Effect of a Short-Term Diet and Exercise Intervention on Oxidative Stress, Inflammation, MMP-9 and Monocyte Chemotactic Activity in Men with Metabolic Syndrome Factors

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Abstract

The present study was designed to examine the effects of lifestyle modification on key contributing factors to atherogenesis, including oxidative stress, inflammation, chemotaxis, and cell adhesion. Obese men (N=31), 15 of whom had metabolic syndrome 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 (for estimation of insulin sensitivity), oxidative stress generating enzyme myeloperoxidase (MPO) and marker 8-isoprostaglandin F$_2\alpha$ (8-iso-PGF$_2\alpha$), the inflammatory protein C-reactive protein (CRP), soluble intracellular adhesion molecule (sICAM)-1 as an indicator of endothelial activation, sP-selectin as a marker of platelet activation, the chemokine macrophage inflammatory protein-1α (MIP-1α) and total matrix metalloproteinase-9 (MMP-9). Using subject sera and human aortic endothelial cell (HAEC) culture systems, vascular cell adhesion molecule-1 (VCAM-1) cell surface abundance and monocyte chemotactic protein-1 (MCP-1), nitric oxide (NO), superoxide, and hydrogen peroxide production were measured *in vitro* by fluorometric detection. Also determined *in vitro* was serum-induced, monocyte adhesion and monocyte chemotactic activity (MCA). After 3 weeks, significant reductions (p<0.05) in BMI, all serum lipids and lipid ratios, fasting glucose, insulin, homeostasis model assessment for insulin resistance, MPO, 8-iso-PGF$_2\alpha$, CRP, sICAM-1, sP-selectin, MIP-1α, and MMP-9 were noted. *In vitro*, serum-stimulated cellular VCAM-1 expression, MCP-1 production, and fluorometric detection of superoxide and hydrogen peroxide production decreased, while a concomitant increase in NO production was noted (all p<0.01). Additionally, both monocyte adhesion (p<0.05) and MCA (p<0.01) decreased. Nine of 15 were no longer positive for metabolic syndrome post-intervention. Intensive lifestyle modification may ameliorate novel CAD risk factors in men with metabolic syndrome factors prior to reversal of obesity.

Keywords: atherosclerosis, lipids, inflammation, cell adhesion molecules, nitric oxide
Introduction

Atherosclerotic disease is the leading cause of mortality in developed countries with coronary artery disease (CAD) being the number one killer of both men and women. Early epidemiological and clinical studies established a link between dietary saturated fat and cholesterol, serum cholesterol and CAD mortality (28). The Framingham Heart Study and MRFIT Study emphasized the relationship between serum cholesterol, especially low-density lipoprotein cholesterol (LDL-C) and CAD (10). Although serum lipid levels have been the focus to explain the incidence of atherosclerosis, other risk factors such as oxidative stress, inflammation, endothelial cell activation, platelet activation and plaque stability are important. For example, levels of the oxidative stress markers 8-isoprostaglandin F_2α (8-iso-PGF_2α) (42) and myeloperoxidase (MPO) (8), inflammation-associated proteins C-reactive protein (CRP) and soluble intracellular adhesion molecule (sICAM)-1 (48), soluble P-selectin (47) and the matrix metalloproteinase MMP-9 (17) all independently predict early risk of cardiovascular disease or myocardial infarction.

Diet and exercise modifications have been documented to ameliorate the classic CAD risk factors, including hyperlipidemia, hypertension and insulin resistance (49). The clustering of multiple CAD risk factors in the same individual, commonly referred to as the metabolic syndrome, is extremely prevalent in Westernized societies, affecting approximately 23% of the population and approximately 43% of those above 60 years of age (20). Accordingly, the examination of the effect of modifying environmental influences (eg. diet, physical activity, cigarette smoking) is warranted. The present study was designed to investigate the hypothesis that a combined intervention consisting of a high-fiber, low-fat diet and physical activity confers reductions in oxidative stress, inflammation, and monocyte-endothelial interactions in obese men at high risk for CAD and related clinical outcomes.

Methods

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 participating subjects was obtained. Serum samples for this study were obtained from 31 overweight/obese middle-aged and older men (age range 46 – 76, mean 63.3 yr) who voluntarily participated in the Pritikin Longevity Center 21-day residential diet and exercise intervention during 2001. All participants were overweight or obese (mean body mass index (BMI) = 33±1.5) and 15 had the metabolic syndrome according to the World Health Organization (WHO) modified criteria (32). There were 13 Type 2 diabetic patients (by fasting glucose), all others had >1 metabolic syndrome factor and 5 had diagnosed CAD. All subjects were free of any viral infections and were able to consume the prescribed diet and to perform physical activity.

Once enrolled in the program, participants underwent a complete medical history and physical examination, prior to a 21-day diet and exercise intervention as previously described (50). 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 (>40g 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 (≥ 5 servings/day), vegetables (≥ 4 servings/day) and fruits (≥ 3 servings/day). Protein was from plant sources, nonfat dairy (up to 2 servings/day) and fish/fowl (3 ½ 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 results, the subjects were provided with an appropriate training heart rate value and given an individualized walking program including both level and graded walking. The exercise regimen consisted of daily treadmill walking at the training heart rate for 45-60 minutes. The training heart rate was defined as 70 to 85% of the maximal heart rate attained during the treadmill test.
Twelve-hour fasting blood samples were drawn from the subjects in 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 \(-80^\circ C\) 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 (m\(^2\)).

**Determination of Serum Lipids, Glucose, Insulin, HOMA\(_{IR}\) and QUICKI**

Total-C, triglycerides (TG) and HDL-C levels were measured using a kit from Sigma Diagnostics (St. Louis, MO). The LDL-C was calculated as described by Friedewald et al. (21). Glucose concentration was determined using standard enzymatic procedures on an Olympus Autoanalyzer (Quest Diagnostics). Fasting insulin concentration was measured by radioimmunoassay (Diagnostic Systems Laboratories). Insulin resistance was evaluated using homeostasis model assessment (HOMA\(_{IR}\)) which has been shown to correlate with insulin sensitivity by the hyperinsulinemic-euglycemic clamp (31). HOMA\(_{IR}\) is calculated as \([\text{fasting insulin (}\mu\text{U/ml}) \times \text{fasting glucose (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 (26). QUICKI = \(1/[\log(\text{fasting insulin (}\mu\text{U/ml)}) + \log(\text{fasting glucose (mg/dl)})]\). Because QUICKI is the reciprocal of the log-transformed product of fasting glucose and insulin, it is a dimensionless index without units.

**Oxidative Stress Measures**

Serum MPO concentration was measured in duplicate with an ELISA kit (Calbiochem) according to the manufacturer’s instructions. Serum 8-iso-PGF\(_{2\alpha}\) was measured in duplicate using an enzyme immunoassay kit (Cayman Chemical).

**Determination of Serum CRP, sICAM-1, sP-selectin, MIP-1\(_\alpha\), and MMP-9**
Serum CRP (Diagnostic Systems Laboratories), soluble ICAM-1 (sICAM-1) and soluble P-selectin (sP-selectin), MIP-1α (Biosource) and MMP-9 (R&D Systems) concentrations were measured in duplicate with specific ELISA kits according to the manufacturer’s instructions.

**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 (40, 52). In brief, the cells were subcultured by trypsinization and grown to confluence in 75cm² 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). 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 hr. HASMC were seeded in the wells at a density of approximately 5 X 10⁴ cells/cm² and were cultured for 2-3 d at 37°C, 95%O₂/5%CO₂ at which time they had reached confluency. For co-cultures, HAEC were subsequently overlaid on top of HASMC at approximately 1 X 10⁵ cells/cm² and were allowed to grow forming a complete monolayer of confluent EC in 2 d.

**Production of MCP-1.** Cocultures were washed with M199 medium containing 1% FBS 3 times. The second wash was incubated for 1-2 hr at 37°C and 95%O₂/5%CO₂; other washes were removed immediately. Washed cocultures were then incubated with M199 medium containing 5% human serum pre- and post-intervention for 4 hr. Subsequently, cultures were centrifuged and culture supernatants harvested and stored at –20°C pending the MCP-1 measurement by ELISA (PharMingen, BD™ OptEIA ELISA Set #555179) following the manufacturer’s instructions. Serum samples were incubated in wells with no cells as a 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.
**VCAM-1 Expression.** A modified ELISA procedure was used to measure cellular VCAM-1 content (44). Human aortic endothelial cells (HAEC) were grown to confluency in 96-well plates. Cultures were then washed 3 times in media containing 1% FBS. The second wash was incubated for 1-2 hr at 37°C, 95%O₂/5%CO₂. Other washes were removed immediately. Washed cultures were then incubated with media containing 10% pre-intervention or post-intervention serum for 4 hr. After incubation, supernatants were removed and HAEC were washed once with 200µl of PBS, fixed in 150µl of 100% methanol for 10 min, air-dried, sealed and stored at 4°C. Dried cells were rehydrated and blocked in 200 µl 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 hr at room temperature a mouse monoclonal IgG antibody against human VCAM-1 (PharMingen Cat.# 555645) diluted 1:500 in PBS/Tween/BSA. The wells were then washed twice in PBS/Tween, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody against mouse IgG from sheep (Amersham Life Sciences Cat. #NA931V) diluted in PBS/Tween/BSA at a 1:1000 dilution. After a 1 hr incubation at room temperature, wells were washed five times in PBS/Tween, and they were subsequently colored using 100µl of tetramethylbenzidine reagent (Sigma Cat. #T0440) as substrate for the bound HRP. After 5 min of incubation, the reaction was stopped by adding 100 µl of 3M H₂SO₄. Absorbance was read at 450 nm. Fetal bovine serum (10% FBS) was used as a control and 10 ng/mL lipopolysaccharide (LPS) was used as a positive control. The data are expressed as percentage of FBS control.

**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 (37). 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 µL serum.
free medium (SFM) three times. The second wash was incubated for 1 hr at 37°C and 95%O₂/5%CO₂. SFM supplemented with 10% test serum (pre- or post intervention) was added onto cells which were then incubated at 37°C and 95%O₂/5%CO₂ for 18 hr. Thereafter, 100 µL of 10 µM DAF-2DA dissolved in SFM was added to all cells except negative control wells. Negative control wells included cells with 100 µL of SFM containing either 10 µM 4 AF-DA (Calbiochem), a non-reactive DAF-2DA analog, or 300 µM 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%O₂/5%CO₂ for 1 hr. After incubation, cells were washed three times with 200 µL of SFM to remove any residual extracellular DAF-2DA probe. Subsequently, 100 µL of SFM with 4 µM bradykinin (Calbiochem) was added to the cells. The DAF-2DA positive control included DAF-2T (Calbiochem) dissolved in SFM. After 10 to 15 min in a light protected incubator at 37°C and 95%O₂/5%CO₂, 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 (10X). A higher magnification (20X) 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.

**Hydrogen Peroxide Production.** The cell permeable fluorescent probe 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes) was used to measure hydrogen peroxide production by HAEC (11). 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 (5). In cultured endothelial cells, DCF is oxidized by H₂O₂, along with other intracellular processes involving reactive oxygen species, making it useful in determining changes in hydrogen peroxide and overall oxidant formation (51). SFM supplemented with 10% test serum (pre- or post intervention) was added onto cells which were then incubated at 37°C and 95%O₂/5%CO₂ for 18 hr. The hydrogen peroxide positive control included cells incubated for 18 hr with 2 ng/mL TNF-α dissolved in SFM. Following incubation, the supernatant was removed from the cells and 100 µL of 10 µM DCFH-DA dissolved in SFM was applied to the cells which were then placed in a light protected incubator at 37°C and 95%O₂/5%CO₂ for 1 hr. Following DCFH-DA incubation, cells were washed three times with 200 µL SFM to remove residual extracellular probe. After washing, 100 µL SFM was applied to the cells which were then placed in a light protected incubator at 37°C and 95%O₂/5%CO₂ 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.

**Superoxide Production.** The cell permeable fluorescent probe dihydroethidium (DHE, Calbiochem) was used to measure superoxide production by HAEC (53). 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%O₂/5%CO₂ for 18 hr. The superoxide positive control included cells incubated with 2 ng/mL TNF-α dissolved in SFM. Following incubation, the supernatant was removed and 100 µL of 25 µM DHE dissolved in SFM was applied to the cells which were then
placed in a light protected incubator at 37° C and 95%O₂/5%CO₂ for 45 min. Following DHE incubation, cells were washed once with 200 µL SFM and then twice with 200 µL HEPES to remove residual extracellular probe. After washing, 100 µL HEPES was applied to the cells which were then placed in a light protected incubator at 37° C and 95%O₂/5%CO₂ 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).

**Monocyte Adhesion Assay.** Confluent monolayers of HAEC were grown and monocyte adhesion was quantified with adaptation of methods from Van Lenten et al. (52). 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 hr. Supernatants were then removed and cultures washed with SFM. A 400 µl THP-1 suspension in M199 providing 2.0 x 10⁵ cells/cm² was then added per well. 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 320x magnification. The pre-intervention and post-intervention serum was compared with 10% FBS and 10 ng/ml LPS as a positive control.

**Monocyte Chemotactic Activity (MCA) Assay.** Monocytes from healthy normal individuals were isolated by a modification of the Recalde procedure (18). The MCA was performed as previously described by Navab et al. (40). Briefly, pre and post-intervention sera were added to confluent monolayers of HAEC. Eighteen hours later, supernatants were collected and tested for MCA as
described previously (38). The supernatants were added to a standard Neuroprobe chamber (NeuroProbe, Cabin John, MD), with isolated human peripheral blood monocytes added to the top. The chamber was incubated for 60 min at 37°C. After the incubation, the chamber was disassembled and the nonmigrated monocytes were wiped off. The membrane was then air dried and fixed with 1% glutaraldehyde and stained with 0.1% crystal violet dye. The number of migrated monocytes was determined microscopically and expressed as the mean ± SD of 12 standardized high power fields counted in quadruple wells. Additionally, we confirmed that this assay is specific to monocytes and chronic inflammation, as opposed to neutrophils and acute inflammation, by measuring E-selectin after a 4 hr incubation, which did not change (2).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism version 4.0 for Windows, GraphPad Software (San Diego, CA) and STATA 9.0 (College Station, TX). Pre-intervention and post-intervention values were compared using matched pair t-tests. Correlations were tested on all available data by determining Pearson’s correlation coefficients for the relationships between MPO, CRP, 8-iso-PGF$_{2\alpha}$, DAF-2A, ethidium and DCF fluorescence and BMI, body weight, fasting insulin, glucose and HOMA-IR. All data are expressed as mean ± SE unless otherwise noted. A p value of < 0.05 was considered statistically significant.

Results

Fasting Lipids, Glucose, Insulin, Blood Pressure and Anthropometry

Anthropometric and metabolic data are presented in Table 1, along with consensus values that the medical community considers normal. The 21-day diet and exercise intervention significantly reduced body weight (p<0.01) and BMI (p<0.01), although as a group, the subjects remained obese (BMI>30 kg/m$^2$) at the end of the intervention. Subjects positive for metabolic syndrome by WHO criteria decreased from 48% to 19%. Noted were significant reductions in Total-C, LDL-C, HDL-C, TG, LDL:HDL and total-C:HDL-C (p<0.01 for all). Significant reductions in serum fasting glucose
(p<0.05), fasting insulin (p<0.01) were also observed after the intervention. The HOMA_{IR} and QUICKI were determined as surrogates of insulin sensitivity. The intervention resulted in a significant decrease in HOMA_{IR} (p<0.01) as well as a significant rise in QUICKI (p<0.01). Correlation analyses indicated that except for a significant correlation between change in CRP and change in insulin (r=0.52, p=0.03), no significant correlation between changes in inflammation or oxidative stress measures and metabolic factors were noted.

**Oxidative Stress Measures**

After the diet and exercise intervention, the enzymatic oxidant MPO was significantly reduced (132.7±11.2 vs. 166.2±19.3 ng/mL, p<0.05, Figure 1a). Additionally, a significant reduction in serum 8-iso-PGF$_{2\alpha}$ (131.3±21.6 vs. 202.6±37.2 pg/mL, p<0.01, Figure 1b) was observed.

**Serum, CRP, sICAM-1, sP-selectin and MMP-9**

As noted in figure 2, after the intervention, there was a reduction in serum concentration of the inflammatory protein CRP (1.46±0.29 vs. 2.39±0.46 mg/L, p<0.01, Figure 2A). We measured the serum concentration of ICAM-1 and sP-selectin as indicators of vascular endothelial cell and platelet activation, respectively. Both ICAM-1 (291.2±33.4 vs. 363.5±45.0 ng/mL, p<0.05, Figure 2B) and sP-selectin (135.3±5.6 vs. 147.5±8.1 ng/mL, p<0.01, Figure 2C) concentrations decreased post-intervention. Additionally, serum total level of the gelatinase MMP-9, an index of plaque stability was reduced post-intervention (625.7±61.3 vs. 779.3±85.2 ng/mL, p<0.01, Figure 2D).

**Monocyte Adhesion, Chemotactic Activity and MIP-1α**

Monocyte adhesion using THP-1 cells, a monocytic cell line, significantly decreased after the intervention (127.7±13.4 vs. 185.3±12.5 adherent THP-1/HPF, p<0.001, figure 3A). The ability of serum to induce MCA was tested by incubating pre- and post-intervention serum with cultured HAEC. Supernatants from these incubations revealed a significant reduction in MCA (11.1±1.1 vs. 16.5±1.3
migrated monocytes/HPF, p<0.01, figure 3B). The chemokine MIP-1α also decreased post-intervention (8.93±1.7 vs. 12.6±1.8 pg/mL, p<0.05, figure 3B), corroborating the drop in MCA.

**In Vitro Production of MCP-1, VCAM-1, Superoxide, Hydrogen Peroxide and NO**

In an effort to identify specific factors to account for the observed reductions in THP-1 adhesion, and chemotaxis, endothelial cell production of MCP-1, VCAM-1, ROS and NO were investigated. When pre- and post-intervention sera were added to cultured HAEC for 4 hr, the expression of the adhesion molecule VCAM-1 was reduced (97.0±5.1% vs. 113.8±7.6% of FBS control, p<0.01, Figure 4A). We determined the production of MCP-1 in a co-culture of HAEC and HASMC as an index of monocyte chemoattraction. Moreover, the addition of post-intervention sera to co-cultures of HAEC and HASMC for 4 hr resulted in a lower production of MCP-1 compared to that noted with pre-intervention (62.4±3.8 vs. 68.0±3.7 ng/ml, p<0.05, Figure 4B).

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 (105.4±5.8% vs. 114.2±7.4%, p<0.01, Figure 5A). Medium plus TNFα (2 ng/mL) induced hydrogen peroxide production (119±3.0% of the FBS control). 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 (84.5±4.8% vs. 92.0±4.5%, p<0.01, figure 5B). These data demonstrate decreased subject sera-stimulated reactive oxygen species generation by HAEC. Finally, 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 (132±5% vs. 144±5% of FBS control, p<0.01, figure 5C), indicating increased NO production from HAEC grown in post-intervention serum. Co-incubation of DAF2-DA with AF-DA or L-NAME abrogated NO production (data not shown).
Discussion

Current recommendations to reduce the risk of CAD include lifestyle modification (exercise and diet), which may have significant benefit in the prevention/treatment of CAD (49). The Adult Treatment Panel III guidelines included recommendations for therapeutic lifestyle change, including diet and physical activity (1). Hence, the present study was designed to investigate the effects of a short-term, intensive diet and exercise intervention on several aspects of atherogenesis in men with metabolic syndrome factors. The primary findings of this study provide evidence that in men with metabolic syndrome, lifestyle may 1) improve the lipid and metabolic profile; 2) decrease oxidative stress and increase NO production; 3) decrease inflammation; 4) decrease endothelial cell activation and platelet activation; 5) decrease monocyte adhesion and MCA and; 6) decrease MMP-9, a marker of plaque destabilization.

For the first time, we 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 reduction in serum-stimulated VCAM-1 expression, serum ICAM-1 and sP-selectin. P-selectin is a cell-surface adhesion molecule involved in leukocyte rolling and attachment as well as platelet activation (36), and thus the reduction in sP-selectin also reflects a reduction in platelet activation. We also noted a decrease in MCA in HAEC after the diet and exercise intervention. The decrease in MCA was confirmed by a reduction in MCP-1 production in a HAEC/HASMC coculture, as MCA is directly related to MCP-1 production (40). Additionally, the decrease in MIP-1α, a chemoattractant that activates monocytes (45), supports the contention that monocyte attraction was mitigated by the intervention. 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 a disease of lipid accumulation, but also a chronic inflammatory process (6) and inflammation is now considered to be a component of the metabolic syndrome (56), with CRP being a well established promoter of atherogenesis. The present study demonstrated a marked reduction
in CRP (~40%) within 3 weeks, a finding likely unrelated to CRP instability (41) or circadian variation (33). We previously reported a reduction in CRP in women after the same diet and exercise intervention (54) as have other groups using caloric restriction (25) or diet and exercise (57). Receptor-mediated CRP uptake is associated with decreased nitric oxide bioavailability in human endothelial cells (14) and induces plasminogen activator inhibitor (15). The observed increase in NO production and/or decrease in reactive oxygen species may be related to the reduction in CRP. Epidemiological studies (19) suggest that regular physical activity is associated with reduced inflammation and Liu et al. (35) have shown that glycemic load is associated with increased plasma CRP concentration.

Vulnerable plaques tend to have a lipid-rich core, with a thin fibrous cap and reduced extracellular matrix and extensive macrophage-derived foam cells that produce MMP enzymes that degrade the collagen and elastin components of the matrix. Recent data suggests that MMPs play an integral role in human atherosclerosis and plaque disruption, as patients with CAD and history of myocardial infarction have elevated MMP-9 serum levels (17, 46). The decreased total MMP-9 post-intervention is in agreement with the reduction in oxidized lipids, which have been shown to upregulate MMP-9 expression in monocyte derived macrophages (55). Previously Koh et al. (30) investigated the effects of diet and simvastatin on MMP-9 levels and noted that while an AHA Step I diet for 14 weeks (reduced cholesterol about ~10%) had no effect on MMP-9 levels, addition of simvastatin led to a reduction in MMP-9.

One potential enzymatic source for generation of NO-derived oxidants is MPO, a hemoprotein abundantly expressed in neutrophils and to a lesser extent by monocytes and macrophages, that displays potent proatherogenic properties. MPO can oxidize LDL cholesterol, thereby propagating its uptake by macrophages and perpetuating foam cell formation (43). MPO is markedly increased within human atherosclerotic lesions (13) and directly utilizes both NO and nitrite as substrates in vitro (16) and participates in both protein nitration and initiation of lipid peroxidation in vivo (9). The decrease in MPO with lifestyle modification provides evidence for a decrease in oxidative stress, data that agrees
with our previous documentation of a reduction in 8-iso PGF2α (50), which we also confirmed. Interestingly, MPO has been shown to activate MMPs and promote destabilization and rupture of the atherosclerotic plaque surface (22). Thus, the reduction in MMP-9 in this study may be mediated, in part, by the reduction in MPO.

We utilized a HAEC culture system and specific fluorescent probes to study subject sera-stimulated NO and ROS production. Both DCF and ethidium fluorescence decreased post-intervention indicating reduced serum-stimulated production of peroxides and superoxide respectively. 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 (50). Additionally, Hamdy et al. (24) 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 by others (12, 23) with diet and/or exercise was due, at least in part, to increasing endothelial cell NO production, decreased NO-scavenging reactive oxygen species production and/or MPO levels, which vary inversely with endothelial dysfunction (3).

The current study has important strengths and limitations that need to be acknowledged. 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 intake 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 (27, 34). Finally, more sophisticated measures of insulin sensitivity such as an oral-glucose tolerance test may be more definite than surrogate measures of insulin sensitivity used in our analysis. Its possible that we underestimated the number of diabetic subjects as we did not perform oral-glucose tolerance tests.

In conclusion, multiple factors can modulate atherosclerotic lesions, and little is known about the effects of lifestyle modification on novel mediators of the atherosclerotic process. 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, adhesion and MCA, and leukocyte production of MMP-9. The reduced monocyte adhesion and MCA observed using the serum after the intervention is a clear indication of increased antioxidant/oxidant balance (39). The changes observed appear to be largely independent of weight loss, as the magnitude of weight loss was minimal (107 to 104 kg, mean BMI change 1 unit), the subjects remained overweight/obese at the conclusion of the study. Furthermore, correlational analysis showed no significant relationship between change in BMI or body weight and any of the parameters measured. These observations suggest that an appropriate diet and daily exercise may confer significant risk reduction despite relatively minimal weight loss. Blair and colleagues have suggested that cardiorespiratory fitness may be a more powerful predictor of risk than body weight per se (7). We suggest that the focus for CAD risk reduction be emphasized through ameliorating metabolic risk as opposed to focusing on body weight changes. An intervention of this type may be of clinical benefit for those desiring rapid cardiovascular risk reduction. Additionally, the changes in selected diabetic-related variables appear to be superior to changes noted with metformin treatment (29), furthering the notion that lifestyle may provide an alternative to traditional drug treatment. The impressive response observed in a relatively short period has been documented to be durable in its effects (4), and highlights the value of intensive lifestyle modification in men at high risk for CAD, which if sustained may mitigate the progression of atherosclerosis and its clinical consequences.
Acknowledgements

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References


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Figure Legends

Figure 1. (A) Effect of diet and exercise intervention on serum MPO (N=21) (B) Effect of diet and exercise intervention on serum 8-iso PGF2α (N=21). Data are expressed as mean ± SE.*P<0.05, †P<0.01 post-intervention vs. pre-intervention.

Figure 2. (A) Effect of intervention on serum concentration of CRP (N=31). (B) Effect of intervention on serum sICAM-1 (N=21). (C) Effect of intervention on serum sP-selectin (N=10). (D) Effect of intervention on serum MMP-9 (N=21). Data are expressed as mean ± SE. *P<0.05, †P<0.01 post-intervention vs. pre-intervention.

Figure 3. (A) Effect of intervention on monocyte adhesion (N=16) (B) monocyte chemotaxis (N=21) and (C) MIP-1α (N=20). Data are expressed as mean ± SE. *P<0.05, †P<0.01 post-intervention vs. pre-intervention.

Figure 4. (A) Effect of intervention on VCAM-1 cell surface expression on HAEC (N=23). (B) Effect of intervention on MCP-1 expression in a HAEC/HASMC coculture system (N=22). Data are expressed as mean ± SE. †P<0.01 post-intervention vs. pre-intervention.

Figure 5. (A) Serum-stimulated ethidium fluorescence (% of FBS control), an index of superoxide production (N=9). (B) Serum-stimulated DCF fluorescence (% of FBS control), an index of peroxide production (N=10). (C) Endothelial cell serum-stimulated DAF-2T fluorescence (% of FBS control), an index of NO production (N=22). Data are expressed as mean ± SE. †p<0.01 post-intervention vs. pre-intervention.
Table 1. Anthropometric and metabolic parameters of subjects (N=31) undergoing a 21-day diet and exercise intervention.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
<th>% Decrease</th>
<th>Consensus values</th>
</tr>
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<tbody>
<tr>
<td>Percent with Metabolic Syndrome</td>
<td>48%</td>
<td>19%</td>
<td>3.6</td>
<td>-</td>
</tr>
<tr>
<td>% Obese (BMI&gt;30)</td>
<td>71%</td>
<td>58%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Overweight (BMI&gt;24.9)</td>
<td>100%</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Diabetic</td>
<td>42%</td>
<td>23%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>106.5±3.9</td>
<td>102.7±4.0†</td>
<td>3.6</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>35.4±1.5</td>
<td>34.4±1.6†</td>
<td>2.8</td>
<td>18.5-24.9</td>
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<tr>
<td>Total-C, mg/dL</td>
<td>201.0±6.6</td>
<td>158.7±6.0†</td>
<td>21.0</td>
<td>&lt;200</td>
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<tr>
<td>LDL-C, mg/dL</td>
<td>118.1±6.6</td>
<td>88.1±5.5†</td>
<td>25.5</td>
<td>&lt;100</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>44.1±1.7</td>
<td>39.5±1.7*</td>
<td>10.5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>215.0±21.5</td>
<td>156.4±15.4†</td>
<td>27.7</td>
<td>&lt;150</td>
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<tr>
<td>Total-C/HDL-C</td>
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<td>4.19±0.20†</td>
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<tr>
<td>LDL-C/HDL-C</td>
<td>2.72±0.15</td>
<td>2.29±0.13†</td>
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<tr>
<td>Insulin, µU/mL</td>
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<td>21.6±2.7†</td>
<td>30.1</td>
<td>-</td>
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<tr>
<td>Blood glucose, mg/dL</td>
<td>125.9±6.4</td>
<td>110.3±4.5†</td>
<td>12.4</td>
<td>&lt;100</td>
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<tr>
<td>HOMA&lt;sub&gt;IR&lt;/sub&gt;</td>
<td>10.2±1.2</td>
<td>6.4±0.8†</td>
<td>32.6</td>
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<tr>
<td>QUICKI</td>
<td>0.28±0.01</td>
<td>0.30±0.01†</td>
<td>-5.9</td>
<td>-</td>
</tr>
</tbody>
</table>

All data are expressed as mean±SE. HOMA<sub>IR</sub>: Homeostasis-model assessment for insulin resistance. QUICKI: Quantitative insulin-sensitivity check index. *P<0.05, †P<0.01 Post vs. Pre.
A.

MPO (ng/mL)

Pre | Post
---|---

B.

8-Isoprostane PGF2α (pg/ml)

Pre | Post
---|---
A. CRP (mg/L)

B. sICAM-1 (µg/L)

C. sP-selectin (ng/mL)

D. MMP-9 (ng/mL)
A.

THP-1 Adhesion (%FBS)

B.

Monocyte Chemotaxis (Migrated Monocytes/HPF)

C.

MIP1α (pg/mL)
A. VCAM-1 Expression (% FBS control)

B. MCP-1 Production (ng/mL)
A. Ethidium Fluorescence (% FBS Control)

B. DCF Fluorescence (% FBS Control)

C. DAF-2T Fluorescence (% FBS Control)