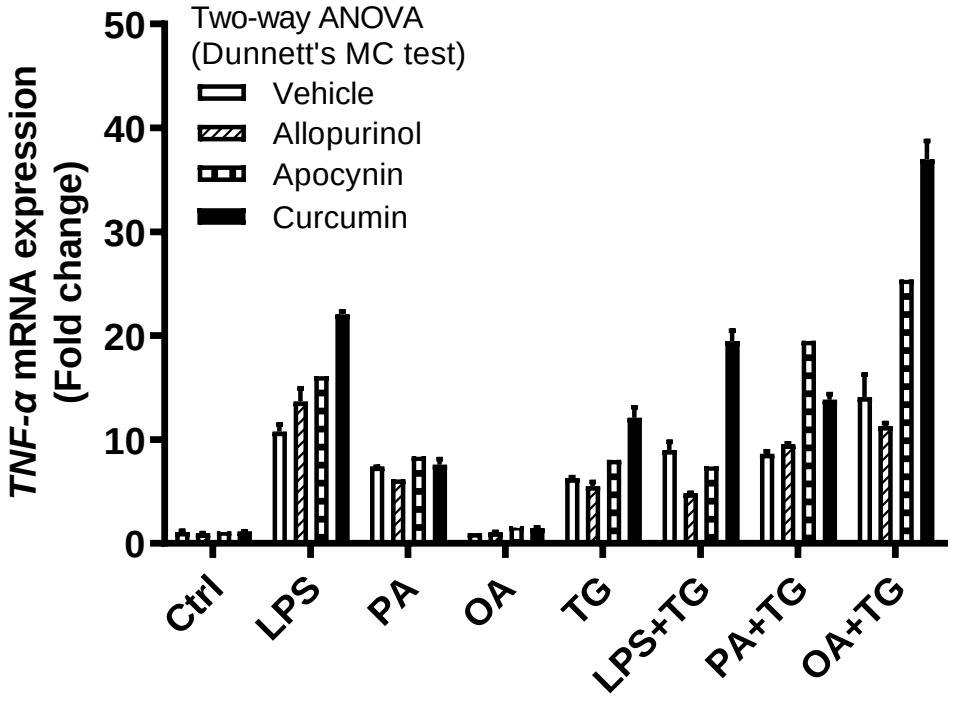
Manuscript titled: Endoplasmic Reticulum Stress Promotes the Expression of TNF-α in Monocytic Cells by Mechanisms Involving ROS/CHOP/HIF-1α and MAPK/NF-κB Pathways



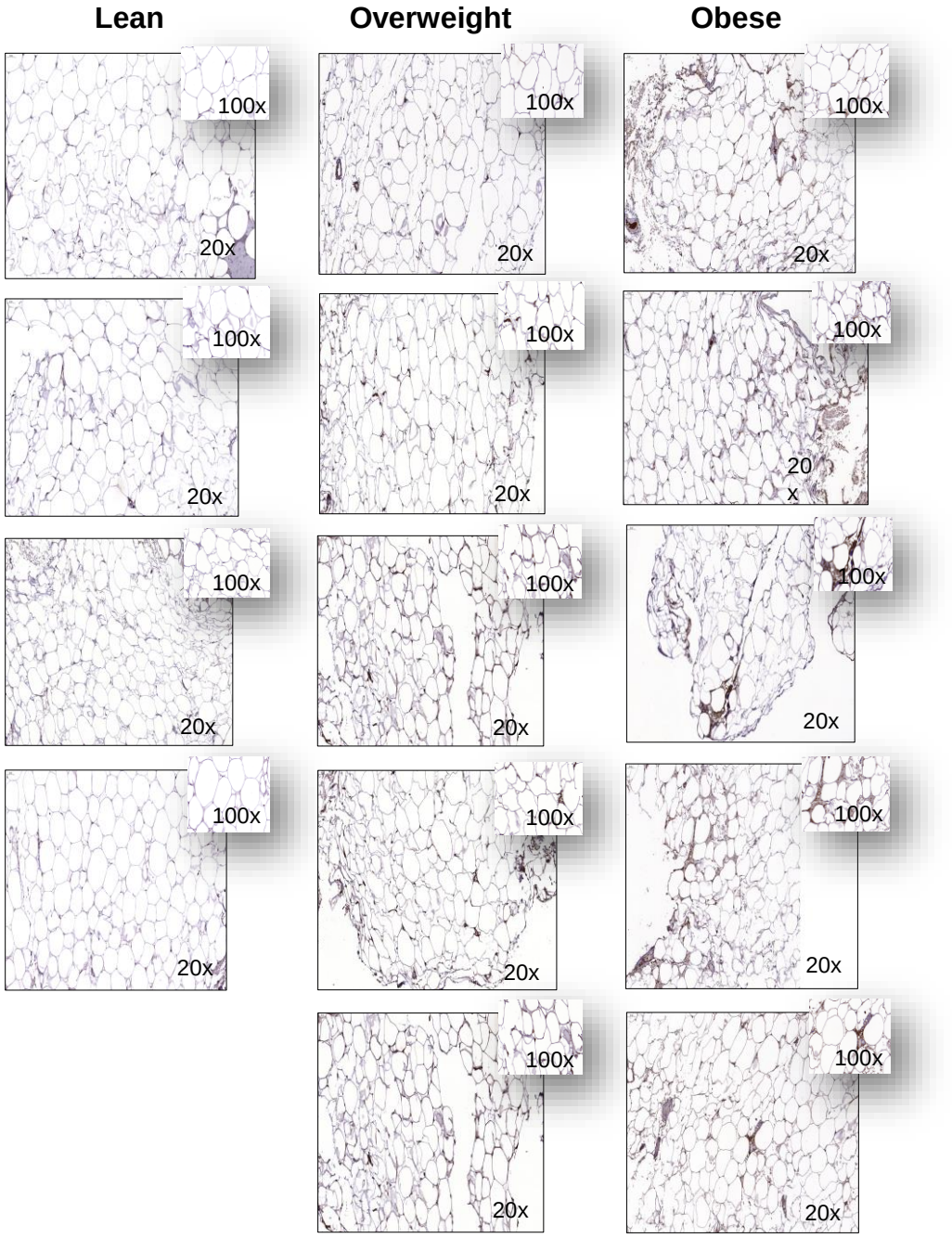
Supplementary Figure S1. Changes in intracellular reactive oxygen species (ROS) are oxidation dependent. THP-1 monocytic cells were plated at a cell density of 1×10^6 cells/mL/well in triplicate wells of 12-well plates and the cells in designated wells were pre-treated (1h) with curcumin and then stimulated with palmitate (PA, 200 μM) and with palmitate (PA, 200 μM) + Thapsigargin (TG, 1 μM), including vehicle (0.1% BSA) treatment as control, and cells were incubated at 37ºC for 24h. Intracellular ROS was measured using DCFH-DA assay and flow cytometry as described in Materials and Methods. Similar results were obtained from three independent experiments and flow cytometry data (expressed as mean±SEM) representing ROS expression were analyzed using One-way ANOVA, Tukey's multiple comparisons test. P<0.05 was considered significant. The histogram and graph data show significant suppression of ROS when cells were stimulated after treatment with curcumin, suggesting that ROS expression was oxidation dependent and not affected by influx/efflux of DCFH probe by lipid stimulant.



Supplementary Figure S2. Superoxide dismutase 2 (SOD2) and nuclear factor erythroid 2-related factor 2 (NRF2) protein expression. THP-1 cells were plated at a cell density of 1×10^6 cells/mL in triplicate wells of 12-well plates and stimulated with lipopolysaccharide (LPS, 10 ng/mL), palmitate (PA, 200 μM), and oleate (OA, 200 μM), in presence or absence of thapsigargin (TG, 1 μM), and control was treated with vehicle (0.1% BSA) only. After 24h incubation at 37^0C, cells were harvested and lysed by incubating for 30 min with lysis buffer containing Tris 62.5 mM (pH 7.5), 1% Triton X-100, and 10% glycerol, lysates were clarified by centrifugation (14000 rpm, 4°C, 10 min), and supernatants were collected. Protein concentrations were measured, and samples were resolved by 12% SDS-PAGE. Blots were probed overnight at 4°C using anti-human SOD2 (ab16956, Abcam) and NRF2 (ab62352, Abcam) antibodies (1:1000 dilution). Blots were washed 3× with TBS wash buffer and incubated (1:2500 dilution) for 2h with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Promega). Bands were developed (Amersham ECL Plus Western Blot Detection System, GE HealthCare, Buckinghamshire, UK) and visualized (ImageDoc™ MP Imaging Systems, BioRad Labs, CA, USA). Band densities expressed as arbitrary units (AU) were determined and data (mean±SEM; n=3) were compared using One-way ANOVA, Dunnett's multiple comparisons test; $P<0.05$ was considered significant. (A) Representative Western blots showing SOD2 and NRF2 protein expression in control (Lane 1) and stimulated cells (Lanes 2-8), using β-actin housekeeping gene expression as internal control. Normalized target gene expression versus respective β-actin expression is shown for (B) SOD2 and (C) NRF2 proteins; asterisks denoting a significantly higher expression compared with control.



Supplementary Figure S3. Effect of antioxidants/ROS scavengers on *TNF-α* gene expression in monocytic cells. THP-1 monocytic cells were plated at a cell density of 1×10^6 cells/mL/well in triplicate wells of 12-well plates and the cells in designated wells were pre-treated (1h) with antioxidants/ ROS scavengers including allopurinol, apocynin, and curcumin and then stimulated with lipopolysaccharide (LPS, 10 ng/mL), palmitate (PA, 200 μM), and oleate (OA, 200 μM), in presence or absence of the ER stressor thapsigargin (TG, 1 μM), while anti-oxidant control wells were pre-treated with vehicle (0.1% BSA) and later stimulated likewise other cells that were pre-treated with antioxidants. After 24h incubation (37ºC), cells were harvested, lysed in RLT buffer, total RNA was purified and *TNF-α* gene expression was determined using qRT-PCR as described in Materials and Methods. Similar results were obtained from three independent experiments. Data (expressed as mean±SEM) were analyzed using two-way ANOVA, Dunnett's multiple comparisons test and $P<0.05$ was considered significant. The representative data show that, compared with respective controls, no antioxidant or ROS scavenger suppressed *TNF-α* at the transcriptional level.



Supplementary Figure S4. TNF-α expression in the adipose tissue. Adipose tissue samples from 4 lean (BMI 18.5 to 24.9 kg/m^2), 5 overweight (BMI 25 to 29.9 kg/m^2), and 5 obese (BMI ≥30 kg/m^2) individuals (cohort 1), were collected by abdominal fat pad biopsy and TNF-α protein expression was determined using immunohistochemistry (IHC) as described in Materials and Methods. The representative IHC images from three independent stainings with similar results are shown (20× magnification, scale bar 50 μm). Image insets (100× magnification) highlight the TNF-α staining area in the adipose tissue.

Supplementary Tables: S1 and S2

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Supplementary Table S1. Characteristics of Study Population (Cohort 1)

Parameter	Lean	Overweight	Obese
Age (Yrs)	45.28±4.92	43.30±5.51	43.70±6.07
Gender (Male/Female)	5/4	6/4	5/5
BMI (kg/m^2)	23.46±0.78	27.66±0.45	35.55±1.23
Blood glucose (mmol/L)	5.09±0.23	5.24±0.33	5.11±0.27
TC (mmol/L)	5.45±1.04	5.08±0.37	5.38±0.51
HDL-c (mmol/L)	1.49±0.30	1.16±0.13	1.14±0.13
LDL-c (mmol/L)	3.22±0.75	3.21±0.35	3.78±0.45
TG (mmol/L)	0.49±0.04	1.55±0.31	1.06±0.19

BMI: Body mass index; TC: Total cholesterol; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; TG: Triglycerides

Supplementary Table S2. Characteristics of Study Population (Cohort 2)

Parameter	Lean	Overweight	Obese
Age (Yrs)	42.20±3.80	36.84±5.06	36.67±5.48
Gender (Male/Female)	7/5	6/6	5/7
BMI (kg/m^2)	21.69±0.56	27.71±0.38	39.51±2.17
Blood glucose (mmol/L)	5.32±0.20	5.16±0.30	5.26±0.19
TC (mmol/L)	4.91±0.82	5.64±0.53	4.62±0.44
HDL-c (mmol/L)	1.47±0.32	1.37±0.18	1.08±0.14
LDL-c (mmol/L)	3.30±0.55	3.25±0.40	3.40±0.36
TG (mmol/L)	0.70±0.13	1.30±0.29	1.13±0.19
TNF-α (pg/mL)	7.35±1.43	8.55±2.53	14.94±2.49

BMI: Body mass index; TC: Total cholesterol; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; TG: Triglycerides; TNF-α: Tumor necrosis factor-α