GLOBAL JOURNAL OF PURE AND APPLIED SCIENCES VOL. 12, NO. 3, 2006: 339 - 342
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EFFECT OF PHENACETIN ON NADH-METHAEMOGLOBIN REDUCTASE OF HUMAN ERYTHROCYTES

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(Received 19, June, 2005; Revision Accepted 8 August, 2006)

ABSTRACT

In vitro studies were carried out to ascertain the level of NADH – methaemoglobin reductase activity and comparatively, the effect of phenacetin on the red cell enzyme in the three human genotypes, HbAA, HbAS and HbSS. The mean ± S.D level of activity of NADH-methaemoglobin reductase in the three genotypes was significantly different ($P<0.05$). The values reported as iu/gHb were: 9.13±0.12, 13.71±0.48 and 21.91±0.41 for HbAA, HbAS and HBSS erythrocytes respectively. The introduction of increasing concentrations of phenacetin in the order of 0.04mM, 0.08mM, 0.12.mM, 0.16mM and 0.22mM into the enzyme assay mixture engendered a decline in the activity of the enzyme in the following range: 9.13±0.11 iu/gHb to 2.28±0.12 iu/gHb, 13.71±0.48 iu/gHb to 3.11±0.47 iu/gHb and 21.91±0.41 iu/gHb to 4.12±0.53 iu/gHb for HbAA, HbAS and HbSS red cells respectively. Further enzyme kinetic analysis showed the mode of inhibition by phenacetin was competitive. The results indicated a genotype dependent variability in the inhibitory property of phenacetin on NADH – methaemoglobin reductase activity in a concentration dependent manner.

KEYWORDS: NADH – methaemoglobin reductase, Genotype, Phenacetin

INTRODUCTION

Phenacetin is an analgesic drug (Clark *et al* 1988). The drug is associated with toxic methaemoglobinemia especially in individuals with reduced capacity to transform phenacetin to acetaminophen, resulting to the formation of alternative metabolic products of 2 – hydroxy phenacetin and 2 – hydroxy phenatidin ("PHENACETIN", 2003 and Clark *et al* 1988).

Concisely, methaemoglobin is formed when the ferrous iron (Fe^{2+}) of deoxyhaemoglobin is oxidized to the ferric state (Fe^{3+}). Methaemoglobin will not bind reversibly with oxygen. Because the red cells are continuously exposed to oxidizing agents and oxygen free radicals, methaemoglobin is formed continuously in plasma. However, plasma level of methaemoglobin rarely exceed 1% of total plasma haemoglobin (Tietz, 1976; Callister, 2003).

Basically there are two enzyme systems that operate to keep the level of this dysfunctional haemoglobin to the minimum. The first mechanism involves Diaphorase I (NADH – methaemoglobin reductase), which is responsible for 95% of the red cells reducing capacity, while 5% is accounted for by Diaphorase II NADPH- methaemoglobin reductase) (Callister, 2003). There are various reducing compounds such as ascorbic acid, glutathione and other sulphydryl compounds that are present in the red cell for reducing oxidizing molecules and free radicals, which can cause the generation of methaemoglobin. But this mechanism probably function with a failure of the enzyme system (Kuma, 1981; Prchal and Jenkin, 2001).

The red cell NADH – methaemoglobin reductase (E.C: 1.6.4.3.1.) has been characterized as NADH diaphorase or Diaphorase I (Gibson, 1984, Breakug *et al* 1951), NADH – ferricyanide reductase (Board, 1981) and NADH cytochrome b5 reductase (Yubisui and Takeshita, 1980). Deficiency of Diaphorase I has long been proposed to be associated with hereditary methaemoglobinemia (Breakug *et al* 1951; Ursula, 1998). The isolation of diaphorase – like enzyme from natural cells by Scott and coworkers provided a direct evidence of its role in the catalytic reduction of methaemoglobin through NADH (Scott and Hospkins, 1958; Scott and Griffith 1959).

Much work has been done on assessing the *in vitro* effect of diverse biochemicals on the activity of the red cell – NADH methaemoglobin reductase. Worthy of mention are the comparative *in vitro* effect of *cajanus canjan* extract, dithionite, phenylalanine, sicklervite ™, α- Tocopherol, 4 – methyl umbelliferon and haemin (Uwakwe, 1991) and ascorbic acid (Jaffe and Neuman 1985, Varley *et al* 1980) on the red cell enzyme, NADH – methaemoglobin reductase activity. These endogenous and exogenous agents were found to either activate or inhibit the enzyme activity.

This work was designed to elucidate the plasma levels of NADH – methaemoglobin reductase activity and ascertain the *in vitro* effect of phenacetin on the activity of the red cell enzyme in the three human genotypes – HbAA, HbAS and HbSS.

MATERIALS AND METHODS

Collection of Blood Samples

Five milliliters of blood samples were collected from subjects/volunteers within the age bracket of 18 – 35 years. All the blood donors are residing in Owerri West Local Government Area of Imo State, Nigeria. The blood samples were stored at 0°C and haemolysed by freeze thawing usually within 6 hours after collection.

Portions of the blood samples were used for electrophoretic genotyping, determinations of haemolysate haemoglobin concentration and NADH methaemoglobin reductase activity.

Determination of Haemolysate Haemoglobin Concentration in HbAA, HbAS and HbSS Blood Samples.

Haemoglobin concentration was measured by adding haemolysate to ferrocyanide- Cyanide reagent to convert the pigment to cyanomet haemoglobin (Van –Kampen and Zijlatra, 1961). A 0.05ml portion of the blood samples were added to 4.95ml of Drabkin reagent (100mg Na CN and 300mg K_4 Fe $(CN)_6$ per litre). The mixture was left to stand for 10 minutes and then read at 540nm against a standard blank (Drabkin reagent only). The values were compared to those of known standards.

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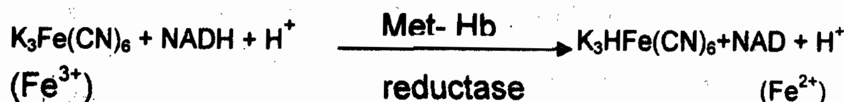
Haemolysate haemoglobin standard (stock) was prepared by dissolving 14.6g of commercal bovin haemoglobin in 100ml of Drabkin reagent.

Determination of Red cell – NADH methaemoglobin Reductase Activity in HbAA, HbAS and HbSS Blood Samples.

NADH- methaemoglobin reductase has a very active NADH – ferrocyanide reductase activity (Board, 1981). This reaction could be followed spectrophotometrically as 340nm and provides a rapid and simple means for determining NADH – methaemoglobin reductase activity. The rate of oxidation of NADH is represented by the aquation.



$$K_3Fe(CN)_6 + NADH + H^+ \xrightarrow[\text{reductase}]{\text{Met- Hb}} K_3HFe(CN)_6 + NAD + H^+$$
$$(Fe^{3+}) \qquad\qquad\qquad (Fe^{2+})$$

Into a test tube, 0.2ml tris - HCl/EDTA buffer, pH = 8.0 and 2.0ml of 0.2mM of NADH was added. A 4.35 mls of distilled water was added next before incubating for 10 minutes at 30^{0}C. The solution was transferred into a cuvette and the enzyme reaction started by adding 0.2ml of 10mM of K_4 Fe $(CN)_6$/0.05ml haemolysate haemoglobin (ratio 4:1). The premixing of the haemolysate and potassium ferrocyanide was necessary to avoid the error resulting from a non – enzymatic reaction between the haemolystate and potassium ferrocyanide.

The increase in the absorbance of the medium as monitored per minute for 10 minutes at 30^{0}C against a blank containing reaction mixture without haemolysate (volume of haemolysate accounted for with distilled water). The reaction system is unstable at higher temperature above 30^{0}C (Beutler, 1984).

Determination of the Effect of Phenacetin on the Activity of Red cell NADH – methaemoglobin Reductase in HbAA, HbAS and HbSS Blood Samples.

The enzyme activity of Red cell NADH-methaemoglobin reductase of the three genotypes was determined in the presence of varied concentrations of phenacetin (0.04mM, 0.08mM, 0.12mM, 0.16mM and 0.22mM). To the five test tubes, 0.2ml tris HCL/EDTA buffer, pH = 8.0 and 0.2ml of 0.2mM of NADH was added. A 4.15ml, 3.95ml, 3.75ml, 3.55ml and 3.35ml of distilled water was added before incubating for 10 minutes at 30^{0}C. This solution was transferred into a cuvette and the reaction started by adding 0.2ml of 10mM $K_4(CN)_6$/0.05ml haemolysate (ratio 4:1) after adding initially 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1.0ml of phenacetin of the above stated concentrations respectively.

The increase in absorbance of the solution was monitored per minute at 30^{0}C against a control sample at 340nm for 10 minutes

RESULTS AND DISCUSSION

Comparative studies of the enzyme activity in the three genotypes showed a variable activity in the order HbSS > HbAS > HbAA (Table 1.0).

The genotype dependent variability of the enzyme activity is suggestive that there was an increased positive activation of the red cell enzyme in HbSS and HbAS as a result of higher level of the oxidant methaemoglobin in the red cell associated with higher production of superoxide compared to those of HbAA erythrocytes (Van Kiujk *et al* 1987, Kirshner – Zilber *et al* 1982).

Enzyme activity was reported in international unit per gramme haemolglobin Iu/gHb of red cell.

n = Number of sample used for each determinations.

Erythrocytes from sicklers have been reported to have higher than normal level of oxidants such as haemin and its effect on the red cell enzyme has been studied (Orjih *et al*, 1985; Uwakwe, 1991). The presence of endogenous oxidants such as haemin has a profound effect in increasing the activity of the enzyme *in vitro*. The efflux of haemin from the fragile sickled erythrocytes is attributable to the high level of haemolytic phenomenon perculiar to these haemoglobin variant cells (Orjih *et al* 1985). The high ferric haem generation therefore triggers a higher oxidation rate of NADH methaemoglobin reductase in their bid to maintain the redox equilibrium. Another plausible explanation for the observed increase in the activity of the red cell enzyme in the order described above in the three genotypes is the possibility of insufficient level of reducing agents resulting from low levels of NADH and NADPH generation in these cells to measure up with the raised oxidant level of superoxide generation.

An overview of the effect of phenacetin on the activity of the red cell enzyme in the three genotypes showed a genotype dependent variability with increasing concentration in phenacetin (Table 1.0). The overall decrease in the level of the red cell enzyme activity was in the order HbAA> HbAS> HbSS. This may not be unconnected with the level of the enzyme activity in the three genotypes as earlier described.

Table 1.0: Relative enzyme activity of NADH – Methaemoglobin reductase with increasing concentration of Phenacetin of the three human genotypes.

GENOTYPE	[Hb]g/dl	PHENACETIN CONCENTRATIONS (mM)					
		Control	0.04	0.08	0.12	0.16	0.22
HbAA n=5	1.76±0.12	9.13±0.11 (100.0)	7.04±0.25 (77.1)	4.39±0.04 (48.1)	3.87±0.24 (42.4)	3.12±1.11 34.2)	2.28±0.12 (24.9)
HbAS n=5	0.82±0.19	13.71±0.48 (100.0)	10.05±0.39 (73.3)	8.00±0.35 (58.4)	6.09±0.91 (44.4)	5.60±0.21 (40.9)	3.11±0.47 (22.7)
HbSS n=5	0.66±0.38	21.91±0.41 (100.0)	17.95±0.19 (81.9)	13.71±0.23 (62.6)ss	10.47±0.05 (47.8)	9.56±0.02 (43.6)	4.12±0.53 (18.8)

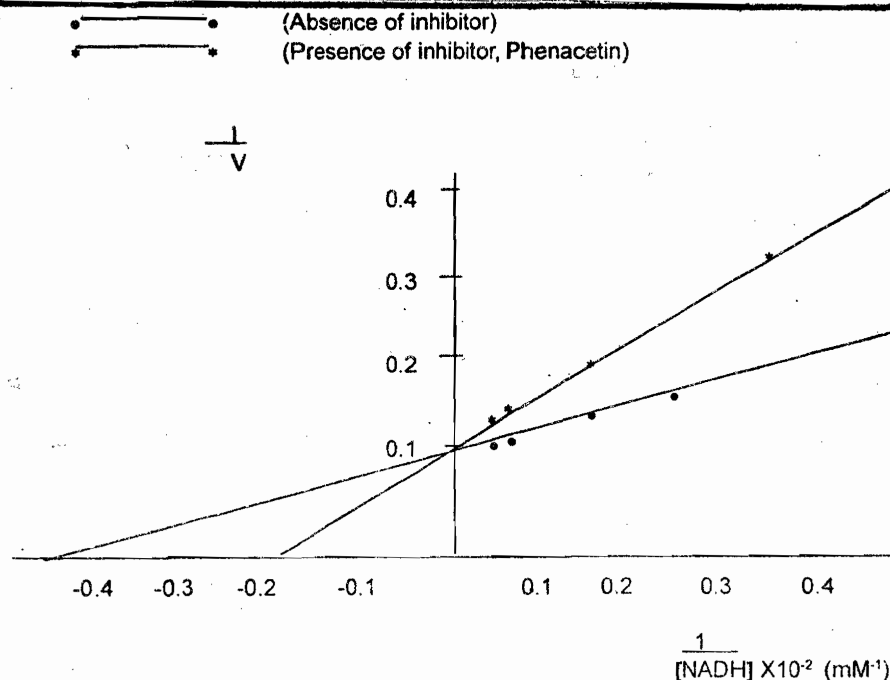


FIGURE 1.0 Double reciprocal plot of the mode of inhibition of NADH-methaemoglobin reductase by phenacetin in HbSS red cells. The micheails constsnt (km) in the absence of inhibitor lo is 0.025×10^{-2}M, while the apparent inhibition constant $K_1 = 0.06 \times 10^{-2}$M.

The nature of the plot above (figure1.0) showed the mode of inhibition of phenacetin on the activity of the red cell enzyme was competitive. Therefore, it is believed that phenacetin competes with the natural cosubstrate (NADH) of the red cell enzyme. The capacity of phenacetin to inhibit NADH - methaemoglobin reductase *in vitro*, had a bearing with the concentration and its structural peculiarities.

This mode of inhibition which was effected by the displacement of NADH by phenacetin reduced that probability of the natural cosubstrate to bind to the red cell enzyme as the concentration of phenacetin increased progressively. This decrease in the activity of NADH - methaemoglobin reductase elicited by phenacetin can be reversed by increasing the concentration of the cosubstrate. Thus, metabolic substrates for the generation of NADH+ H^+ could relieve this inhibitory action of phenacetin on the red cell enzyme. Also, antioxidants and scavengers of free radicals and superoxides can serve as agents to reduce the oxidant levels of the red cells and a corresponding decline in NADH - methaemoglobin reductase activity as observed especially in HbSS erythrocytes. Example of such agents are a - tocopherol, ascorbic acid, mendione, methyleneblue, glutathione and other sulphydryl group containing molecules (Jaffe and Neuman, 1985).

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