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# GLUCOSE CHALLENGE STIMULATES REACTIVE OXYGEN SPECIES (ROS) GENERATION BY LEUCOCYTES

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## Abstract

Diabetes mellitus is associated with increased ROS generation, oxidative injury and obesity. To elucidate the relationship between nutrition and ROS generation, we have investigated the effect of glucose challenge on ROS generation by leucocytes, $p47^{phox}$ protein, a key protein in the enzyme NADPH oxidase and α-tocopherol levels. Blood samples were drawn from 14 normal subjects prior to, at 1, 2 and 3 h following ingestion of 75 g glucose. ROS generation by polymorphonuclear leucocytes (PMNL) and mononuclear cells (MNC) increased to a peak of 244 ± 42% and 233 ± 34% of the basal respectively at 2h. The levels of $p47^{phox}$ in MNC homogenates increased significantly at 2 h and 3 h after glucose intake. α-Tocopherol levels decreased significantly at 1 h, 2 h and 3 h. We conclude that glucose intake stimulates ROS generation and $p47^{phox}$ of NADPH oxidase; increases oxidative load and causes a fall in α-tocopherol concentration.

## Introduction

Diabetes mellitus is associated with an increase in ROS generation by mononuclear cells[1] and an increased oxidative load resulting in oxidative damage to lipids[2], proteins[3] and DNA[4]. Acute hyperglycemia has been shown to result in an increase in blood pressure, which is prevented by antioxidants[5]; this suggests that acute hyperglycemia probably causes increased generation of ROS and thus a diminished bioavailability of nitric oxide (NO) [5-7]. We have also shown that ROS generation by leucocytes falls after weight loss (unpublished data). This raises the possibility that nutrition may modulate ROS generation. These facts suggest that chronic hyperglycemia (diabetes mellitus) and acute hyperglycemia may result in increased ROS generation (unpublished data).

We therefore, embarked on a study to investigate the effect of glucose challenge on leukocytic ROS generation. We also investigated the levels of $p47^{phox}$, the key protein component of NADPH oxidase [8-9] to determine whether the NADPH units increase in number following glucose challenge. Plasma concentration of thiobarbituric acid reacting substances (TBARS) was measured since it is an index of lipid peroxidation.

## Subjects, Materials and Methods

Fourteen normal subjects (7 males and 7 females; age range: 25-42 years; weight range: 53-80 kg; mean BMI: 24.2 ± 0.6 $kg/m^2$) participated in the study. They were given 75 g of glucose dissolved in 300 ml water (Glucola) to drink over 5 minutes. Controls (n=6) drank 300 ml water containing saccharin. Blood samples were obtained at 0,1,2 and 3h. PMNL and MNC isolation and ROS generation were carried out as previously described [10]. $p47^{phox}$ was measured by Western blotting using anti-$p47^{phox}$ antibody from Transduction Labs (Lexington, KY). TBARS were measured as described by Ohkawa et al [11]. α.-Tocopherol was measured in plasma by HPLC as described previously [12]. Insulin levels were determined using an ELISA kit from Diagnostic Systems Laboratories Inc. (Webster, TX).

Statistical analysis was carried out by one way analysis of variance (ANOVA) for repeated measures. The data on ROS generation were analyzed by reducing the basal readings to 100%. Values are expressed as mean ± S.E.

## Results

The mean plasma glucose and insulin concentrations peaked at 1h to 108.5 ± 9.4 mg/dl and 66.6 μU/ml (Table 1). The mean ROS generation by PMNL in the fasting state (basal) was 117 ± 19 mV (100%). It increased to a peak of 244 ± 42% over the basal (Figure 1)($p<0.005$). The mean ROS generation by MNC in the fasting state (basal) was 213 ± 40 mV (100%); it increased to a peak of 233 ± 34% over the basal at 2h (Figure 2)($p<0.001$). There was no change in ROS generation by leucocytes following a drink of 300 ml water or saccharine containing water. The levels of $p47^{phox}$ in MNC homogenates, representative of NADPH oxidase, did not alter at 1 h, but increased significantly at 2 h and 3 h after glucose intake (Figure 3) with a peak effect at 2 h.

TBARS increased from a basal level of 1.07 ± 0.10 μmol/L (100%) to 116.7 ± 7.2% of basal at 1 h, 120.6 ± 8.3% of basal at 2 h and 108 ± 12.9% of basal at 3 h ($p=0.065$) (Figure 4). The mean fasting α-tocopherol level was 8.7 ± 0.4 μg/ml (basal, 100%). α-tocopherol levels fell to 99% ± 0.4%, 96. 1 % ± 0.6% and 96 ± 0.6% of the basal at 1, 2 and 3 h respectively ($p<0.001$) (figure 5).

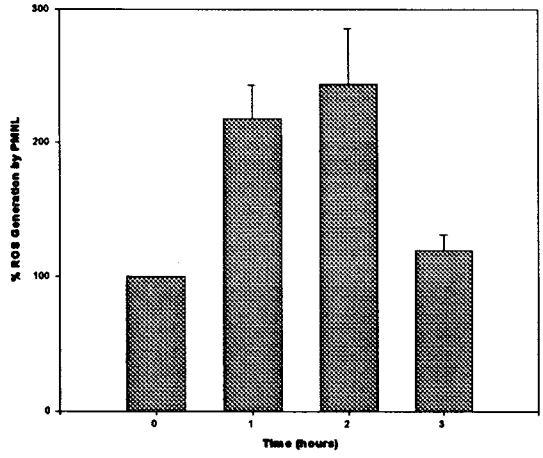


**Figure 1:** ROS generation by PMNL prior to and following glucose challenge (75 g) at 1, 2 and 3 h. Results are expressed as % increase over fasting basal (100%). Note that the ROS generation increased significantly at 1 h and increased further at 2 h and then decreased at 3 h.

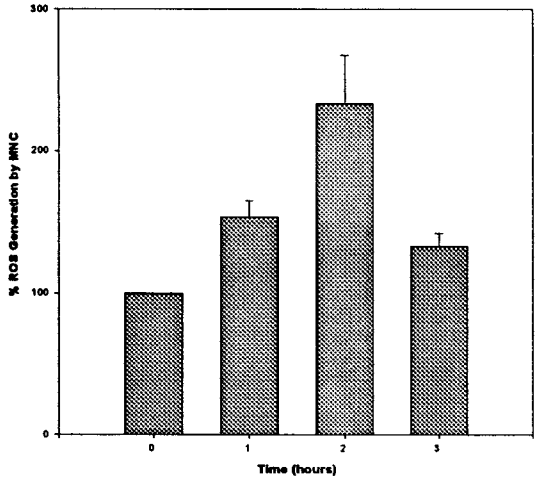


**Figure 2:** ROS generation by MNC prior to and following glucose challenge (75 g) at 1, 2 and 3 h. Results are expressed as % increase over fasting basal (100%). Note that the ROS generation is increased significantly at 1 h and increased further at 2 h and then declined at 3 h.

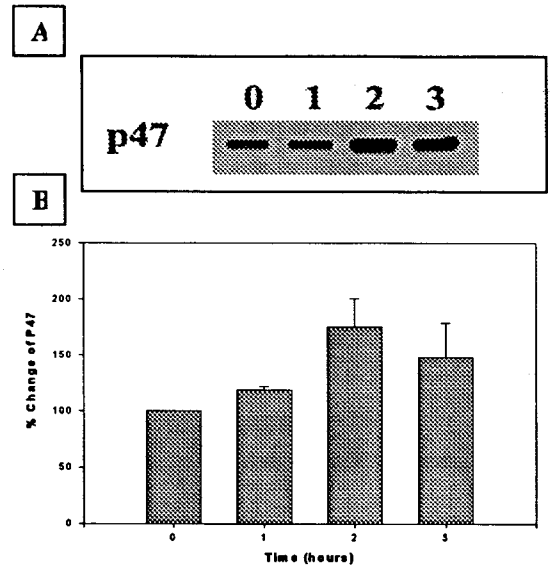


**Figure 3:** A) Western Blot showing the relative expression of $p47^{phox}$ in MNC. Note that $p47^{phox}$ expression is induced at 1 h after glucose intake. Maximum induction is observed at 2 h. This blot is a representative of four different experiments with four different subjects. B) Densitometric semi-quantitative analysis of $p47^{phox}$ protein levels in MNC. Note the significant increase at 2 h ($p<0.05$).

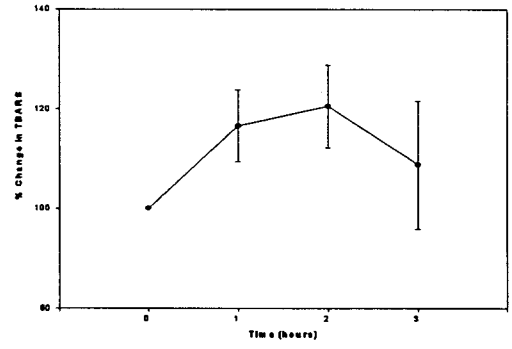


**Figure 4:** Change in TBARS at 1, 2 and 3 h after glucose challenge. Results are expressed as mean ± S.E increase over the basal value (100%). Note that the increase at 1 h and 2 h was significant with paired t-test.

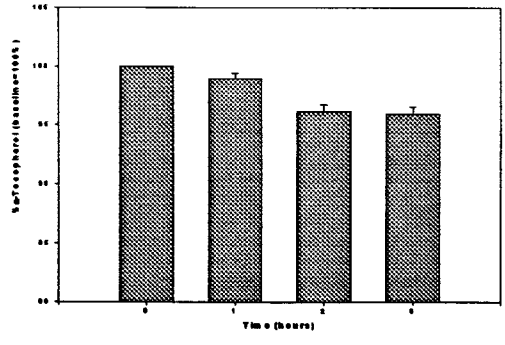


**Figure 5:** Change in α-tocopherol levels at 1, 2 and 3 h after glucose challenge. Values are expressed as mean ± S.E.% of baseline, baseline being normalized as 100%. Note that the fall in α-tocopherol was significant at 2 h and 3 h.

## Discussion

Our data demonstrate clearly for the first time that a challenge with glucose results consistently in an increase in ROS generation by leucocytes in normal subjects. No increase was observed following the intake of 300 ml of water containing saccharine. There was also an increase in the expression of $p47^{phox}$, the key protein component of NADPH oxidase. The increase in $p47^{phox}$ was observed at 2 and 3 h with no significant change at 1 h. Since ROS generation by MNC increased at 1 h, while $p47^{phox}$ increased only at 2 and 3 h, it would appear that the initial increase in ROS generation is a function of increased activity of pre-existent NADPH oxidase units, while the further increases at 2 and 3 h may receive a contribution from the formation of additional new NADPH oxidase units. Clearly, there is no direct quantitative relationship between $p47^{phox}$ expression and ROS generation; however, there is a qualitative relationship between the two and thus, the increase of 70% in $p47^{phox}$ reflecting an increase in the enzyme NADPH oxidase, may contribute to increased superoxide generation. It is possible that glucose is an important modulator of $p47^{phox}$ gene expression.

There is a recent demonstration of a depletion in antioxidant reserve following an oral glucose tolerance test in normal subjects and in patients with type 2 diabetes [13, 14]. The previous studies did not investigate ROS generation, although the depletion of antioxidant reserve (α-tocopherol) is consistent with an increase in ROS generation. Our study demonstrates increased ROS generation by PMNL and MNC, it is also possible that increased ROS generation is more generalized. Glucose and/or food intake stimulate the metabolic rate [15] and therefore probably stimulate metabolic activity at the cellular and mitochondrial level, which may result in increased ROS generation. Triiodothyronine administration also stimulates metabolism and has recently been shown by us to increase ROS generation by PMNL and MNC [16].

We have recently demonstrated that carvedilol (a β blocker) administration to normal subjects for 1week results in a significant decrease in ROS generation by PMNL and MNC with a parallel reduction in oxidative damage to amino acids [17]. Thus, β-adrenergic modulation of NADPH oxidase is a possibility. It is therefore possible that nor-epinephrine, known to be released following glucose intake, may stimulate ROS generation by leucocytes. Another leucocyte membrane enzyme, Na-K ATPase, has previously been shown by us to be stimulated by glucose challenge [18] and $T_3$ administration [19] and to be inhibited by β-blockade [20].

The possibility that insulin may play a role in the stimulation of ROS generation also has to be considered. MNC are known to have insulin receptors [21] but glucose transport in MNC appears to be primarily mediated by GLUT-1 rather than GLUT-4 [22]. There is at least one study demonstrating insulin stimulated GLUT-1 mediated glucose transport by MNC [22]. On the other hand, insulin may have a direct effect on ROS generation. Indeed, we have now shown that insulin infusion inhibits ROS generation by leucocytes [23]. Thus, it is unlikely that insulin contributed to the stimulation of ROS generation following glucose challenge.

The small (4%) but significant fall in α-tocopherol concentration following a 75 g glucose (≡300 k calories) challenge is consistent with an increase in oxidative load due to increased ROS generation. All subjects tested demonstrated this fall. These data are consistent with those of Ceriello et al [13], who demonstrated a similar fall in α-tocopherol after glucose challenge. It is unlikely that these small changes affect ROS generation, since much larger changes (180%) in concentrations of α-tocopherol associated with large doses of this vitamin have been shown to alter ROS (60% inhibition) generation over a prolonged period of vitamin E administration [24].

The link between nutrition and oxidative damage is an important one since oxidative damage of lipids, the LDL particle in particular, may contribute to atherosclerosis [25]. Oxidized LDL, which is internalized via the scavenger receptor of the circulating monocytes and the subendothelial macrophage which form the foam cells; and the latter cells form the fatty streak, the initial lesion of atherosclerosis [26]. Our work opens the way for the investigation of the effect of other macronutrients on ROS generation and oxidative damage

In conclusion, glucose challenge induces a consistent increase in ROS generation by leucocytes, an increase in $p47^{phox}$, an integral protein component of NADPH oxidase, and a decrease in α-tocopherol concentrations in plasma.

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| | 0 hour | 1 hour | 2 hours | 3 hours |
|---|---|---|---|---|
| Glucose (mg/dl) | 83 ± 7.8 | 108 ± 9.4 | 107 ± 8.8 | 70 ± 3.4 |
| Triglycerides (mg/dl) | 142 ± 28 | 136 ± 22 | 133 ± 22 | 151 ± 50 |
| Cholesterol (mg/dl) | 195 ± 11 | 191 ± 10 | 189 ± 10 | 197 ± 1 |
| Insulin (μIU/ml) | 7.9 ± 1.8 | 66.6 ± 15.7 | 32.8 ± 10.3 | 16.5 ± 9 |

**Table 1:** Glucose, triglyceride, cholesterol, and insulin concentrations prior to and following glucose intake.