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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

Christian K. Roberts,¹ Dean Won,¹ Sandeep Pruthi,¹ Silvia Kurtovic,¹
Ram K. Sindhu,² Nosratola D. Vaziri,³ and R. James Barnard¹

¹Department of Physiological Science, University of California, Los Angeles; ²Division of Endocrinology, Metabolism and Molecular Medicine, Charles R. Drew University, Los Angeles; and ³Division of Nephrology and Hypertension, Department of Medicine, University of California, Irvine, California

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Roberts, Christian K., Dean Won, Sandeep Pruthi, Silvia Kurtovic, Ram K. Sindhu, Nosratola D. Vaziri, and R. James Barnard. 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. *J Appl Physiol* 100: 1657–1665, 2006. First published December 15, 2005; doi:10.1152/jappphysiol.01292.2005.—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-wk residential program where food was provided ad libitum and daily aerobic exercise was performed. In each subject, pre- and postintervention fasting blood was drawn for circulating levels of serum lipids, glucose and insulin (for estimation of insulin sensitivity), oxidative stress-generating enzyme myeloperoxidase and marker 8-isoprostaglandin $F_{2\alpha}$, the inflammatory protein C-reactive protein, soluble ICAM-1 as an indicator of endothelial activation, sP-selectin as a marker of platelet activation, the chemokine macrophage inflammatory protein-1 α , and total matrix metalloproteinase-9. Using subject sera and human aortic endothelial cell culture systems, we measured VCAM-1 cell surface abundance and monocyte chemotactic protein-1, nitric oxide, superoxide, and hydrogen peroxide production in vitro by fluorometric detection. Also determined in vitro was serum-induced, monocyte adhesion and monocyte chemotactic activity. After 3 wk, significant reductions ($P < 0.05$) in body mass index, all serum lipids and lipid ratios, fasting glucose, insulin, homeostasis model assessment for insulin resistance, myeloperoxidase, 8-isoprostaglandin $F_{2\alpha}$, C-reactive protein, soluble ICAM-1, soluble P-selectin, macrophage inflammatory protein-1 α , and matrix metalloproteinase-9 were noted. In vitro, serum-stimulated cellular VCAM-1 expression, monocyte chemotactic protein-1 production, and fluorometric detection of superoxide and hydrogen peroxide production decreased, whereas 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 postintervention. Intensive lifestyle modification may ameliorate novel coronary artery disease risk factors in men with metabolic syndrome factors before reversal of obesity.

atherosclerosis; lipids; cell adhesion molecules; nitric oxide; matrix metalloproteinase-9

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 Multiple-Risk Factor Intervention Trial Study emphasized the relationship between serum cholesterol, especially low-density lipoprotein cholesterol (LDL-C), and CAD (9). 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 $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$) (42) and myeloperoxidase (MPO) (7), 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 ~23% of the population and ~43% of those above 60 years of age (20). Accordingly, the examination of the effect of modifying environmental influences (e.g., 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

Address for reprint requests and other correspondence: C. K. Roberts, Dept. of Physiological Science, UCLA, 4101 Life Sciences Bldg. 621 Charles E. Young Dr. South, Los Angeles, CA 90095-1606 (e-mail: croberts@ucla.edu).

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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 modified criteria (32). There were 13 Type 2 diabetic patients (by fasting glucose); all others had >1 metabolic syndrome factor and five 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, followed by 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-to-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 (≥ 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 ($3\frac{1}{2}$ oz. portions 1 day/wk and in soups or casseroles 2 days/wk).

Before 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. On the basis of 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 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 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, and the serum was separated by centrifugation and stored at -80°C until analyzed. Weight was measured by using a scale from Pennsylvania Medical Scales (model no. 7500). Height was measured with a stadiometer from Seca, attached to the wall. BMI was calculated as weight (kg)/height (m^2).

Determination of Serum Lipids, Glucose, Insulin, Homeostasis Model Assessment, and Quantitative Insulin-Sensitivity Check Index

Total cholesterol (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 by use of standard enzymatic procedures on an Olympus Autoanalyzer (Quest Diagnostics). Fasting insulin concentration was measured by radioimmunoassay (Diagnostic Systems Laboratories). Insulin resistance was evaluated by 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 [fasting insulin ($\mu\text{U}/\text{ml}$) \times 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). $\text{QUICKI} = 1/\{\log[\text{fasting insulin } (\mu\text{U}/\text{ml})] + \log[\text{fasting glucose } (\text{mg}/\text{dl})]\}$. 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, Soluble P₁-Selectin, Macrophage Inflammatory Protein-1 α , and MMP-9

Serum CRP (Diagnostic Systems Laboratories), sICAM-1 and soluble P-selectin (sP-selectin), macrophage inflammatory protein-1 α (MIP-1 α ; 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 75 cm^2 flasks in M199 medium (Invitrogen) supplemented with 20% vol/vol FBS, 0.8 ml heparin, and 2 mg endothelial cell growth factor/100 ml media (Becton-Dickinson); 1% vol/vol penicillin-streptomycin-glutamine (GIBCO-BRL); and 1% vol/vol 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 h. HASMC were seeded in the wells at a density of $\sim 5 \times 10^4$ cells/ cm^2 and were cultured for 2–3 days at 37°C , 95% O_2 -5% CO_2 , at which time they had reached confluency. For cocultures, HAEC were subsequently overlaid on top of HASMC at $\sim 1 \times 10^5$ cells/ cm^2 and were allowed to grow forming a complete monolayer of confluent HAEC in 2 days.

Production of monocyte chemoattractant protein-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_2 -5% CO_2 ; other washes were removed immediately. Washed cocultures were then incubated with M199 medium containing 5% human serum pre- and postintervention for 4 h. Subsequently, cultures were centrifuged and culture supernatants harvested and stored at -20°C pending the monocyte chemoattractant protein-1 (MCP-1) measurement by ELISA (PharMingen, BD OptEIA ELISA set no. 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). HAEC were grown to confluency in 96-well plates. Cultures were then washed three times in media containing 1% FBS. The second wash was incubated for 1–2 h at 37°C , 95% O_2 -5% CO_2 . Other washes were removed immediately. Washed cultures were then incubated with media containing 10% preintervention or postintervention serum for 4 h. After incubation, supernatants were removed and HAEC were washed once with 200 μl of PBS, fixed in 150 μl 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 h at room temperature with a mouse monoclonal IgG antibody against human VCAM-1 (PharMingen cat. no. 555645) diluted 1:500 in PBS-Tween-BSA. The wells were then washed twice in PBS-Tween, followed by incubation with a horseradish peroxidase-conjugated secondary antibody against mouse IgG from sheep (Amersham Life Sciences cat. no. NA931V) diluted in PBS-Tween-BSA at a 1:1,000 dilution. After a 1-h incubation at room temperature, wells were washed five times in PBS-Tween, and they were subsequently colored with 100 μl of tetramethylbenzidine re-

agent (Sigma cat. no. T0440) as substrate for the bound horseradish peroxidase. After 5 min of incubation, the reaction was stopped by adding 100 μ l of 3 M H_2SO_4 . Absorbance was read at 450 nm. FBS (10%) was used as a control and 10 ng/ml LPS was used as a positive control. The data are expressed as percentage of FBS control.

Nitric oxide 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 percentage of FBS control. The cell-permeable fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA, Calbiochem) was used to measure nitric oxide (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 μ l serum-free medium (SFM) three times. The second wash was incubated for 1 h at 37°C and 95% O_2 -5% CO_2 . SFM supplemented with 10% test serum (pre- or post-intervention) was added onto cells that were then incubated at 37°C and 95% O_2 -5% CO_2 for 18 h. Thereafter, 100 μ l of 10 μ M DAF-2DA dissolved in SFM was added to all cells except negative control wells. Negative control wells included cells with 100 μ l of SFM containing either 10 μ M 4 AF-DA (Calbiochem), a nonreactive DAF-2DA analog, or 300 μ 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_2 -5% CO_2 for 1 h. After incubation, cells were washed three times with 200 μ l of SFM to remove any residual extracellular DAF-2DA probe. Subsequently, 100 μ l of SFM with 4 μ 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_2 -5% CO_2 , fluorescence intensity was read and quantified in a fluorescence microplate reader at 495-nm excitation wavelength and 515-nm emission wavelength. The optical density (OD) readings pre- and postintervention were expressed as percentage of FBS control. Pictures of the DAF-2T fluorescence in the HAECs from the representative pre- and posttest 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 ($\times 10$). A higher magnification ($\times 20$) and light wavelength filter were 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'-dichloro-dihydrofluorescein (DCF) diacetate (DCFH-DA, Molecular Probes) was used to measure hydrogen peroxide production by HAEC (10). 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, that can be used to detect enzymatic generation of reactive oxygen species (ROS) (4). In cultured endothelial cells, DCF is oxidized by hydrogen peroxide, along with other intracellular processes involving ROS, making it useful in determining changes in hydrogen peroxide and overall oxidant formation (51). SFM supplemented with 10% test serum (pre- or postintervention) was added onto cells that were then incubated at 37°C and 95% O_2 -5% CO_2 for 18 h. The hydrogen peroxide positive control included cells incubated for 18 h with 2 ng/ml TNF- α dissolved in SFM. After incubation, the supernatant was removed from the cells and 100 μ l of 10 μ 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_2 -5% CO_2 for 1 h. After DCFH-DA incubation, cells were washed three times with 200 μ l of SFM to remove residual extracellular probe. After being washed, 100 μ l SFM was applied to the cells, which were then placed in a light-protected incubator at 37°C and 95% O_2 -5% CO_2 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 postintervention 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 postintervention) was added onto cells, which were then incubated at 37°C and 95% O_2 -5% CO_2 for 18 h. The superoxide positive control included cells incubated with 2 ng/ml TNF- α dissolved in SFM. After incubation, the supernatant was removed and 100 μ l of 25 μ 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_2 -5% CO_2 for 45 min. After DHE incubation, cells were washed once with 200 μ l of SFM and then twice with 200 μ l of HEPES to remove residual extracellular probe. After being washed, 100 μ l of HEPES were applied to the cells, which were then placed in a light-protected incubator at 37°C and 95% O_2 -5% CO_2 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 were 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 were 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 postintervention serum (10% final concentration) in M199 media for 20 h. Supernatants were then removed and cultures washed with SFM. A 400- μ l monocytic cell line (THP-1) suspension in M199 providing 2.0×10^5 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 $\times 320$ magnification. The preintervention and postintervention sera were compared with 10% FBS and 10 ng/ml LPS as a positive control.

Monocyte chemotactic activity assay. Monocytes from healthy normal individuals were isolated by a modification of the Recalde procedure (18). The monocyte chemotactic activity (MCA) was performed as previously described by Navab et al. (40). Briefly, pre- and postintervention 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 Neuro Probe chamber (Neuro Probe, 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%

Table 1. Anthropometric and metabolic parameters of subjects undergoing a 21-day diet and exercise intervention

	Pre	Post	%Decrease	Consensus Values
%With metabolic syndrome	48%	19%		
%Obese (BMI >30)	71%	58%		
%Overweight (BMI >24.9)	100%	100%		
%Diabetic	42%	23%		
Body weight, kg	106.5±3.9	102.7±4.0†	3.6	
BMI	35.4±1.5	34.4±1.6†	2.8	18.5–24.9
Total-C, mg/dl <200	201.0±6.6	158.7±6.0†	21.0	
LDL-C, mg/dl	118.1±6.6	88.1±5.5†	25.5	<100
HDL-C, mg/dl	44.1±1.7	39.5±1.7*	10.5	>40
TG, mg/dl	215.0±21.5	156.4±15.4†	27.7	<150
Total-C/HDL-C	4.74±0.21	4.19±0.20†	11.4	
LDL-C/HDL-C	2.72±0.15	2.29±.13†	15.8	
Insulin, μ U/ml	30.9±3.3	21.6±2.7†	30.1	
Blood glucose, mg/dl	125.9±6.4	110.3±4.5†	12.4	<100
HOMA _{IR}	10.2±1.2	6.4±0.8†	32.6	
QUICKI	0.28±0.01	0.30±0.01†	-5.9	

All data are expressed as means \pm SE ($n = 31$ subjects). BMI, body mass index; Total-C, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HOMA_{IR}, homeostasis-model assessment for insulin resistance; QUICKI, quantitative insulin-sensitivity check index. * $P < 0.05$, † $P < 0.01$, after (Post) vs. before (Pre) intervention.

glutaraldehyde and stained with 0.1% crystal violet dye. The number of migrated monocytes was determined microscopically and expressed as the mean \pm SE of 12 standardized high-power fields (HPF) 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 h incubation, which did not change (1).

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). Preintervention and postintervention values were compared by 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 α} , DAF-2A, ethidium and DCF fluorescence and BMI, body weight, fasting insulin, glucose, and HOMA_{IR}. All data are expressed as means \pm 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/m²) at the end of the intervention. Subjects positive for metabolic syndrome by World Health Organization criteria decreased from 48 to 19%. Noted were significant reductions in total-C, LDL-C, HDL-C, TG, LDL-to-HDL ratio, and total-C-to-HDL-C ratio ($P < 0.01$ for all). Significant reductions in serum fasting glucose ($P < 0.05$) and 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, $P < 0.05$, Fig. 1A). Additionally, a significant reduction in serum 8-iso-PGF_{2 α} (131.3 ± 21.6 vs. 202.6 ± 37.2 pg/ml, $P < 0.01$, Fig. 1B) was observed.

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

As noted in Fig. 2, after the intervention, there was a reduction in serum concentration of the inflammatory protein

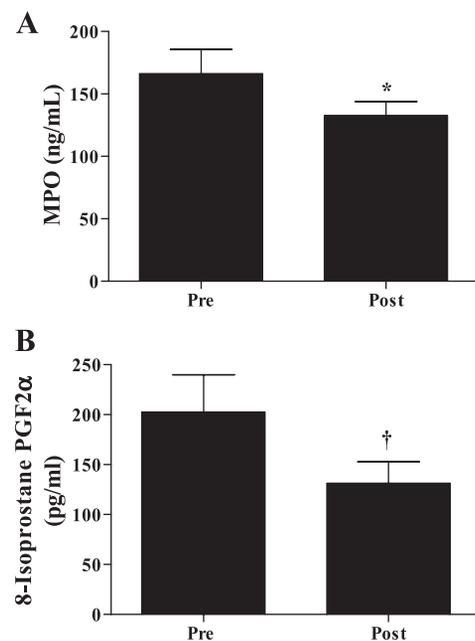


Fig. 1. A: effect of diet and exercise intervention on serum myeloperoxidase (MPO; $n = 21$). B: effect of diet and exercise intervention on serum 8-iso-prostaglandin F_{2 α} (8-iso-PGF_{2 α}) ($n = 21$). Data are expressed as means \pm SE. * $P < 0.05$, † $P < 0.01$ postintervention vs. preintervention.

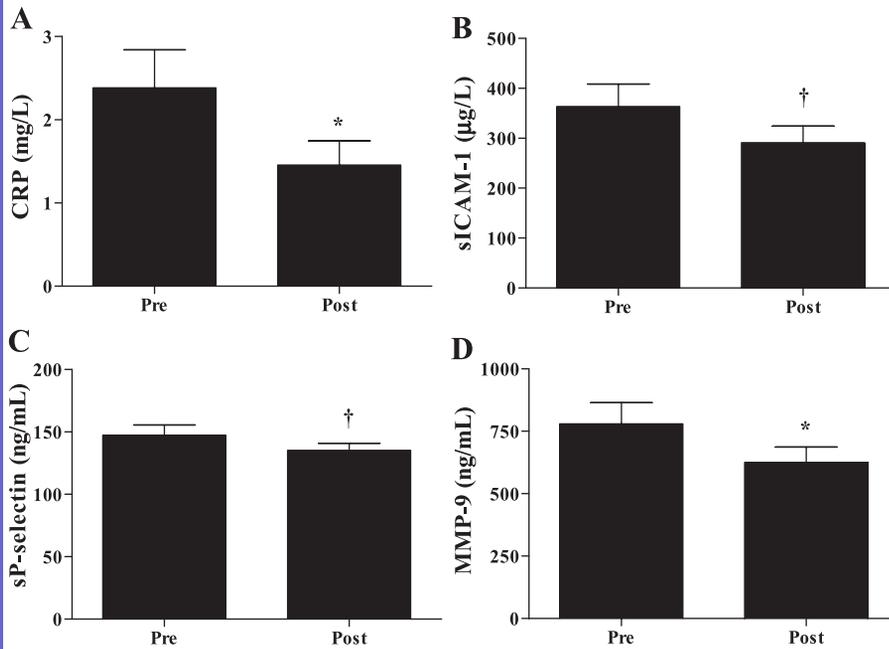


Fig. 2. A: effect of intervention on serum concentration of C-reactive protein (CRP; $n = 31$). B: effect of intervention on serum soluble ICAM-1 (sICAM-1; $n = 21$). C: effect of intervention on serum soluble P-selectin (sP-selectin; $n = 10$). D: effect of intervention on serum matrix metalloproteinase-9 (MMP-9; $n = 21$). Data are expressed as means \pm SE. * $P < 0.05$, † $P < 0.01$ postintervention vs. preintervention.

CRP (1.46 ± 0.29 vs. 2.39 ± 0.46 mg/l, $P < 0.01$, Fig. 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, $P < 0.05$, Fig. 2B) and sP-selectin (135.3 ± 5.6 vs. 147.5 ± 8.1 ng/ml, $P < 0.01$, Fig. 2C) concentrations decreased postintervention. Additionally, serum total level of the gelatinase MMP-9, an index of plaque stability, was reduced postintervention (625.7 ± 61.3 vs. 779.3 ± 85.2 ng/ml, $P < 0.01$, Fig. 2D).

Monocyte Adhesion, Chemotactic Activity, and MIP-1 α

Monocyte adhesion using THP-1 cells, a monocytic cell line, significantly decreased after the intervention (127.7 ± 13.4 vs. $185.3.1 \pm 12.5$ adherent THP-1/HPF, $P < 0.001$, Fig. 3A). The ability of serum to induce MCA was tested by incubating pre- and postintervention 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, $P < 0.01$, Fig. 3B). The chemokine MIP-1 α also decreased postintervention (8.93 ± 1.7 vs. 12.6 ± 1.8 pg/ml, $P < 0.05$, Fig. 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 postintervention sera were added to cultured HAEC for 4 h, the expression of the adhesion molecule VCAM-1 was reduced (97.0 ± 5.1 vs. $113.8 \pm 7.6\%$ of FBS control, $P < 0.01$, Fig. 4A). We determined the production of MCP-1 in a coculture of HAEC and HASMC as an index of monocyte chemoattraction. Moreover, the addition of postintervention sera to cocultures of HAEC and HASMC

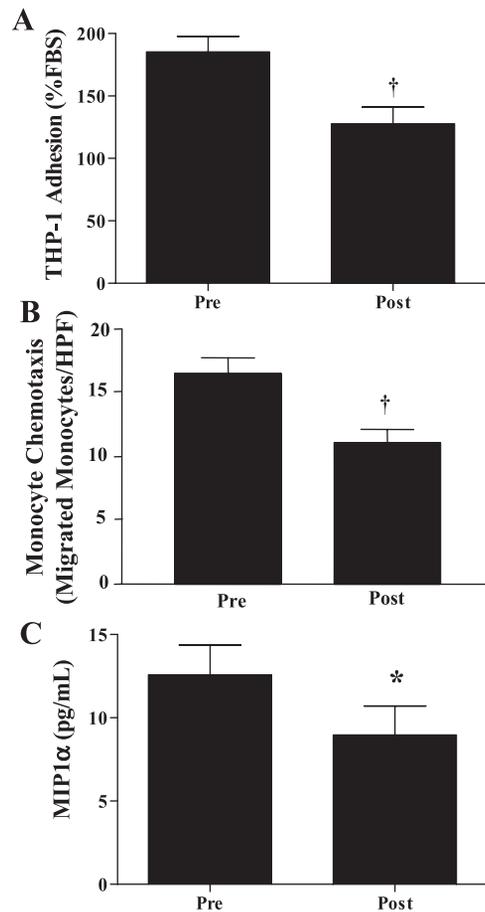


Fig. 3. Effect of intervention on monocyte adhesion ($n = 16$; A), monocyte chemotaxis ($n = 21$; B), and macrophage inflammatory protein-1 α (MIP-1 α ; $n = 20$; C). Data are expressed as means \pm SE. * $P < 0.05$, † $P < 0.01$ postintervention vs. preintervention. HPF, high power field; THP-1, monocytic cell line.

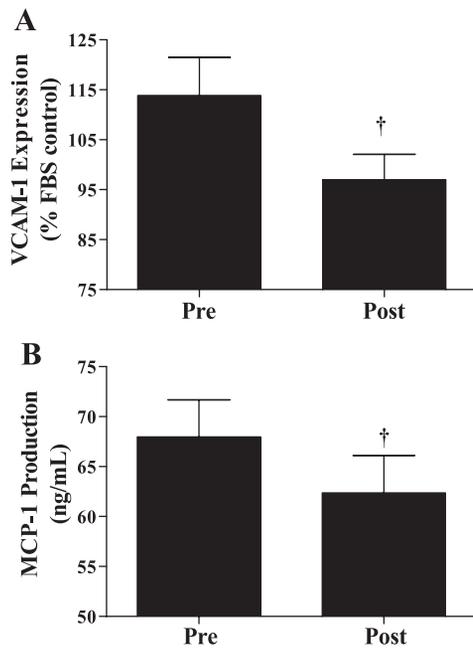


Fig. 4. A: effect of intervention on VCAM-1 cell surface expression on human aortic endothelial cells ($n = 23$). B: effect of intervention on monocyte chemoattractant protein-1 (MCP-1) expression in a human aortic endothelial cells/human aortic smooth muscle cell coculture system ($n = 22$). Data are expressed as means \pm SE. [†] $P < 0.01$ postintervention vs. preintervention.

for 4 h resulted in a lower production of MCP-1 compared with that noted with preintervention (62.4 ± 3.8 vs. 68.0 ± 3.7 ng/ml, $P < 0.05$, Fig. 4B).

The probe DCF was used as an index of ROS production, primarily as hydrogen peroxide. Postintervention, there was a significant reduction in DCF fluorescence compared with preintervention, indicating a decrease in hydrogen peroxide formation (105.4 ± 5.8 vs. $114.2 \pm 7.4\%$, $P < 0.01$, Fig. 5A). Medium plus TNF- α (2 ng/ml) induced hydrogen peroxide production ($119 \pm 3.0\%$ 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 postintervention (84.5 ± 4.8 vs. $92.0 \pm 4.5\%$, $P < 0.01$, Fig. 5B). These data demonstrate decreased subject sera-stimulated ROS 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 postintervention vs. preintervention (132 ± 5 vs. $144 \pm 5\%$ of FBS control, $P < 0.01$, Fig. 5C), indicating increased NO production from HAEC grown in postintervention serum. Coincubation 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 and treatment of CAD (49). The Adult Treatment Panel III guidelines included recommendations for therapeutic lifestyle change, including diet and physical activity (16). 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 postintervention 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

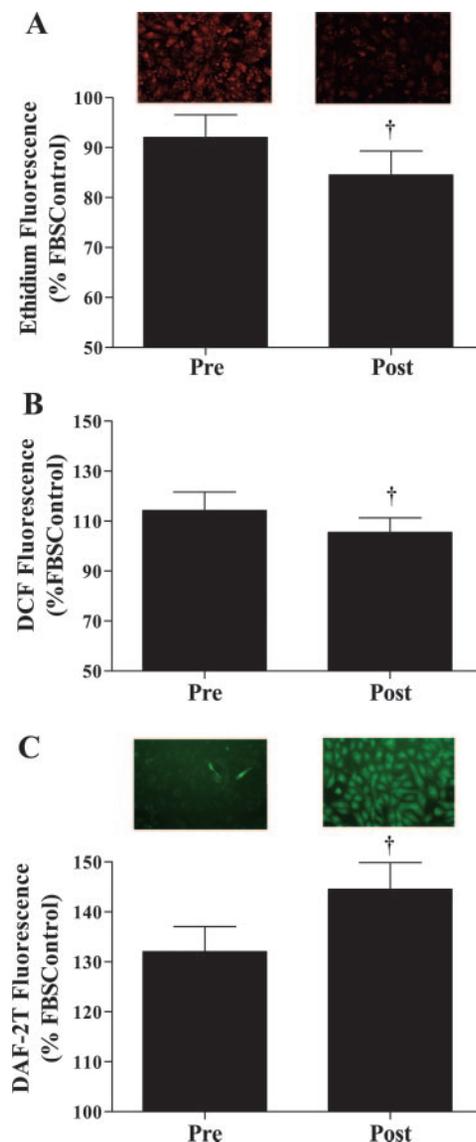


Fig. 5. A: serum-stimulated ethidium fluorescence (% of FBS control), an index of superoxide production ($N = 9$). B: serum-stimulated dichlorodihydrofluorescein (DCF) fluorescence (% of FBS control), an index of peroxide production ($N = 10$). C: endothelial cell serum-stimulated 4,5-diaminofluorescein trazol (DAF-2T) fluorescence (% of FBS control), an index of nitric oxide production ($N = 22$). Data are expressed as means \pm SE. [†] $P < 0.01$ postintervention vs. preintervention.

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, because MCA is directly related to MCP-1 production (40). Additionally, the decrease in MIP-1 α , a chemoattractant that activates monocytes (45), supports the contention that monocyte attraction was mitigated by the intervention. The effect of postintervention 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 (5), 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 wk, a finding likely unrelated to CRP instability (41) or circadian variation (33). Our laboratory previously reported (54) a reduction in CRP in women after the same diet and exercise intervention, 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 (13) and induces plasminogen activator inhibitor (14). The observed increase in NO production and/or decrease in ROS 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 suggest that MMPs play an integral role in human atherosclerosis and plaque disruption, because patients with CAD and history of myocardial infarction have elevated MMP-9 serum levels (17, 46). The decreased total MMP-9 postintervention 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 whereas a National Cholesterol Education Program Step I diet for 14 wk (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-C, thereby propagating its uptake by macrophages and perpetuating foam cell formation (43). MPO is markedly increased within human atherosclerotic lesions (12) and directly utilizes both NO and nitrite as substrates in vitro (15) and participates in both protein nitration and initiation of lipid peroxidation in vivo (8). The decrease in MPO with lifestyle modification provides evidence for a decrease in oxidative stress, data that agree with our laboratory's previous documentation of a reduction in 8-iso-PGF_{2 α} (50), which we also confirmed. Interestingly, MPO has been shown to activate MMPs and promote destabilization and rupture of the athero-

sclerotic 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 postintervention, 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 postintervention, indicating reduced serum-stimulated production of peroxides and superoxide, respectively. It is plausible that the improvement in endothelial function noted by others (11, 23) with diet and/or exercise was due, at least in part, to increasing endothelial cell NO production, decreased NO-scavenging ROS production, and/or MPO levels, which vary inversely with endothelial dysfunction (2).

The present 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. Furthermore, 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. It is possible that we underestimated the number of diabetic subjects because 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 when 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 Brodney (6) have

suggested that cardiorespiratory fitness may be a more powerful predictor of risk than body weight per se. 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 (3) 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.

GRANTS

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