Increase in intranuclear nuclear factor κB and decrease in inhibitor κB in mononuclear cells after a mixed meal: evidence for a proinflammatory effect\textsuperscript{1–3}

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ABSTRACT

Background: In view of the stimulatory effect of glucose on reactive oxygen species (ROS) generation, we investigated the possibility that a mixed meal stimulates ROS generation and possibly induces concomitant proinflammatory changes.

Objective: The objective was to determine whether the intake of a 900-kcal mixed meal induces an increase in ROS generation by leukocytes and an inflammatory response at the cellular level.

Design: Nine normal-weight subjects were given a 900-kcal mixed meal, and 8 normal-weight subjects were given 300 mL water after an overnight fast. Blood samples were collected at 0, 1, 2, and 3 h. ROS generation by mononuclear cells and polymorphonuclear leukocytes and the expression of p47\textsubscript{phox} subunit were measured. Intranuclear nuclear factor κB (NF-κB) binding and the expression of inhibitor κBα (IκBα), IκB kinase α (IKK\textalpha), and IκB kinase β (IKKβ) were measured. Plasma concentrations of C-reactive protein (CRP) and soluble intercellular adhesion molecule were also measured.

Results: ROS generation by mononuclear cells and polymorphonuclear leukocytes and p47\textsubscript{phox} expression increased significantly. The expression of IKK\textalpha and IKKβ and DNA-binding activity of NF-κB increased significantly, whereas IκBα expression decreased. Plasma CRP concentrations increased. The intake of 300 mL water did not induce a change in any of the above indexes.

Conclusions: These data show that the intake of a mixed meal results in significant inflammatory changes characterized by a decrease in IκBα and an increase in NF-κB binding, plasma CRP, and the expression of IKK\textalpha, IKKβ, and p47\textsubscript{phox} subunit. These proinflammatory changes are probably relevant to the state of chronic hypertension and obesity and to its association with atherosclerosis. Am J Clin Nutr 2004;79:682–90.

KEY WORDS Nuclear transcription factor κB, inhibitor κB, mononuclear cells, mixed meal, inflammation, reactive oxygen species

INTRODUCTION

We recently showed that glucose (1) and lipid and protein (2) intakes cause an increase in the generation of reactive oxygen species (ROS) by leukocytes and that each of these macronutrients induces a distinct pattern of increase in ROS generation. Thus, glucose induces a peak in ROS generation by both mononuclear cells (MNCs) and polymorphonuclear leukocytes (PMNLs) at 2 h, whereas cream (lipid) produces a peak at 1 h. The peak increase in ROS generation is the greatest with glucose, whereas it is the least with casein (protein). On the other hand, cream intake causes a prolonged increase in lipid peroxidation. Consistent with these observations, we also showed that a 48-h fast results in a marked decrease in ROS generation by leukocytes and oxidative damage of amino acids (3). Thus, we suggest that nutritional intake may be the major modulator of ROS generation. Indeed, we showed recently that the state of obesity reflecting chronic hypernutrition is associated with marked oxidative stress, as reflected in an increase in indexes of lipid peroxidation, protein carbonylation, and oxidative damage of amino acids, which diminishes after dietary restriction over a relatively short period of time, eg, 4 wk (4).

Because our studies to date have not dealt with real food, but instead with pure macronutrients, we have now undertaken our first study with food items. We chose to study the effect of a mixed meal composed of carbohydrates, protein, and fats. Such a mix was previously shown to impair endothelium-mediated vasodilatation in the brachial artery (5, 6). In addition, Ceriello et al (7) showed an increase in oxidative stress and LDL oxidation in diabetes after a meal challenge.

We hypothesized that the intake of a mixed meal causes an increase in oxidative stress and proinflammatory activity in circulating MNCs. In this study we investigated the effect of the intake of this mixed meal on ROS generation by PMNLs and MNCs. Because we previously showed that glucose intake results in an increase in the p47\textsubscript{phox} subunit, the key protein of NADPH oxidase in leukocytes, an increase in intranuclear nuclear factor κB (NF-κB), and a decrease in IκBα (8), we decided to also investigate the effect of the mixed meal on intranuclear NF-κB binding activity and the expression of inhibitor κBα (IκBα), IκB kinase α (IKK\textalpha), IκB kinase β (αIKKβ), and p47\textsubscript{phox} subunit in MNC. Plasma C-reactive protein (CRP), soluble intercellular adhesion molecule 1 (sICAM-1), and α-
tocopherol were also measured. These indexes would allow us to explore a possible link between nutrition, oxidative stress, and inflammation.

SUBJECTS AND METHODS

Subjects

Nine nondiabetic subjects aged 29–38 y (\(\bar{x} \pm\) SEM: 32 \(\pm\) 3 y) of normal weight [body mass index (in kg/m\(^2\)): 25.4 \(\pm\) 2.7; \(\bar{x} \pm\) SEM] were included in the study. The subjects came to the Clinical Research Center of the Diabetes–Endocrinology Center of Western New York after an overnight fast between 0800 and 0900. A fasting blood sample was obtained, and the subjects were asked to eat a mixed meal containing 910 kcal (egg-muffin and sausage-muffin sandwiches and 2 hash browns, which contained 81 g carbohydrate, 51 g fat, and 32 g protein) over 15 min. Additional blood samples were obtained 1, 2, and 3 h after the meal was eaten. The control subjects (age: range, 26–50 y; \(\bar{x} \pm\) SD, 35 \(\pm\) 8 y; body mass index: 24.3 \(\pm\) 2.0) were 8 volunteers who were given 300 mL water to drink in the fasting state. Blood samples were obtained before and 1, 2, and 3 h after the drink. The protocol was approved by the Institutional Review Board of the State University of New York at Buffalo based at The Millard Fillmore Hospital. All participants gave their written informed consent.

Isolation of PMNLs and MNCs

Blood samples were collected in tubes containing Na-EDTA as an anticoagulant; 3.5 mL of the anticoagulated blood sample was carefully layered over 3.5 mL of the PMNL isolation medium (Robbins Scientific Corp, Sunnyvale, CA). Samples were centrifuged at 450 \(\times\) g in a swing rotor for 30 min at 22 °C. At the end of centrifugation, 2 bands separate out at the top of the red blood cell pellet. The top band consists of MNCs, whereas the bottom band consists of PMNLs. The MNC and PMNL bands were harvested with a Pasteur pipette, repeatedly washed with Hank’s balanced salt solution, and reconstituted to a concentration of 4 \(\times\) 10\(^5\) cells/mL in Hank’s balanced salt solution.

NF-\(\kappa B\) electrophoretic mobility shift assay

DNA-binding protein extracts were prepared from MNCs with the method described by Andrews and Faller (9). Total protein concentrations were determined with the bicinchoninic acid protein assay (Pierce, Rockland, IL). The NF-\(\kappa B\) gel retardation assay was performed with the use of a NF\(\kappa B\) binding protein detection kit (Life Technologies, Long Island, NY). Briefly, the double-stranded oligonucleotide containing a tandem repeat of the consensus sequence for the NF-\(\kappa B\) binding site was radiolabeled with \(\gamma\)-\(P\) by T4 kinase. Then, 5 \(\mu\)g of the nuclear extract was mixed with the incubation buffer, and the mixture was preincubated at 4 °C for 15 min. Labeled oligonucleotide (60 000 cpm) was added, and the mixture was incubated at room temperature for 20 min. Samples were then applied to wells of 6% nondenaturing polyacrylamide gel. The gel was dried under vacuum and exposed to X-ray film. Densitometry was performed with the use of Bio-Rad molecular analysis software (Hercules, CA).

\(\text{p47}^{\text{phox}}\) Subunit, IkB\(\alpha\), IKK\(\alpha\), and IKK\(\beta\) Western blotting

Western blotting was carried out as previously described. Briefly, total protein concentrations were determined with the use of a bicinchoninic acid protein assay (Pierce, Rockland, IL); 20 \(\mu\)g MNC homogenate was electrophoresed on sodium dodecyl sulfate polyacrylamide gels for the \(\text{p47}^{\text{phox}}\) subunit. Forty micrograms of total homogenates was used for IkB\(\alpha\) (Cell Signaling Technology Inc, Beverly, MA), IKK\(\alpha\) (Pharmingen, San Diego), and IKK\(\beta\) (Transduction Labs, Lexington, KY) Western blotting.

Measurement of reactive oxygen species generation

Five hundred microliters of PMNLs or MNCs (2 \(\times\) 10\(^5\) cells) were delivered into a Chronolog Lumi-Aggregate cuvette. Fifteen microliters of 10 mmol luminol/L was then added, followed by 1.0 \(\mu\)L of 10 mmol formylmethylion leucinyl phenylalanine/L. Chemiluminescence was recorded for 15 min (a protracted record after 15 min did not alter the relative amounts of chemiluminescence produced by various blood 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 after 15 min did not alter the relative amounts of chemiluminescence produced by various blood 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 as well as diphenylene iodonium (data not shown), which is a spe-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Glucose (mg/dL)</th>
<th>Baseline (0 h)</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>82 (\pm) 3</td>
<td>95 (\pm) 6</td>
<td>89 (\pm) 6</td>
<td>93 (\pm) 3</td>
<td></td>
</tr>
<tr>
<td>Insulin ((\mu)U/mL)</td>
<td>6.1 (5.7–6.6)</td>
<td>28.8 (22.7–72.6)</td>
<td>17.5 (14.1–32.0)</td>
<td>19.8 (13.4–14.6)</td>
</tr>
<tr>
<td>Triacylglycerol (mg/dL)</td>
<td>97 (\pm) 14</td>
<td>121 (\pm) 13</td>
<td>146 (\pm) 17</td>
<td>175 (\pm) 20</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>57 (\pm) 14</td>
<td>54 (\pm) 12</td>
<td>52 (\pm) 10</td>
<td>53 (\pm) 12</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>104 (\pm) 8</td>
<td>90 (\pm) 7</td>
<td>85 (\pm) 8</td>
<td>81 (\pm) 11</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>180 (\pm) 12</td>
<td>168 (\pm) 11</td>
<td>165 (\pm) 9</td>
<td>169 (\pm) 12</td>
</tr>
<tr>
<td>(\alpha)-Tocopherol ((\mu)g/mL)</td>
<td>15.3 (\pm) 2.5</td>
<td>15.2 (\pm) 2.5</td>
<td>14.6 (\pm) 2.3</td>
<td>14.7 (\pm) 1.8</td>
</tr>
</tbody>
</table>

\(n = 9\), \(\bar{x} \pm\) SEM (all such values), \(\bar{x} \pm\) interquartile range in parentheses.

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**Significantly different from baseline, \(P < 0.05\) (one-factor ANOVA for repeated measures followed by Dunnett’s test).**
cific inhibitor of NADPH oxidase—the enzyme responsible for the production of superoxide radicals. The specific inhibitory effect of diphenylene iodonium on NADPH oxidase was established by Hancock and Jones (13).

Measurement of plasma vitamin E, sICAM-1, CRP, and insulin

α-Tocopherol was measured in plasma by HPLC as described previously (1, 4). Plasma sICAM-1 was assayed with an enzyme-linked immunosorbent assay kit from R&D Systems (Minneapolis). The intraassay CV for sICAM-1 is 4.6%, whereas the interassay CV is 6.1%. The CRP (intraassay CV: 4%; interassay CV: 5%) and insulin (intraassay CV: 2.6%; interassay CV: 6.2%) enzyme-linked immunosorbent assay kits were purchased from Diagnostic Systems Laboratories Inc (Webster, TX).

Statistical analysis

The statistical analysis was carried out by using SIGMASTAT software (version 2.03; Jandel Scientific, San Rafael, CA). The analysis was carried out with one-factor repeated-measures analysis of variance (ANOVA) with the use of Dunn’s test for comparisons against the baseline (0 h) for normally distributed data. Dunn’s test was used for the nonparametric data. Two-factor ANOVA was used to evaluate the interaction between treatment (water compared with the mixed meal) and time. Student’s t test was used to compare the area under the curves...
(AUCs). For the comparisons of ROS generation by PMNLs and MNCs before and after the meal challenge, the data were normalized to a baseline value of 100% because the baseline ROS generation varied markedly from one person to another. We previously showed this same level of variability while showing a consistency (reproducibility) in ROS generation within individuals (10). The AUC for all ROS generation was then measured because the peak of ROS generation for both PMNLs and MNCs varied from one subject to another, whereas an increase occurred in all subjects. The AUC was calculated for NF-κB binding activity, IκBα, IKKα, IKKβ, and p47phox and then compared between the meal-fed and control subjects by using Student’s t test for unpaired data. CRP and sICAM-1 concentrations were also normalized to a baseline value of 100% because the baseline concentrations varied markedly from one subject to another. A P value < 0.05 was used to assess the significance for all statistical analyses. The results are presented as means ± SEMs.

RESULTS

Glucose, lipid, and insulin concentrations

Plasma glucose concentrations did not change significantly after the meal challenge (Table 1). Plasma insulin concentration increased significantly at 1 h and remained elevated at 2 and 3 h when compared with baseline. Plasma triacylglycerol concentrations increased significantly 2 and 3 h after the meal challenge.

NF-κB binding activity

NF-κB binding to the consensus sequence oligonucleotide showed 2 bands on electrophoretic mobility shift assay. The upper band (I) was supershifted with the antibodies to both p65 and p50, whereas the second band (II) was supershifted with an antibody against p50 but not against p65 (Figure 1). NF-κB (p65:p50 heterodimer) binding activity in MNC nuclear extracts increased significantly 1 and 2 h after the meal challenge and returned to baseline at 3 h (P < 0.05; Figure 1). The decrease in the p50:p50 complex (II) was not consistent in all the subjects. Only 4 subjects showed this decrease, whereas the other subjects showed no change in complex II formation.

IκBα, IKKα, and IKKβ concentrations

IκBα protein concentrations, measured by Western blotting, decreased significantly 1 h after meal intake, as shown in Figure 2. This decrease persisted at 2 and 3 h (P < 0.05) when compared with baseline (0 h) and with the values for the control subjects (water intake). IKKα protein concentrations, measured by Western blotting, showed a significant increase 1, 2, and 3 h after meal intake when compared with both baseline and with the values for the control subjects (P < 0.05; Figure 3). IKKβ protein concentrations did not increase after the meal challenge when com-
pared with baseline ($P = 0.28$; Figure 4). However, the AUC for IKKβ was statistically significant after the meal challenge. This finding suggests that the increase in nuclear NF-κB was due to the inhibition of expression of IκB, which in turn was due to the induction of IKKα and IKKβ, which phosphorylate IκBα and cause its degradation.

p47phox Subunit concentrations and ROS generation

The protein quantities of the p47phox subunit of NADPH oxidase in MNC homogenates increased significantly at 1 h and remained elevated until 3 h when compared with both baseline and with the values for the control subjects ($P < 0.05$; Figure 5). All 9 subjects showed an increase in ROS generation by MNCs after meal intake. However, the time of the peak increase varied from one subject to another: in 1 subject the peak was at 1 h, in 5 subjects it was at 2 h, and in 3 subjects it was at 3 h. Baseline ROS generation by MNCs was 256 ± 140 mV (100%). Because of the interindividual variation in ROS generation, the AUC for all ROS generation was measured. The AUC for ROS generation by MNCs increased significantly (Figure 6; $P < 0.05$) after the mixed-meal challenge when compared with the AUC of ROS generation after the water challenge. Eight of 9 subjects showed an increase in ROS generation by PMNLs; the peak increase varied from one subject to another. Baseline ROS generation by PMNLs was 108 ± 55 mV. The AUC for ROS generation by PMNLs also increased significantly (Figure 6; $P < 0.05$) after the mixed-meal challenge when compared with the AUC of ROS generation after the water challenge.

**Plasma CRP, sICAM-1, and α-tocopherol concentrations**

After the meal challenge, the increase in absolute plasma CRP concentrations was not significant. However, when values were normalized to a baseline concentration of 100% because of the interindividual variation in plasma CRP concentrations, this increase was significant ($P < 0.05$) when compared with one-factor ANOVA for the repeated measures followed by Dunn’s test (Table 2). Water intake, on the other hand, did not cause any change in absolute or normalized plasma CRP concentrations. There was a significant interaction between treatment (water compared with the mixed meal) and time when CRP concentrations were compared between the 2 groups with two-factor ANOVA ($P < 0.05$). Plasma sICAM-1 concentrations did not change significantly after the meal challenge ($P = 0.119$) or water intake (Table 2). Plasma α-tocopherol concentrations did not change after the meal challenge (Table 1).

**DISCUSSION**

Our data show that after the intake of a 900-kcal mixed meal, there is a significant increase in intranuclear NF-κB binding...
activity by the proinflammatory p65:p50 heterodimer in MNCs. The increase was significant 1 and 2 h after the meal challenge and decreased 3 h after the meal challenge. This is important because the p65:p50 heterodimer is thought to be responsible for the transcription of proinflammatory genes, whereas the p50:p50 homodimer inhibits the activation of proinflammatory genes. Associated with this increase in NF-κB binding activity in the nucleus was a decrease in cellular IκB. IκBα is present in the cytosol, where it binds to NF-κB and prevents its translocation into the nucleus (14, 15). It is noteworthy that proinflammatory stimuli such as endotoxin and cytokines (eg, tumor necrosis factor α) induce an increase in intranuclear NF-κB and a decrease in IκBα (16–18). On the other hand, antiinflammatory agents cause an increase in IκBα and a decrease in NF-κB (18). The intake of 300 mL water did not cause a significant change in NF-κB, IκB, or any other index measured in the study.

The increase in IKKα and IKKβ after the caloric challenge of the mixed meal is of interest because this enzyme is induced by endotoxin challenge and is responsible for the phosphorylation, ubiquitination, and eventual proteasomal degradation of IκBα. This decrease in IκB releases NF-κB to translocate into the nucleus. There are recent data that show that IKKα may have an additional function: it may translocate into the nucleus and act as a coactivator of NF-κB–induced tumor necrosis factor α and other proinflammatory gene expression (19).

p47phox Subunit expression increased significantly at 1 h and persisted at an elevated concentration for 3 h. This suggests that NADPH oxidase activity increased significantly after the meal, which is consistent with our previous data on the effects of glucose intake (8). Our data are thus consistent with the NF-κB–mediated modulation of the p47phox subunit. We previously showed that the suppression of intranuclear NF-κB is associated with a decrease in p47phox subunit expression, as observed after hydrocortisone (20), rosiglitazone (P Mohanty, A Aljada, H Ghanim et al, unpublished observations, 2001), troglitazone (21), and insulin (22, 23) injections, whereas an increase in NF-κB is associated with a parallel increase in p47phox subunit expression as observed after glucose intake (8). The essential role of NADPH oxidase and superoxide radical in the pathogenesis of atherosclerosis was recently shown. ApoE−/− mice, in whom the p47phox gene has been deleted, do not develop atherosclerotic lesions even when fed a high-cholesterol diet (24). Thus, it is of interest that p47phox and superoxide radical get induced with each meal and that obesity is characterized by a marked increase in oxidative stress (4).

The magnitude of the peak increase in the p47phox subunit after the intake of the mixed meal was similar to that observed after glucose intake, but the peak increase in ROS generation was at different times in the subjects studied. The lack of a significant increase in ROS generation at a single time point contrasts with the increases we previously showed after glucose (1), cream, and casein (8) challenges. This may be the result of the variability caused by the variable digestion of food before absorption and the peak of ROS generation being blunted by the innate antioxi-
dants in the mixed meal, including vitamins C and E (25). However, when the AUC of an increase from baseline in ROS generation at 1, 2, and 3 h was combined and comparisons were made between the mixed-meal group and the control subjects, there was a significant increase in ROS generation by both MNCs and PMNLs in the mixed-meal group compared with the control subjects, who drank 300 mL water.

Our observations have relevance to the pathogenesis of atherosclerosis, in which inflammation may play a role through leukocyte-endothelial interactions. Such damage is of special relevance to diabetic patients because glycated proteins in these patients may trigger inflammation through advanced glycation end product receptors (26, 27). The proinflammatory changes induced by a meal may act in combination with the increase in oxidative stress, which is known to be increased in diabetes. Similarly, macrovascular disease in obesity may be related to increased oxidative stress, which we recently showed in obese subjects (4), and to the proinflammatory changes induced by meal intake. Thus, these observations may be relevant to the pathogenesis of atherosclerosis, which is a chronic inflammatory state of the arterial wall (28, 29), in both diabetes and obesity. It is also relevant to the reversal of atherosclerosis and the improvement in myocardial perfusion, which was shown after intense lifestyle changes by Ornish et al (30).

These observations may also help explain the abnormalities in brachial artery reactivity observed after consumption of the 900-kcal meal in the current study. Vogel et al (6) showed that such a meal results in a significant reduction in postischemic flow-
mediated vasodilation of the brachial artery. Furthermore, they showed that prior intake of vitamins E and C prevents this abnormality (31). They concluded that the abnormality in vascular reactivity induced by the meal was mediated through an increase in ROS generation and a possible reduction in the bioavailability of endothelial nitric oxide. Our observations provide actual evidence that confirms their hypothesis in terms of increased ROS generation and altered endothelium-mediated vasodilatation probably through reduced nitric oxide bioavailability.

The concomitant increase in IKKα and IKKB expression, decrease in IkBα, and increase in intranuclear NF-κB are consistent with leukocytic activation and a proinflammatory effect of the intake of a moderate-sized meal. It is of interest that these processes get triggered within 1 h of a meal and remain active for ≥ 3 h and that CRP also increases after such a meal. The duration of this proinflammatory effect is relevant because the time for another meal is usually 4–5 h after a meal, with the potential for further NF-κB activation, ROS load, and potential proinflammatory changes. It is possible that chronic overeating may result in permanent increases in NF-κB binding activity and in total expression as a protein in the cell. This area needs further investigation because the state of obesity is known to be associated with persistent increases of proinflammatory transcription factor α, interleukin 6, and CRP (32–34).

The fact that insulin is secreted in response to the meal raises the theoretical possibility that it may mediate this proinflammatory effect. However, we already showed that insulin has a potent and rapid antiinflammatory effect both in vivo (22, 23) and in vitro (35, 36). Insulin suppresses intranuclear NF-κB, induces IkBα, and suppresses the p47phox subunit of NADPH oxidase, ICAM-1, MCP-1, and CRP, all of which are proinflammatory.

Our data are relevant to the concept that, whereas insulin and insulin sensitizers of the thiazolidinediones class have an ROS-suppressive and a profound antiinflammatory effect, macronutrient intake induces oxidative stress and proinflammatory changes. Thus, our current understanding of the relation between macronutrient intake and insulin, which contains metabolic implications only, has to incorporate the respective inflammatory and antiinflammatory effects of the macronutrient intake and insulin action.

In conclusion, a moderate-sized mixed meal results in a significant increase in the proinflammatory transcription factor NF-κB in the nucleus, a decrease in IkBα (which is probably a consequence of the increase in IKKα and IKKB), and a parallel increase in the cellular p47phox subunit, which is the key protein component of NADPH oxidase and in ROS generation. These changes occur within 1 h of meal consumption and last for ≥ 3 h. These observations are consistent with potent inflammatory changes and raise fundamental issues about the relation between food intake, oxidative damage, inflammation, and atherosclerosis and about the ideal way to eat and modify lifestyle.

### REFERENCES

7. Ceriello A, Bortolotti N, Motz E, et al. Meal-induced oxidative stress and...