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Inhibitory Effect of a Two Day Fast on Reactive Oxygen Species (ROS) Generation by Leucocytes and Plasma Ortho-Tyrosine and Meta-Tyrosine Concentrations

Paresh Dandona, Priya Mohanty, Wael Hamouda, Husam Ghanim, Ahmad Aljada, Rajesh Garg, Vikramjeet Kumar

Division of Endocrinology, State University of New York at Buffalo and Kaleida Health, 3 Gates Circle, Buffalo, New York 14209

Abstract

Since glucose intake acutely increases reactive oxygen species (ROS) generation by polymorphonuclear leucocytes (PMN) and mononuclear cells (MNC), we have now investigated whether a fast over a period of 48h reduces ROS generation by these cells. Eight normal subjects were fasted for 48h. Blood samples were obtained at 0, 24h and 48h. ROS generation by PMN fell significantly at 24h (66.1 ± 19.5 % of basal) and further at 48h (45.9 ± 23.0 % of basal; $p<0.001$). ROS generation by MNC fell to 62.4 ± 16.5 % at 24h and by 48.4 ± 16.5 % ($p<0.001$) by 48h. The level of $p47^{phox}$ subunit, an index of NADPH oxidase, the enzyme converting molecular oxygen to superoxide ($O^{\cdot}_2{}^-$) radical, also fell in parallel. Plasma *o*-tyrosine/phenylalanine ratio fell significantly from 0.326 ± 0.053 mmol/mol to 0.303 ± 0.055 mmol/mol at 48h and *m*-tyrosine/phenylalanine ratio fell from 0.363 ± 0.063 mmol/mol to 0.340 ± 0.064 mmol/mol ($p<0.05$). Thus, a 48h fast may reduce ROS generation, total oxidative load and oxidative damage to amino acids.

Introduction

We have recently demonstrated that glucose intake causes an increase in ROS generation by PMN and MNC (1). This increase is probably mediated by the stimulation of the leucocyte membrane enzyme NADPH oxidase, which converts molecular O_2 into $O^{\cdot}_2{}^-$ radical, since the system in which we assay ROS generation mainly measures $O^{\cdot}_2{}^-$ generation by NADPH oxidase (2, 3). Indeed, we have also shown that the expression of $p47^{phox}$, the key component of NADPH oxidase, is also increased following glucose challenge, also suggesting an upregulation of NADPH oxidase (1). We have now carried out a study on the effect of a 48 hour fast in normal subjects to determine whether abstinence from food results in a fall in ROS generation by leucocytes. We have previously used this model of ROS generation by leucocytes to investigate the effect of drugs (2, 4)and pathological states like diabetes mellitus (5) and Bloom's Syndrome (6). In addition to ROS generation, we also investigated the effect of a 48 hour fast on: 1) phenylalanine oxidation to *o*-tyrosine and *m*-tyrosine, 2) linoleic acid peroxidation to 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE); 3) thiobarbituric acid reacting substances (TBARS) as a general index of lipid peroxidation. The conversion of linoleic acid to 9-HODE and 13-HODE and that of phenylalanine to *o*-tyrosine and *m*-tyrosine are thought to be due to ROS attack on the parent compound.

Subjects, Materials and Methods

Subjects

Eight normal subjects (age range: 27-56 years; mean: 37 ± 9.8 years; body weight: 71.2 ± 7.7 kg) volunteered for this study. None of the subjects were on antioxidants or non-steroidal anti-inflammatory drugs. Each subject was asked to come to our clinical research unit between 8 and 9:00 a.m., having not eaten after 10:00 p.m. the previous night. This was to ensure that the baseline blood sample (day 0) for ROS generation by leucocytes was not affected by nutrient intake. The subjects were then asked to abstain from any nutrient intake for a further period of 48h. They were only allowed a free intake of water and 0 calorie non-caffeine drinks for this period. Two further blood samples were obtained at 24h (day 1) and 48h (day 2) and two weeks after the basal blood sample and again between 8 and 9:00 a.m. Thus, the total period of restriction of food was 10h + 48h, with the basal sample being obtained 10h after the last ingestion of food. Blood samples were collected in Na EDTA. The protocol of this study was approved by the Institutional Review Board of the State University of New York at Buffalo, based at the Millard Fillmore Hospital. All participating subjects gave their written informed consent for the study.

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Methods

PMN and MNC Isolation and ROS generation assay were carried out as previously described (2, 7, 8). *o*-tyrosine, *m*-tyrosine and phenylalanine concentration measurements were carried out as previously described by an HPLC technique (9). TBARS were measured using the method described by Yagi et al (10). $p47^{phox}$ subunit was measured as previously described (1).

Statistical Analysis

All data on ROS generation were normalized to a baseline of 100% in view of the inter-individual variability in ROS generation. The results are expressed as mean ± SD. Statistical analysis was carried out using paired t-test or one way ANOVA for repeated measures using the Sigma Stat software.

Results

ROS Generation by Leucocytes: ROS generation by PMN at baseline (day 0) was 481 ± 269 mV/10^6 cells (100%). During the fast, ROS generation fell to 66.1 ± 19.5% at 24h and to 45.9 ± 23.0% at 48h ($p<0.001$, Figure 1). ROS generation levels returned to basal levels after two weeks (recovery). ROS generation by MNC at baseline (day 0) was 549 ± 230 mV/10^6 cells (100%). During the fast, ROS generation fell significantly to 62.4 ± 16.5% at 24h and to 48.4 ± 16.5% at 48h ($p<0.001$) as shown in figure 1 and returned to basal levels at 2 weeks.

p47phox Subunit: $p47^{phox}$ Subunit level in MNC fell significantly and remained low at 48 h (Figure 2).

Plasma o-Tyrosine, m-Tyrosine and Phenylalanine Concentrations: Plasma *o*-tyrosine concentration fell in all subjects from a mean of 3.79 ± 0.62 ng/ml to 3.59 ± 0.66 ng/ml at 48h. Plasma *m*-tyrosine concentration also fell from 4.21 ± 0.68 ng/ml to 4.00 ± 0.69 ng/ml. Plasma phenylalanine concentrations increased slightly from 10.47 ± 0.60 μg/ml to 10.67 ± 0.64 μg/ml; this increase was not significant.

o-Tyrosine/phenylalanine ratio fell significantly from 0.326 ± 0.053 mmol/mol to 0.303 ± 0.055 mmol/mol at 48h (Figure 3; $p<0.05$). This fall was 7 % from the basal levels. *m*-Tyrosine/phenylalanine ratio fell significantly from 0.363 ± 0.063 mmol/mol to 0.340 ± 0.064 mmol/mol at 48h. This fall was 6.6 % from the basal and was significant ($p<0.05$; Figure 3). *o*-tyrosine/phenylalanine ratio returned to basal levels at two weeks. However, *m*-tyrosine/phenylalanine ratio at 2 weeks was still lower than the baseline levels.

Plasma TBARS, 9-HODE/linoleic acid ratio and 13-HODE/ Linoleic Acid ratio: Plasma TBARS concentrations did not change significantly after 48h fast. Plasma 9-HODE, 13-HODE and linoleic acid concentrations started to increase at 24h and were significantly higher than baseline levels at 48h ($p<0.05$). However, 9-HODE/ linoleic acid and 13-HODE/ linoleic acid ratios did not alter significantly (Table 1).

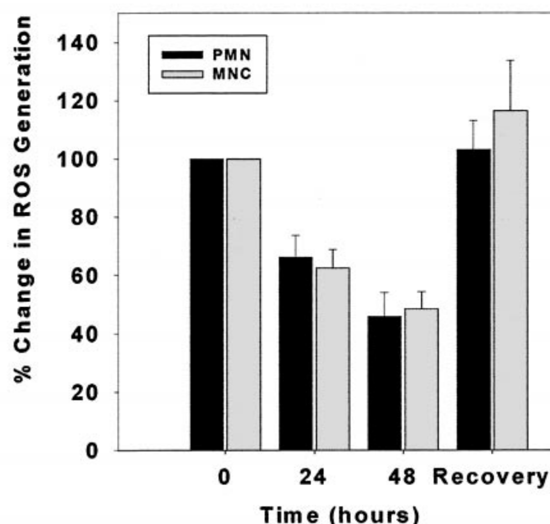
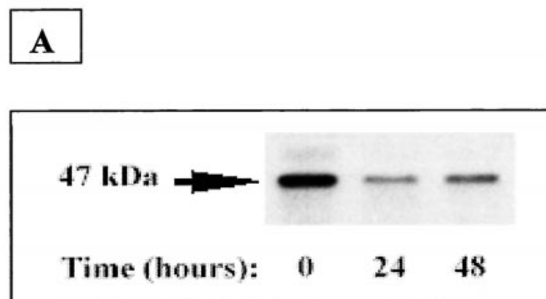


Figure 1: ROS generation by PMN and MNC following 48h fast. ROS generation was significantly inhibited at 24h and 48h. ROS generation levels returned to basal levels at 2 weeks (recovery).



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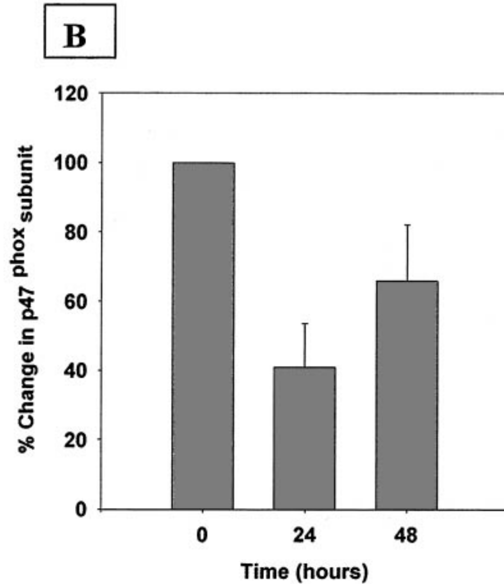

Figure 2: A) Western Blot showing the relative expression of $p47^{phox}$ subunit in MNC. **B)** Densitometric analysis of $p47^{phox}$ subunit protein levels in MNC.

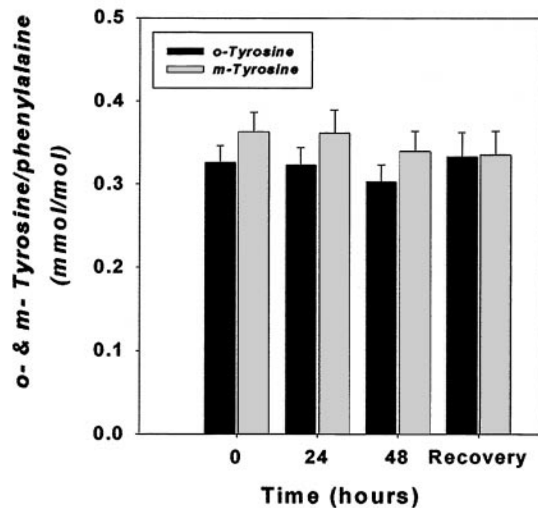

Figure 3: *o*- and *m*-Tyrosine/phenylalanine ratios following 48h fast. This ratio did not change at 24h and fell significantly at 48h ($p<0.05$).

Discussion

Our data demonstrate for the first time that ROS generation by both PMN and MNC decreases markedly after a 24 hour fast with a further fall after 48 h (>50% inhibition). Consistent with this fall in ROS generation was reduction in $p47^{phox}$ subunit, the cardinal component of NADPH oxidase, the enzyme responsible for the conversion of molecular O_2 to $O^{\cdot}{}_2^-$ radical. We have previously demonstrated that glucose intake acutely induces an increase in ROS generation by PMN and MNC and also a parallel increase in $p47^{phox}$ subunit. Thus, our present study is consistent with our previously suggested concept that ROS generation by leucocytes and the expression of NADPH oxidase is modulated by nutritional factors.

The fall in *o*-tyrosine and *m*-tyrosine within 2 days of fast is indicative of diminished oxidative damage to phenylalanine and is consistent with a rapid fall in overall oxidative load. *o*-tyrosine and *m*-tyrosine are known to be products of ROS attack on phenylalanine in contrast to *p*-tyrosine, which is formed by specific enzymatic action on phenylalanine. Thus, the decrease in their concentration in association with the fall in ROS generation is probably causally related to the later. Changes in *o*- and *m*-tyrosine concentrations are now accepted as indicators of oxidative damage caused by the hydroxyl radical. In contrast to the fall in *o*- and *m*-tyrosine, there was a small, but significant increase in linoleic acid, 9-HODE and 13-HODE. However, when corrected for the concentration of linoleic acid, 9-HODE/linoleic acid and 13-HODE/linoleic acid ratios did not alter. It is possible that during the fast, accelerated lipolysis resulted in an increase in linoleic acid concentrations, providing an increased substrate for the formation of its oxidatively damaged products: 9-HODE and 13-HODE. A more prolonged fast may be associated with a fall in the indices of lipid peroxidation.

The fall in ROS generation may also have implications for vascular reactivity since $O^{\cdot}{}_2^-$ radical binds to NO and reduces its bioavailability. Thus, with the reduction in $O^{\cdot}{}_2^-$ generation, the bioavailability of NO may increase and vasodilatory responses may also improve. Indeed, Vogel et al were able to demonstrate a decrease in flow mediated vasodilation of the brachial artery reactivity following an 800 calorie meal (11); this decrease in brachial arterial reactivity was

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prevented by prior administration of vitamin E. Hitherto, the approach to reduce oxidative load has been based on the administration of antioxidant like vitamin E (12). Indeed, vitamin E not only reduces oxidative damage to lipids (13, 14), but also the chances of developing coronary heart disease related events in prospective long-term observational studies in subjects not known to have clinical manifestations of atherosclerosis (15, 16). We too have previously demonstrated falls in ROS generation by leucocytes following vitamin E (17), carvedilol (8), a β-blocker, and glucocorticoids (2, 4, 7). Thus, hitherto, the approach to ROS induced damage has been through the administration of drugs and not through nutritional restriction.

Ornish has shown that lifestyle modifications reduce the frequency of cardiovascular events in patients with CHD, with evidence of a concomitant improvement in myocardial perfusion and a reversal of atherosclerotic lesions in coronary ateries (18). It is possible that dietary modifications, including the reduction of fat intake to less than 10% of total, contributed to diminished ROS generation, reduced oxidative damage and diminished lipid peroxidation. This may have an inhibitory effect on atherogenesis, since the formation of oxidized LDL (19) is important and critical to formation of foam cells and the fatty streak (20).

In conclusion, nutritional restriction has a profound inhibitory effect on ROS generation by leucocytes. Allied with previous data on the acute stimulatory effect of nutrient intake on ROS generation, we suggest that nutrition is a major modulator of ROS generation and oxidative load. Thus, lifestyle changes with restricted nutritional intake may have beneficial effects on ROS generation and oxidative injury and may therefore affect outcomes in relation to atherosclerosis and aging.

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