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The Suppressive Effect of Dietary Restriction and Weight Loss in the Obese on the Generation of Reactive Oxygen Species by Leukocytes, Lipid Peroxidation, and Protein Carbonylation*

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ABSTRACT

Increased reactive oxygen species generation by the leukocytes of the obese may be responsible for increased oxidative injury to lipids and proteins and, hence, atherosclerosis. We have investigated whether reactive oxygen species generation by leukocytes and other indexes of oxidative damage in the body fall with short-term dietary restriction and weight loss. Nine nondiabetic obese subjects (body mass index, 32.5–64.4 kg/m^2), not taking any antioxidants, were put on a 1000-Cal diet. Fasting blood samples were taken at 0, 1, 2, 3, and 4 weeks and at 12 weeks after the cessation of dietary restriction. Blood samples were also obtained at 1 and 2 h after administration of 75 g oral glucose at 0 and 4 weeks. Mononuclear cells (MNC) and polymorphonuclear leukocytes (PMN) were isolated, and reactive oxygen species generation was measured. Plasma concentrations of thiobarbituric acid-reactive species (TBARS), 13-hydroxyoctadecadienoic acid (13-HODE), 9-hydroxyoctadecadienoic acid (9-HODE), carbonylated proteins, *o*-tyrosine, and *m*-tyrosine as indexes of oxidative damage to lipids, proteins and amino acids, respectively, were measured. Antioxidant vitamins were measured as indexes of antioxidant reserves. Plasma tumor necrosis factor-α concentrations were also measured. Mean weight loss was 2.4 ± 0.6 kg at week 1, 2.5 ± 1.7 kg at week 2, 3.9 ± 0.8 kg at week 3, and 4.5 ± 2.8 kg at week 4 ($P < 0.05$). Reactive oxygen species generation by PMN fell from 236.4 ± 95.8 to 150.9 ± 69.0, 125.9 ± 24.3, 96.0 ± 39.9, and 103.1 ± 35.7 mV at weeks 1, 2, 3, and 4, respectively ($P < 0.001$). It increased 3 months after the cessation of dietary restriction to 270.0 ± 274.3 mV. Reactive oxygen species generation by MNC fell from 187.8 ± 75.0 to 101.7 ± 64.5, 86.9 ± 42.8, 63.8 ± 14.3, and 75.1 ± 32.2 mV and increased thereafter to 302.0 ± 175.5 mV at 1, 2, 3, 4, and 16 weeks, respectively ($P < 0.005$). Reactive oxygen species generation by PMN and MNC increased in response to glucose; the relative increase was greater at 4 weeks than that at week 0 due to a fall in the basal levels of reactive oxygen species generation. Consistent with the fall in reactive oxygen species generation, there was a reduction in plasma TBARS from 1.68 ± 0.17 μmol/L at week 0 to 1.47 μmol/L at 4 weeks ($P < 0.05$). The 13-HODE to linoleic acid ratio fell from a baseline of 100% to 56.4 ± 36.1% at 4 weeks ($P < 0.05$), and the 9-HODE to linoleic acid ratio fell from a baseline of 100% to 60.5 ± 37.7% at 4 weeks ($P < 0.05$). Carbonylated proteins fell from 1.39 ± 0.27 μg/mg protein at week 0 to 1.17 ± 0.12 μg/mg protein at week 4 ($P < 0.05$); *o*-tyrosine fell from 0.42 ± 0.03 mmol/mol phenylalanine at week 0 to 0.36 ± 0.02 mmol/mol phenylalanine at 4 weeks ($P < 0.005$), and *m*-tyrosine fell from 0.45 ± 0.04 mmol/mol phenylalanine at week 0 to 0.40 ± 0.03 mmol/mol phenylalanine at 4 weeks ($P < 0.05$). The basal concentrations of TBARS, 9-HODE, 13-HODE, carbonylated proteins, *o*-tyrosine, and *m*-tyrosine in the obese were significantly greater than those in normal subjects. On the other hand, tumor necrosis factor-α concentrations did not change during this 4-week period, nor was there any change in antioxidant vitamins. This is the first demonstration of 1) an increase in reactive oxygen species-induced damage in lipids, proteins, and amino acids in the obese compared with normal subjects; and 2) a decrease in reactive oxygen species generation by leukocytes and oxidative damage to lipids, proteins, and amino acids after dietary restriction and weight loss in the obese over a short period. (*J Clin Endocrinol Metab* **86:** 355–362, 2001)

OBESITY IS NOW recognized as a major risk factor for atherosclerosis (1). The initial lesion of atherosclerosis is a fatty streak, which is formed by a large collection of foam cells in the arterial intima underneath the endothelium. Foam cells are formed by monocyte/macrophages after the internalization of large quantities of oxidized, low density lipoprotein (LDL). Native LDL is oxidatively damaged by reactive oxygen species generated by the monocytes/macrophages and the endothelium (2). Thus, reactive oxygen species generation and lipid peroxidation are cardinal to atherogenesis.

We previously demonstrated that reactive oxygen species generation by mononuclear cells (MNC) in the obese is relatively resistant to inhibition by diphenylene iodonium (DPI), a specific inhibitor of NADPH oxidase, the enzyme that generates the superoxide ($O^{\cdot}_2{}^-$) radical, compared with that in normal subjects (3). We also demonstrated that plasma tumor necrosis factor-α (TNFα) concentrations are elevated in the obese and fall with weight loss (4). As TNFα stimulates leukocytes in inflammatory processes (5) and may stimulate reactive oxygen species generation by leukocytes (6), it is probable that with weight loss, there may occur a fall in leukocytic reactive oxygen species generation. Moreover, in a set of preliminary experiments we observed that the high

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concentrations of TNFα found in the obese stimulate reactive oxygen species generation by leukocytes *in vitro* (unpublished observations). The state of obesity is associated with an increased metabolic rate. It is possible that an increased rate of metabolism and accelerated metabolic processes may be associated with an increase in reactive oxygen species generation. It is also possible that in obesity those effects of acute nutritional intake may be magnified. We therefore embarked on a study to determine whether obesity is associated with increased reactive oxygen species generation and whether weight loss results in a fall in reactive oxygen species generation by leukocytes. As we have recently demonstrated that glucose intake acutely induces increased reactive oxygen species generation by leukocytes in normal subjects (7), we also investigated this effect of glucose challenge in the obese and the effect of weight loss on their response to glucose.

As increased or nonsuppressible reactive oxygen species generation in the obese may result in increased oxidative damage to lipids and proteins, we also tested for possible associated changes in lipid peroxidation, protein carbonylation, and antioxidant reserves. Lipid peroxidation was assessed by the measurement of 1) plasma thiobarbituric acid-reactive species (TBARS) concentrations, the classically used index; and 2) plasma concentrations of 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), the specific oxidative products of reactive oxygen species attack on linoleic acid (8). Oxidative damage of phenylalanine was also investigated through measurement of plasma concentrations of *o*-tyrosine, *m*-tyrosine, and phenylalanine. Unlike *p*-tyrosine, which is formed by enzymatic conversion of phenylalanine, *o*-tyrosine and *m*-tyrosine are formed by the attack of reactive oxygen species on phenylalanine (9); therefore, the concentrations of these two metabolites as well as the ratio of these metabolites to their precursor phenylalanine provide useful indexes of oxidative damage of phenylalanine.

Subjects and Methods

Patients

Nine obese patients, 31–66 yr (mean, 45.3 ± 13.2 yr), all with body mass index (BMI) greater than 32 kg/m^2 (BMI range, 32.5–64.4; mean, 40.7 ± 9.4 kg/m^2) were included in this study (Table 1). All patients had a fasting venous plasma glucose less than 110 mg/dL. None of the obese subjects was taking vitamin A, E, or C; any other antioxidant therapy; or glucocorticoids. Five subjects were taking L-T_4 (0.1–0.15 mg/day). All five had been taking T_4 for longer than 1 yr, were clinically euthyroid, and had normal thyroid function tests, including serum T_4, T_3 resin uptake, and TSH concentrations. They continued on T_4 treatment throughout the 4-week period of this experiment at doses they had been stabilized on for over 1 yr. The control group composed of 12 normal subjects, 30–55 yr, with a BMI of 22.5 ± 1.0. One of the normal subjects was hypothyroid and receiving replacement therapy with L-T_4. His serum T_4, T_3 resin uptake, and TSH levels were normal, and he was clinically euthyroid.

The institutional review board of Millard Fillmore Hospitals and the State University of New York (Buffalo, NY) approved the study. Written informed consent was obtained from all subjects.

Dietary regimen

All subjects were given a diet of 1000 Cal/day, consisting of 200 Cal Sweet Success (Nestle, Glendale, CA) for breakfast and lunch and a self-cooked 600-Cal dinner. The dinner consisted of three servings of bread/starch (240 Cal), two servings of lean protein (110 Cal), one serving of fruit (60 Cal), two servings of vegetables (50 Cal), one serving of skim milk (90 Cal), and one serving of fat (45 Cal). The subjects were encouraged to drink water and low calorie drinks freely. No vitamins were provided, and patients were specifically asked not to take any vitamin preparations, especially vitamins A, C, and E. All subjects continued on this regimen for 4 weeks. The subjects were not given any instruction regarding exercise, except to continue to be as active as they normally would be.

The study was carried out on an out-patient basis with subjects returning to our center at weekly intervals to be weighed, to have blood taken, and to be given Sweet Success supplies. Blood samples were also obtained 3 months after the cessation of dietary restriction. The samples were collected in sodium-ethylenediamine tetraacetate and processed immediately for polymorphonuclear leukocytes (PMN) and MNC isolation.

Glucose tolerance tests

All obese patients were administered an oral glucose tolerance test (OGTT) with 75 g glucose dissolved in 300 mL water (glucola), consumed over 5 min. Glucose tolerance tests were carried out at the beginning of the study and at the end of the study at week 4. At the end of weeks 1, 2, and 3, only fasting blood samples were obtained; OGTTs were not performed. Fasting blood samples were obtained from all subjects between 0800–0900 h. For the OGTT, blood samples were obtained before and 1 and 2 h after glucose challenge.

PMN and MNC preparation

Blood samples were collected in sodium-ethylenediamine tetraacetate-containing tubes. Three and a half milliliters of the anticoagulated blood sample were carefully layered over 3.5 mL PMN medium (Robbins Scientific Corp., Sunnyvale, CA). Samples were centrifuged at 450 × *g* in a swing-out rotor for 30 min at 22 C. At the end of centrifugation, two bands separated out at the top of the red blood cell pellet. The top band consisted of MNC, and the bottom band consisted of PMN. The bands were harvested with a Pasteur pipette. The harvested cells were repeatedly washed with HBSS and reconstituted to a concentration of 4×10^5 cells/mL in HBSS. This method provides yields of greater than 95% pure PMN and MNC suspensions.

TABLE 1. Demographic data on the obese patients included in the study

Patient no.	Age (yr)	Sex	Wt (kg)	Ht (cm)	BMI (kg/m^2)	Blood pressure	Medications
1	52	F	164.7	160	64.4	120/80	Omeprazole, T_4
2	46	F	104.1	175	33.9	130/80	T_4
3	33	F	94.8	160	37.0	120/80	T_4
4	31	F	109.8	168	39.1	120/80	None
5	56	M	129.7	175	42.2	150/90	None
6	52	F	99.3	157	40.1	130/82	T_4
7	33	F	96.8	152	41.7	144/88	T_4
8	33	F	83.2	130	32.5	120/80	None
9	66	M	110	175	35.8	120/80	None

Reactive oxygen species generation assay

Five hundred microliters of PMN or MNC (2×10^5 cells) were delivered into a Chronolog LumiAggregometer cuvette. Fifteen microliters of 10 mmol/L luminol were then added, followed by 1.0 μL 10 mmol/L formylmethionyl leucinyl phenylalanine. Chemiluminescence was recorded for 15 min. A more protracted record after 15 min did not alter the relative amounts of chemiluminescence produced by various cell samples. Our method, developed independently (10, 11), is similar to that published by Tosi and Hamedani (12). In this assay system the release of superoxide radical, as measured by chemiluminescence, has been shown to be linearly correlated with that measured by the ferricytochrome *c* method (12). We further established that in our assay system, there is a dose-dependent inhibition of chemiluminescence by superoxide dismutase and catalase. DPI (data not shown), a specific inhibitor of NADPH oxidase, the enzyme responsible for the production of the superoxide radical, inhibited chemiluminescence at nanomolar concentrations. The specific inhibitory effect of DPI on NADPH oxidase has been established by Hancock and Jones (13). The interassay coefficient of variation of this assay is 8%. We have further established that the biological variation in reactive oxygen species generation in normal subjects is approximately 6% for readings obtained 1–2 weeks apart. Similarly, the variation in reactive oxygen species generation in the obese over a period of 1–2 weeks is less than 8%.

Assay of TBARS, 9-HODE, and 13-HODE

TBARS were measured using the method described by Ohkawa *et al.* (14). Hydroxypolyunsaturated fatty acids, 9-HODE and 13-HODE, were measured by a modification of the method of Brown and Armstrong (8) as adapted from previous work (15, 16).

Assay of carbonylated proteins

Carbonyl contents were measured by the method described by Levine *et al.* (17) with modifications. Briefly, 400 μL 10 mmol/L 2,4-dinitrophenylhydrazine (DNPH) in 2.5 mol/L HCl or 400 μL 2.5 mol/L HCl were added to 100 μL serum and incubated for 2 h. Samples were vortexed every 15 min, extracted with 500 μL 20% trichloroacetic acid, and then spun for 5 min at $14{,}000 \times g$. The precipitates were washed once with 1 mL 10% trichloroacetic acid, followed by three washes with 1 mL ethanol-ethyl acetate (1:1) to remove the free DNPH and lipid contaminants. Precipitates from DNPH-treated samples were dissolved in 2 mL 6 mol/L guanidine hydrochloride solution at 37 C for 10 min. The carbonyl contents were calculated from the peak absorbance (355–390 nm) using an absorption coefficient of 22,000 mol/L·cm against pellets derived from the 2.5 mol/L HCl-treated samples, which were also used to calculate the protein contents against a BSA standard dissolved in 6 mol/L guanidine hydrochloride and read at 280 nm.

Assay of plasma o-tyrosine and m-tyrosine

o-Tyrosine and *m*-tyrosine determinations in serum were performed using high performance liquid chromatography (HPLC)-fluorometric detection as described by Ishimitsu *et al.* (18) with modification. Briefly, a 0.5-mL serum sample was deproteinized by the addition of 125 μL 1.0 mol/L trichloroacetic acid. The mixture was centrifuged at $14{,}000 \times g$ for 10 min at 4 C. The supernatant was collected and filtered through a 0.22-μm pore size membrane filter (Millipore Corp., Bedford, MA). The filtrate was lyophilized and then dissolved in 200 μL deionized water; 100 μL of this solution were injected directly into a Shimadzu HPLC system equipped with a 3-μm Supelco reverse phase C_{18} column (id, 4.6 mm; od, 25 cm; Supelco, Bellefonte, PA) operated under isocratic conditions with an aqueous solution containing 250 mmol/L KH_2PO_4-H_3PO_4, pH 3.0, with 4% (vol/vol) methanol at a flow rate of 1 mL/min. Detection of the compounds was accomplished by a fluorometer (RF-10A, Shimadzu Scientific Instruments, Colombia, MD) with excitation at 275 nm and emission at 305 nm for *p*-, *m*-, and *o*-tyrosine and with excitation at 258 nm and emission at 288 nm for phenylalanine. Analysis was carried out in duplicate, and the mean value was reported. Peaks were identified by coinjection with reference compounds.

Assay of plasma TNFα

Plasma TNFα concentrations were measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN).

Assay of fat-soluble antioxidant vitamins

Retinol, α-tocopherol, and carotenoids were measured in plasma simultaneously by HPLC on an LC-7A liquid chromatograph with SPD-M6A photodiode array (Shimadzu Scientific Instruments). HPLC grade acetonitrile, methanol, dichloromethane, and triethylamine were purchased from J.T. Baker (Phillipsburg, NJ). Tocopherol acetate, α-tocopherol, lycopene, and β-carotene were purchased from Sigma (St. Louis, MO). Vitamin A-alcohol (>95% *cis*-retinol) was purchased from Fluka Chemical Co. (Ronkonkoma, NY). Lutein and zeaxanthin were purchased from Indofine Pure Biochemicals (Somerville, NJ).

Vitamins were extracted from 300 μL absolute ethanol containing 0.03% butylated hydroxytoluene and 50 μg/mL tocopherol acetate as an internal standard. Samples were extracted twice with 3.0 mL hexane, and the upper phases were aspirated, pooled, and evaporated to dryness under nitrogen. The sample was dissolved in 300 μL mobile phase and injected onto a reverse phase Supelcosil LC-18 column (150×4.6 mm; Supelco). Vitamins were eluted isocratically at a flow rate of 1.5 mL/min using a mobile phase of acetonitrile/methanol/dichloromethane (60:25:15, vol/vol/vol), which contained 0.1% triethylamine and 0.05 mol/L ammonium acetate. The eluent was monitored at 284, 292, 326, 450, and 478 nm, and each peak was quantified against a calibration curve of pure standard. The accuracy of this method was verified against The National Institute of Standards and Technology serum SRM 968a fat-soluble vitamin control material and The National Institute of Standards round robin proficiency testing. Results are expressed as micrograms per mL. Lutein and zeaxanthin were quantified as a single peak and reported as total lutein and zeaxanthin.

Statistical analysis

The sequential data were analyzed by ANOVA of ranks for repeated measures. Widely scattered data (13-HODE and 9-HODE) were normalized to a baseline of 100%, and comparisons were carried out thereafter. The paired *t* test was used for analysis of paired data at weeks 0 and 4.

Results

Weight loss

The mean weight of the patients at baseline was 110.5 ± 24.2 kg. The mean weight loss was 2.4 ± 0.6 kg at week 1, 2.5 ± 1.7 kg at week 2, 3.9 ± 0.8 kg at week 3, and 4.5 ± 2.8 kg at week 4 ($P < 0.05$). All patients lost weight at the end of week 1. Some of them remained static, and one patient regained some weight at week 2; most of them continued to lose weight steadily until the end of the 4-week period (Fig. 1).

Reactive oxygen species generation

The mean reactive oxygen species generation by PMN was 236.4 ± 95.8 mV, and that by MNC was 187.8 ± 75.0 mV before weight loss. These values were not significantly different from those in normal subjects. Reactive oxygen species generation decreased significantly after this diet regimen and weight loss. This decrease was evident even after dieting for 1 week and continued until week 4. Reactive oxygen species generation by PMN fell to 150.9 ± 69.0 mV at week 1, 125.9 ± 24.3 mV at week 2, 96.0 ± 39.9 mV at week 3, and 103.1 ± 35.7 mV at week 4 ($P < 0.001$; Fig. 2). Reactive oxygen species generation by PMN increased to 270.0 ± 274.3 mV 3 months after the cessation of caloric restriction. Reactive oxygen species generation by MNC fell to 101.7 ± 64.5 mV at week 1,

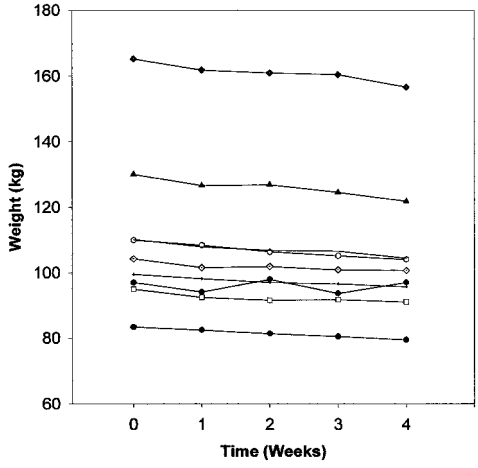

FIG. 1. Sequential weight of each patient at 0, 1, 2, 3, and 4 weeks. The mean basal weight was 110.5 ± 24.2 kg; the mean weight loss was 4.5 ± 2.8 kg at week 4.

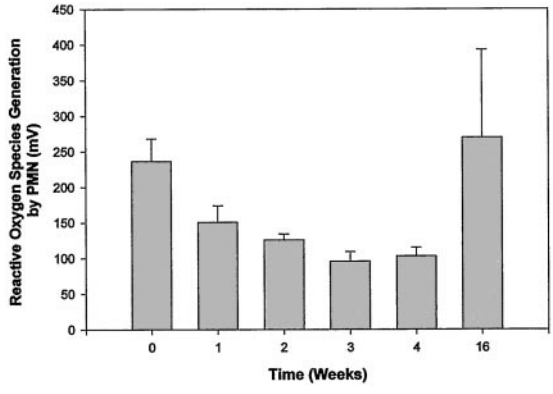

FIG. 2. Reactive oxygen species generation by PMN after 0, 1, 2, 3, and 4 weeks of a 1000-Cal diet. Note that a significant fall in reactive oxygen species generation occurred at week 1; a gradual fall continued thereafter until week 3. Reactive oxygen species inhibition persisted at week 4. Reactive oxygen species generation increased 12 weeks after cessation of dietary restriction.

86.9 ± 42.8 mV at week 2, 63.8 ± 14.3 mV at week 3, and 75.1 ± 32.2 mV at week 4 ($P < 0.005$; Fig. 3). Reactive oxygen species generation by MNC increased to 302.0 ± 175.5 mV 3 months after the cessation of dietary restriction. The most impressive fall was at week 1 for both PMN leukocytes and MNC. Reactive oxygen species generation in normal or obese subjects measured 1–2 weeks apart did not differ by more than 6% in the absence of weight change or any intervention. Thus, the magnitude of the fall in reactive oxygen species generation was far greater than that expected from biological variation.

Reactive oxygen species generation during OGTT

Glucose challenge resulted in an increase in reactive oxygen species generation by PMN as well as MNC. At week 0, reactive oxygen species generation by PMN increased from 236 ± 95.8 to 326.0 ± 115.5 mV (144.2 ± 48.7%) at 1 h and 327.0 ± 89.4 mV (149.5 ± 47.3%) at 2 h ($P < 0.05$). At week 4, reactive oxygen species generation by PMN increased from a baseline of 103.1 ± 35.7 mV (46.8 ± 19.2% of the basal) to 188.6 ± 102.7 mV (81.9 ± 37.6% of the basal) at 1 h and 206.1 ± 89.4 mV (95.2 ± 54.4% of the basal) at 2 h ($P < 0.05$; Fig. 4). Reactive oxygen species generation by MNC increased from 187.8 ± 75.1 mV to 244.6 mV (132.4 ± 26.7% of the basal) at 1 h and 248.9 ± 68.5 mV (146.2 ± 58.8% of the basal) at 2 h after glucose challenge at week 0, whereas it increased from a baseline of 75.1 ± 32.2 mV (48.5 ± 31.1% of the basal) to 135.0 ± 46.0 mV (96.4 ± 83.8% of the basal) at 1 h and 162.8 ± 43.0 mV (103.3 ± 59.3% of the basal) at 2 h at week 4 (Fig. 5).

Plasma TBARS

At baseline, the mean plasma TBARS concentration was 1.68 ± 0.17 μmol/L in obese subjects compared with that in age-matched controls (1.29 ± 0.12 μmol/L; $P < 0.05$). The mean TBARS concentration fell to 1.47 ± 0.13 μmol/L (87.9 ± 5.8% of the basal) at 4 weeks ($P < 0.05$). This concentration was still significantly greater than that in normal subjects ($P < 0.05$).

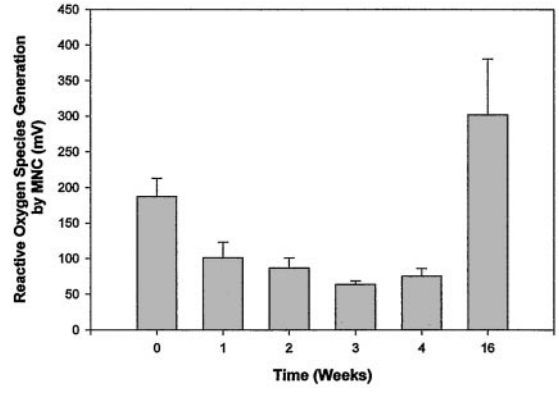

FIG. 3. Reactive oxygen species generation by MNC after 0, 1, 2, 3, and 4 weeks of a 1000-Cal diet. Note that a significant fall in reactive oxygen species generation occurred at week 1; a gradual fall continued thereafter until week 3. Reactive oxygen species inhibition persisted at week 4. Reactive oxygen species generation increased 12 weeks after cessation of dietary restriction.

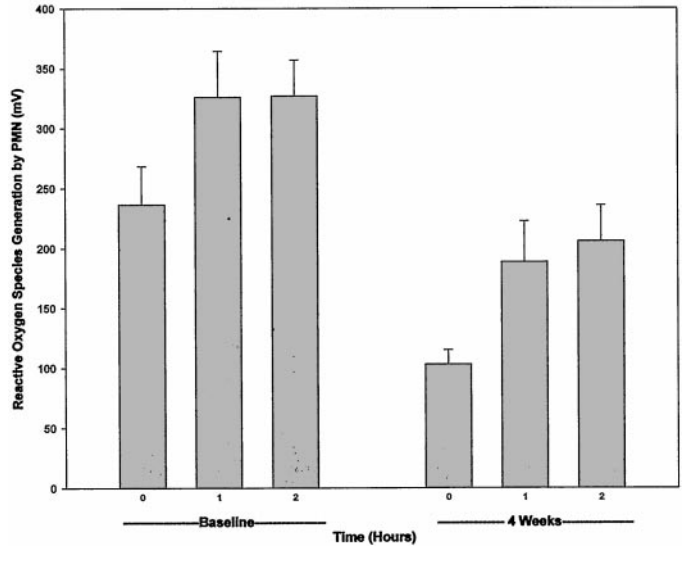

FIG. 4. Reactive oxygen species generation by PMN in response to glucose challenge at 0 and 4 weeks.

Linoleic acid, 13-HODE, and 9-HODE

The mean ± SD linoleic acid concentration was 4.30 ± 1.38 μmol/L at baseline. The concentration fell to 3.54 ± 1.16 μmol/L (83.2 ± 14.2% of the basal level) at week 4 ($P < 0.001$). The mean basal concentration of 13-HODE in the obese was 6.67 ± 3.85 μmol/L, which was significantly greater than that in control subjects (2.04 ± 0.72 μmol/L; $P < 0.001$); after weight loss, it fell to 4.15 ± 5.62 μmol/L (49.3 ± 38.7% of the basal level) at week 4. The basal concentration of 9-HODE was 7.10 ± 3.88 μmol/L; this was significantly greater than observed in normal controls (1.97 ± 0.84 μmol/L; $P < 0.0001$). 9-HODE fell to 4.5 ± 5.7 μmol/L (52.5 ± 39.5% of the basal level) at week 4 (Fig. 6). The decreases in 9-HODE, 13-HODE, 9-HODE/linoleic acid ratio, and 13-HODE/LA ratio were not significant when expressed in absolute values, but were highly significant when expressed as the percent change over the basal level, because the data were scattered.

As 13-HODE and 9-HODE are oxidatively derived from linoleic acid, the concentration of which had fallen significantly over 4 weeks, we calculated the ratios of 13-HODE and 9-HODE to linoleic acid. The ratio of 13-HODE to linoleic acid at baseline was 6.45 ± 4.90 mmol/mol (100%); it fell to 4.00 ± 4.91 mmol/mol (56.4 ± 36.1% of the basal level; $P < 0.05$) at week 4. The ratio of 9-HODE to linoleic acid at baseline was 6.85 ± 5.04 mmol/mol (100%), and it fell to 4.38 ± 4.99 mmol/mol (60.5 ± 37.7% of the basal level; $P < 0.05$) at week 4 (Figs. 7 and 8).

Plasma carbonylated proteins

Carbonylated proteins were 1.39 ± 0.27 μmol/mg protein at week 0 and fell to 1.17 ± 0.12 μmol/mg protein at 4 weeks. This change was significant at 2, 3, and 4 weeks ($P < 0.05$; Table 2). The carbonylated protein concentration was sig-

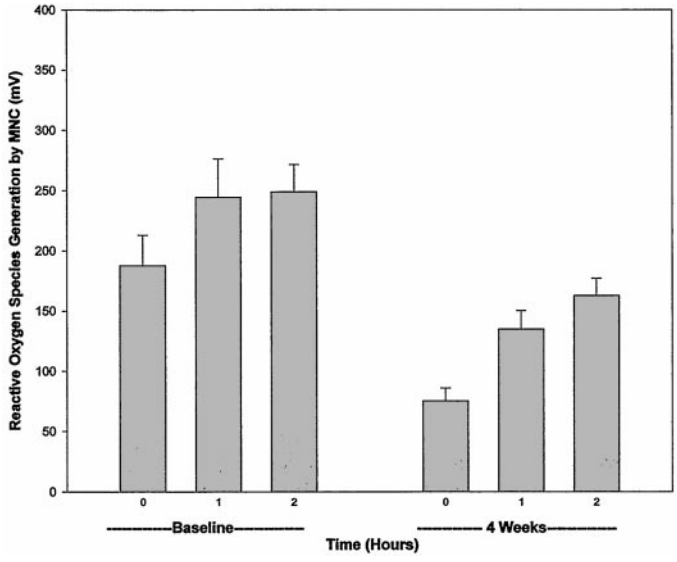

FIG. 5. Reactive oxygen species generation by MNC in response to glucose challenge at 0 and 4 weeks.

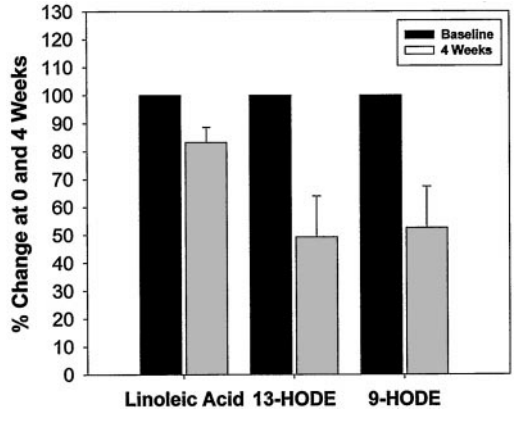

FIG. 6. Percent fall in plasma concentrations of linoleic acid, 13-HODE, and 9-HODE after 4 weeks of a 1000-Cal diet and weight loss. There was a significant fall in all three indexes. Note that the magnitude of the fall in 13-HODE and 9-HODE was greater than that in linoleic acid. The decreases in 13-HODE and 9-HODE were significant when expressed as the percent change over the basal level (100%).

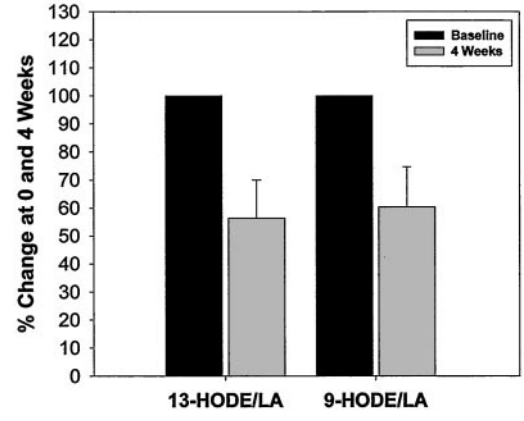

FIG. 7. Percent fall in ratios of 13-HODE and 9-HODE to linoleic acid after 4 weeks of a 1000-Cal diet. Note that both 13-HODE/LA and 9-HODE/LA ratios fell after weight loss. LA, Linoleic acid. The fall was significant when expressed as the percent change over the basal level (100%).

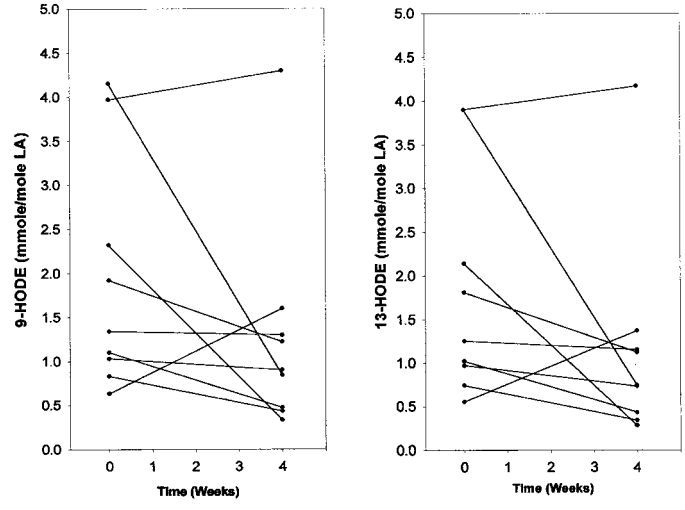

FIG. 8. 13-HODE/linoleic acid and 9-HODE/linoleic acid ratios after 4 weeks of a 1000-Cal diet. LA, Linoleic acid. This change was not significant because of the scatter of the basal data.

TABLE 2. Plasma concentrations of TBARS, carbonylated proteins, *o*-tyrosine/phenylalanine ratios, *m*-tyrosine/phenylalanine ratios, TNFα, α-tocopherol, β-carotene, lycopene, and lutein/zeaxanthin before and after weight loss

	Baseline	Week 1	Week 2	Week 3	Week 4	Week 16	*p*
TBARS (μmol/L)	1.68 ± 0.17	ND	ND	ND	1.47 ± 0.13	ND	0.04
Carbonylated proteins (μmol/mg)	1.39 ± 0.27	1.13 ± 0.13	1.15 ± 0.19	1.09 ± 0.21	1.17 ± 0.12	ND	0.035
o-Tyrosine/phenylalanine ratio (μmol/mol)	0.42 ± 0.03	0.41 ± 0.03	0.37 ± 0.05	0.38 ± 0.03	0.36 ± 0.02	0.52 ± 0.09	0.001
m-Tyrosine/phenylalanine ratio (μmol/mol)	0.45 ± 0.04	0.44 ± 0.04	0.42 ± 0.03	0.41 ± 0.03	0.40 ± 0.03	0.54 ± 0.1	0.01
Phenylalanine (μg/mL)	12.17 ± 0.99	11.96 ± 0.90	11.87 ± 0.74	11.80 ± 0.88	11.71 ± 0.83	11.34 ± 0.87	NS
TNFα (pg/mL)	2.01 ± 0.86	2.31 ± 1.00	2.18 ± 0.70	2.07 ± 1.16	2.18 ± 0.91	ND	NS
α-Tocopherol (μg/mL)	11.15 ± 4.90	12.67 ± 5.71	11.91 ± 4.67	12.35 ± 5.04	11.07 ± 5.40	ND	NS
β-Carotene (μg/mL)	0.08 ± 0.05	0.09 ± 0.06	0.11 ± 0.10	0.11 ± 0.08	0.10 ± 0.11	ND	NS
Lycopene (μg/mL)	0.48 ± 0.23	0.48 ± 0.26	0.52 ± 0.27	0.52 ± 0.42	0.43 ± 0.29	ND	NS
Lutein/zeaxanthin (μg/mL)	0.13 ± 0.06	0.14 ± 0.08	0.14 ± 0.09	0.13 ± 0.10	0.14 ± 0.13	ND	NS

ND, Not determined.

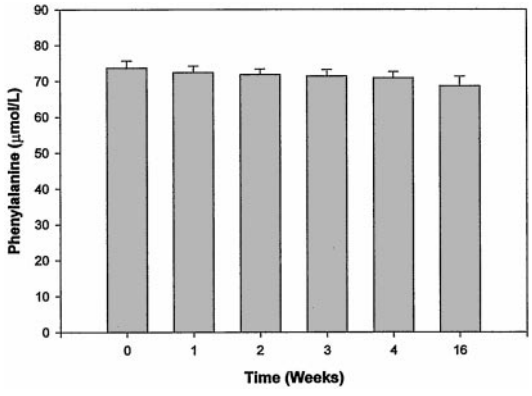

FIG. 9. Phenylalanine concentrations after 0, 2, 3, and 4 weeks of a 1000-Cal diet and at 12 weeks after the cessation of dietary restriction. Note that phenylalanine concentrations did not change significantly during this period.

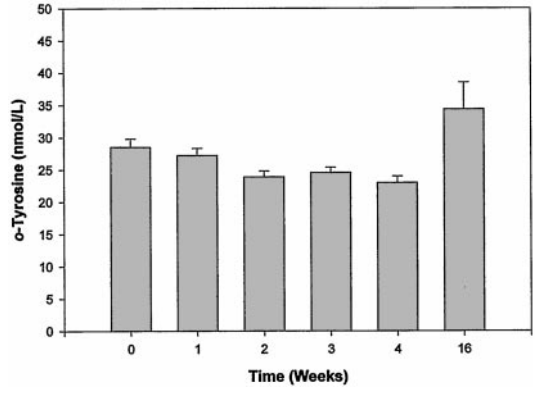

FIG. 10. *o*-Tyrosine concentrations after 0, 1, 2, 3, and 4 weeks of a 1000-Cal diet and at 3 months after the cessation of dietary restriction. Note that *o*-tyrosine concentrations fell with dietary restriction and weight loss; *o*-tyrosine/phenylalanine ratios also fell during this period. *o*-Tyrosine concentrations increased 12 weeks after the cessation of calorie restriction.

nificantly higher in obese subjects than in normal subjects (0.60 ± 0.10 μmol/mg; $P < 0.005$).

Phenylalanine and plasma o-tyrosine and m-tyrosine concentrations

The mean phenylalanine concentration was 73.7 ± 6.0 μmol/L at baseline (week 0). The concentration of phenylalanine was 72.4 ± 5.4 μmol/L at week 1, 71.8 ± 4.5 μmol/L at week 2, 71.4 ± 5.3 μmol/L at week 3, 70.9 ± 5.1 μmol/L at week 4, and 68.6 ± 5.2 μmol/L 3 months after cessation of calorie restriction (Fig. 9). The mean basal concentration of *o*-tyrosine was 28.5 ± 3.9 nmol/L; during weight loss it fell to 27.2 ± 3.3 nmol/L at week 1, 23.9 ± 2.8 nmol/L at week 2, 24.6 ± 2.4 nmol/L at week 3, and 23.0 ± 3.0 nmol/L at week 4 ($P < 0.005$; Fig. 10). The plasma *o*-tyrosine concentration increased to 34.4 ± 8.3 nmol/L 3 months after cessation of dietary restriction. The basal concentration of *m*-tyrosine was 30.5 ± 4.7 nmol/L; it fell to 29.3 ± 4.2 nmol/L at week 1, 27.5 ± 3.4 nmol/L at week 2, 24.7 ± 5.3 nmol/L at week 3, and 26.1 ± 2.9 nmol/L at week 4 ($P = 0.067$; Fig. 11). The plasma *m*-tyrosine concentration increased to 35.5 ± 4.5 nmol/L 3 months after cessation of dietary restriction.

As *o*-tyrosine and *m*-tyrosine are formed by oxidative damage of phenylalanine after reactive oxygen species attack, the ratios of *o*-tyrosine and *m*-tyrosine to phenylalanine were calculated to correct for any changes in plasma phenylalanine concentrations. The plasma concentration ratio of

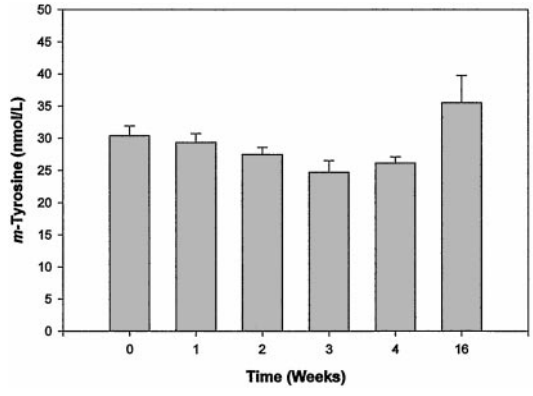

FIG. 11. *m*-Tyrosine concentrations after 0, 1, 2, 3, and 4 weeks of a 1000-Cal diet and at 3 months after the cessation of dietary restriction. Note that *m*-tyrosine concentrations fell with dietary restriction and weight loss; *m*-tyrosine/phenylalanine ratios also fell during this period. *m*-Tyrosine concentrations increased 12 weeks after the cessation of calorie restriction.

o-tyrosine to phenylalanine in the obese was significantly greater than that in the controls (0.42 ± 0.03 *vs.* 0.28 ± 0.02 mmol/mol; $P < 0.005$). This ratio decreased during weight loss from 0.42 ± 0.03 mmol/mol phenylalanine at week 0 to 0.41 ± 0.03 at week 1, 0.37 ± 0.05 at week 2, 0.38 ± 0.03 at week 3, and 0.36 ± 0.02 mmol/mol phenylalanine at week

4 ($P < 0.001$; Table 2). The ratio then increased after the cessation of dietary restriction to 0.52 ± 0.09.

The plasma concentration ratio of *m*-tyrosine to phenylalanine was 0.45 ± 0.04 mmol/mol phenylalanine at week 0. This was significantly greater than that in controls (0.27 ± 0.03 mmol/mol; $P < 0.001$). During weight loss, the *m*-tyrosine/phenylalanine ratio fell to 0.44 ± 0.04 at week 1, 0.42 ± 0.03 at week 2, 0.41 ± 0.03 at week 3, and 0.40 ± 0.03 at week 4 ($P < 0.05$; Table 2). The ratio then increased after the cessation of dietary restriction to 0.54 ± 0.10.

TNFα levels

The plasma TNFα concentration did not change over the 4 weeks of weight loss (Table 2).

Fat-soluble antioxidant vitamins

There were no significant changes in levels of β-carotene, zeaxanthin, lycopene, and α-tocopherol after 4 weeks of a 1000-Cal diet and weight loss (Table 2).

Discussion

Our data demonstrate for the first time that key indexes of lipid peroxidation (TBARS, 9-HODE, and 13-HODE) as well as those of oxidative damage to proteins and amino acids (carbonylated proteins, *o*-tyrosine, and *m*-tyrosine) are significantly greater in the obese than in normal subjects and that they fall significantly after dietary restriction and weight loss.

Our data also show clearly, for the first time, that reactive oxygen species generation by PMN and MNC falls markedly in association with dietary restriction and weight loss after the institution of a 1000-Cal diet. This effect is evident at 1 week and persists over 4 weeks, the duration of this experiment. The biological variation in reactive oxygen species generation in normal and obese subjects is 6% over a period of 1–2 weeks. Thus, the marked fall (>50%) in reactive oxygen species generation after dietary restriction and weight loss is far greater than that observed with biological variation. Furthermore, reactive oxygen species generation after the cessation of dietary restriction and at 3 months not only reversed, but exceeded, that at baseline for both PMN and MNC. Our study also shows a fall in lipid peroxidation and oxidative damage to amino acids and proteins over this short period. The demonstration of a parallel fall in reactive oxygen species generation and lipid peroxidation has important implications for oxidative mechanisms underlying atherosclerosis, as MNC-mediated reactive oxygen species generation may be the mechanism underlying oxidative damage to LDL. The more than 50% decrease in reactive oxygen species generation, the 13% decrease in TBARS, and the more than 40% decrease in HODE/LA ratios, indexes of lipid peroxidation over 4 weeks, are important in that they represent a dramatic reversal in cardinal processes affecting atherogenesis without the use of any drug or antioxidant. Whether the difference in the magnitude of effects on reactive oxygen species generation and TBARS represents a lag in terms of the effect of diminished reactive oxygen species generation is not clear. It is of interest, however, that the 13-HODE/linoleic acid ratio fell by 45% and the 9-HODE/linoleic acid ratio also fell by 45%, values close to the magnitude of the fall in reactive oxygen species generation. The absence of change in the levels of antioxidant vitamins shows that the diminution in reactive oxygen species generation by leukocytes is independent of these vitamins and that the concentrations of these vitamins are not altered by the reduced oxidative load following dietary restriction and weight loss.

Our data also provide the first demonstration of increased oxidative damage of amino acids in the obese. It is possible that these amino acid residues were oxidatively damaged even as a part of proteins, but our method only measures free *o*- and *m*-tyrosine in plasma. Indeed, there was also a reduction in carbonylated protein levels after dietary restriction, indicative of a diminution in oxidative damage to proteins consistent with a fall in *o*- and *m*-tyrosine concentrations. These indexes fell significantly after a dietary regimen that led to weight loss. Plasma concentrations of *o*-tyrosine and *m*-tyrosine increased after the cessation of dietary restriction, and at 3 months they exceeded the respective concentrations at baseline. The increases in reactive oxygen species generation, *o*-tyrosine, and *m*-tyrosine over the initial baselines are consistent with an increase in food intake by these subjects, as they all gained weight after the cessation of dietary restriction.

It should be emphasized that each of the obese subjects included in this study responded to dietary restriction by diminished reactive oxygen species generation and oxidative damage to lipids, proteins, and amino acids. Despite the wide variation in BMI, the changes were consistent and are, therefore, intrinsic to the process of dietary restriction and weight loss in the obese population. Our data do not distinguish between the effects of dietary restriction and weight loss; however, they do establish a role for an effect of nutrition on reactive oxygen species generation, as glucose challenge stimulated reactive oxygen species generation both before and after weight loss. The increase in oxidative damage in obesity may therefore reflect consistent overeating by obese subjects. Our data are more consistent with dietary intake as the major modulator of reactive oxygen species generation and the related oxidative damage, as the maximum effect on reactive oxygen species generation was observed within 1 week of dietary restriction before marked weight loss occurred, and it increased back to baseline despite the fact that body weight did not return to baseline.

An important question raised by our findings is whether the increased reactive oxygen species generation and oxidative damage in the obese are a function of overnutrition or are due to obesity *per se*. This question was partially answered by the reactive oxygen species response after OGTT. The increase in ROS generation after glucose ingestion suggests that nutritional intake has a role to play in causing increased reactive oxygen species load in the obese in a manner similar to that observed in normal subjects recently reported by us (7). The small changes in weight compared with overall body weight over 4 weeks would result in relatively small reductions in total adipose tissue mass. This supports the hypothesis that nutritional intake is a major factor affecting reactive oxygen species generation and total oxidative load. There are studies to show that oxidative damage to lipids increases and antioxidant reserves decrease after

nutritional intake (7, 19, 20). Persistent overconsumption of calories in the obese may be exposing them to excessive oxidative damage besides maintaining the state of obesity. It is noteworthy that a 48-h fast causes a fall in reactive oxygen species generation by PMN and MNC similar to that observed after 4 weeks of caloric restriction and weight loss in the obese (21).

There may be other determinants of increased reactive oxygen species load in the obese apart from nutritional intake alone. Nutrition may be modifying these other, as yet unidentified, determinants. TNFα is one of the possible agents in this regard. However, its role was ruled out in this study because its concentrations did not change. We have previously reported a fall in serum TNFα concentrations after weight loss over periods longer than 1 yr (4). It is possible that TNFα concentrations change only after prolonged periods of weight loss.

We have previously demonstrated that sodium, potassium-adenosine triphosphatase (Na,K-ATPase), a membrane enzyme that is increased in obesity, falls with weight loss (22) and is acutely increased by glucose intake in a fashion similar to that seen in reactive oxygen species generation (23). As reactive oxygen species generation assayed in our system is largely the superoxide radical, which is generated by NADPH oxidase, a membrane enzyme, it is possible that both of these enzymes are dependent on similar mediators or metabolic factors. Metabolic rates are known to fall with weight loss, and this may mediate the decrease observed in both NADPH oxidase and Na,K-ATPase.

Weight loss is associated with a fall in serum TSH and T_3, and an increase in rT_3 (24). It is possible that T_3 may modulate NADPH oxidase activity, similar to the way it modulates Na,K-ATPase in leukocytes (25). Indeed, we recently demonstrated an increase in reactive oxygen species generation by PMN and MNC after a short-term treatment with T_3 (26). Thus, a fall in T_3 may contribute to a fall in NADPH oxidase activity and ROS generation.

Our observations also have relevance to the work of Ornish *et al.* (27), who have shown that lifestyle modifications reduce the frequency of cardiovascular events in patients with CHD with a concomitant improvement in myocardial perfusion and a reversal of atherosclerosis lesions in coronary arteries. It is possible that dietary modification contributes to a reduction in reactive oxygen species generation and a corresponding reduction in indexes of oxidative damage, including lipid peroxidation.

In conclusion, the obese demonstrate increases in lipid peroxidation, protein carbonylation, and oxidative damage to amino acids. Dietary restriction and weight loss in the obese induce a significant decrease in reactive oxygen species generation by PMN and MNC. This decrease is associated with decreases in TBARS, 13-HODE, 9-HODE, 13-HODE/linoleic acid ratio, and 9-HODE/linoleic acid ratio, all indexes of lipid peroxidation. There are concomitant decreases in *o*-tyrosine, *m*-tyrosine, and carbonylated proteins, indexes of oxidative damage to amino acids and proteins. Thus, oxidative load and damage in the obese can be reduced with dietary restriction and weight loss without antioxidant therapy. This decrease may have implications in the pathogenesis of lipid peroxidation and other aspects of oxidative injury in the obese, which may contribute to atherogenesis.

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