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Glucose ingestion induces an increase in intranuclear nuclear factor κB, a fall in cellular inhibitor κB, and an increase in tumor necrosis factor α messenger RNA by mononuclear cells in healthy human subjects

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Abstract

Because hyperglycemia is a major detrimental factor in the prognosis of acute cardiovascular conditions such as acute myocardial infarction (AMI) and stroke, and because an acute glucose challenge in healthy subjects has been shown to induce oxidative stress in mononuclear cells (MNCs), we have now investigated whether glucose induces inflammatory stress at the cellular and molecular level. Glucose ingestion (75 g in 300 mL water) in healthy human subjects resulted in an increase in intranuclear nuclear factor κB (NF-κB) binding, the reduction of inhibitor κB α (IκBα) protein, and an increase in the activity of inhibitor κB kinase (IKK) and the expression of IKKα and IKKβ, the enzymes that phosphorylate IκBα, in MNCs. Glucose intake caused an increase in NF-κB binding to NF-κB2, NF-κB2a, and NF-κB3 sequences in the promoter site of tumor necrosis factor α (TNF-α) gene along with an increase in the expression of TNF-α messenger RNA in MNCs. Membranous $p47^{phox}$ subunit, an index of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression and activation, also increased after glucose intake. We conclude that glucose intake induces an immediate increase in intranuclear NF-κB binding, a fall in IκBα, an increase in IKKα, IKKβ, IKK activity, and messenger RNA expression of TNF-α in MNCs in healthy subjects. These data are consistent with profound acute pro-inflammatory changes in MNCs after glucose intake.
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1. Introduction

Hyperglycemia has emerged as a major predictor of morbidity and mortality in acute myocardial infarction (AMI), stroke, and in patients undergoing coronary artery bypass graft surgery [1-8]. Mortality in AMI, stroke, and intensive care unit (ICU) patients increases by 100% with significant hyperglycemia and by more than 6 times in patients with hyperglycemia without a prior diagnosis of diabetes [4,5,9]. Morbidity and mortality can be reduced markedly by the restoration of normoglycemia in patients in ICU and in patients undergoing coronary artery bypass graft surgery [8,10,11]. In patients with AMI too, insulin infusion has shown a fall in morbidity and mortality provided hyperglycemia is not induced by the glucose and insulin infusion [12-14].

We have previously demonstrated that glucose (75 g) intake leads to an acute increase in reactive oxygen species (ROS) generation by leukocytes and an increase in plasma thiobarbituric acid–reacting substance (TBARS) concentration. Because the superoxide radical (O_2^-) can cause the activation of the key pro-inflammatory transcription factor, nuclear factor κB (NF-κB), we have now investigated whether glucose intake activates NF-κB and whether this leads to an increase in the transcription of tumor necrosis factor α (TNF-α) gene. Tumor necrosis factor α is a major pro-inflammatory cytokine that is regulated by NF-κB. We have also investigated whether glucose causes an increase in inhibitor κB kinase α (IKKα) and inhibitor κB kinase β (IKKβ) expression and IKK activity and whether there is a fall in inhibitor κB (IκB). These steps are involved in the activation of NF-κB [15-17]. The increase in NF-κB binding induced by endotoxin is at NF-κB2 and NF-κB2a sites, 2 of the 4 κB binding sites in TNF-α promoter. The relationship of glucose-induced binding to these κB binding sites is not known.

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The hypotheses that we have investigated are (1) glucose intake, which causes an increase in superoxide generation, leads to NF-κB activation and an increase in TNF-α gene transcription; (2) the activation of NF-κB is mediated through an increase in IKKα and IKKβ expression and activity with an associated fall in IκB; and (3) NF-κB binding sites in the promoter region of TNF-α activated after glucose intake are different from those activated by lipopolysaccharide.

2. Subjects, materials, and methods

2.1. Subjects

Eight healthy subjects (5 males and 3 females; age range, 31-39 years; weight range, 56.7-90.7 kg; mean body mass index, 25.6 ± 3.1 kg/m^2) participated in the study. Subjects with blood pressure of higher than 130/80, fasting blood glucose concentration greater than 100 mg/dL, and cholesterol concentrations greater than 200 mg/dL were excluded. The subjects were given 75 g of glucose dissolved in 300 mL water (Glucola) to drink over 5 minutes. Four weeks later, the same subjects were given 300 mL of water sweetened with saccharine. Blood samples were obtained at 0, 1, 2, and 3 hours. The institutional review board of the State University of New York at Buffalo approved the study. Written informed consent was obtained from all subjects.

2.2. Mononuclear cell isolation

Blood samples were collected as previously described [18-20]. For endotoxin-induced activation of NF-κB acti-

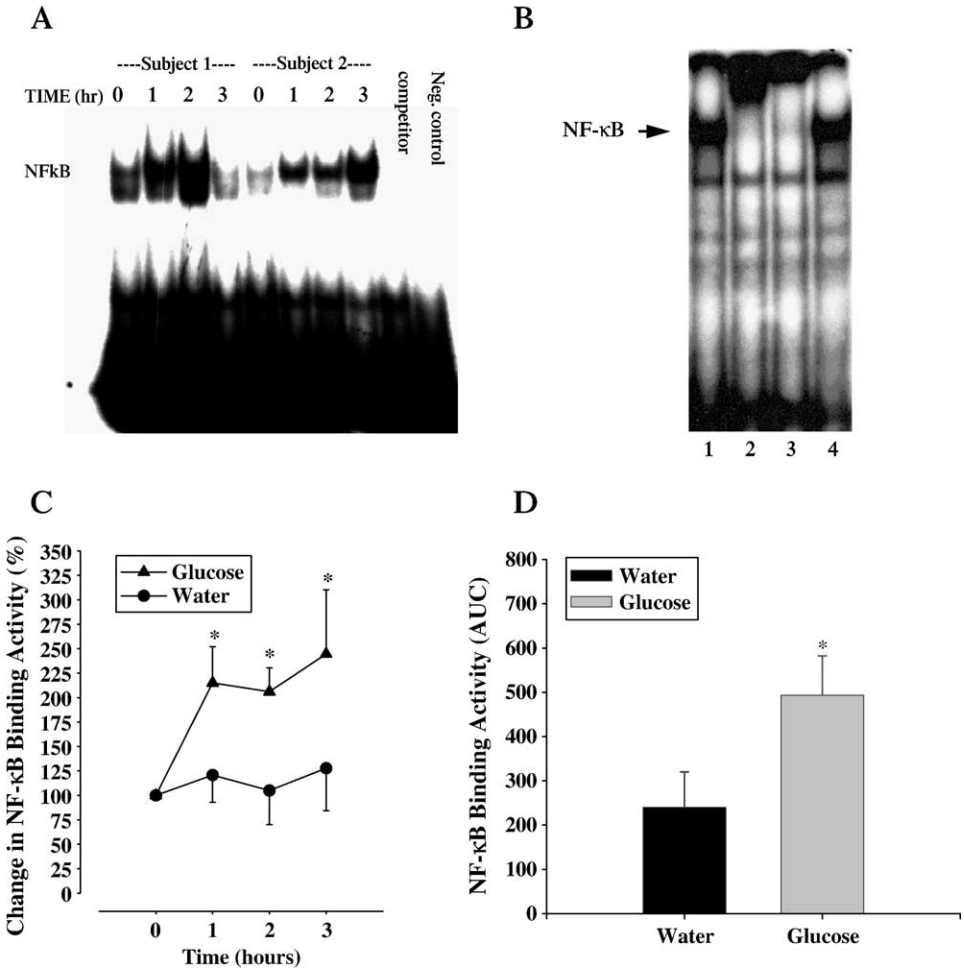

Fig. 1. A, A representative electrophoretic mobility shift assay gel showing NF-κB binding levels in MNC nuclear protein extracts for 2 subjects. The first subject showed an increase in NF-κB level at 1 hour, continued to increase at 2 hours, and levels declined thereafter at 3 hours. The second subject showed a gradual increase in intranuclear NF-κB levels at 1, 2, and 3 hours after glucose challenge. The sequence specificity of the protein-DNA interactions was determined using a specific unlabeled competitor oligonucleotide for NF-κB binding site. Lane "competitor" shows the inhibition of NF-κB binding using 2 μL of competitor oligonucleotide in subject 1 baseline sample nuclear protein extract. Lane "Neg. control" is radiolabeled NF-κB double-stranded oligonucleotide binding site without any nuclear extract. B, Lane 1 shows NF-κB binding activity in the baseline sample nuclear extract for one of the subjects; lane 2, supershift using an antibody against p50 subunit of NF-κB; lane 3, against p65 subunit of NF-κB; lane 4, against p75 (c-Rel) subunit of NF-κB. C, Relative NF-κB binding to double-stranded oligonucleotide containing NF-κB DNA binding site. All values were normalized to 100% for baseline time point, and the following values were expressed as percentage of basal level. Statistical analysis was carried out with 1-factor ANOVA for the repeated measures using Dunnett test for comparisons against the baseline (0 hour). *P < .05 when compared with baseline (n = 8). D, Area under the curve for NF-κB binding activity after water and glucose intake. Paired t test was used for AUC comparisons. *P < .05.

vation, freshly isolated MNCs were resuspended in RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 50 μmol/L β-mercaptoethanol, 10 mmol/L HEPES, 50 μg/mL penicillin, and 50 U/mL streptomycin (Gibco), and 5 mmol/L glucose. Cells were centrifuged and immediately resuspended in the media containing endotoxin (30 ng/mL) for 1 hour. Control wells containing cells in media only were also incubated for 1 hour. Cells were then lysed in buffer A and nuclear extract prepared as described below.

2.3. Nuclear factor κB electrophoretic mobility shift assay

DNA-binding protein extracts were prepared from MNCs by the method described by Andrews and Faller [21] with modifications [19,22]. Super shift assays were performed with antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA) against p50, p65, and p75 (c-Rel) subunits of NF-κB.

2.4. Nuclear factor κB binding activities to NF-κB binding sites in the TNF-α promoter region

Differential NF-κB binding to the 4 NF-κB binding sites in the TNF-α promoter region was performed as described above. Double-stranded oligonucleotides for each of the NF-κB binding sites were radiolabeled with γ-P32 by T4 kinase: κB1 (F: agctGAGTATGGGGACCCCCCCTTAA; R: agctTTAAGGGGGGGTCCCCATACTC); κB2 (F: agctGGGTCTGTGAATTCCCGGGGGT; R: agctACCCCCGGGAATTCACAGACCC; κB2a (F: agctTCCCCGGGGCTGTCCCAGGCTT; R: agctAAGCCTGGGACAGCCCCGGGGA; and κB3 (F: agctGCTCATGGGTTTCTCCACCAAG; R: agctCTTGGTGGAGAAACCCATGAGC).

2.5. In vitro IKK activity assay

The kinase assays were performed with glutathione S-transferase (GST)-IκBα (1-317) protein in 20 μL of kinase buffer containing 10 μmol/L adenosine triphosphate (ATP), 3 μCi of γ^{32}P ATP, and protease inhibitors. Half of the immunoprecipitated IKKα complex was incubated in the kinase buffer with 2.5 μg of the substrate for 30 minutes at 37°C. The reaction was stopped by boiling the samples for 5 minutes in 2× sodium dodecyl sulfate (SDS) loading buffer. Samples were resolved by 10% SDS–polyacrylamide gel electrophoresis and autoradiography. The remaining half of the immunoprecipitated samples were run on a separate 10% SDS–polyacrylamide gel electrophoresis and were Western blotted with the IKKα antibody to check for equality of loading.

2.6. Preparation of subcellular monocytic fractions

Mononuclear cell subcellular fractions were prepared by differential centrifugation. Mononuclear cell pellets were resuspended in 1 mL ice-cold HEPES buffer (10 mmol/L HEPES, 100 mmol/L KCl, 3 mmol/L NaCl, 3.5 mmol/L $MgCl_2$, 1 mmol/L ATP (Na_2), and 0.1% Triton X-100, pH 7.3, 1 mmol/L phenylmethylsulfonyl fluoride, 100 U/mL aprotinin). Cells were disrupted with a Potter homogenizer and the nuclei and unbroken cells were removed by centrifugation (15 minutes, 500*g*). Then, the supernatant was centrifuged for 15 minutes at 17 000*g* to obtain the plasma membranes and mitochondria-containing fraction. The pellet and the crude cytosol were then solubilized with lysis buffer (1% SDS, 1 mmol/L sodium orthovanadate, 10 mmol/L Tris, pH 7.4).

2.7. p47Phox subunit, phosphorylated IκBα, IKKα, and IKKβ Western blotting

Western blotting was carried out as previously described [23,24].

2.8. Total RNA isolation and real-time RT-PCR

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method. The quality and quantity of the isolated RNA were determined before using the RNA. One microgram of total RNA was reverse transcribed using Advantage RT-for-PCR Kit (Clontech, Mountain View, CA). Real-time RT-PCR was done using Cepheid

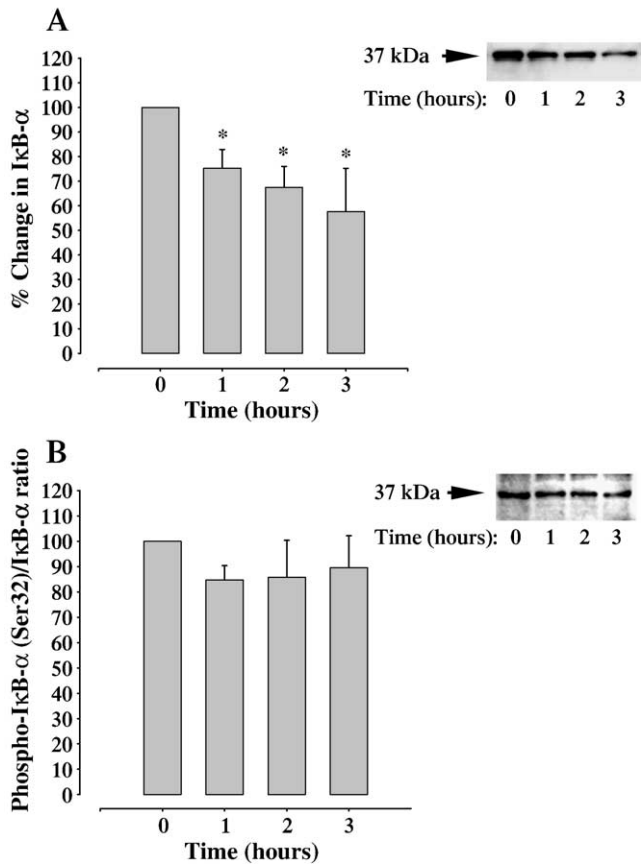

Fig. 2. A, Western blot showing the relative expression of IκBα in MNC lysate after glucose challenge. Note that IκBα protein levels decreased gradually after glucose intake. *$P < .05$. B, Western blot showing the relative protein expression of phosphorylated IκBα (Ser32) after glucose ingestion. Note that there was no significant changes in phosphorylated IκBα (Ser32). The results are presented as mean ± SE. *$P < .05$ (n = 8).

Smart Cycler (Sunnyvale, CA), containing 2 μL complementary DNA, 10 μL Sybergreen Master mix (Qiagen, Valencia, CA) along with 0.5 μL of 20 μmol/L TNF-α specific primers (Invitrogen, Carlsbad, CA); sense 5′-AAG AGTTCCCCAGGGACCTCT-3′ and antisense primer 5′-CC TGGGAGTAGATGAGGTACA-3′. The specificity and the size of the PCR products were tested by adding a melt curve at the end of the amplifications and by running it on a 2% agarose gel. All values were normalized to actin expression.

2.9. Plasma insulin and glucose measurements

Insulin levels were determined using an ELISA kit from Diagnostic Systems Laboratories (Webster, TX). Glucose levels were measured in whole blood by a Hemocue glucose analyzer (Hemocue, Mission Viejo, CA).

2.10. Statistical analysis

Statistical analysis was carried out using SigmaStat software (Jandel Scientific, San Rafael, CA). All data are expressed as mean ± SE. Analysis was carried out with 1-factor analysis of variance for repeated measures using Dunnett test for comparisons against the baseline. Area under the curve (AUC) was calculated for NF-κB binding activity, IκBα, IKKα, *IKKβ*, and $p47^{phox}$, and then compared between the glucose intake and water intake using paired *t* test.

3. Results

3.1. Plasma glucose and insulin concentrations

Plasma glucose concentrations after glucose challenge increased from 93.5 ± 6.4 to 128.6 ± 21.0, 109.0 ± 20.1, and 94.1 ± 10.1 mg/dL, respectively, at 1, 2, and 3 hours ($P < .05$, analysis of variance [ANOVA]). Plasma insulin concentration increased from 9.6 ± 1.9 to 50.4 ± 12.6, 20.6 ± 7.1, and 10.6 ± 4.2 μU/mL, respectively, at 1, 2, and 3 hours ($P < .01$, ANOVA). There was no significant change in plasma glucose or insulin concentration after challenge with water sweetened with saccharine.

3.2. NF-κB binding activity level

Nuclear factor κB binding increased by 215.0% ± 36.9%, 206.1% ± 24.3%, and 244.7% ± 65.7% of the basal level at 1, 2, and 3 hours, respectively ($P < .05$; ANOVA, Fig. 1A and C). There was no significant change in NF-κB binding activity after water challenge. Area under the curve for NF-κB binding was also significantly greater after glucose intake when compared with AUC for water intake ($P < .05$, *t* test, Fig. 1D).

Supershift assays to determine the composition of NF-κB complex demonstrated that antibodies to p50 and p65 caused a supershift, whereas that to p75 (c-Rel) did not (Fig. 1B).

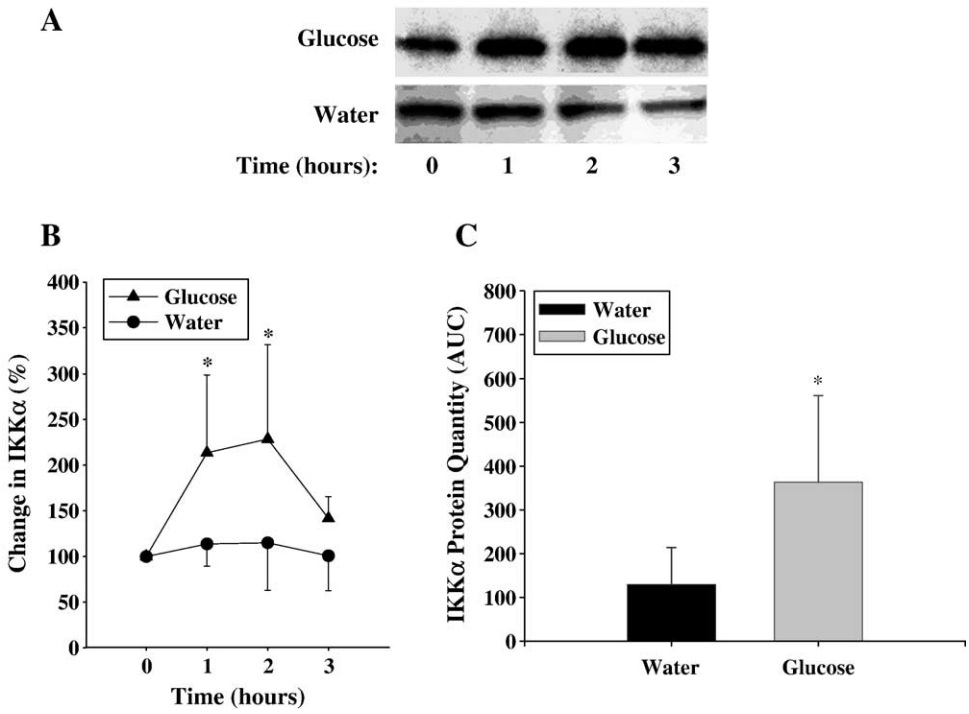

Fig. 3. A, Representative Western blots showing the relative expression of IKKα in MNC homogenate after glucose and water intake. B, Densitometric quantitative analysis of IKKα. There was a marked increase in IKKα after glucose intake. *$P < .05$ when compared with the baseline using 1-factor ANOVA for repeated measures and Dunnett test for comparisons against the baseline (0 hour). There was no significant change in IKKα after water challenge. C, Area under the curve for IKKα protein quantity after water and glucose intake. Results are presented as mean ± SE (n = 8). *$P < .05$.

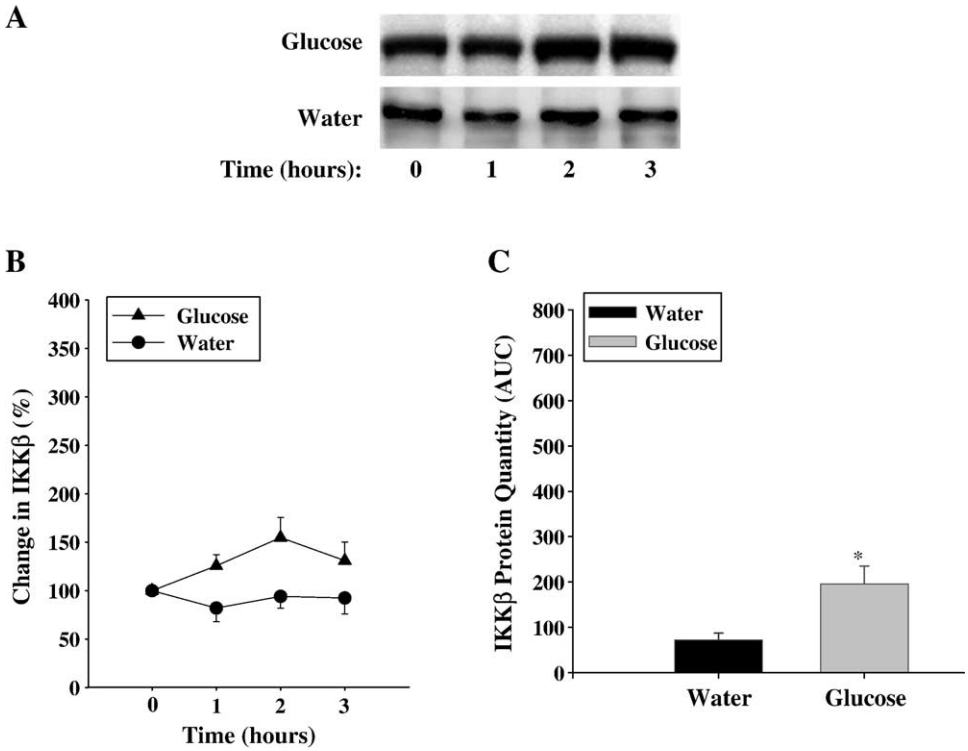

Fig. 4. A, A representative Western blot showing the relative IKKβ quantity in MNC homogenate after glucose or water challenge. B, IKKβ densitometry (n = 8). Note that IKKβ levels did not increase significantly largely because of variation in the magnitude of change after water intake. Results are presented as mean ± SEM. C, Area under the curve for IKKβ protein expression after water and glucose intake. Paired *t* test was used for AUC comparisons. *$P < .05$.

3.3. Total IκB and phosphorylated IκBα (Ser32) levels

Inhibitor κB α protein concentration in total MNC homogenate decreased significantly at 1, 2, and 3 hours ($P < .05$; Fig. 2A). Phosphorylated IκBα (Ser32) in total MNC homogenate did not change (Fig. 2B). Water intake did not induce any significant changes in IκBα or phosphorylated IκBα (Ser32).

3.4. Inhibitor κB kinase α and IKKβ levels

Inhibitor κB kinase α protein levels increased significantly at 1, 2, and 3 hours when compared with the baseline (Fig. 3). Inhibitor κB kinase β protein showed a trend toward an increase after glucose intake when compared with the baseline ($P = .1$; Fig. 4). However, AUC for IKKβ was significantly greater after glucose intake than after water intake (Fig. 4C). Water challenge did not cause any significant change in IKKα or IKKβ protein levels.

3.5. Inhibitor κB kinase activity in MNC homogenates

Precipitate from whole MNC cell lysate showed a significant increase in IKK activity after glucose intake ($P < .05$; Fig. 5), whereas water intake did not induce any changes.

3.6. Messenger RNA expression of TNF-α in MNCs

Expression of TNF-α in MNCs increased significantly after glucose challenge ($P < .05$) when compared with water intake (Fig. 6).

3.7. Differential NF-κB binding activities to the 4 binding sites of TNF-α promoter

Glucose induced a significant increase in binding activity for 3 of the 4 NF-κB binding sites with maximal binding at 2 hours: κB2 (Fig. 7B; $P < .05$); κB2a (Fig. 7C; $P < .05$); and κB3 (Fig. 7D; $P < .01$). There was no significant change in κB1 binding activity (Fig. 7A). Water intake (control) did not affect NF-κB binding activity to any of the κB binding sites in human MNCs. Endotoxin was able to induce NF-κB activation and binding activity to 3 of the 4 sites (Fig. 7E) in freshly isolated MNCs: κB1 showed the maximal increase, followed by κB2 and κB2a. Antibodies against the p65 and p50 subunits of NF-κB confirmed that the upper band for all the κB binding sites is the p65:p50 heterodimer. Endotoxin did not induce NF-κB binding at site κB3 (Fig. 7). Thus, the pattern of activation of NF-κB binding at the 4 TNF-α promoter sites was different for glucose when compared with endotoxin.

3.8. $p47^{phox}$ subunit protein levels

$p47^{phox}$ subunit protein levels in the membrane fraction showed a significant increase at 1, 2, and 3 hours after glucose challenge (Fig. 8). Area under the curve for $p47^{phox}$ subunit protein levels was also statistically significant after glucose intake when compared with AUC for water intake ($P < .05$). On the other hand, $p47^{phox}$ subunit protein levels in the crude cytosolic fractions of MNCs did not increase.

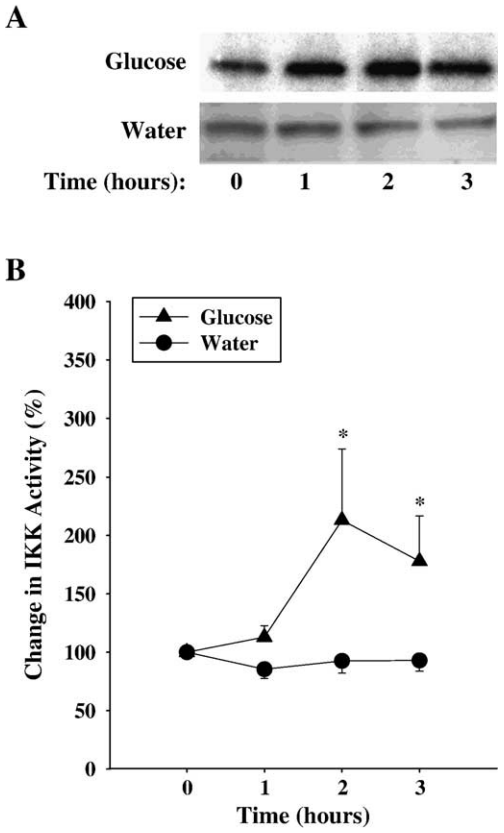

Fig. 5. A, Representative gel showing the relative IKK activity in MNC total cell lysate after glucose and water. B, Note that IKK activity increased significantly after glucose intake (upper gel) when compared with water intake. Results are presented as mean ± SE. *$P < .05$.

After water intake, there was no change in $p47^{phox}$ subunit protein concentration.

4. Discussion

Our data demonstrate clearly that there was a significant increase in NF-κB binding in the nucleus at 1, 2, and 3 hours after glucose intake. There was a parallel increase in IKK activity and IKKα and IKKβ expression and a decrease in total IκBα protein. There was a concomitant increase in messenger RNA (mRNA) expression of TNF-α and $p47^{phox}$ subunit in the membrane. These changes are consistent with glucose-induced oxidative and inflammatory stress and with our previous demonstration of an increase in ROS generation after glucose [25].

The sequence of IKK activation and the translocation of cytosolic NF-κB into the nucleus are very similar to that induced by endotoxin and other pro-inflammatory cytokines. The mechanism by which glucose mimics the pro-inflammatory effect of cytokines, TNF-α, interleukin 1, and endotoxin, is not clear. However, it is possible that the increase in ROS, particularly the superoxide radical, results in the activation of NF-κB. These data are also relevant to diabetes because chronic hyperglycemia, in vivo, may also induce the translocation of NF-κB into the nucleus and potentially lead to the transcription of cytokines, adhesion molecules, and enzymes mediating ROS generation [26]. Acute hyperglycemia induced by an intravenous infusion of glucose has been shown to induce an increase in plasma TNF-α concentration, but only when endogenous insulin secretion is concomitantly inhibited by somatostatin [27]. This latter finding is of interest because insulin, secreted in response to glucose intake, has an anti-inflammatory effect as reflected in NF-κB binding suppression, increase in IκBα, and the suppression of ROS generation [19]. Insulin also suppresses early growth response 1, another pro-inflammatory transcription factor, which regulate tissue factor and plasminogen activator inhibitor 1 genes. Insulin also suppresses matrix metalloproteinase (MMP) 9 and vascular endothelial growth factor [28]. Clearly, the pro-inflammatory response to 75-g glucose overwhelms the anti-inflammatory effect of insulin secreted in response to it. The possibility that the pro-inflammatory effect of glucose intake is mediated by the insulin secreted in response to glucose intake is highly unlikely because when insulin is infused in patients while maintaining euglycemia, an anti-inflammatory effect is observed, not a pro-inflammatory effect.

There are, however, certain aspects of our observations that are distinct from the previously described sequence of inflammatory changes after endotoxin. Although there was a fall in IκB expression after glucose intake there was no increase in phosphorylated IκBα. We have previously reported that an increase in plasma free fatty acid concentrations induces an increase in NF-κB binding independently of any change in IκBα or phosphorylated IκBα [29].

It is relevant that IKKβ has been shown to mediate insulin resistance caused by fatty acids and that aspirin, an inhibitor of IKKβ, prevents the induction of insulin resistance in mice. Similarly, IKKβ knockout mice do not

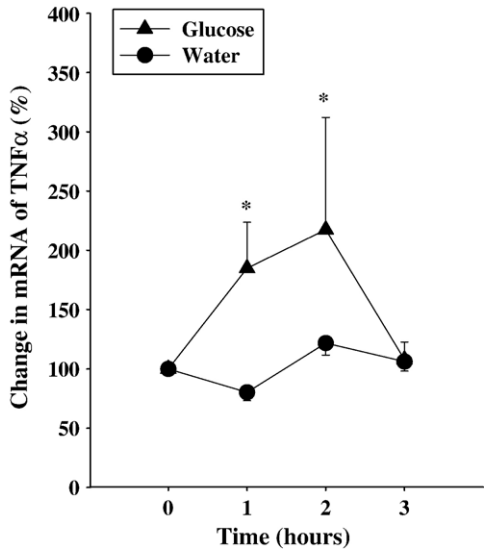

Fig. 6. Relative mRNA expression of TNF-α in MNCs after glucose or water challenge. Note that mRNA expression increased significantly after glucose intake. Results are presented as mean ± SEM. *$P < .05$.

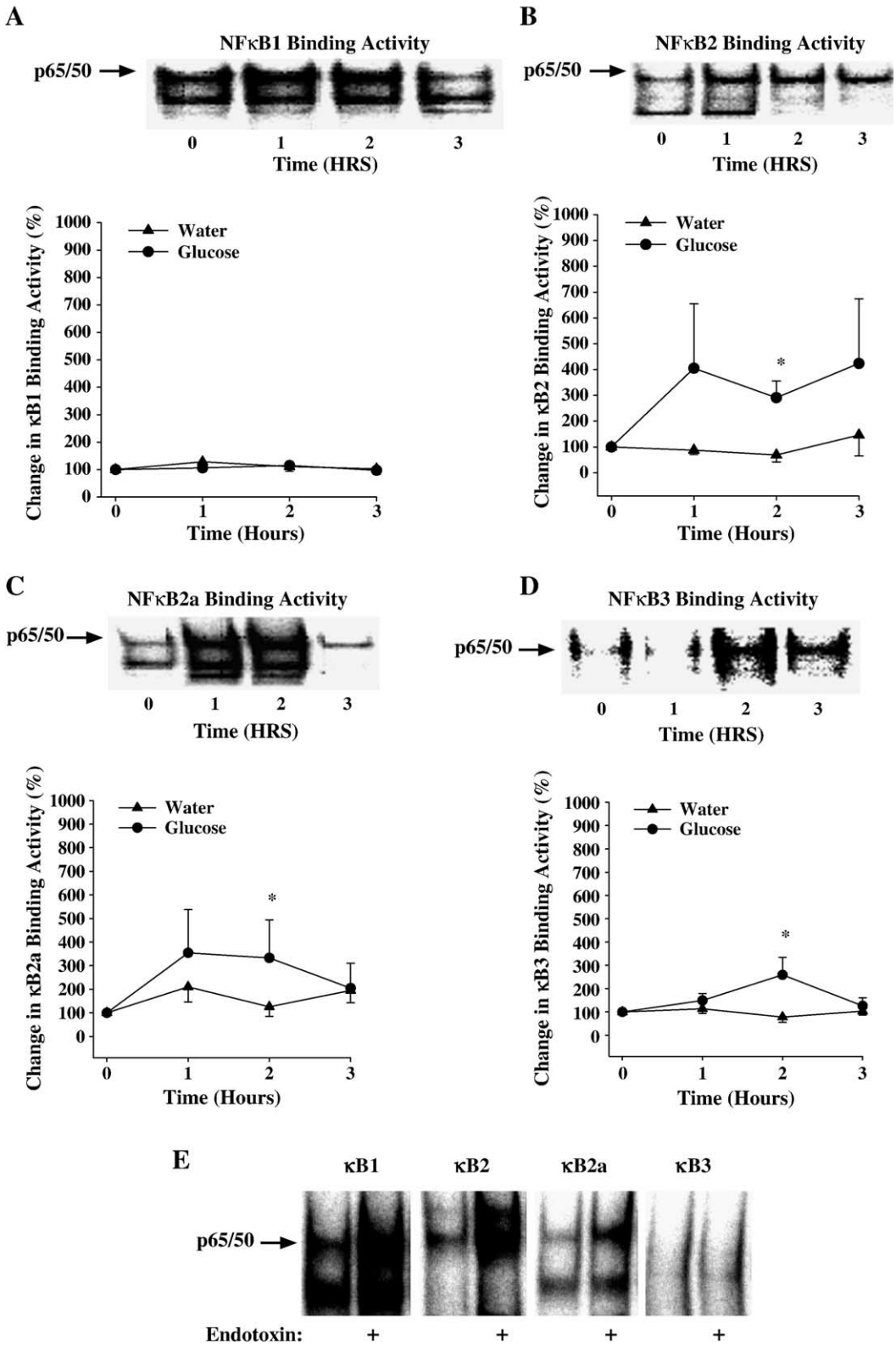

Fig. 7. Differential NF-κB binding activities to the 4 binding sites of TNF-α promoter: (A) NF-κB1; (B) NF-κB2; (C) NF-κB2a, and (D) NF-κB3. E, Representative gel for in vitro experiment assessing NF-κB (p65/p50) binding activity to binding sites κB1, κB2, κB2a, and κB3 when freshly isolated MNCs are exposed to endotoxin (30 ng) for 1 hour.

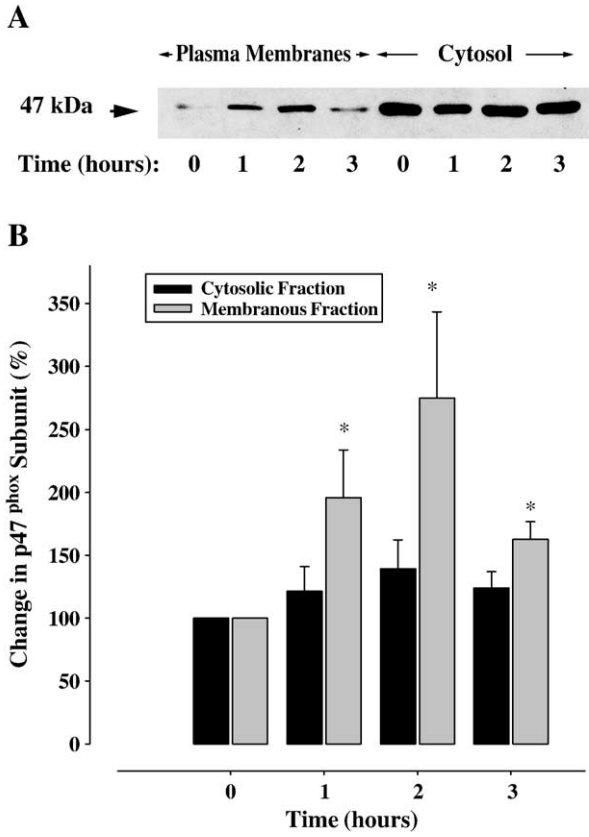

Fig. 8. A, A representative Western blot showing the relative protein levels of $p47^{phox}$ subunit in plasma membranes and crude cytosolic fractions of MNCs after glucose intake. Note that there was an increase of $p47^{phox}$ subunit in the membrane fraction at 1 and 2 hours; membrane $p47^{phox}$ subunit fraction declined at 3 hours, but was still greater than that at baseline. On the other hand, $p47^{phox}$ subunit levels increased slightly at 1 and 2 hours. B, Densitometric analysis of $p47^{phox}$ subunit protein levels in membranous and cytosolic fractions. Note the significant increase in membranous fractions at 1, 2, and 3 hours (*$P < .05$). The results are presented as mean ± SE (n = 8).

develop insulin resistance after lipid infusion [30]. Conversely, transgenic IKKβ mice show evidence of insulin resistance [31]. Thus, IKKs could be important mediators of insulin resistance/sensitivity in experimental animals. Our observations may therefore be relevant to hyperglycemia-induced insulin resistance. In our experiments, we were able to demonstrate the increase of IKK activity and the expression of both IKKα and IKKβ. This may be relevant to the mechanism underlying glucotoxicity-associated insulin resistance.

It has been shown previously that the incubation of endothelial cells and monocytic cell lines with high concentrations of glucose leads to NF-κB activation in vitro [32-34]. This has been proposed as one mechanism underlying the pathogenesis of microvascular complications of diabetes. In contrast, our current observations on oral glucose-induced NF-κB activation followed an oral glucose load in healthy subjects leading to a modest increase in glucose concentration without the occurrence of gross hyperglycemia.

Our data also demonstrate that glucose intake induces an increase in TNF-α mRNA. This is probably mediated through enhanced NF-κB binding activity to 3 out of 4 TNF-α gene promoter sites tested (κB2, κB2a, and κB3). These sites are different than those to which lipopolysaccharide (κB1, κB2 and κB2a) increases binding. This difference in the pattern of activation of TNF-α promoter sites has 2 implications. Firstly, factors beyond the translocation of NF-κB into the nucleus regulate specific gene transcription and, secondly, the magnitude or the duration of activation of a gene may differ according to the type of pro-inflammatory challenge.

We have previously shown that glucose intake has been shown to increase activator protein 1 and early growth response binding activities along with an increase in the expression of MMPs, MMP-2 and MMP-9, and tissue factor, the genes regulated by these 2 transcription factors [35]. These changes add to the pro-inflammatory effect of glucose and also provide a mechanism for enhanced thrombosis through tissue factor increase.

Our data show that there is a significant increase in the expression of $p47^{phox}$ in membrane fraction of the MNCs after glucose challenge. This is consistent with an increase in NADPH oxidase–dependent O_2 generation previously observed after glucose and the increase in total cellular $p47^{phox}$ expression. The increase in membrane and total $p47^{phox}$ expression without a change in the cytosol is consistent with a translocation of $p47^{phox}$ from the cytosol to the membrane of the MNCs.

The fact that glucose is pro-inflammatory and prothrombotic, in vivo, at the cellular level in the human has several ramifications in clinical medicine. Firstly, morbidity and mortality after AMI and stroke are related to hyperglycemia in a dose-response fashion. Mortality after cardiac surgery is also related to hyperglycemia. Secondly, both fasting and postprandial blood glucose concentrations are known to predict atherosclerotic complications including coronary morbidity and mortality [36,37]. Thirdly, hyperglycemia at the time of hospital admission in nondiabetic patients predicts mortality and the duration stay in the hospital [9]. Fourthly, the pathogenesis of microvascular complications of diabetes is now known to be related to hyperglycemia [38]. Finally, hyperglycemia in ICUs predicts mortality, renal failure, systemic infectious, and duration of stay in the hospital [10], and the treatment of hyperglycemia in intensive care [10] and coronary care units [13] reduces morbidity and mortality.

In conclusion, glucose intake induces an acute increase in IKKα and IKKβ expression, IKK activity, and a fall in IκBα protein levels. This is associated with an increase in intranuclear NF-κB and a parallel marked increase in TNF-α mRNA expression and membranous and total $p47^{phox}$ subunit. These changes are potentially relevant to the relationship of hyperglycemia and glucose-induced inflammation, which may promote atherogenesis in the long term and worsen prognosis markedly in acute clinical syndromes such as AMI and stroke.

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