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The presence of higher level of endogenous free radical reaction products in the erythrocyte ghost membrane (EGM) of Non-insulin-dependent diabetes mellitus (NIDDM) subjects compared with that of normal healthy controls has been demonstrated. The EGMs of NIDDM subjects were also shown to be more susceptible to exogenously generated oxidative stress than those of normal healthy individuals. The decreased level of reactive thiol groups in the EGM of NIDDM individuals supported this observation. We propose that the presence of significant levels of non-heme iron in the EGM of NIDDM subjects is an indication of the potential for iron-catalysed production of hydroxy and other toxic radicals which could cause continuous oxidative stress and tissue damage. Oxygen free radicals could therefore be responsible for most of the erythrocyte abnormalities associated with non-insulin-dependent diabetes and could indeed be intimately involved in the mechanism of tissue damage in diabetic complications.

KEY WORDS: Oxidative stress; non-heme iron; erythrocyte membranes; Non-insulin-dependent diabetes mellitus.

INTRODUCTION

Diabetes mellitus, a syndrome characterized by fasting hyperglycemia, glycosuria and osmotic diuresis [1], is generally known to be caused by a faulty production of, or sub-normal tissue response to insulin. Type II or Non-insulin-dependent diabetes mellitus (NIDDM) which is mostly characterized by sub-normal tissue response to elevated blood insulin is among the commonest metabolic disorders both in the developed and developing countries [2, 3]. Both type I insulin-dependent diabetes (IDDM) and type II are known to be prone to develop complications relating to elevated blood glucose concentrations, including atherosclerosis, retinal damage, cataract and neuropathy [1].

The excessive amount of glucose in the blood of diabetics has apparently converted this beneficial compound into a toxic one. Glucose toxicity could be manifested through sorbitol production [4], glycation of proteins and deoxyribonucleic acid (DNA) [5] and production of reactive oxygen species in the presence of metal

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catalysts [6]. Indeed, the biochemical mechanisms involved in these three pathways suggest that oxidative stress could be an important feature of diabetes mellitus. For instance, the excessive activity of aldose reductase in the sorbitol pathway places a burden on cellular NADPH, and may reduce the availability of this reduced coenzyme for glutathione reductase activity and hence affect the maintenance of cellular reduced glutathione. Furthermore, the non-enzymic glucosylation of erythrocyte Cu–Zn superoxide dismutase greatly accounts for the lower activity of this enzyme in the blood of diabetics [7], and hence tilts the balance towards oxidative stress when reactive oxygen species are oxidatively generated by glucose, in the presence of trace amounts of metal catalysts [6].

The association of oxidative stress with diabetes mellitus is further supported by the report that diabetics in comparison with normal individuals consume ascorbate faster, have higher concentrations of dehydroascorbate in their plasma [8], have lower levels of vitamin E in their platelets, [7] and have decreased levels of GSH in their blood [10]. Other studies have also shown increased levels of plasma lipid peroxidation products in diabetic individuals [11].

In this study, we demonstrate the presence of lipid peroxidation products in the red cell membranes of NIDDM patients as well as the relatively higher susceptibility of these plasma membranes to exogenously-generated oxidant stress. We also present evidence for the presence of significant amounts of non-heme iron and low levels of thiols in these membranes.

MATERIALS AND METHODS

3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), 5,5′-Dithio-is(2-nitrobenzoic acid), Malonaldehyde bis (dimethylacetal), Thiobarbituric acid, Thiourea, bovine serum albumin, glutathione and glycine were purchased from Sigma Chemical Co., Poole, Dorset, U.K. All other reagents were purchased from British Drug Houses, U.K.

Isolation of Haemoglobin-free Ghost Membranes

Blood samples were collected into acid-citrate dextrose buffer from individuals who were newly identified as having Non-insulin dependent diabetes mellitus at the diabetes unit of the Department of Medicine, University College Hospital, Ibadan, after informed verbal consent. These patients had not received any medication or dietary therapy prior to the time blood was collected. Normal human blood was collected from healthy adult donors and was used as control. All blood samples were stored at 4°C and used within 12 hr. All steps of the membrane isolation were carried out at 4°C.

Blood samples were centrifuged at 2500 rpm for 10 mins followed by removal of the plasma and buffy layers by aspiration. The packed cells were suspended in 10 volumes of cold isotonic phosphate buffer pH 7.4 and centrifuged at same speed for 10 mins. This was repeated once and any remaining buffy coat was carefully aspirated.

The packed cells were then resuspended in 10 volumes of cold hypotonic 5 mM phosphate buffer pH 7.4 and allowed to stand on ice for 30 mins. Centrifugation was later carried out at 16,000 rpm for 10 mins at 4°C. The supernatant as well as the dense pellet at the bottom of the tubes were carefully aspirated. The membranes obtained were washed four more times in 10 volumes of the same buffer and later resealed by resuspension in 10 volumes of the isotonic phosphate buffer pH 7.4 followed by centrifugation at 16,000 rpm for 10 mins. The membranes were stored in the same buffer at 4°C and used within 12 hr.

Protein Determination

Membrane concentration was assessed by determining protein concentration according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard [12].

Assessment of Lipid Peroxidation in Erythrocyte Ghost Membranes of Normal and Non-insulin Dependent Diabetic Individuals

Membrane lipid peroxidation was assayed by a modification of the method described by Rice-Evans *et al.* (1986) [13]. Membranes were suspended in isotonic phosphate buffer at a final concentration of 1 mg/ml and incubated at 37°C in a shaking water bath for five hours. In another experiment, the haemoglobin-free membranes were incubated in an isotonic phosphate buffer pH 7.4 containing iron(II) sulphate (100 μM), ascorbic acid (1 mM) and hydrogen peroxide (200 μM), under the same conditions as above. 0.5 ml of 0.75% Thiobarbituric acid in 0.1 M HCl was later added to 0.5 ml of the incubation mixture already quenched with 0.5 ml of 10% trichloroacetic acid. The mixture was heated at 90–95°C for 20 mins and after cooling, centrifuged for 10 mins at 2500 rpm. The supernatant was transferred into acid resistant tubes and centrifuged at 16,000 rpm for 10 mins. The pink chromophore in the resulting clear solution was assayed spectrophotometrically at 532 nm. Standards were run simultaneously under the same conditions utilizing malondialdehyde (50 μM) prepared by acid (0.1 M HCl) hydrolysis of malonaldehyde bis (dimethylacetal).

Determination of Erythrocyte Ghost Membrane-bound Non-heme Iron

Membrane-bound non-heme iron was determined according to a modified method of Ceriotti and Ceriotti (1980) [14]. Final concentration of reagents used were 38 mM ascorbate, 0.67% sodium dodecyl sulphate, 10 mM phosphate buffer pH 7.4, 150 mM NaCl and 1.28 mM ferrozine (3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid) 1,2,4-triazine) in a total volume of 3 ml. The intensity of the pink-colored complex formed was determined by measuring absorbance at 562 nm after six hours. Standards were simultaneously run with a stock solution of 25 mg/L iron.

Estimation of Reactive Thiol Content of Erythrocyte Ghost Membranes

The reactive thiol groups of the erythrocyte ghost membranes were estimated spectrophotometrically at 412 nm by a modification of the Ellman procedure (1959) using GSH as standard [15]. Reaction medium contained in final concentrations: 364 mM phosphate buffer pH 8.0, 0.91% SDS and 200–300 μg protein, in a total volume of 3.3 ml. The mixture was incubated at 37°C for 1 hr in a shaking water bath after addition of 0.091 mM DTNB, the assay was run in duplicate.

RESULTS

The procedure used in this study to determine the extent of lipid peroxidation involves the interaction of thiobarbituric acid with the breakdown products of lipid hydroperoxides, under acid conditions. The results as presented in Fig. 1 indicate the presence of higher level of lipid peroxidation products in the erythrocyte ghost membrane (EGM) of NIDDM in comparison with EGM of healthy individuals. In fact, the extent of lipid peroxidation products in the EGM of NIDDM subjects is about four times higher than levels observed for EGM of healthy individuals. Furthermore, significant increases in the amount of lipid peroxidation products were observed in both membranes when exposed to exogenously generated reactive oxygen species using a mixture of Iron(II), ascorbate and hydrogen peroxide. In this regard, the amount of thiobarbituric acid reactive (TBAR) products increased by 3.5- and 5-fold respectively for EGM of normal and NIDDM individuals.

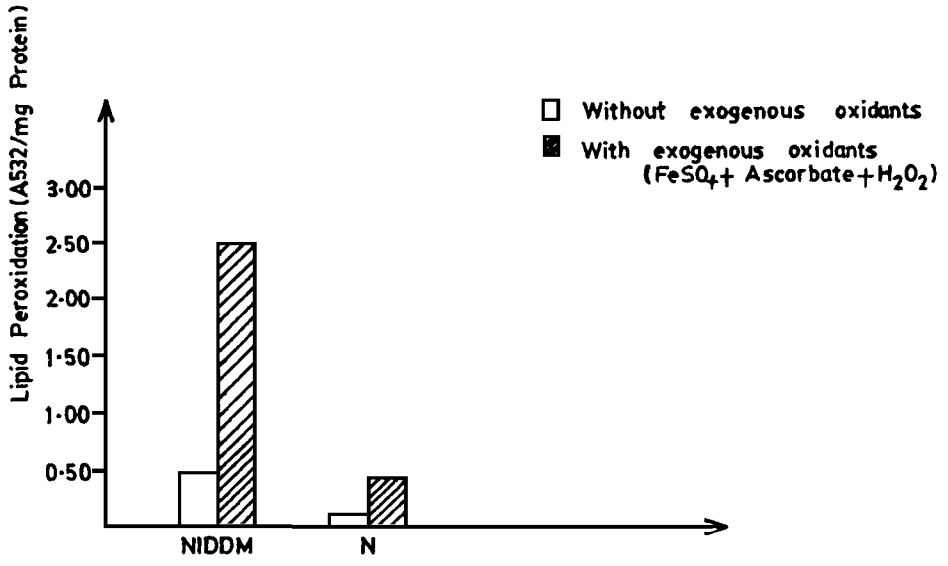

Fig. 1. Degree of lipid peroxidation of erythrocyte ghost membranes of healthy (N) and non-insulin dependent diabetes mellitus (NIDDM) individuals in the presence and absence of exogenously generated reactive oxygen species (ROS). Lipid peroxidation was estimated as described in Materials and Methods and expressed as A532/mg protein.

Table 1. Levels of Non-Heme Iron Bound to Ghost Membranes Prepared from Erythrocytes of Healthy and NIDDM Subjects

Erythrocyte ghost membranes of	Non-haem iron (nmol mg protein^{-1})
Healthy subjects	0.0 ± (n = 13)
NIDDM subjects	17.2 ± 5.3 (n = 15)

Non-heme iron was estimated by a modification of the ferrozine assay described by Ceriotti and Ceriotti (1980) and expressed as nmoles non-heme iron per mg protein. Mean and standard deviation of 15 preparations are given.

In view of the considerably high level of lipid peroxidation products in the EGM of NIDDM subjects in the absence of exogenously generated free radicals, the presence of non-heme iron in these membranes was investigated since this metal iron invariably catalyzes the generation of highly reactive free radicals. Non-heme iron was determined by a procedure based on the ability of ascorbic acid to reduce iron. Addition of ferrozine to the mixture results in the formation of a complex with iron that is intensely colored [14]. Sodium dodecyl sulphate (SDS) was included in the assay to ensure solubilization and denaturation of erythrocyte membrane proteins. Although the absorbance of the stable pink chromophore was read 6 hr and 18 hr after addition of ferrozine, the results obtained (Table 1) did not differ significantly thus indicating that all the non-heme iron have been released into solution. The data shown in Table 1 indicate the absence of non-heme iron in haemoglobin-free EGM prepared from red cells of healthy subjects. In contrast, non-heme iron is present in significant amounts in erythrocyte ghost membranes of NIDDM subjects even though there was considerable variation in the amounts observed among the ghost membranes of these individuals.

Determination of the membrane antioxidant status in terms of free thiol groups show that the level of free thiol groups (Table 2) in the EGM of NIDDM subjects is 17% lower than that of the normal healthy control subjects.

DISCUSSION

Speculation about involvement of reactive oxygen species (ROS) in the pathophysiologic process of diabetes mellitus emanated from the observation that alloxan induces diabetes in rats via the generation of these species. Alloxan injected into rats

Table 2. Membrane Thiol Content in Erythrocytes of Healthy and NIDDM Subjects

	Membrane thiol content (nmoles SH/mg protein)
Healthy subjects	82.5 ± 2.6 (n = 6)
NIDDM subjects	68.6 ± 5.5 (n = 13)

Red cell membrane reactive thiol levels were determined spectrophotometrically at 412 nm with 5,5′-dithiobis-(2-nitrobenzoic acid and expressed as nmole SH per mg protein. Mean and standard deviation of indicated number of preparations are given.

accumulate only in the liver and the islet of Largerhans where it generates superoxide radical from oxygen during the oxidation of its unstable reduction product-dialuric acid [16]. The oxidation step depends on the presence of trace amounts of transition metal and also results in the generation of hydrogen peroxide and hydroxyl radicals by Fenton-type reactions [17]. The poor activities of enzymic antioxidants in the islet cells enhance the accumulation and toxicity of these species causing degeneration of B-cells which normally produce insulin. The report of Thornalley and Stern (1984) [6] that oxidation of monosaccharides can lead to the generation of reactive oxygen species in the presence of catalytic amounts of iron or copper ions further fortified the suspicion that ROS could play an important role in the pathology of diabetic complications.

The importance of erythrocytes in assessing the pathophysiologic mechanisms in diabetes mellitus has become very evident from the several abnormal features demonstrated for the red cells of diabetic patients. In particular, these include altered oxygen transport and metabolism [18], increased glycation of haemoglobin [19], a feature now generally used as an index of blood glucose control [20], glycation of membrane and cytosolic proteins [20], decreased deformability [21] altered membrane phospholipid asymmetry [22] decreased membrane lipid fluidity [23], decreased total GSH level [24], increased aggregation [25], reduced $Na^+ + K^+$-ATPase activity [26], decreased Ca^{2+}-pumping ATPase activity [27] increased adherence to endothelial cells [28] and decreased life span [29]. Significantly, many of these abnormalities are associated with the red cell membrane which incidentally has also been unequivocally demonstrated to contain insulin receptor [30]. Interestingly, similar abnormal features of erythrocytes have been previously demonstrated in some pathological states particularly sickle cell anaemia [31] in which these cells have also been demonstrated to be under severe oxidative stress [32]. It has been shown by several workers that oxidative stress-induced membrane lipid peroxidation could result in altered membrane phospholipid asymmetry, decreased survival of cells and increased adherence to vascular endothelium [33].

Attempts have therefore been made in this study to assess the possibility that erythrocytes in diabetic patients are under oxidative stress and determine the factors that might have contributed to this.

The results obtained in this study illustrate that red cell membranes of NIDDM subjects show a greater tendency to lipid peroxidation and the formation of secondary breakdown products which are thiobarbituric acid reactive compared with erythrocyte ghost membranes of normal healthy individuals (Fig. 1). It seems likely that subsequent modification of the phospholipid fatty acyl chains following peroxidation may be the basis of some of the observed erythrocyte membrane abnormalities such as decreased membrane fluidity [23], decreased deformability [21] and Ca^{2+}-pumping functions [27]. Furthermore, we have shown in this study that exposure of the EGM to exogenous oxidative stress could result in additional accumulation of lipid breakdown products (Fig. 1). A probable explanation is that the levels of membrane-bound antioxidants particularly the free thiol groups and Vitamin E content might be lower in the EGM of diabetics than in EGM of healthy individuals. Although total GSH concentration has been reported to be lower in erythrocytes of diabetic patients [24], it was not known whether the decrease is also reflected in the

membranes. The data obtained (Table 2) may be explained in terms of glucose oxidation through the sorbitol pathway which causes NADPH to be inadequately available for the effective resynthesis of reduced glutathione normally utilized to combat the rising oxidative stress in the cell. Consequently, the EGM might have undergone abnormal oxidation of protein thiols. Graf *et al.* (1982) have suggested that maintenance of optimal Ca^{2+}-ATPase activity depends, at least in part, upon preservation of membrane thiols in their reduced state since erythrocyte Ca^{2+}-ATPase is a reasonably thiol-rich protein [35]. Hence, activated oxygen species generated in erythrocytes of NIDDM subjects might possibly be responsible for the reduced activity of Ca^{2+}-ATPase through mechanisms involving lipid peroxidation and formation of disulphide bonds [36].

The detection of significant amounts of lipid breakdown products in a membrane after oxidative stress induced by incubation gives the impression that ROS capable of initiating peroxidation of membrane phospholipids are being generated in the membrane. Although lipid hydroperoxides are fairly stable at physiological temperatures, transition metal complexes in the form of heme, haemoglobin, methemoglobin, non-heme iron proteins etc. certainly play a role in the decomposition of these peroxides to alkoxy and peroxy radicals which are incapable of initiating lipid peroxidation [37]. The initiation of peroxidation requires highly reactive radical species such as hydroxyl radical, the generation of which requires the presence of a transitional metal catalyst such as iron near the site of its action. It therefore follows that even in its compromised state of decreased Cu–Zn superoxide dismutase activity and reduced erythrocyte GSH level, erythrocytes in diabetic state still require the presence of iron in a form that can catalyze the generation of these highly reactive free radical species in order to be under oxidative stress.

Experiments designed to determine the amount of non-heme iron bound to the red cell membranes of healthy and NIDDM subjects were carried out using a modification of the ferrozine assay described by Ceriotti and Ceriotti [14]. The result obtained (Table 1) is consistent with the findings of Ergstrom *et al.* (1990) that the iron content in the bone marrow of hyperglycaemia and hyperinsulinaemia obese mice was substantially greater in comparison to control mice, an indication of increased iron content in the bone marrow of NIDDM individuals [38]. Obviously, it will be interesting to know the precise nature and source of the non-heme iron associated with the erythrocyte ghost membranes. In this connection, the most probable candidate seems to be the glycosylated haemoglobin which may be bound to the membranes but there is no evidence as yet to support this assertion. It is also not unlikely that this iron is contained within ferritin, high serum levels of which has been reported in some type II diabetic patients [39]. Studies by Van der Kraaij *et al.* have shown that iron could be released from ferritin by superoxide radicals and that peroxidation of liver microsomes can be induced by ferritin in the presence of superoxide radicals [40]. Iron atoms derived from intracellular ferritin can also auto-oxidize to form lipid-soluble iron-oxygen complexes that could catalyze the formation of highly reactive free radicals whose variety of action result in alteration of membrane properties leading to tissue damage.

While the biochemical mechanisms underlying the development of long-term complications of diabetes mellitus remain largely speculative, it is not absolutely

clear whether the results obtained in this study could be extended to other tissues of the body particularly in view of the unique presence of haemoglobin in erythrocytes, and the non-nucleated natures of these short lived cells. However, the idea that oxidative stress contribute to diabetic pathogenesis and complications appears quite attractive and relevant in view of the fact that hyperglycemia, the primary cause of diabetic tissue damage [41], constitute the genesis and manifestations of oxidant stress respectively. In this connection, glucose has been demonstrated to fragment protein, cause the peroxidation of both liposomes and low density lipoproteins [42, 43] and cause embryonic dysmorphogenesis *in vitro* by generation of oxygen free radicals [44]. Its auto-oxidation, though very slow is known to have direct bearing on non-enzymic protein glycosylation [45].

It is therefore reasonable to postulate that the abnormalities in levels of catalytic iron demonstrated in this study together with the normally elevated levels of glucose makes oxidative stress a predisposing factor in the pathogenesis of diabetes and its complications.

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