

# Effect of a diet and exercise intervention on oxidative stress, inflammation and monocyte adhesion in diabetic men

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## Abstract

Diabetes increases the risk of coronary artery disease. We examined the effects of lifestyle modification on key contributing factors to atherogenesis, including oxidative stress, inflammation and cell adhesion. Diabetic men ( $N = 13$ ) were placed on a high-fiber, low-fat diet in a 3-week residential program where food was provided ad libitum and daily aerobic exercise was performed. In each subject, pre- and post-intervention fasting blood was drawn for circulating levels of serum lipids, glucose and insulin, oxidative stress marker 8-isoprostaglandin  $F_{2\alpha}$  (8-iso-PGF $_{2\alpha}$ ), the inflammatory protein C-reactive protein (CRP), and soluble intracellular adhesion molecule (sICAM)-1 and sE-selectin as indicators of endothelial activation. Using subject sera and human aortic endothelial cell (HAEC) culture systems, serum-induced monocyte adhesion, ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and cell surface abundance, and monocyte chemotactic protein-1 (MCP-1) production were determined. Nitric oxide (NO), superoxide, and hydrogen peroxide production were measured in vitro by fluorometric detection. After 3 weeks, significant reductions ( $p < 0.05$ ) in BMI, all serum lipids including total cholesterol (pre:  $188.9 \pm 10.1$  mg/dL versus post:  $146.3 \pm 3.8$  mg/dL) and low-density lipoprotein ( $103.1 \pm 10.2$  mg/dL versus  $76.4 \pm 4.3$  mg/dL), fasting serum glucose ( $157.5 \pm 10.1$  mg/dL versus  $126.7 \pm 8.7$  mg/dL), insulin ( $33.8 \pm 4.0$   $\mu$ U/ml versus  $23.8 \pm 3.4$   $\mu$ U/ml), homeostasis model assessment for insulin resistance, 8-iso-PGF $_{2\alpha}$ , CRP, sICAM-1, and sE-selectin were noted. In vitro, serum-stimulated monocyte adhesion, cellular ICAM-1 and VCAM-1 expression ( $p < 0.05$ ), and fluorometric detection of superoxide and hydrogen peroxide production decreased, while a concomitant increase in NO production was noted (all  $p < 0.01$ ). A combination of diet and exercise ameliorates oxidative stress, inflammation, and monocyte-endothelial interaction. Intensive lifestyle modification may improve novel CAD risk factors in men with diabetes.

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## 1. Introduction

The estimated prevalence of diabetes increased 600% from 1958 to 1993 [1] and has continued to rise in

the last 10 years [2,3]. Epidemiological studies have consistently shown an association between diabetes and coronary heart disease (CHD) in the U.S. population [4], and Stamler et al. [5] noted a two- to four-fold increase in risk for CHD in diabetics. Emerging risk factors such as oxidative stress, inflammation and endothelial cell activation are thought to play integral roles in the development of atherosclerotic lesions. In diabetics, levels of the oxidative stress marker 8-isoprostaglandin  $F_{2\alpha}$  (8-iso-PGF $_{2\alpha}$ ) [6] and peroxide

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production are increased [7], while nitric oxide (NO) availability is diminished [8]. Inflammation-associated proteins including C-reactive protein (CRP) [9], soluble intracellular adhesion molecule (sICAM)-1 [10], and cell adhesion molecules such as vascular cell adhesion molecule (sVCAM-1) [11] and soluble E-selectin (sE-selectin) [12] are elevated in diabetics and independently predict early risk of myocardial infarction or cardiovascular disease.

Diet and exercise modifications have been documented to control type 2 diabetes and to ameliorate the classic CAD risk factors, including hyperlipidemia and hypertension [13]. However, the effects of lifestyle modification on emerging risk factors in diabetics is largely unknown. The aim of this study was to investigate the effects of a 3-week diet and exercise intervention consisting of a high-fiber, low-fat diet and physical activity on emerging atherosclerotic risk factors in diabetic men. Three hypotheses were tested: diet and exercise would (1) improve insulin sensitivity; (2) reduce serum lipids and markers of oxidative stress, inflammation and adhesion; and (3) reduce in vitro production of reactive oxygen species (ROS) while increasing NO production.

## 2. Methods

### 2.1. Diet and exercise intervention

The study protocol was approved by the Human Subjects Protection Committee of the University of California, Los Angeles and informed consent of all participants was obtained. Serum samples for this study were obtained from 13 diabetic men (age range 55–74, mean 64.6 year) who voluntarily participated in the Pritikin Longevity Center 21-day residential diet and exercise intervention. All participants were overweight or obese with a mean body mass index (BMI) =  $38.7 \pm 2.5$  and were free of any viral infections during the study (CRP < 10 mg/L). Three subjects were using HMG-CoA reductase inhibitors, three were using metformin and two were using insulin.

Once enrolled in the program, participants underwent a complete medical history and physical examination, prior to the 21-day diet and exercise intervention as previously described [14]. From dietary analysis software, prepared meals contained 12–15% of calories from fat (polyunsaturated/saturated fatty acid ratio = 2.4:1), 15–20% of calories from protein, and 65–70% of calories from primarily unrefined-carbohydrate, high in dietary fiber (>40 g per/day). The program is designed to allow the subjects ad libitum eating without control of calories, only restricting the type of foods. Carbohydrates were primarily in the form of high-fiber whole grains ( $\geq 5$  servings/day), vegetables ( $\geq 4$  servings/day) and fruits ( $\geq 3$  servings/day). Protein was from plant sources, non-

fat dairy (up to two servings/day) and fish/fowl (3.5 oz. portions 1 day/week and in soups or casseroles 2 days/week). Prior to starting the exercise training, subjects underwent a graded treadmill stress test according to a modified Bruce protocol to determine the appropriate individual level of exercise intensity. Based on the treadmill results, the subjects were provided with an appropriate training heart rate value and given an individualized walking program. The exercise regimen consisted of daily treadmill walking at the training heart rate for 45–60 min. The training heart rate was defined as 70–85% of the maximal heart rate attained during the treadmill test.

Twelve-hour fasting blood samples were drawn from the subjects into vacutainers (Becton–Dickinson Vacutainer Systems) containing SST clot activating gel between 6:30 and 8:00 am on days 1 and 21 of the intervention. The blood was transported on ice to the laboratory, the serum was separated by centrifugation and stored at  $-80^{\circ}\text{C}$  until analyzed. Weight was measured using a scale from Pennsylvania Medical Scales (Model# 7500). Height was measured using a stadiometer from Seca Inc., attached to the wall. BMI was calculated as weight (kg)/height ( $\text{m}^2$ ).

### 2.2. Determination of serum lipids, glucose, insulin, $\text{HOMA}_{\text{IR}}$ and QUICKI

Total-cholesterol (Total-C), triglycerides (TG) and high-density lipoprotein (HDL-C) levels were measured using a kit from Sigma Diagnostics (St. Louis, MO). Low-density lipoprotein (LDL-C) was calculated as described by Friedewald et al. [15]. Glucose concentration was determined using standard enzymatic procedures on an Olympus Autoanalyzer (Quest Diagnostics). Fasting insulin concentration was measured by radioimmunoassay (Diagnostic Systems Laboratories Inc. #DSL-10-1600). This assay has an intra-assay coefficient of variation of  $\pm 2\%$ , and has a minimum detection limit of  $0.26 \mu\text{IU/mL}$ . Insulin resistance was evaluated using homeostasis model assessment ( $\text{HOMA}_{\text{IR}}$ ) which has been shown to correlate with insulin sensitivity by the hyperinsulinemic-euglycemic clamp [16,17].  $\text{HOMA}_{\text{IR}}$  is calculated as  $[\text{fasting insulin } (\mu\text{U/ml}) \times \text{fasting glucose } (\text{mmol/L})] / 22.5$ . Quantitative insulin-sensitivity check index (QUICKI) is determined from a mathematical transformation of fasting blood glucose and plasma insulin levels and has been shown to be a surrogate for insulin sensitivity that correlates well with the minimal model and the hyperinsulinemic-euglycemic clamp [18].  $\text{QUICKI} = 1 / [\log (\text{fasting insulin } (\mu\text{U/ml})) + \log (\text{fasting glucose } (\text{mg/dl}))]$ . Because QUICKI is the reciprocal of the log-transformed product of fasting glucose and insulin, it is a dimensionless index without units.

### 2.3. Determination of serum 8-iso-PGF<sub>2 $\alpha$</sub> , CRP, sICAM-1 and sE-selectin

Serum 8-iso-PGF<sub>2 $\alpha$</sub>  was measured in duplicate using an enzyme immunoassay kit (Cayman Chemical, #516351).

According to the manufacturer's insert, this assay has an intra-assay coefficient of variation of  $\pm 8\%$ , and has a minimum detection limit of 5 pg/mL. Serum high sensitivity CRP was determined from an ELISA kit from Diagnostic Systems Laboratories. Serum soluble ICAM-1 (sICAM-1) concentrations were measured with a specific ELISA kits according to the manufacturer's instructions (BD Biosciences, PharMingen, #551424). This assay has an intra-assay coefficient of variation of  $\pm 5\%$  with a detection limit of 1.6 ng/mL for ICAM-1. Additionally, sE-selectin (R&D Systems, #BBE2B) was determined; this assay has an intra-assay coefficient of variation of  $\pm 5\%$ , and a minimum detection limit of 0.47 ng/mL.

#### 2.4. *In vitro* cell culture studies

Human aortic endothelial cells (HAEC) alone and HAEC and human aortic smooth muscle cells (HASMC) were cocultured as previously described [19,20]. In brief, the cells were subcultured and grown to confluence in 75 cm<sup>2</sup> flasks in M199 medium (Invitrogen) supplemented with 20% (v/v) FBS, 0.8 ml heparin and 2 mg endothelial cell growth factor/100 ml media (Becton–Dickinson), 1% (v/v) penicillin–streptomycin–glutamine (Gibco BRL), and 1% (v/v) sodium pyruvate (Gibco BRL) followed by trypsinization. HASMC were cultured in the same media without addition of endothelial cell growth factor or heparin. Subsequently, wells in a 96-well plate were treated with 0.1% gelatin for at least 1 h. HASMC were seeded in the wells at a density of approximately  $5 \times 10^4$  cells/cm<sup>2</sup> and were cultured for 2–3 days at 37 °C, 95% O<sub>2</sub>/5% CO<sub>2</sub> at which time they had reached confluency. For co-cultures, HAEC were subsequently overlaid on top of HASMC at approximately  $1 \times 10^5$  cells/cm<sup>2</sup> and were allowed to grow forming a complete monolayer of confluent EC in 2 days.

##### 2.4.1. Monocyte adhesion assay

Confluent monolayers of HAEC were grown and monocyte adhesion was quantified with adaptation of methods from Van Lenten et al. [20]. HAEC were grown in gelatin coated-48 well microtiter plates and were treated with pre- and post-intervention serum (10% final concentration) in M199 media for 20 h. Supernatants were then removed and cultures washed with serum-free medium (SFM). A 400  $\mu$ l THP-1 suspension in M199 providing  $2.0 \times 10^5$  cells/cm<sup>2</sup> was then added per well, and incubated for 15 min. Extreme care was taken to avoid drying out of the HAEC during the changes and throughout the assay. The loosely adherent cells were washed off with PBS, the cultures fixed with 1% glutaraldehyde and the adherent THP-1 cells enumerated under 320 $\times$  magnification. Each well was scored in four different areas for a total of 12 counts per subject. The pre- and post-intervention serum was compared with 10% FBS and 10 ng/ml LPS was used as a positive control.

##### 2.4.2. VCAM-1 and ICAM-1 expression

A modified ELISA procedure was used to measure cell surface expression of ICAM-1 and VCAM-1 [21]. Human

aortic endothelial cells (HAEC) were grown to confluency in 96-well plates. Cultures were then washed three times in media containing 1% FBS. Washed cultures were then incubated with media containing 10% pre- or post-intervention serum for 4 h (VCAM-1) or 20 h (ICAM-1). After incubation, supernatants were removed and HAEC were washed 3 $\times$  with 200  $\mu$ l of PBS, fixed in 150  $\mu$ l of 100% methanol for 10 min, air-dried, sealed and stored at 4 °C. Dried cells were rehydrated and blocked in 200  $\mu$ l of PBS containing 0.1% Tween-20 and 0.5% BSA (PBS/Tween/BSA) for 30 min and then washed twice in PBS/Tween. HAEC were then incubated for 2 h at room temperature a mouse monoclonal IgG antibodies against either human VCAM-1 (PharMingen #555645) or human ICAM-1 (R&D Systems #BBA3) at 1 (g/mL in PBS/Tween/BSA. The wells were then washed 3 $\times$  in PBS/Tween, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody against mouse IgG from sheep (Amersham Life Sciences #NA931V) diluted in PBS/Tween/BSA at a 1:1000 dilution. After a 1 h incubation at room temperature, wells were washed five times in PBS/Tween, and they were subsequently colored using 100  $\mu$ l of tetramethylbenzidine reagent (Sigma #T0440) as substrate for the bound HRP. After 5 min of incubation, the reaction was stopped by adding 100  $\mu$ l of 3 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450 nm. Fetal bovine serum (10% FBS) was used as a control, 10 ng/mL lipopolysaccharide (LPS) was used as a positive control and all samples were run in triplicate. The data are expressed as percentage of FBS control.

##### 2.4.3. Production of MCP-1

Cocultures were washed with M199 medium containing 1% FBS three times. The second wash was incubated for 1–2 h at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub>; other washes were removed immediately. Washed cocultures were then incubated with M199 medium containing 5% human serum pre- and post-intervention for 4 h. Subsequently, cultures were centrifuged and culture supernatants harvested and stored at –20 °C pending the MCP-1 measurement by ELISA (PharMingen, BD<sup>TM</sup> OptEIA ELISA Set #555179) following the manufacturer's instructions. Serum samples were incubated in wells with no cells as a negative control and LPS at 10 ng/mL in medium was used as a positive control. Endothelial cell production of MCP-1 was determined by subtracting no cell control supernatants from supernatants incubated with cocultures. All tests were run in triplicate.

##### 2.4.4. NO production

All steps involving the fluorescent probe were performed without direct light. The average of quadruplicate values for each condition was then taken and the final values for each test condition reported as a percent of FBS control. The cell permeable fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA, Calbiochem) was used to measure NO production by HAEC [22]. DAF-2DA is converted to DAF-2 by intracellular esterases, trapping the probe inside the cell. DAF-2 reacts with NO to form the highly fluorescent compound DAF-

2T. After the HAEC in 96-well plates reached confluency, growth medium was removed and the cells were washed with 200  $\mu\text{L}$  SFM three times. The second wash was incubated for one hour at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub>. SFM supplemented with 10% test serum (pre- or post-intervention) was added onto cells, which were then incubated at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub> for 18 h. Thereafter, 100  $\mu\text{L}$  of 10  $\mu\text{M}$  DAF-2DA dissolved in SFM was added to all cells except negative control wells. Negative control wells included cells with 100  $\mu\text{L}$  of SFM containing either 10  $\mu\text{M}$  4 AF-DA (Calbiochem), a non-reactive DAF-2DA analog, or 300  $\mu\text{M}$  N-nitro-L-arginine methyl ester (L-NAME, Sigma), an inhibitor of NO synthesis. The cells were then placed in a light protected incubator at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub> for 1 h. After incubation, cells were washed three times with 200  $\mu\text{L}$  of SFM to remove any residual extracellular DAF-2DA probe. Subsequently, 100  $\mu\text{L}$  of SFM with 4  $\mu\text{M}$  bradykinin (Calbiochem) was added to the cells. The DAF-2DA positive control included DAF-2T (Calbiochem) dissolved in SFM. After 10–15 min in a light protected incubator at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub>, fluorescence intensity was read and quantified in a fluorescence microplate reader at 495 nm excitation wavelength and 515 nm emission wavelength. The OD readings pre- and post-intervention were expressed as percentage of FBS control. Pictures of the DAF-2T fluorescence in the HAECs from the representative pre- and post-test conditions were taken with a camera connected to a fluorescent microscope (Zeiss Axiovert 135 microscope) and a computer, using Axiovision software. Picture (black and white) location within each well was standardized through finding a cluster of cells indicated by a dark spot at the center of each well with low magnification (10 $\times$ ). A higher magnification (20 $\times$ ) and light wavelength filter was then used to identify the intracellular fluorescence of a group of cells within this cluster. The filter allowed the view of light only within the same wavelength range as the light emitted by the fluorescent probe. The color corresponding to the same wavelength was added to the black and white picture using Axiovision software. The picture files were converted to JPEG format using Adobe Photoshop.

#### 2.4.5. Hydrogen peroxide production

The cell permeable fluorescent probe 2',7'-dichloro-dihydrofluorescein diacetate (DCFH-DA, Molecular Probes) was used to measure hydrogen peroxide production by HAEC [23]. For DCFH-DA to emit a fluorescent signal the diacetate group is first cleaved by cytoplasmic esterases to form DCFH. DCFH reacts with hydrogen peroxide to form a highly fluorescent compound, DCF, which can be used to detect enzymatic generation of reactive oxygen species [24]. In cultured endothelial cells, DCF is oxidized by H<sub>2</sub>O<sub>2</sub>, along with other intracellular processes involving reactive oxygen species, making it useful in determining changes in hydrogen peroxide and overall oxidant formation [25]. SFM supplemented with 10% test serum (pre- or post-intervention) was added onto cells which were then incubated at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub> for 18 h. The hydrogen peroxide positive control included cells incubated for 18 h with 2 ng/mL TNF- $\alpha$  dissolved in SFM. Following incubation, the

supernatant was removed from the cells and 100  $\mu\text{L}$  of 10  $\mu\text{M}$  DCFH-DA dissolved in SFM was applied to the cells which were then placed in a light protected incubator at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub> for 1 h. Following DCFH-DA incubation, cells were washed three times with 200  $\mu\text{L}$  SFM to remove residual extracellular probe. After washing, 100  $\mu\text{L}$  SFM was applied to the cells which were then placed in a light protected incubator at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub> for 30 min allowing time for the intracellular probe to be cleaved and oxidized as described above. The DCFH-DA probe positive control included the fluorescent oxidized form of DCFH in SFM. The negative control included SFM on cells without any probe application. The fluorescence intensity was then read and quantified in a fluorescence microplate reader with 485 nm excitation wavelength, 530 nm emission wavelength and 515 nm cutoff. The OD readings pre- and post-intervention were expressed as a percentage of FBS control.

#### 2.4.6. Superoxide production

The cell permeable fluorescent probe dihydroethidium (DHE, Calbiochem) was used to measure superoxide production by HAEC [26]. DHE has blue fluorescence and after DHE is oxidized to ethidium by superoxide it intercalates with the cell's DNA staining the nucleus a bright fluorescent red. SFM supplemented with 10% test serum (pre- or post-intervention) was added onto cells, which were then incubated at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub> for 18 h. The superoxide positive control included cells incubated with 2 ng/mL TNF- $\alpha$  dissolved in SFM. Following incubation, the supernatant was removed and 100  $\mu\text{L}$  of 25  $\mu\text{M}$  DHE dissolved in SFM was applied to the cells which were then placed in a light protected incubator at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub> for 45 min. Following DHE incubation, cells were washed once with 200  $\mu\text{L}$  SFM and then twice with 200  $\mu\text{L}$  HEPES to remove residual extracellular probe. After washing, 100  $\mu\text{L}$  HEPES was applied to the cells which were then placed in a light protected incubator at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub> for 10 min. The negative control included HEPES buffer without any probe application. The fluorescence intensity was then read and quantified in a fluorescence microplate reader. A 518 nm excitation wavelength and 605 nm emission wavelength with 590 nm autocutoff filter was used to detect and quantify the fluorescence of the probe that reacted with superoxide. A 355 nm excitation wavelength and 425 nm emission wavelength with 420 nm autocutoff filter was used to detect and quantify the fluorescence of the remaining probe that did not react with superoxide. The average of the quadruplicate values was taken for each condition. The final data points were reported as a percent of the 10% FBS condition. Pictures of the ethidium fluorescence were taken in the same manner as for NO detection (above).

#### 2.5. Statistical analysis

Statistical analyses were performed with Graph Pad Prism (GraphPad, San Diego, CA). Pre- and post-intervention values were compared using matched paired t-tests. CRP pre- and

post-intervention values were compared using matched paired Wilcoxon signed-rank tests for non-parametric data, and was graphed using box plots with median and interquartile ranges. All data are expressed as mean  $\pm$  S.E. unless otherwise noted. A  $p$  value of  $<0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Fasting lipids, glucose, insulin and anthropometry

Anthropometric and metabolic data are presented in Table 1. The 21-day diet and exercise intervention significantly reduced body weight ( $p < 0.01$ ) and body mass index (BMI) ( $p < 0.01$ ), although as a group, the subjects remained obese (BMI  $> 30$  kg/m<sup>2</sup>) at the end of the intervention. Noted were significant reductions in total-C ( $p < 0.001$ ), LDL-C ( $p < 0.001$ ), HDL-C ( $p < 0.05$ ), TG ( $p < 0.05$ ), and the LDL-C:HDL-C ratio ( $p < 0.05$ ). The reduction in the total-C:HDL-C ratio ( $p = 0.06$ ) did not reach statistical significance. Significant reductions in serum fasting glucose ( $p < 0.05$ ) and insulin ( $p < 0.01$ ) were also observed after the intervention. The HOMA<sub>IR</sub> and QUICKI were determined as surrogates of insulin sensitivity. The intervention resulted in a significant decrease in HOMA<sub>IR</sub> ( $p < 0.01$ ) as well as a significant rise in QUICKI ( $p < 0.01$ ), and 6 of the 13 subjects were not classified as diabetic post-intervention by fasting blood glucose level and others had their medication dosages reduced.

Table 1  
Anthropometric and metabolic parameters of subjects ( $N = 13$ ) undergoing a 21-day diet and exercise intervention

	Pre	Post	% Decrease
Body weight (kg)	111.9 $\pm$ 6.4	107.1 $\pm$ 6.6 <sup>†</sup>	4.3
BMI	38.7 $\pm$ 2.5	34.4 $\pm$ 3.7 <sup>†</sup>	11.1
Total-C (mg/dL)	188.9 $\pm$ 10.1	146.3 $\pm$ 3.8 <sup>†</sup>	22.6
LDL-C (mg/dL)	103.1 $\pm$ 10.2	76.4 $\pm$ 4.3 <sup>†</sup>	25.8
HDL-C (mg/dL)	43.2 $\pm$ 3.2	37.7 $\pm$ 2.8*	12.8
TG (mg/dL)	208.5 $\pm$ 28.2	163.6 $\pm$ 24.8*	17.3
Total-C/HDL-C	4.61 $\pm$ 0.35	4.15 $\pm$ 0.35	10.0
LDL-C/HDL-C	2.50 $\pm$ 0.18	2.13 $\pm$ 0.15*	14.8
Insulin ( $\mu$ U/mL)	33.8 $\pm$ 4.0	23.8 $\pm$ 3.4 <sup>†</sup>	29.6
Blood glucose (mg/dL)	157.5 $\pm$ 10.1	126.7 $\pm$ 8.7*	19.6
HOMA <sub>IR</sub>	12.62 $\pm$ 1.55	7.68 $\pm$ 0.96 <sup>†</sup>	39.1
QUICKI	0.27 $\pm$ 0.004	0.29 $\pm$ 0.004 <sup>†</sup>	-7.3

All data are expressed as mean  $\pm$  S.E. HOMA<sub>IR</sub>: homeostasis-model assessment for insulin resistance. QUICKI: quantitative insulin-sensitivity check index. All data are expressed as mean  $\pm$  S.E.,  $N = 13$ .

\*  $p < 0.05$ .

<sup>†</sup>  $p < 0.01$ , post vs. pre.

#### 3.2. Serum 8-iso-PGF<sub>2</sub> $\alpha$ CRP, sICAM-1 and sE-selectin

After the diet and exercise intervention, a significant reduction in serum 8-iso-PGF<sub>2</sub> $\alpha$  ( $p < 0.05$ , Fig. 1A) was observed. As noted in Fig. 1B, after the intervention, there was a reduction in serum concentration of the inflammatory protein CRP ( $p < 0.05$ ). We measured the serum concentration of sICAM-1 and sE-selectin as indicators of vascular endothelial cell activation and both sICAM-1 ( $p < 0.05$ , Fig. 1C) and sE-selectin ( $p < 0.01$ , Fig. 1D) concentrations decreased post-intervention.

#### 3.3. Monocyte adhesion and in vitro production of VCAM-1, ICAM-1 and MCP-1

Monocyte adhesion to cultured HAEC, using THP-1 cells (a monocytic cell line) significantly decreased after the 20 h incubation with post-intervention vs. pre-intervention serum ( $187 \pm 4.8$  to  $134 \pm 5.8\%$  of FBS control,  $p < 0.01$  Fig. 2A). Medium plus LPS 10 ng/mL induced adhesion that was  $908 \pm 47.9\%$  of the FBS control. In an effort to identify specific factors to account for the observed reductions in THP-1 adhesion, endothelial cell production of VCAM-1, ICAM-1, and MCP-1, were investigated. When pre- and post-intervention sera were added to cultured HAEC, the expression of the adhesion molecules VCAM-1 ( $p < 0.01$  Fig. 2B) and ICAM-1 post-intervention ( $p < 0.001$ , Fig. 2C) was reduced. LPS (10 ng/mL) induced expression of VCAM-1 and ICAM-1 that was  $193 \pm 13.8\%$  and  $362 \pm 18.6\%$  of the FBS control, respectively. We determined the production of MCP-1 in a co-culture of HAEC and HASMC as a marker of monocyte chemoattraction. Moreover, the addition of post-intervention sera to co-cultures of HAEC and HASMC for 4 h resulted in a non-significant reduction in MCP-1 production compared to that noted with pre-intervention ( $p = 0.09$ , Fig. 2D).

#### 3.4. In Vitro superoxide, hydrogen peroxide and NO production

Incubation of subject sera with cultured HAEC in concert with use of the fluorometric probe DAF-2DA was used to detect NO production. DAF-2T quantitated fluorescence, increased significantly as a percentage of FBS control post-intervention versus pre-intervention ( $p < 0.01$ , Fig. 3A), indicating increased NO production from HAEC grown in post-intervention serum. Co-

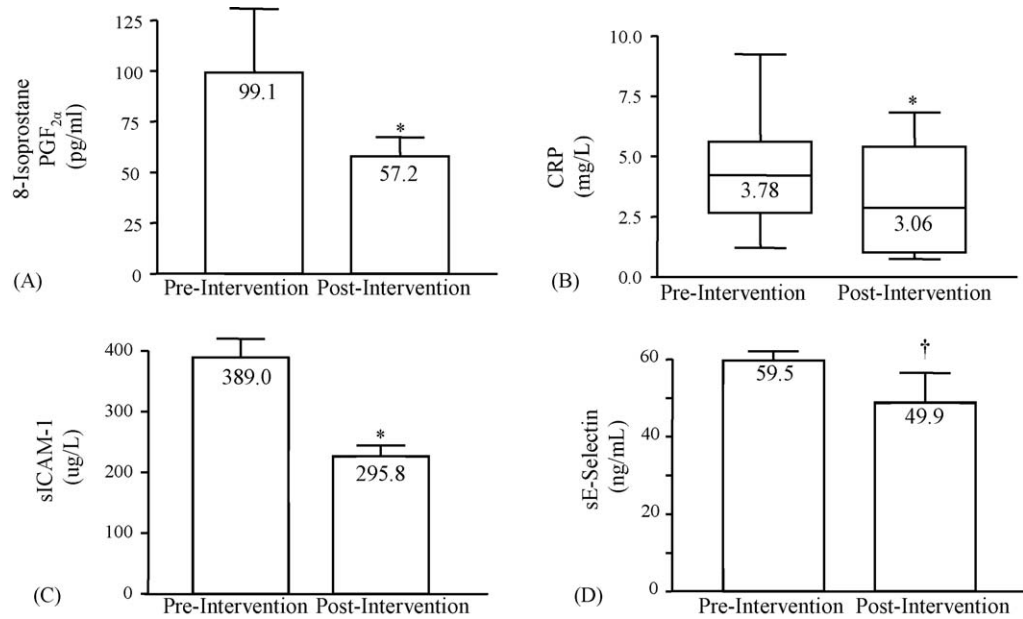


Fig. 1. (A) Effect of diet and exercise intervention on serum concentration of 8-iso- $\text{PGF}_{2\alpha}$  (mean  $\pm$  S.E.,  $p < 0.05$ ), (B) CRP (box plot demonstrates median, 25th, and 75th percentile values), (C) sICAM-1 (mean  $\pm$  S.E.,  $p < 0.05$ ) and (D) sE-selectin (mean  $\pm$  S.E.,  $p < 0.01$ ).

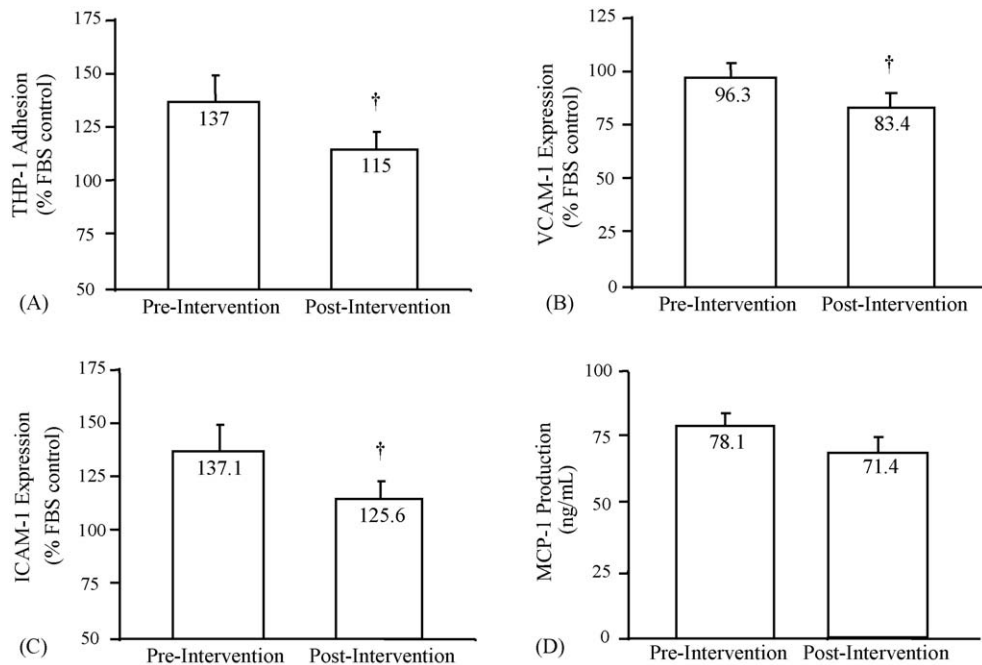


Fig. 2. (A) Effect of serum pre- or post-intervention on THP-1 adhesion. Stimulated cultures were washed and then incubated with THP-1 cells as described in Section 2. Adherent THP-1 cells were counted under light microscopy. (B) Cell surface expression of VCAM-1 and (C) ICAM-1. HAEC cultures were incubated with serum pre- and post-intervention. After incubation, stimulated cultures were washed and fixed as described in Section 2. Data from each subject are presented pre- and post-intervention as a percent of FBS control. The mean  $\pm$  S.E. of pre- and post-intervention data are reported. (D) Effect of serum pre- or post-intervention on coculture production of MCP-1. Cocultures of HASMCs overlaid with HAECs were incubated with M199 medium containing 5% serum pre- or post-intervention. Supernatants from these incubations were analyzed for MCP-1 content by ELISA. The mean  $\pm$  S.E. of pre- and post-intervention data are reported. †  $p < 0.01$  post-intervention vs. pre-intervention.

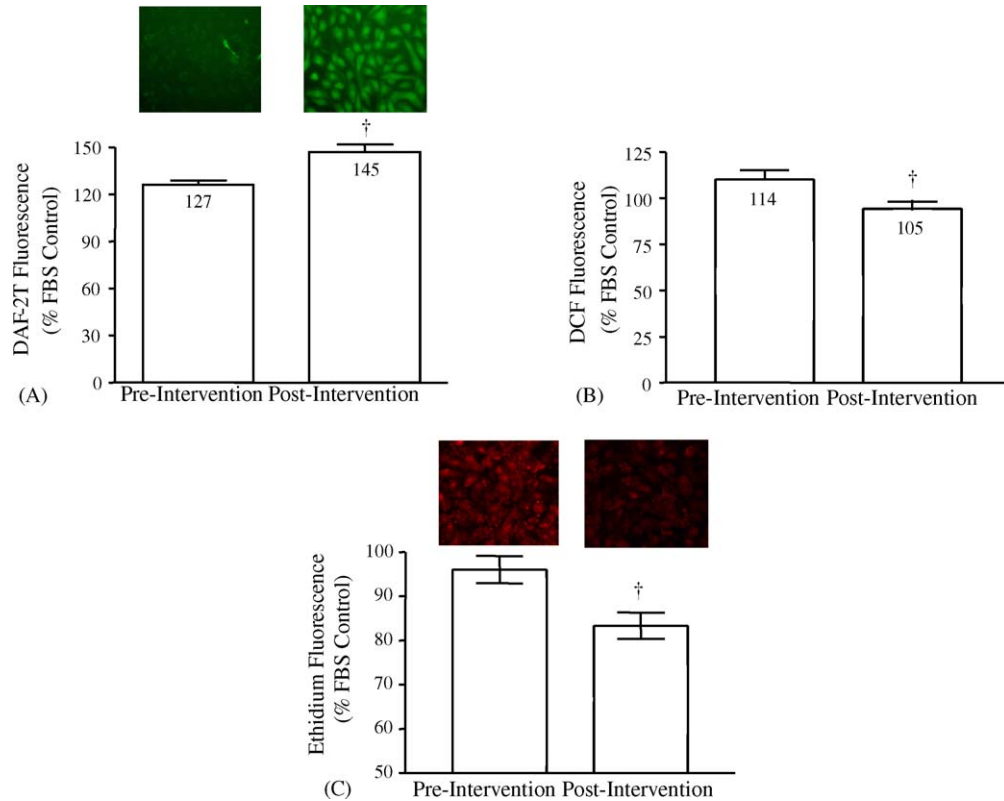


Fig. 3. (A) Endothelial cell serum-stimulated DAF-2T fluorescence (% of FBS control), an index of NO production. (B) Serum-stimulated DCF fluorescence (% of FBS control), an index of peroxide production. (C) Serum-stimulated ethidium fluorescence (% of FBS control), an index of superoxide production. Data are expressed as mean  $\pm$  S.E. \*  $p < 0.05$ , †  $p < 0.01$  post-intervention vs. pre-intervention.

incubation of DAF2-DA with AF-DA or L-NAME abrogated NO production (data not shown). The probe DCF was used as an index of ROS production, primarily as hydrogen peroxide. Post-intervention, there was a significant reduction in DCF fluorescence compared to pre-intervention, indicating a decrease in hydrogen peroxide formation ( $p < 0.01$ , Fig. 3B). Medium plus TNF- $\alpha$  (2 ng/mL) induced H<sub>2</sub>O<sub>2</sub> production that was  $119 \pm 3.0\%$  of the FBS control. Finally, DHE was used to detect superoxide production in HAEC incubated with subject sera. The ethidium fluorescence, an indicator of reacted DHE decreased significantly post-intervention ( $p < 0.01$ , Fig. 3C). Medium plus TNF $\alpha$  (2 ng/mL) induced hydrogen peroxide and superoxide production. These data demonstrate decreased subject sera-stimulated reactive oxygen species generation by HAEC.

#### 4. Discussion

Insulin resistance in muscle is the primary defect leading to type 2 diabetes [27] and the CDC has estimated that 40% of US adults have impaired fasting

glucose and/or impaired glucose tolerance [28]. In parallel, diabetes has increased dramatically and to epidemic levels in recent years with the estimated prevalence increasing 600% from 1958 to 1993 [1]. Currently, ~20 million people in the US have diabetes, with 18% of those 60 years or older having the disease [29]. Furthermore, the risk of CVD is elevated long before the clinical manifestation of diabetes [30]. Diet quality and fitness can predict risk of diabetes. For instance, in the Nurses' Health Study, a higher consumption of refined grains was associated with an increased risk of diabetes [31]. Additionally, several studies indicate that low fitness increases the risk of diabetes and increased physical activity is effective in preventing diabetes [32,33]. In men with diabetes, low cardiorespiratory fitness and physical inactivity independently predict mortality risk compared with fit men [34]. Lifestyle interventions have been documented to improve insulin sensitivity (as reviewed by Roberts and Barnard [13]), however the effects on emerging CVD risk factors is largely unknown. Hence, the present study was designed to investigate the effects of a short-term, intensive diet and exercise intervention on novel

contributors to atherogenesis in diabetics. The primary findings of this study provide evidence that in men with type 2 diabetes, lifestyle change may (1) increase insulin sensitivity; (2) improve the lipid profile; (3) decrease oxidative stress as determined by 8-iso-PGF<sub>2α</sub>, superoxide and hydrogen peroxide production, and increase NO production; (4) decrease inflammation; (5) decrease endothelial cell activation; and (6) decrease monocyte adhesion.

A significant decrease in total-C, LDL-C and TG levels was noted post-intervention. Evidence from previous investigations using the same intervention in diabetics [35,36] is in agreement with these findings. The decreases are likely due to increased fiber/unrefined carbohydrate and reduced saturated fat/trans fat/cholesterol consumption [37], as well as exercise [38]. The observed drop in HDL-C is consistent with earlier reports using the same intervention [39,40] as well as a report by Brinton et al. [41] using a low-saturated fat, low-cholesterol diet. However, the decrease in HDL-C was coupled with large reductions in both LDL-C and total-C and a marked reduction in the LDL-C:HDL-C ratio, suggesting a decrease in CAD risk. Further, it is now apparent that HDL, which can prevent LDL-induced monocyte transmigration [19], may actually be pro-inflammatory, independent of the level of HDL-C [20,42,43].

As expected, there was an improvement in insulin sensitivity. Exercise training ameliorates insulin resistance through several mechanisms, by direct effects on the muscle, such as enhancing insulin receptor autophosphorylation [44], increasing GLUT-4 content [45–48] and glucose transport-phosphorylation [49], as well as by reducing visceral obesity [46]. Regarding specific dietary components, dietary fiber consumption is associated with reduced diabetes risk [50–52] and lower fasting insulin [50].

Previously, elevations in sVCAM-1, sICAM-1 and sE-selectin have been reported in type 2 diabetics [53–55]. One caveat regarding soluble CAM determination is that it is not known how accurately the serum levels represent the expression level in the activated endothelium. For example, ICAM-1 is also expressed in non-endothelial cell types including macrophages, monocytes, lymphocytes, and intimal smooth muscle cells [56,57]. Hence, in addition to soluble adhesion molecules we measured HAEC serum-stimulated production of ICAM-1 and VCAM-1, and found both to be reduced post-intervention in agreement with a prior long-term lifestyle trial in which a decrease in sICAM-1 was noted [58]. We also noted a significant decrease in THP-1 cell adhesion to endothelial cell

cultures incubated with post-intervention serum. This was likely due in part to the decreases in VCAM-1 or ICAM-1. The effect of post-intervention serum on HAEC could be due, in part, to increased antioxidant capacity, decreased LDL oxidation and/or reduced LDL substrate, resulting in less endothelial cell activation.

Atherosclerosis is not only as a disease of lipid accumulation, but also a chronic inflammatory process [59,60] and inflammation is now considered to be a component of the metabolic syndrome [61,62], with CRP being a well established promoter of atherogenesis. The present study demonstrated a reduction in CRP, a finding unrelated to CRP instability [63] or circadian variation [64]. Previously, the Diabetes Prevention Program noted reduced CRP with lifestyle intervention [65]. Receptor-mediated CRP uptake is associated with decreased nitric oxide bioavailability in human endothelial cells [66–68] and induces plasminogen activator inhibitor [69]. The observed increase in NO production and/or decrease in reactive oxygen species may be related to the reduction in CRP.

Non-enzymatic oxidation products of arachidonic acid by reactive oxygen species, denote the presence of oxidative stress and are inducers of inflammation and promote vascular endothelial cell and platelet activation. Urinary levels of F<sub>2</sub>-isoprostanes have been found to be an accurate, direct measure of *in vivo* oxidative stress in type 2 diabetics [70]. The diet and exercise intervention resulted in a reduction in 8-iso-PGF<sub>2α</sub>, providing evidence for decreased lipid peroxidation. Thompson et al. [71] documented a 35% reduction in urinary 8-iso-PGF<sub>2α</sub> after 14 days of consuming a diet with an array of fruits and vegetables. Additionally, we utilized a HAEC culture system and specific fluorescent probes to study subject sera-stimulated NO and ROS production. As determined by DAF2-DA fluorescence, we noted increased NO production by HAEC *in vitro* in agreement with previous data documenting improved urinary NO metabolite excretion with lifestyle intervention [14]. Additionally, Hamdy et al. [58] noted increases in both insulin sensitivity and flow-mediated dilation in subjects with impaired glucose tolerance and diabetes. Both DCF and ethidium fluorescence decreased post-intervention indicating reduced serum-stimulated production of peroxides and superoxide, respectively. It is plausible that the improvement in endothelial function noted in diabetics with diet and/or exercise [72], was due, at least in part to increasing endothelial cell NO production, decreased NO-scavenging reactive oxygen species production, which correlate inversely with endothelial dysfunction in humans [73].

The current study has important strengths and limitations to consider. The major strength is the supervised nature of the study. Supervising food intake and physical activity removes the need to question compliance or to rely on food and activity questionnaires. Further, all exercise sessions were supervised and adherence to the diet and activities was essentially 100%. Conversely, the study was not randomized and the subjects were motivated to take part in the intervention; hence, we cannot extrapolate adherence to the general population. Nevertheless, the findings document that benefits are possible in motivated subjects. Caloric intake was not determined with consumption of the ad libitum diet, (except for animal protein). However, increasing fiber and reducing the fat content of the diet without specific efforts to maintain body weight has been reported to result in a spontaneous decrease in caloric intake and weight loss [74,75]. Finally, more sophisticated measures of insulin sensitivity such as an oral-glucose tolerance test may be more definitive than surrogate measures of insulin sensitivity used in our analysis.

In conclusion, implementation of a high-fiber, low-fat diet, combined with daily aerobic exercise, results in significant reductions in serum lipids, insulin, oxidative stress, inflammation, leukocyte-endothelial interactions, and adhesion in diabetic men. The reduced monocyte adhesion after the intervention is indicative of increased antioxidant/oxidant balance of the serum [42,43]. The diabetic and lipid responses observed in a relatively short period have been documented to be durable for 2–3 years follow-up in diabetics [35]. An intervention of this type may help reduce atherosclerosis progression and risk of myocardial infarction in patients with type 2 diabetes.

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