Effect of Vitamin E and *N*-Acetylcysteine on Phosphatidylserine Externalization and Induction of Coagulation by High-Glucose–Treated Human Erythrocytes

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This study examines the effect of high glucose levels on the markers of oxidative stress, phosphatidylserine (PS) externalization, and induction of coagulation by high-glucose–treated red blood cells (RBCs). Washed normal RBCs were suspended to 15% hematocrit in phosphate-buffered saline and incubated with different concentrations of glucose for 24 hours in a shaking water bath at 37°C. This treatment caused depletion of vitamin E and accumulation of vitamin E–quinone and malondialdehyde ([MDA] an end product of lipid peroxidation), externalization of PS in the membrane bilayer, and induction of coagulation by RBCs. Pretreatment of RBCs with *N*-acetylcysteine (NAC) and vitamin E reduced membrane lipid peroxidation, PS externalization, and the tendency of high-glucose–treated RBCs to clot plasma. This study provides further evidence for the increased oxidative stress in RBCs exposed to high glucose levels. In addition, it suggests a role for membrane lipid peroxidation in the PS externalization in the membrane bilayer and in the induction of clotting by RBCs exposed to hyperglycemia. It also suggests that certain antioxidants can decrease cellular damage and restore certain cellular functions in diabetes.

IN NORMAL ERYTHROCYTES, phosphatidylserine (PS) is present only in the inner membrane bilayer.[1] Previous studies have documented that membrane lipid peroxidation can result in the movement of PS from the inner to the outer membrane bilayer and cause the red blood cell (RBC) to enhance the coagulation of plasma.[2-6] In this regard, other studies have documented the accumulation of malondialdehyde ([MDA] an end product of lipid peroxidation) both in RBCs after treatment with high glucose in vitro and in RBCs from diabetic animals and patients.[7-12]

This study was undertaken to examine the effect of high glucose levels on vitamin E and vitamin E–quinone (the oxidation product of vitamin E) and to test whether membrane lipid peroxidation has any effect on PS externalization and coagulability in high-glucose–treated RBCs.

MATERIALS AND METHODS

Blood from normal human volunteers was collected into tubes containing EDTA according to a protocol approved by the Institutional Human Experiments Review Committee. The EDTA blood was centrifuged, and the clear plasma and the buffy coat were discarded. The RBC suspension was filtered through cotton wool to remove any leftover leukocytes. The resultant cells were washed with a cold 0.15-mol/L sodium chloride solution three times after a 1:10 dilution.

In Vitro Treatment With Glucose

Washed RBCs were suspended to 15% hematocrit in phosphate-buffered saline[7] containing 6 mmol/L glucose. Aliquots of the cell suspension were placed in Erlenmeyer flasks, and a freshly prepared stock solution of glucose or 3-*O*-methylglucose was added to the flasks. Concentrations are expressed in terms of the total cell suspension. The flask contents were incubated in a shaking water bath at 37°C for 24 hours. In certain experiments, the RBC suspension was preincubated with *N*-acetylcysteine (NAC) or vitamin E for 30 minutes before the addition of glucose to the suspension. Hemolysis was less than 1% in all incubations. Treated RBCs were washed two times after a 1:10 dilution with 0.15 mol/L NaCl before biochemical analyses. All incubations contained 10 μL penicillin-streptomycin solution/mL cell suspension, to prevent microbial growth during the overnight incubations. The penicillin-streptomycin solution contained 300 mg penicillin G and 500 mg streptomycin in 10 mL buffer.

Vitamin E and vitamin E–quinone levels were measured using the high-performance liquid chromatography (HPLC) method of Hatam and Kayden.[13] Washed RBCs were stored in 2% pyrogallol-ethanol at −70°C. All samples were analyzed within 1 month of storage. A 0.2-mL aliquot of RBCs was mixed with 1 mL pyrogallol, and then 0.05 mL 10N KOH was added. This mixture was incubated in a water bath at 70°C for 30 minutes and then cooled on ice, and 0.75 mL water and 2.2 mL hexane were added. The samples were vortexed three times for 40 seconds; they were kept on ice for 2 to 3 minutes between the periods of mixing. The tubes were centrifuged at 2,000 rpm for 15 minutes. Two milliliters of the upper (hexane) layer were carefully placed into another tube. The hexane was dried with nitrogen gas, and the resulting residue was dissolved in 0.1 mL cold methanol. A 0.02-mL aliquot of either the extract of RBCs or a vitamin E standard was injected into a Nova-Pak C-18 reverse-phase column (60 A°, 4 μm, 3.9 × 150 mm; Waters, Milford, MA). Vitamin E and vitamin E–quinone were separated using a 95% methanol solvent system with the detector set at 292 nm. In separate assays of the sample extract, absorbance peaks were also measured at 265 nm to determine the level of tocopherol-quinone.[14]

Membrane lipid peroxidation was determined by HPLC analysis of the thiobarbituric acid–MDA complex. The complex was chromatographed using an ion-exclusion and reverse-phase Shodex KC-811 column (Waters) with the detector set at 532 nm according to the method of Esterbauer et al.[15]

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PS externalization was determined by treatment of RBCs with bee venom phospholipase A_2, which specifically hydrolyzes phosphatidylcholine, phosphatidylethanolamine, and PS, present only at the outer bilayer, to their respective lysolipids.[3]

Induction of coagulation by RBCs in vitro was determined by the ability of RBCs to shorten the clotting time of normal human platelet-poor plasma in the presence of Russel's viper venom (Sigma, St Louis, MO), as described by Zwaal et al.[16]

All chemicals were purchased from Sigma Chemical unless stated otherwise. The data were analyzed using one-way ANOVA and the Wilcoxon signed rank sum test with Sigma Plot statistical software (Jandel Scientific, San Rafael, CA). A P value less than .05 was considered significant.

RESULTS

The effects of high-glucose treatment on vitamin E, vitamin E–quinone, and MDA levels in RBCs are listed in Table 1. Vitamin E levels were significantly lower and vitamin E–quinone and MDA levels significantly higher in high-glucose–treated RBCs at 24 hours. This suggests that RBCs exposed to high levels of glucose are more susceptible to lipid peroxidation and accumulation of MDA and vitamin E–quinone, markers of increased oxidative stress. There was a significant increase in lactate formation in high-glucose–treated RBCs compared with controls. However, treatment of cells with lactate alone did not affect the levels of MDA, vitamin E, or vitamin E–quinone. Our previous study[7] showed that lactate levels are higher in high-glucose–treated RBCs, but treatment of cells with exogenous lactate also did not increase membrane lipid peroxidation. Similarly, preincubation of cells with fluoride inhibited glucose metabolism and the increased membrane lipid peroxidation in high-glucose–treated RBCs.[7]

Table 2 shows that high-glucose treatment causes the externalization of a portion of PS from the inner to the outer membrane bilayer and shortens the in vitro clotting time of plasma by RBCs. The effect of NAC and vitamin E on membrane lipid peroxidation, PS externalization, and coagulability in high-glucose–treated RBCs is listed in Table 3. Both NAC and vitamin E blocked membrane lipid peroxidation. These antioxidants also caused a reduction in the externalization of PS and in the ability of RBCs to induce clotting. This suggests a relationship between membrane lipid peroxidation, PS externalization, and hypercoagulability of high-glucose–treated RBCs. In comparison to fresh untreated RBCs, a large percentage of the original vitamin E (92%) was present at the end of the 24-hour incubation. Vitamin E–supplemented RBCs showed a threefold increase in vitamin E at the end of a 24-hour incubation period (results not shown). NAC levels were not measured in NAC-supplemented RBCs.

Table 1. Effect of High-Glucose Treatment on Vitamin E, Vitamin E–Quinone and MDA Levels in RBCs

Parameter	Treatment (mmol/L)			
	6 G (control)	20 G	40 G	40 OMG
Vitamin E (μg/g Hb)	2.20 ± 0.10	2.10 ± 0.10	1.93 ± 0.11*	2.12 ± 0.14
Vitamin E–quinone (μg/g Hb)	0.40 ± 0.12	0.41 ± 0.10	0.51 ± 0.11*	0.42 ± 0.12
MDA (nmol/mL)	2.0 ± 0.2	2.4 ± 0.3*	2.6 ± 0.3†	2.2 ± 0.3

NOTE. Values are the mean ± SE of 6 experiments.

Abbreviations: G, glucose; Hb, hemoglobin; OMG, 3-*O*-methylglucose.

*$P < .05$ *v* control.

†$P < .02$ *v* control.

Table 2. Effect of High-Glucose Treatment on PS Externalization and Induction of Coagulation by RBCs

Parameter	Glucose (mmol/L)		
	6 (control)	20	40
PS externalization (%)	0	4 ± 2*	7 ± 2*
Coagulability (% of saline)	32 ± 2	28 ± 1*	25 ± 2*

NOTE. Values are the mean ± SE of 6 experiments.

*$P < .01$ *v* control.

DISCUSSION

The delicate balance of PS across the inner membrane bilayer of the RBC is maintained by the enzyme flipase.[1] This enzyme contains an essential oxidizable thiol group,[17,18] which could be inactivated as a result of oxidative damage. Inactivation of the flipase would result in translocation of PS from the inner to the outer bilayer surface (PS externalization). Considerable evidence suggests that the conversion of prothrombin to thrombin by a prothrombinase complex in the clotting cascade requires a negatively charged phospholipid membrane surface, such as PS.[16] Thus, even a small fraction of PS externalization in RBCs would have an effect on the PS surface available for hemostasis and thus promote coagulability.

Among several abnormalities, RBCs of diabetic patients show increased levels of membrane lipid peroxidation and PS externalization in the outer membrane bilayer.[19] These patients also experience blood hypercoagulability.[20] However, it is not known whether there is any link between membrane lipid peroxidation, PS externalization, and hyperglycemia. Treatment of RBCs with elevated glucose concentrations can cause PS externalization in the membrane bilayer.[21] The present study also shows that high glucose concentrations can induce depletion of vitamin E and accumulation of vitamin E–quinone and lipid peroxidation products in RBCs. High glucose levels can generate oxygen radicals by several mechanisms, including autoxidation, reduced activity due to glycation of antioxidative enzymes, or stimulation of cytochrome P-450–like activity by the glucose metabolites NADPH or NADH.[7,8,20] This can lead to increased oxidative stress and generation of MDA, a product of lipid peroxidation. It is possible that NAC prevents lipid peroxidation by scavenging the free radicals generated by high glucose concentrations. PS externalization in glucose-treated RBCs could be due to inactivation of the enzyme flipase as a result of oxidative damage to lipids and an essential thiol group.[17,18] This view is indirectly supported by the protective effects of NAC and vitamin E on membrane lipid peroxidation, PS externalization, and coagulation induction by glucose-treated RBCs.

Table 3. Effect of NAC and Vitamin E on Membrane Lipid Peroxidation, PS Externalization, and Induction of Coagulation by High-Glucose–Treated RBCs

Parameter	G (6 mmol/L)	NAC (1 mmol/L)	E (0.1 mmol/L)	G (40 mmol/L)	G + NAC	G + E
MDA (nmol/mL cells)	2.0 ± 0.2*	2.0 ± 0.1	1.9 ± 0.2	2.7 ± 0.2	2.3 ± 0.2†	1.8 ± 0.1†
PS externalization (%)	0	0	0	8 ± 2	4 ± 1†	3 ± 1†
Coagulability (% of control)	32 ± 2	33 ± 2	34 ± 3	24 ± 3	29 ± 1†	33 ± 2†

NOTE. Values are the mean ± SE of 5 experiments.
Abbreviations: G, glucose; E, vitamin E.
*$P < .02$ *v* glucose 40 mmol/L.
†$P < .02$ *v* glucose 40 mmol/L.

This study suggests a role for membrane lipid peroxidation in PS externalization in the membrane bilayer and coagulation induction by RBCs exposed to hyperglycemia.[19-21] Induction of coagulation by RBCs may contribute to the generalized activation of blood coagulation known to occur in diabetic patients.[20] This study also provides a basis for the idea that certain antioxidants may be useful in reducing cellular damage and dysfunction in diabetes.

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