Hyperglycemia Can Cause Membrane Lipid Peroxidation and Osmotic Fragility in Human Red Blood Cells*

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The present study has examined the effect of elevated glucose levels on membrane lipid peroxidation and osmotic fragility in human red blood cells (RBC). Defibrinated whole blood or RBC were incubated with varying concentrations of glucose at 37 °C for 24 h. RBC incubated with elevated levels of glucose showed a significantly increased membrane lipid peroxidation when compared with control RBC. A significant positive correlation was observed between the extent of glucose-induced membrane lipid peroxidation and the osmotic fragility of treated RBC. Glucose-induced membrane lipid peroxidation and osmotic fragility were blocked when RBC were pretreated with fluoride, an inhibitor of glucose metabolism; with vitamin E, an antioxidant; with para-chloromercuribenzoate and metyrapone, inhibitors of the cytochrome P-450 system; or with dimethylfuran, diphenylamine, and thiourea, scavengers of oxygen radicals. RBC treated with elevated glucose concentrations also showed an increase in NADPH levels. Exogenous addition of NADPH to normal RBC lysate induced membrane lipid peroxidation similar to that observed in the glucose-treated RBC. These data suggest that elevated glucose levels can cause the peroxidation of membrane lipids in human RBC.

Elevated levels of glucose in the medium or blood are known to cause membrane damage and cell death of cultured pericytes, endothelial cells, kidney cells, retinal cells, and red blood cells (RBC) (1–5). However, the biochemical mechanism that results in membrane damage and cell death is not known. It has been proposed that nonenzymatic glycosylation of certain proteins results in their irreversible cross-linking, which may contribute to the loss of elasticity characteristic of cells exposed to hyperglycemia (6). Membrane lipids are vital for the maintenance of cellular integrity and survival. Peroxidation of membrane lipids can result in the inactivation of enzymes and cross-linking of membrane lipids and proteins and in cell death (7–11). By using human RBC, the present study has documented that elevated glucose levels per se can cause peroxidation of membrane lipids and increased membrane osmotic fragility. This glucose-induced membrane lipid peroxidation apparently involves NADPH and cytochrome P-450-like activity.

MATERIALS AND METHODS

Blood from adult volunteers was collected into tubes with and without EDTA (10.5 mg/ml). The blood without EDTA was immediately defibrinated by rotating hardwood applicator sticks in the tubes so that fibrin adhered to the sticks before clotting. This defibrinated blood was used as such in some experiments. Blood with EDTA was filtered through cotton wool to remove leukocytes and then centrifuged at 2000 rpm for 7 min in a refrigerated centrifuge. The RBC were washed with cold 0.15 M NaCl solution 3 times after 1:10 dilution.

Treatment with Glucose—Defibrinated blood or washed RBC suspensions were treated with varying concentrations of glucose. The effects of glucose on membrane lipid peroxidation and osmotic fragility of washed RBC were measured by the TBA reactivity and osmotic fragility assays. Because glucose may interfere in the TBA reactivity and osmotic fragility assays, washed RBC were measured for glucose to make sure that they were glucose-free.

Treatment with Malondialdehyde (MDA)—RBC suspensions (40–45% hematocrit) in PBS were treated with various concentrations of MDA in Erlenmeyer flasks for 24 h in a shaking water bath at 37 °C (12). MDA was freshly prepared by acid hydrolysis of bis(dimethylacetal) (Aldrich) as described previously (12) and then neutralized with NaOH to bring its pH to 7. MDA-treated RBC were washed 3 times with cold 0.15 M NaCl before lipid extraction or TBA reactivity measurement.

Measurement of Lipid Peroxidation—Membrane lipid peroxidation in the glucose-treated RBC was determined by the TBA reactivity of MDA, an end product of fatty acid peroxidation (13). For this purpose, 0.2 ml of packed RBC were suspended in 0.8 ml of PBS (made up of 8.1 g of NaCl, 2.302 g of NaHPO₄, 0.194 g of NaH₂PO₄, pH 7.4) were taken in Erlenmeyer flasks and incubated with varying glucose concentrations in a shaking water bath at 37 °C for 24 h. Glucose was estimated in RBC suspensions at 0 h, i.e. immediately after the addition of stock glucose and again after 24 h of incubation. Control RBC suspensions contained 0 mM glucose because normal blood glucose level is 4–6 mM. In the experiments with blood, flasks without exogenous glucose (i.e. those with normal glucose levels) were used as controls. Glucose levels in flasks containing blood were slightly different than those in flasks containing RBC in buffer. Flasks containing 30 and 45 mM glucose showed between 1 and 1.5% hemolysis; other flasks showed less than 0.5% hemolysis at the end of 24 h of incubation. The extent of hemolysis on glucose treatment was similar in blood and PBS-RBC. At the end of treatment, RBC were washed 3 times after 1:10 dilution with 0.15 M NaCl for biochemical and osmotic fragility analyses. Because glucose may interfere in the TBA reactivity and osmotic fragility assays, washed RBC were measured for glucose to make sure that they were glucose-free.

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The abbreviations used are: RBC, red blood cells; MDA, malondialdehyde; TBA, thiobarbituric acid; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; PS, phosphatidylserine; pCMB, para-chloromercuribenzoate.
were determined using the extinction coefficient of the MDA-TBA complex at 532 nm = 1.56 x 10^5 per cm per molar solution. Packed cell volume was determined by using an Autocrit centrifuge; Hb was determined using the Coulter Counter.

Measurement of MDA by the TBA reactivity is the method most widely used to assess lipid peroxidation (14). Using an additional new method of oxidative membrane damage was also assessed by determining the molar ratio of serine to phosphorus and amino acids when expressed per g of Hb, respectively. Mean cell volume (μm^3) = 85.8 ± 0.6, 85.1 ± 0.7, 85.8 ± 0.2, and 86.0 ± 0.3, and mean cell hemoglobin (pg) = 28.4 ± 1.1, 28.9 ± 0.7, 28.5 ± 1.2, 29.1 ± 0.8, 28.6 ± 1.3 (mean ± S.D.) of RBC after treatment of RBC-PBS suspension with 5, 15, 25, 35, and 45 mM glucose. This shows that elevated glucose level treatment does not have any effect on MCV and mean cell hemoglobin of RBC. Percentage change in MDA values in glucose-treated RBC compared with control RBC was similar when values are expressed per g of Hb (Fig. 1, lower panel) compared with per ml of RBC (Fig. 1, middle panel).

Fig. 2 illustrates TLC of lipids of RBC treated with varying concentrations of glucose for 24 h. TLC shows the appearance of a new lipid marked as an adduct between PE and PS in RBC-PBS treated with elevated glucose concentrations. Co-chromatography ruled out this new spot being an intermediate of glucose metabolism such as glucose-6-PO_4, fructose-6-PO_4, or glyceraldehyde-3-PO_4. This new lipid spot was phosphorus-positive and ninhydrin- and sugar-negative on spraying with specific reagents. Further characterization by acid hydrolysis suggested that the new phospholipid spot contained PS, PE, and MDA. The molar ratio of serine to phosphorus and ethanolamine to phosphorus was 0.40 and 0.47, respectively, and the TBA reactivity to phosphorus in the eluate of phospholipid adduct was 0.54. It appears that the new lipid spot in glucose-treated RBC is formed by the cross-linking of PE, PS, and MDA, but the exact nature of the cross-linking between these membrane components is not known and needs further study.

Fig. 2 also shows that a similar new phospholipid can be formed when normal RBC are treated in vitro with authentic MDA. TBA reactivity of MDA-treated washed RBC was 1.0 ± 0.3, 2.9 ± 0.8, 5.9 ± 1.8, 12.2 ± 4.3, 17.7 ± 6.1, and 24.1 ± 6.2. Fluorescence measurements were made in the Perkin-Elmer spectrofluorometer, model 650-10. Osmotic fragility was determined by measuring the extent of hemolysis in hypotonic NaCl solution (19). Osmotic fragility of glucose-treated RBC is expressed as percent of control value of glucose-treated RBC, the lipid was scraped into a tube and eluted with chloroform/methanol (2:1). The eluate was dried with nitrogen and resolved into a known amount of chloroform for quantitation of phosphorus and amino acids. For amino acid determination, a portion of chloroform extract was dried in a Teflon-stoppered tube and then hydrolyzed with 1 ml of 6 N HCl in an oven at 100 °C for 4 h. The acid hydrolysate was neutralized with KOH. Control tubes containing standard serine and ethanolamine (Sigma) were simultaneously treated with acid. Separation of amino acid in the extract was done by thin layer chromatography on Silica Gel G 300 glass plates (Brinkmann) using the solvent system, butanol/pyridine/acetic acid/water (30:30:10:10, v/v). Amino acids were visualized with ninhydrin spray and quantitated spectrophotometrically as described by Pataki (17).

Treatment with NADPH—The RBC in PBS were lysed by freezing (1 h, −20 °C) and thawing. The hemolysate was treated with 2 mM authentic NADPH (Sigma) for 24 h at 37 °C. The membrane lipid peroxidation was determined as described for RBC.

Glucose utilization in the cell suspension was determined by measuring the decrease in the glucose levels at 0 h and after 24 h of incubation. Glucose was quantitated using o-toluidine as described in Sigma kit no. 63. NADPH was measured by the enzymatic cycling method of Lowry et al. (18). Concentration of NADPH in 20 μl of the cell suspension or standard NADPH solution was determined by using a molar extinction coefficient of ε_600nm = 6.22. Fluorescence measurements were made in the Perkin-Elmer spectrofluorometer, model 650-10. Osmotic fragility was determined by measuring the extent of hemolysis in hypotonic NaCl solution (19). Osmotic fragility of glucose-treated RBC is expressed as percent of control value because RBC from different individuals are known to have a large variation in osmotic fragility. RBC sorbitol was determined using the sorbitol dehydrogenase method as described by Malone et al. (20) and Williams-Ashman (21). Mean cell volume and mean cell hemoglobin of glucose-treated RBC were determined using the Coulter Counter.

All incubations contained 10 μl of pen-strep/ml of cell suspension to vitiate any microbial growth during overnight incubations. Pen-strep contained 300 mg of penicillin G and 500 mg of streptomycin per ml of distilled water. All biochemicals were purchased from Sigma. Data were analyzed using the nonpaired Student's t test.
9.8 nmol/ml packed cells; phospholipid-MDA adduct formation was 0.1 ± 0.1, 0.3 ± 0.1, 0.5 ± 0.2, 0.7 ± 0.2, 1.2 ± 0.5, 1.5 ± 0.3 (percent of total phospholipid) when RBC were treated in vitro for 24 h with 0, 2.5, 5, 10, 15, 20 mM or μmol/ml MDA, respectively.

Glucose treatment for 24 h also caused accumulation of lactic acid to a level of 20.2 ± 0.6 mM in 45 mM glucose-treated and 12.7 ± 0.6 mM in 5 mM glucose-treated (control) cell-PBS suspensions. As illustrated in Fig. 2 (right) and by TBA reactivity of treated cells, lactic acid (25 mM) treatment to RBC by itself does not seem to contribute to the lipid peroxidation and phospholipid-MDA adduct formation. Glucose treatment also resulted in a fall in the pH of the medium from 7.4 to 6.78 in comparison with 6.88 in controls. The effect of pH in glucose-induced lipid peroxidation was ruled out because lactic acid treatment also caused a fall in pH to 6.81 without inducing lipid peroxidation.

Fig. 3 illustrates that the osmotic fragility of treated RBC increased with increasing concentrations of glucose. At similar glucose levels, osmotic fragility was higher when RBC were incubated in whole blood in comparison with PBS-buffer. This could be due to other factors in plasma formed during the incubation, such as lysolecithin, which can increase osmotic fragility of RBC. A significant positive correlation between osmotic fragility at 0.55% NaCl and the amount of phospholipid-MDA adduct formation (r = 0.89) and TBA reactivity (r = 0.91) was observed in RBC buffer treated with elevated glucose levels.

Treatment of RBC with elevated levels of glucose can trigger aldose reductase activity, which results in the accumulation of sorbitol (22). Sorbitol does not accumulate in RBC treated with sodium barbital, an inhibitor of aldose reductase (22). Data in Table I show that pretreatment of RBC-PBS with sodium barbital blocked accumulation of sorbitol; however, there was no effect on osmotic fragility and lipid peroxidation in RBC treated with elevated glucose levels. This indicates that the increase in the osmotic fragility of glucose-treated RBC may not be due to sorbitol accumulation. Sodium barbital did not have any effect on glucose utilization by RBC.

Table II shows an initial accumulation of NADPH in RBC-PBS suspension treated with high glucose. This increase in
the NADPH was not apparent at 24 h of incubation. Glycolysis in RBC, even under aerobic conditions, usually terminates in lactate. The net accumulation of NADPH is a result of its formation and utilization during the incubation of RBC with glucose. Although maximum NADPH accumulation in glucose-treated RBC was observed at 1 h, an increase in the TBA reactivity was observed only at 4 h. MDA levels (TBA reactivity) in RBC treated with 5 and 45 mM glucose for 4 h were 1.1 ± 0.2 and 1.6 ± 0.3 nmol/ml RBC (mean ± S.D.), respectively. Thus, there seems to be a lag between the generation of maximum NADPH levels and the occurrence of measurable membrane lipid peroxidation. Phospholipid-MDA adduct formation in RBC was not observed at 4 h of treatment with elevated levels of glucose, which may be because MDA cross-linking with PE and PS proceeds slowly and is time-dependent (8).

The effect of sodium fluoride and vitamin E on the membrane lipid peroxidation and osmotic fragility of glucose-treated RBC is given in Table III. Glucose levels were the same before and after incubation in flasks containing sodium fluoride (data not given), showing that fluoride blocked glucose utilization completely. It also blocked the initial increase in the NADPH, glucose-induced lipid peroxidation, and osmotic fragility. Vitamin E did not have any effect on glucose utilization (data not given) and NADPH generation but did block the accumulation of MDA in RBC and its osmotic fragility. This suggests that increased lipid peroxidation in glucose-treated RBC is associated with the oxidation of glucose and accumulation of NADPH. Further, an effect of antioxidant vitamin E on the glucose-induced changes in TBA reactivity and phospholipid-MDA adduct suggests that lipid peroxidation does indeed occur in glucose-treated RBC.

To delineate the role of NADPH in glucose-induced lipid peroxidation, an attempt was made to load RBC with NADPH by treating them in vitro with exogenous NADPH. However, this was not successful; it seems that NADPH is not permeable across the RBC membrane. A second attempt was made in which RBC were lysed by freezing the thawing and the hemolysate treated with authentic NADPH. Fig. 4 shows that NADPH (2 mM) alone can peroxidize RBC membrane lipids and form the phospholipid-MDA adduct. Greater lipid peroxidation was also confirmed by the increase in TBA reactivity in NADPH-treated hemolysate. The phospholipid-MDA adduct was 0.2 ± 0.1 and 0.8 ± 0.3% (mean ± S.D.) of total phospholipids, and TBA reactivity was 7.7 ± 2.8 and 12.7 ± 6.3 (mean ± S.D.) nmol/g Hb in control and NADPH-treated hemolysates, respectively. TBA reactivity and phospholipid-MDA adduct formation were blocked in the presence of vitamin E. This suggests that excess NADPH can induce peroxidation of erythrocyte membrane lipids.

Table IV shows the effect of para-chloromercuribenzoate (pCMB) and metyrapone on glucose-induced membrane lipid peroxidation and osmotic fragility. pCMB and metyrapone are known inhibitors of the cytochrome P-450 system (23, 24). Both glucose-induced increased lipid peroxidation and osmotic fragility were blocked in the presence of pCMB and metyrapone, which suggests that cytochrome P-450-like activity may be involved in the glucose-induced lipid peroxidation processes in RBC. Glucose-induced lipid peroxidation was also blocked when RBC were preincubated with dimethylfuran, diphenylamine, and thiourea (Table V). These agents are known to scavenge singlet oxygen and hydroxyl radicals (25, 26), thereby preventing oxygen radical attack on membrane lipids and, thus, lipid peroxidation and osmotic fragility. Dimethylfuran, which scavenges both singlet oxygen and hydroxyl radicals, had a greater effect in blocking lipid peroxidation than thiourea, which scavenges only hydroxyl radicals. This suggests that both hydroxyl radicals and singlet oxygen may be generated in RBC during treatment with elevated glucose levels.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PL-MDA adduct</th>
<th>MDA</th>
<th>NADPH</th>
<th>Osmotic fragility</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total PL</td>
<td>nmol/ml RBC</td>
<td>nmol/ml RBC</td>
<td>% increase of control at 0.55% NaCl</td>
<td></td>
</tr>
<tr>
<td>5 mM glucose (control)</td>
<td>0.17 ± 0.09*</td>
<td>1.2 ± 0.2</td>
<td>0.38 ± 0.12*</td>
<td></td>
</tr>
<tr>
<td>+NaF (3 mM)</td>
<td>0.19 ± 0.14</td>
<td>1.2 ± 0.2</td>
<td>0.31 ± 0.17</td>
<td>7 ± 5</td>
</tr>
<tr>
<td>+Vitamin E</td>
<td>0.21 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>0.45 ± 0.11</td>
<td>9 ± 6</td>
</tr>
<tr>
<td>45 mM glucose</td>
<td>0.71 ± 0.18*</td>
<td>2.7 ± 0.3*</td>
<td>0.85 ± 0.12*</td>
<td>95 ± 11*</td>
</tr>
<tr>
<td>+NaF</td>
<td>0.29 ± 0.17*</td>
<td>1.2 ± 0.2</td>
<td>0.37 ± 0.09*</td>
<td>11 ± 9*</td>
</tr>
<tr>
<td>+Vitamin E</td>
<td>0.26 ± 0.10*</td>
<td>1.5 ± 0.3</td>
<td>0.92 ± 0.19*</td>
<td>38 ± 8*</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of five observations. Values for RBC treated with elevated levels of glucose and inhibitors are significantly lower than RBC treated with elevated levels of glucose without any inhibitor (p < 0.01). pCMB and metyrapone were dissolved in alcohol and layered on the bottom of flasks. Alcohol was dried with nitrogen before adding the RBC suspension to flasks. RBC were preincubated for 15 min with pCMB and metyrapone before glucose treatment for 24 h. Osmotic fragility of RBC was measured at 0.55% NaCl.
TABLE V
Effect of dimethylfurane, thioura, and diphenylamine on membrane lipid peroxidation and osmotic fragility of RBC after treatment of RBC-PBS with elevated levels of glucose

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PL-MDA adduct</th>
<th>MDA</th>
<th>Osmotic fragility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% total PL</td>
<td>nmol/ml RBC</td>
<td>% increase of control</td>
</tr>
<tr>
<td>5 mm glucose (control)</td>
<td>0.2 ± 0.1</td>
<td>1.18 ± 0.23</td>
<td>-</td>
</tr>
<tr>
<td>+Thioura (2 mm)</td>
<td>0.1 ± 0.1</td>
<td>ND</td>
<td>-4 ± 8</td>
</tr>
<tr>
<td>+Dimethylfurane (2 mm)</td>
<td>0.2 ± 0.1</td>
<td>1.05 ± 0.30</td>
<td>8 ± 5</td>
</tr>
<tr>
<td>+Diphenylamine (1 mm)</td>
<td>0.2 ± 0.1</td>
<td>1.07 ± 0.27</td>
<td>-9 ± 6</td>
</tr>
<tr>
<td>45 mm glucose</td>
<td>0.8 ± 0.3</td>
<td>2.15 ± 0.34</td>
<td>96 ± 26</td>
</tr>
<tr>
<td>+Thioura</td>
<td>0.5 ± 0.2</td>
<td>ND</td>
<td>21 ± 16</td>
</tr>
<tr>
<td>+Dimethylfurane</td>
<td>0.3 ± 0.1</td>
<td>1.31 ± 0.38</td>
<td>13 ± 9</td>
</tr>
<tr>
<td>+Diphenylamine</td>
<td>0.3 ± 0.2</td>
<td>1.48 ± 0.28</td>
<td>16 ± 11</td>
</tr>
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</table>

DISCUSSION

This study has demonstrated that elevated levels of glucose can result in the peroxidation of membrane lipids in RBC. Glucose-induced lipid peroxidation does not seem to be due to the accumulation of lactic acid or to a change in pH over the course of incubation, because exogenous addition of lactic acid alone to the extent formed in glucose treatment did not have any effect on lipid peroxidation in RBC.

Fluoride is known classically to shut down glycolysis by inhibiting enolase. The inhibitory effect of fluoride on glucose oxidase is also known (27). In the present study, there was complete inhibition of glucose metabolism by fluoride, also suggesting that fluoride can inhibit enzymes other than enolase. Inhibition of glucose utilization by fluoride could also be due to decreased ATP due to the effect of fluoride on glycolysis. Both dimethylfurane and thioura blocked glucose-induced lipid peroxidation and osmotic fragility of RBC, with dimethylfurane having a greater effect than thioura. Although the specificity of these scavengers is not clear, they have been used as a tool to examine involvement of oxygen radicals in biochemical reactions (25, 26), which may indirectly suggest a possible generation of oxygen radicals and their contribution in the lipid peroxidation in RBC treated with elevated glucose levels.

The exact mechanism that leads to the glucose-induced membrane lipid peroxidation in human RBC is not known. Other studies (28, 29) in a cell-free system have suggested that glucose can enolize and thereby reduce molecular oxygen yielding α-keto aldehydes and free radical intermediates. In the present study, significantly elevated levels of NADPH were formed in RBC treated with elevated glucose levels. Since RBC do not need insulin for glucose uptake, it seems likely that increased glucose oxidation leads to the accumulation of glucose metabolites such as NADPH. It is well known that NADPH can promote membrane lipid peroxidation of RBC in the presence of the cytochrome P-450 system of liver microsomes (23, 30). The cytochrome P-450 system consists of enzymes of the drug-metabolizing system and can initiate lipid peroxidation (31). Microsomal lipid peroxidation depends upon NADPH-cytochrome P-450 reductase, and it is likely to involve reduction of free or chelated ferric to ferrous. Microsomal lipid peroxidation is specific for NADPH, although isolated microsomal NADPH-cytochrome P-450 reductase can also cause peroxidation of liposomes in the presence of chelated iron. Other enzymes, such as peroxidases and hemoglobin, can also initiate lipid peroxidation by reducing ferric to ferrous. Recent studies (32–34) have shown that oxymemoglobin in RBC can act like cytochrome P-450 in the presence of NADPH and exhibit aniline hydroxylase activity. The present study has shown that in vitro treatment of RBC hemolysates with standard NADPH can result in the formation of phospholipid-MDA adduct and TBA reactivity. Also, glucose-induced peroxidation in RBC was blocked in the presence of para-chloromercurobenzoate and metyrapone, inhibitors of cytochrome P-450. This may suggest the presence of a NADPH-dependent cytochrome P-450-like activity and its involvement in the glucose-induced lipid peroxidation in the human RBC. The effect of carboxyhemoglobin in drug metabolism or glucose-induced lipid peroxidation is not known. An increase in the cytochrome P-450 activity has been reported in certain tissues of diabetic animals (35).

Apparently in hyperglycemia greater NADPH formation stimulates the NADPH-dependent cytochrome P-450 system-like activity of the hemoglobin, which may form oxygen radicals and result in the membrane lipid peroxidation of RBC observed in the present study.

A significant positive correlation between the glucose-induced membrane lipid peroxidation and the increased osmotic fragility of RBC shows that the observed glucose-induced lipid peroxidative damage can cause changes in the properties of the RBC membrane.

Previous studies have reported elevated levels of lipid peroxidation products in RBC, plasma, and retina (4, 36–42) of diabetic patients and animals. The present findings suggest that membrane lipid peroxidation induced by elevated glucose levels may have a role in the increased lipid peroxidation products found in diabetes. This study also may be the basis for a biochemical mechanism by which hyperglycemia induces cellular injury known to occur in various tissues in diabetics.

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REFERENCES

Hyperglycemia and Membrane Lipid Peroxidation