

Both lipid and protein intakes stimulate increased generation of reactive oxygen species by polymorphonuclear leukocytes and mononuclear cells¹⁻³

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ABSTRACT

Background: It was recently shown that glucose challenge leads to increased generation of reactive oxygen species (ROS) by polymorphonuclear leukocytes (PMNs) and mononuclear cells (MNCs).

Objective: To further elucidate the relation between nutrition and ROS generation, we investigated the effect of lipid and protein challenges on ROS generation by leukocytes.

Design: After having fasted overnight, one group of healthy subjects consumed a carbohydrate- and protein-free cream preparation (1257 kJ) and another group of healthy subjects consumed an equienergetic pure preparation of casein. Sequential blood samples were obtained after the intake of cream and casein. ROS were measured by chemiluminescence after stimulation by *N*-formyl-methionyl-leucyl-phenylalanine. Lipid peroxidation was measured as thiobarbituric acid-reactive substances (TBARS) and α -tocopherol was measured by HPLC.

Results: ROS generation by MNCs and PMNs increased significantly 1, 2, and 3 h after cream intake and 1 h after protein intake. Cholesterol concentrations did not change significantly, whereas triacylglycerol concentrations increased significantly 2 h after cream intake. Total TBARS concentrations increased 1 h after cream intake and remained elevated 3 h after intake, but the increase was not significant when corrected for changes in triacylglycerol. After casein intake, total cholesterol, triacylglycerol, and TBARS concentrations did not change significantly. α -Tocopherol concentrations did not change significantly after either cream or casein intake.

Conclusions: Both fat and protein intakes stimulate ROS generation. The increase in ROS generation lasted 3 h after cream intake and 1 h after protein intake. Cream intake also caused a significant and prolonged increase in lipid peroxidation. These data are important because increased ROS generation and lipid peroxidation are key events in atherogenesis. *Am J Clin Nutr* 2002;75:767-72.

KEY WORDS Lipids, proteins, reactive oxygen species, leukocytes, lipid peroxidation, aging, thiobarbituric acid-reactive substances, TBARS, polymorphonuclear leukocytes, mononuclear cells, α -tocopherol

INTRODUCTION

Oxidative damage to lipids is considered to be important in the pathogenesis of atherosclerosis and microvascular complica-

tions of diabetes (1, 2). It has been suggested that hyperglycemia may modulate oxidative damage in diabetes mellitus (3, 4). It was also shown that antioxidant administration may improve atherosclerosis- or diabetes-related endothelial, leukocytic, and platelet damage and vascular reactivity (5-8). The factors that determine reactive oxygen species (ROS) generation require further investigation because ROS determine oxidative damage to proteins (9), lipids (4), and DNA (10).

We recently showed that a glucose challenge is followed by an increase in ROS generation by polymorphonuclear leukocytes (PMNs) and mononuclear cells (MNCs) and a decrease in plasma α -tocopherol concentrations (11). Glucose and meal challenges also increase lipid peroxidation and decrease antioxidant reserves (12, 13). These findings raise the question of whether macronutrients other than carbohydrates may also increase ROS generation and oxidative load.

In the present study we examined whether lipid or protein challenge also increases ROS generation by leukocytes and whether there are differences in response to these 2 nutrients when given in equienergetic amounts. We also examined the effect of lipid or protein challenge on a commonly used index of lipid peroxidation, ie, the plasma concentration of thiobarbituric acid-reactive substances (TBARS). This index of lipid peroxidation is relatively crude but provides a readily measurable way of assessing this process that is cardinal to the formation of a fatty streak and atherosclerosis. In our recent study on the effect of glucose on ROS generation, we observed a trend toward an increase in TBARS and a significant decrease in plasma α -tocopherol concentrations. Therefore, we included α -tocopherol concentration as one of the indexes measured.

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SUBJECTS AND METHODS

Subjects

Fifteen healthy subjects (10 men and 5 women) aged 28–65 y (mean age: 36 ± 10.7 y) were included in the study. All subjects had normal body weights [body mass index (BMI; in kg/m^2): 25.3 ± 2.0], normal blood pressure measurements (<140 mm Hg systolic and <85 mm Hg diastolic), and normal serum cholesterol concentrations (<5.2 mmol/L) and none smoked cigarettes.

All subjects arrived at the Clinical Research Center of the Diabetes-Endocrinology Center between 0800 and 0900 after having fasted overnight. All subjects had a blood sample obtained from the antecubital vein. Eight subjects were then given 100 mL dairy cream (gourmet heavy whipping cream; Land O Lakes Inc, Arden Hills, MN) to drink. This preparation of dairy cream has 33 g fat and 1257 kJ (300 kcal) per 100 mL cream. The saturated fat content of the cream preparation was 70% (28% unsaturated fat), the protein content was $<2\%$, and the glucose content was 0%. Seven subjects were given 75 g (1257 kJ) protein as casein (Spectrum Quality Products, Gardena, CA). Casein is a pure protein that contains no energy from fat or carbohydrate. Blood samples were then obtained 1, 2, and 3 h after intake of the cream or casein. All blood samples were collected in EDTA-containing evacuated tubes. Four of the subjects were also given 300 mL water to drink. Blood samples were collected before and 1, 2, and 3 h after water intake. All subjects gave their written, informed consent to participate in the protocol, which was approved by the internal review board of the State University of New York at Buffalo, based at Millard Fillmore Hospital.

PMN and MNC preparation

A portion (3.5 mL) of the anticoagulated blood sample was carefully layered over 3.5 mL PMN medium (Robbins Scientific Corp, Sunnyvale, CA). The sample was centrifuged at $450 \times g$ for 30 min at 22°C to separate out 2 bands at the top of the red blood cell pellet. The top band consists of MNCs and the bottom consists of PMNs. The bands were harvested, repeatedly washed with Hank's Balanced Salt Solution (HBSS; Life Technologies Inc, Rockville, MD), and reconstituted to a concentration of 4×10^8 cells/L in HBSS. This method yields $>95\%$ pure PMN and MNC suspensions. The purity of the PMN and MNC preparations was checked randomly, once every 5–10 preparations.

Measurement of plasma, glucose, insulin, and lipids

The effects of cream and casein intakes on glucose, insulin, cholesterol, and triacylglycerol concentrations were measured at the central laboratories at Kaleida (Buffalo, NY) with the use of standard techniques to provide a background of changes in relation to which the other indexes were measured.

Measurement of reactive oxygen species generation by leukocytes

Five hundred microliters of PMNs or MNCs (2×10^5 cells) were delivered into a Chronolog Lumi-Aggregometer cuvette; 15 μL of 10 mmol luminal/L was then added, followed by the addition of 1.0 μL of 10 mmol formyl-methionyl-leucyl-phenylalanine/L. Chemiluminescence was recorded for 15 min. (Recording for >15 min did not alter the relative amounts of chemiluminescence produced by various blood samples.) Our method, developed independently (14, 15), is similar to that published by Tosi and Hamedani (16). 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 (16). We further established, in our assay system, that there is a dose-dependent inhibition of chemiluminescence by superoxide dismutase and catalase and by diphenyleiodonium (data not shown), a specific inhibitor of NADPH oxidase—the enzyme responsible for the production of superoxide radicals. The specific inhibitory effect of diphenyleiodonium on NADPH oxidase was established by Hancock and Jones (17).

Plasma thiobarbituric acid–reactive substances

Plasma TBARS concentrations were measured with the use of the method described by Ohkawa et al (18). Briefly, 100 μL plasma was precipitated with an equal volume of 8.1% sodium dodecyl sulfate. The reaction mixture was acidified with 0.75 mL of 20% acetic acid (pH 3.5). Subsequently, 0.75 mL of 0.53% thiobarbituric acid was added to the reaction mixture and allowed to form adducts with various oxidative products of lipid peroxidation at 95°C for 1 h. After being cooled, the sample was centrifuged at $800 \times g$ for 5 min at room temperature, and the fluorescence was read at excitation and emission wavelengths of 535 and 552 nm, respectively. 1,1,3,3-Tetramethoxypropane was used as a standard.

α -Tocopherol assay

α -Tocopherol was measured in plasma by HPLC on an LC-10A liquid chromatograph with an SPD-10A ultraviolet-visible detector (Shimadzu Scientific Instruments, Columbia, MD). HPLC-grade acetonitrile, methanol, dichloromethane, and triethylamine were purchased from JT Baker (Phillipsburg, NJ). Vitamins were extracted from 300 μL EDTA-treated plasma by precipitation with 300 μL absolute ethanol containing 0.03% butylated hydroxytoluene and 50 mg tocopherol acetate/L as an internal standard. Samples were extracted twice with 2.0 mL hexane, and the upper phases were aspirated, pooled, and evaporated to dryness. The samples were dissolved in 300 μL mobile phase and injected onto a reversed-phase Supelcosil LC-18 column (250 mm \times 4.6 mm, 5 μm internal diameter; Supelco Inc, Bellefonte, PA.) Samples were eluted isocratically at a flow rate of 1.5 mL/min at a mobile phase of acetonitrile:methanol:dichloromethane (60:25:15, by vol) that contained 0.1% triethylamine and 0.05% ammonium acetate. The eluent was monitored at 292 nm and the peak was quantified against a calibration curve of pure standard.

Statistical analysis

The statistical analysis was carried out with the use of SIGMA-STAT software (Jandel Scientific, San Rafael, CA). An analysis of variance was conducted, and all data are expressed as means \pm SDs. When there was a significant *F* value, a Tukey's test was conducted to detect significantly different means. For the comparison of ROS generation by PMNs and MNCs before and after the lipid or protein challenge, the data were normalized to a baseline of 100% because baseline ROS generation varied markedly between the subjects. We previously showed interindividual variability in ROS generation but reproducibility in ROS generation within individuals (15). A *P* value ≤ 0.05 indicated significance.

RESULTS

Plasma variables

Plasma glucose, insulin, cholesterol, triacylglycerol, and α -tocopherol concentrations at baseline (0 h) and 1, 2, and 3 h after



TABLE 1Plasma glucose, triacylglycerol, cholesterol, insulin, and α -tocopherol concentrations before and 1, 2, and 3 h after cream or casein intake¹

	Baseline (0 h)	Time after intake		
		1 h	2 h	3 h
Cream intake (<i>n</i> = 8)				
Glucose (mmol/L)	5.16 ± 0.34	4.74 ± 0.42	4.86 ± 0.33	4.86 ± 0.26
Insulin (pmol/L)	39.6 ± 20.4	49.2 ± 33.0	37.2 ± 14.4	30.0 ± 14.4
Cholesterol (mmol/L)	4.82 ± 0.72	4.97 ± 0.77	4.90 ± 0.65	4.87 ± 0.68
Triacylglycerol (mmol/L)	1.48 ± 0.42	1.63 ± 0.42	2.12 ± 0.60 ²	2.04 ± 0.60 ²
α -Tocopherol (μ mol/L)	26.7 ± 13.3	27.9 ± 16.6	28.0 ± 16.1	26.0 ± 12.5
α -Tocopherol:triacylglycerol ³	17.3 ± 3.3	16.4 ± 5.1	13.5 ± 6.2	13.1 ± 4.8
Casein intake (<i>n</i> = 7)				
Glucose (mmol/L)	5.30 ± 0.17	4.37 ± 0.49	4.88 ± 0.56	4.83 ± 0.34
Insulin (pmol)	39.0 ± 15.6	108.6 ± 69.6 ²	117.0 ± 77.4 ²	90.0 ± 31.2 ²
Cholesterol (mmol/L)	4.40 ± 0.67	4.40 ± 0.75	4.38 ± 0.68	4.35 ± 0.65
Triacylglycerol (mmol/L)	1.09 ± 0.42	1.20 ± 0.42	1.24 ± 0.50	1.31 ± 0.52
α -Tocopherol (μ mol/L)	27.2 ± 4.9	27.6 ± 5.6	28.8 ± 5.6	28.3 ± 5.8
α -Tocopherol:triacylglycerol ³	27.6 ± 10.5	25.2 ± 8.7	26.3 ± 10.3	24.3 ± 8.6

¹ \bar{x} ± SD.²Significantly different from baseline, *P* < 0.05 (Tukey's test).³ α -Tocopherol in mmol; triacylglycerol in mol.

intake of cream or casein are shown in **Table 1**. Plasma glucose concentrations did not change significantly after intake of either cream or casein. Insulin concentrations did not change significantly after cream intake but increased significantly from 39.0 ± 15.6 pmol/L at baseline to 108.6 ± 69.6, 117.0 ± 77.4, and 90.0 ± 31.2 pmol/L, respectively, 1, 2, and 3 h after casein intake.

Total cholesterol concentrations did not change significantly after intake of either cream or casein. Triacylglycerol concentrations increased, but not significantly, from 1.48 ± 0.42 mmol/L at baseline to 1.63 ± 0.42 mmol/L 1 h after cream intake but increased significantly to 2.12 ± 0.60 mmol/L 2 h after and to 2.04 ± 0.60 mmol/L 3 h after cream intake. Triacylglycerol concentrations did not change significantly after casein intake.

Plasma α -tocopherol concentrations did not change significantly after intake of cream or casein. When corrected for the

increase in triacylglycerol concentrations, the ratio of α -tocopherol (mmol) to triacylglycerol (mol) tended to decrease from baseline (17.3 ± 3.3) to 16.4 ± 5.1, 13.5 ± 6.2, and 13.1 ± 4.8, respectively, 1, 2, and 3 h after cream intake, but none of the differences were significant. The ratio of α -tocopherol to triacylglycerol did not change significantly after casein intake.

Generation of reactive oxygen species

ROS generation by MNCs increased after cream intake in all subjects (**Figure 1**). All values were normalized to a baseline concentration of 100% because of the interindividual variation in ROS generation. ROS generation by MNCs increased significantly from baseline by 169.0 ± 24.4% (baseline reading of 118 ± 16 mV = 100% in further comparisons) at 1 h, by 195 ± 66.1% at 2 h, and by 131 ± 12.7% at 3 h. ROS generation

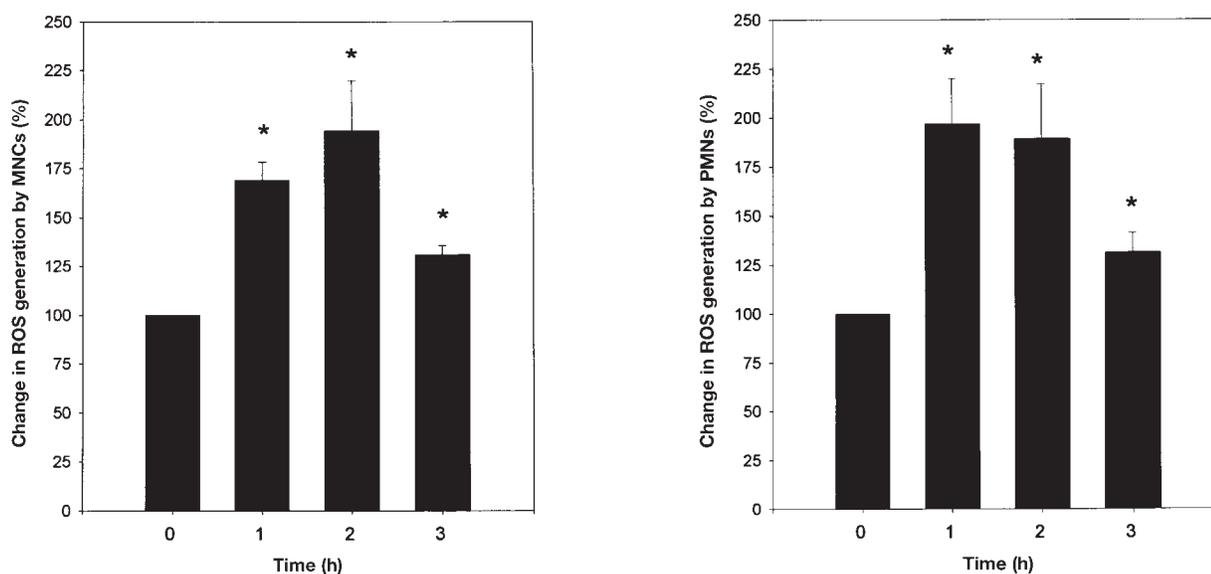


FIGURE 1. Mean (\pm SD) generation of reactive oxygen species (ROS) by mononuclear cells (MNCs) and polymorphonuclear leukocytes (PMNs) before (0 h, baseline) and 1, 2, and 3 h after cream intake (100 mL). *Significantly different from baseline, *P* < 0.05 (Tukey's test).

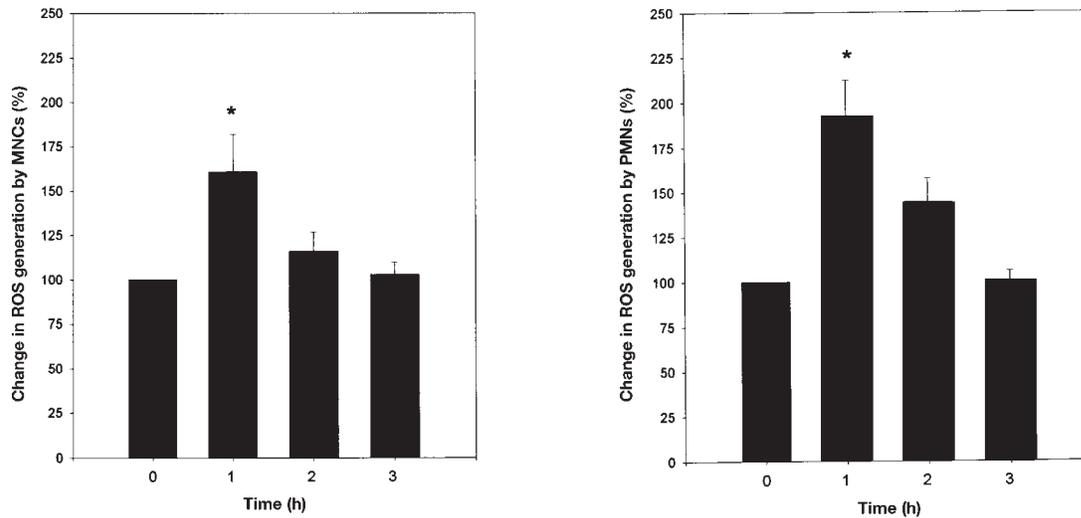


FIGURE 2. Mean (\pm SD) generation of reactive oxygen species (ROS) by mononuclear cells (MNCs) and polymorphonuclear leukocytes (PMNs) before (0 h, baseline) and 1, 2, and 3 h after casein intake (75 g). *Significantly different from baseline, $P < 0.05$ (Tukey's test).

by PMNs also increased after cream intake in all subjects. ROS generation by PMNs increased significantly from baseline (101 ± 15 mV = 100%) by $197 \pm 60.8\%$ at 1 h, by $189 \pm 73.3\%$ at 2 h, and by $131 \pm 25.9\%$ at 3 h.

Six of 7 subjects showed a significant increase in ROS generation by MNCs after casein intake (**Figure 2**). ROS generation by MNCs increased from baseline (385 ± 47 mV = 100%) by $161.0 \pm 59.4\%$ at 1 h ($P < 0.05$), by $116 \pm 30.2\%$ at 2 h (NS), and by $103.0 \pm 19.7\%$ at 3 h (NS). The increase in ROS generation by MNCs was not significant when expressed in absolute values (mV) and when not normalized to the baseline value because of the wide scatter of baseline values and because of the decrease in ROS generation by MNCs in one subject. ROS generation by PMNs increased in all subjects after casein intake. ROS generation by PMNs increased from baseline (143 ± 45 mV = 100%) by $193 \pm 55.5\%$ at 1 h ($P < 0.05$), by $144 \pm 37.5\%$ at 2 h (NS), and by $101 \pm 14.9\%$ at 3 h (NS). The increase in ROS generation at 1 h was significant whether expressed as mV/ 10^6 cells or as the increase over baseline corrected to 100%. There was no significant change in ROS generation by leukocytes after a drink of water (300 mL).

Plasma thiobarbituric acid–reactive substances

The increase in plasma TBARS concentrations was significantly greater than the increase in triacylglycerol concentrations after cream intake. Plasma TBARS concentrations increased significantly from baseline (1.09 ± 0.13 μ mol/L) to 1.62 ± 0.38 μ mol/L at 1 h, to 1.47 ± 0.12 μ mol/L at 2 h, and to 1.41 ± 0.25 μ mol/L at 3 h. The increase in TBARS was greatest at 1 h, whereas the increase in triacylglycerol was greatest at 2 h. When TBARS concentrations were corrected for the change in triacylglycerol concentrations, this increase was not significant. TBARS concentrations (μ mol/mmol triacylglycerol) were 0.773 ± 0.206 at baseline, 1.041 ± 0.348 at 1 h, 0.771 ± 0.329 at 2 h, and 0.767 ± 0.206 at 3 h. Plasma TBARS concentrations did not change significantly after casein intake. The difference in the peak of TBARS and triacylglycerols and the statistical trend toward an increase in the ratio of the 2 substances suggest that the increase in TBARS was not totally dependent on the increase in triacylglycerols.

DISCUSSION

Our data show for the first time that both the cream and casein challenges consistently result in a significant increase in ROS generation by both PMNs and MNCs. The peak effect occurred between 1 and 2 h after cream intake and 1 h after casein intake. Thereafter, ROS generation decreased, but was still higher than that at baseline 3 h after cream intake, whereas it reverted to baseline 3 h after casein intake. We previously showed an increase in ROS generation by PMNs and MNCs after a glucose challenge (11). Thus, we have produced evidence that all 3 major macronutrients (carbohydrate as glucose, fat as cream, and protein as casein) induce an increase in ROS generation. Our data also show that different nutrients produce distinct patterns of stimulation of ROS generation after their intake. Of the 3 nutrients, glucose induced the greatest ROS generation (the peak increase being 2 h after intake), followed in decreasing order by cream and by casein (the peak increase being 1 h after intake). Whether these differences in ROS generation are due to differences in metabolic stimulation after the energetic challenge or whether it reflects different mediators being released after the intake of different nutrients is not clear. We were unable to specify the mediators that may underlie the increase in ROS generation on the basis of our current experiments. Plasma insulin concentrations increased significantly after casein intake, but not after cream intake. Thus, it appears that insulin is not the mediator of increased ROS generation. Furthermore, we recently showed that insulin inhibits ROS generation by MNCs and PMNs in vivo (19).

The fact that both lipid and protein intakes cause an acute increase in ROS generation similar to that induced by glucose indicates that nutrient intake is probably a major modulator of ROS generation. This indication is consistent with our previous observation that ROS generation by MNCs from obese persons is relatively nonsuppressible (20). It is possible that the nonsuppressible ROS generation in obese persons may partly be a reflection of excessive nutrient intakes rather than of obesity itself. In addition, we recently showed that the indexes of lipid peroxidation—protein carbonylation and oxidative damage to amino acids—are elevated in obese persons and that dietary restriction and weight loss will result in the normalization of



these indexes without the use of antioxidants (21). Furthermore, we also showed that ROS generation and oxidative damage to amino acids decreased significantly in healthy subjects after a 48-h fast (22). Thus, nutritional intake is an important determinant of ROS generation and ROS load. This finding raises the issue of the relative effect of different diets on ROS generation, ROS load, and potential oxidative damage. We are currently investigating the effect of comprehensive diets (eg, the American Heart Association diet, in which the fat content decreases progressively, and diets recommended by the American Diabetes Association) on ROS generation. Clearly, a progressive decrease in fat content should reduce the potential for lipid peroxidation. Similarly, the 10% fat content in the diet proposed by Ornish et al (23) should reduce the oxidative load further. Indeed, this diet was shown previously to reverse atherosclerosis and to reduce the risk of myocardial ischemia.

The significant increase in plasma TBARS concentrations after the cream challenge (which was evident at 1 h and persisted until 3 h) is important because it indicates a protracted increase in oxidatively damaged lipids. The TBARS concentration in cream was 5.6 $\mu\text{mol/L}$. Because the volume of cream administered to volunteers was 100 mL, the load of TBARS provided was 0.56 μmol per 100 mL cream. This would produce a maximal increase of 0.15 $\mu\text{mol/L}$ if we assume that TBARS is distributed only in plasma. Because the distribution volume of TBARS is likely to be larger, the increase in TBARS concentrations as a result of cream intake would probably be even smaller. Thus, the increase in plasma TBARS after the cream challenge was probably due to lipid peroxidation *in vivo*.

The mechanism underlying the increase in ROS generation by leukocytes requires further investigation. In a previous study, we showed that glucose causes an increase in NADPH oxidase in leukocytic membranes as reflected in the p47^{phox} (ie, a component of NADPH oxidase in which "phox" is phagocyte oxidase) subunits (11), which are essential to the formation of NADPH oxidase complex in the membrane. NADPH oxidase is the enzyme that generates the superoxide radical (24, 25). It is possible that lipid and protein intakes also increase the activity of NADPH oxidase and its content in leukocytic membranes.

The link between nutrition, oxidative load, and lipid peroxidation is an important one because the oxidative damage of lipids, particularly of the LDL particle, may result in the formation of foam cells, the fatty streak, and atherogenic plaque (26). Oxidized LDL is internalized via the scavenger receptor of the subendothelial macrophage, which forms the foam cells; the foam cells form the fatty streak—the initial lesion of atherosclerosis (27). The association between nutritional intake and oxidative damage is important in terms of aging. It was previously shown that there is progressive oxidative damage in the aging process in small mammals (28). Gene transfection-induced increases in catalase and superoxide dismutase result in both a reduction in oxidative damage and in a slowing of the aging process in *Drosophila melanogaster* (29). Although these data cannot be extrapolated to humans, it is important that oxidative damage appears to modify aging across the species. Thus, overnutrition may be both a risk of atherosclerosis and accelerated aging.

In conclusion, both lipid and protein intakes induce a reproducible increase in ROS generation by PMNs and MNCs as previously reported for glucose. The protracted significant increase in TBARS (lipid peroxidation) over 3 h after a large lipid load may potentially accelerate foam cell formation and the process

of atherosclerosis. The rapid increase in TBARS observed after the cream challenge is of interest because the peak increase (1 h after intake) preceded the peak increase in triacylglycerol (2 h after intake) and coincided with the time of peak ROS generation. Clearly, cream intake increased lipid peroxidation, whereas casein intake did not. This has important implications for atherogenesis in subjects consuming diets with a high fat content because lipid peroxidation is a key step in atherogenesis. 

REFERENCES

- Hoeschen RJ. Oxidative stress and cardiovascular disease. *Can J Cardiol* 1997;13:1021–5.
- Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999;340:115–26.
- Ceriello A. Hyperglycaemia: the bridge between non-enzymatic glycation and oxidative stress in the pathogenesis of diabetic complications. *Diabetes Nutr Metab* 1999;12:42–6.
- Jain SK. Hyperglycemia can cause membrane lipid peroxidation and osmotic fragility in human red blood cells. *J Biol Chem* 1989;264:21340–5.
- Parthasarathy S. Role of lipid peroxidation and antioxidants in atherogenesis. *J Nutr Sci Vitaminol (Tokyo)* 1992;(spec no):183–6.
- Gisinger C, Jeremy J, Speiser P, Mikhailidis D, Dandona P, Scherthaner G. Effect of vitamin E supplementation on platelet thromboxane A₂ production in type I diabetic patients. Double-blind crossover trial. *Diabetes* 1988;37:1260–4.
- Devaraj S, Jialal I. Low-density lipoprotein postsecretory modification, monocyte function, and circulating adhesion molecules in type 2 diabetic patients with and without macrovascular complications: the effect of alpha-tocopherol supplementation. *Circulation* 2000;102:191–6.
- Maxwell SR. Prospects for the use of antioxidant therapies. *Drugs* 1995;49:345–61.
- Aljada A, Thusu K, Armstrong D, Nicotera T, Dandona P. Increased carbonylation of proteins in diabetes mellitus. *Diabetes* 1995;44(suppl)113A (abstr).
- Dandona P, Thusu K, Cook S, et al. Oxidative damage to DNA in diabetes mellitus. *Lancet* 1996;347:444–5.
- Mohanty P, Hamouda W, Garg R, Aljada A, Ghanim H, Dandona P. Glucose challenge stimulates reactive oxygen species (ROS) generation by leukocytes. *J Clin Endocrinol Metab* 2000;85:2970–3.
- Ceriello A, Bortolotti N, Crescentini A, et al. Antioxidant defences are reduced during the oral glucose tolerance test in normal and non-insulin-dependent diabetic subjects. *Eur J Clin Invest* 1998;28:329–33.
- Ceriello A, Bortolotti N, Motz E, et al. Meal-generated oxidative stress in type 2 diabetic patients. *Diabetes Care* 1998;21:1529–33.
- Dandona P, Thusu K, Hafeez R, Abdel-Rahman E, Chaudhuri A. Effect of hydrocortisone on oxygen free radical generation by mononuclear cells. *Metabolism* 1998;47:788–91.
- Dandona P, Karne R, Ghanim H, Hamouda W, Aljada A, Magsino CH Jr. Carvedilol inhibits reactive oxygen species generation by leukocytes and oxidative damage to amino acids. *Circulation* 2000;101:122–4.
- Tosi MF, Hamedani A. A rapid, specific assay for superoxide release from phagocytes in small volumes of whole blood. *Am J Clin Pathol* 1992;97:566–73.
- Hancock JT, Jones OT. The inhibition by diphenyleneiodonium and its analogues of superoxide generation by macrophages. *Biochem J* 1987;242:103–7.
- Ohkawa H, Ohishi N, Yagi K. Reaction of linoleic acid hydroperoxide with thiobarbituric acid. *J Lipid Res* 1978;19:1053–7.
- Dandona P, Aljada A, Mohanty P, et al. Insulin inhibits intranuclear nuclear factor κB and stimulates I κB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect? *J Clin Endocrinol Metab* 2001;86:2899–902.

20. Mohanty P, Khurana U, Chaudhuri A, Thusu K, Aljada A. Non-suppressibility of reactive oxygen species (ROS) generation by mononuclear cells (MNC) in obesity. *Diabetes* 1996;45(suppl):171A (abstr).
21. Dandona P, Mohanty P, Ghanim H, et al. 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. *J Clin Endocrinol Metab* 2001;86:355–62.
22. Dandona P, Mohanty P, Hamouda W, et al. Inhibitory effect of a two day fast on reactive oxygen species (ROS) generation by leucocytes and plasma ortho-tyrosine and meta-tyrosine concentrations. *J Clin Endocrinol Metab* 2001;86:2899–902.
23. Ornish D, Scherwitz LW, Billings JH, et al. Intensive lifestyle changes for reversal of coronary heart disease. *JAMA* 1998;280:2001–7.
24. Heyworth PG, Curmutte JT, Nauseef WM, et al. Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. *J Clin Invest* 1991;87:352–6.
25. Park JW, Benna JE, Scott KE, Christensen BL, Chanock SJ, Babior BM. Isolation of a complex of respiratory burst oxidase components from resting neutrophil cytosol. *Biochemistry* 1994;33:2907–11.
26. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989;320:915–24.
27. Ross R. The pathogenesis of atherosclerosis—an update. *N Engl J Med* 1986;314:488–500.
28. Stadtman ER. Protein oxidation and aging. *Science* 1992;257:1220–4.
29. Orr WC, Sohal RS. Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science* 1994;263:1128–30.

