Impaired production of nitric oxide, superoxide, and hydrogen peroxide in glucose 6-phosphate-dehydrogenase-deficient granulocytes

Kan-Jen Tsai[1,a], Iou-Jih Hung[b], Ching Kwong Chow[c], Arnold Stern[d], Sin Sin Chao[a], Daniel Tsun-Yee Chiu[a,*]

[a] *Graduate Institute of Basic Medical Sciences and School of Medical Technology, Chang Gung University, 259 Wen-Hua 1st Road, Kwei-san, Tao-yuan, Taiwan*

[b] *Department of Hematology, Chang Gung Children's Hospital, Kwei-san, Tao-yuan, Taiwan*

[c] *Department of Nutrition and Food Science, University of Kentucky, Lexington, KY, USA*

[d] *Department of Pharmacology, New York University Medical Center, New York, NY, USA*

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Abstract Since the generation of superoxide and hydrogen peroxide by NADPH oxidase and nitric oxide (NO) by NO synthase (NOS) in granulocytes is NADPH-dependent, we investigated the production of NO, superoxide and H_2O_2 in glucose 6-phosphate dehydrogenase (G6PD)-deficient human granulocytes. Our results showed that upon stimulation with either 5 μg/ml of lipopolysaccharide (LPS) or 10 μM of phorbol 12-myristate 13-acetate (PMA), the production of nitrite in normal granulocytes was elevated, 252 ± 135% and 239 ± 72%, respectively, compared to the resting stage. In contrast, G6PD-deficient granulocytes did not produce more nitrite upon stimulation with either LPS or PMA compared to the resting stage. Western blot analysis indicated a normal expression pattern of inducible NOS in G6PD-deficient granulocytes. In addition, the production of H_2O_2 and superoxide was also significantly impaired in G6PD-deficient granulocytes compared to control cells. These data demonstrate that G6PD deficiency causes an impairment in the production of NO, superoxide and H_2O_2.

Key words: G6PD deficiency; Nitric oxide production; Superoxide production; Granulocyte

1. Introduction

Glucose 6-phosphate dehydrogenase (G6PD) deficiency is one of the most common enzymopathies affecting over 200 million people world-wide [1,2]. The deficiency predisposes subjects to neonatal jaundice, drug or infection mediated hemolytic crisis, favism and, less commonly, to chronic non-spherocytic hemolytic anemia [3,4]. Since its discovery, many advances have been made in the field of G6PD research, but the advances have been unbalanced. The molecular biology of G6PD deficiency has largely been elucidated [5–10]. In contrast, the pathophysiology of G6PD deficiency, particularly the chronic effects of G6PD deficiency, is far from completely understood.

Since the major biochemical function of G6PD in human cells is to generate NADPH, the clinical relevance of G6PD deficiency may well be related to the changes in redox status in G6PD-deficient cells. Such a disturbance in redox status can affect certain cellular processes leading to clinical manifestations. For example, neutrophil dysfunction and increased susceptibility to infection have been reported in severe G6PD deficiency [11,12]. The detailed mechanism of such dysfunction has not been fully elucidated. Nitric oxide (NO) generated from L-arginine by the inducible NO synthase (iNOS) is regarded as a defense effector molecule with cytotoxic/cytostatic and microbicidal/microstatic activity. The major outer membrane component of Gram-negative bacteria, lipopolysaccharide (LPS), is a potent activator of macrophage responses involved in the host defense against infection [13]. Since the generation of NO by NOS and other reactive oxygen species (ROS) by NADPH oxidase in human cells is NADPH-dependent [11–13], we investigated the production of NO and ROS by G6PD-deficient granulocytes and normal controls after the addition of LPS or phorbol myristate acetate (PMA) as a stimulant.

2. Materials and methods

2.1. Samples and other materials

After obtaining informed consent, blood specimens were collected in tubes containing anticoagulant from normal and G6PD-deficient individuals. G6PD-deficient individuals selected in this study all had the Taiwan-Hakka variant ($G6PD^{1376T}$) [8,9]. This means that they have the same molecular defect with a mutation of G to T in the cDNA at bp 1376 of their G6PD gene. A blood specimen from a patient with chronic granulomatous disease (CGD), a hereditary defect in NADPH oxidase, was also obtained for comparison. Unless stated otherwise, all biochemicals were purchased from Sigma (St. Louis, MO, USA). L-[2,3,4,5-^{3}H]Arginine hydrochloride was obtained from Amersham International (Amersham, Bucks., UK).

2.2. Isolation of granulocytes

To separate granulocytes, 3.5 ml of Histopaque-1119 (Sigma) was added to 5 ml of whole blood in a 15 ml centrifuge tube and the mixture was centrifuged at 700×*g* for 25 min. The middle layer containing mostly granulocytes was collected. The granulocytes were washed twice with phosphate buffered saline (PBS), pH 7.4, prior to subsequent experiments.

2.3. G6PD activity

The G6PD activity in fresh blood cells was quantitatively measured using a No. 345-B kit supplied by Sigma as previously described [9].

2.4. Measurement of nitrite production as an assay of NO synthesis

Nitrite production was measured in the supernatant of granulocytes by the Griess reaction [14]. Briefly, 10^7 granulocytes cultured in phenol red-free DMEM were stimulated with various concentrations of LPS or PMA for 24 h. The medium was centrifuged and aliquots of the supernatant were mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylenediamine dihydrochloride/2.5% H_3PO_4) and then incubated at room temperature for 10 min. The

*Corresponding author. Fax: (886) (3) 328-3031.
E-mail: dtychiu@mail.cgu.edu.tw

[1] Present address: School of Medical Technology, Chung Shan Medical College, Tai-Chung, Taiwan.

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nitrite concentration in the medium was determined with sodium nitrite as standard. Absorbance spectroscopy of the samples was performed at 550 nm using a Multiskan II plate reader (Titertek).

2.5. Western blot analysis of iNOS

Granulocytes grown in 35 mm dishes were incubated with LPS for 24 h in DMEM containing serum. Cells were washed twice with ice-cold PBS, and lysed in 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin). The cells were then sonicated for 5 min and centrifuged at $14\,000 \times g$ at 4°C for 10 min. The supernatant was collected and protein content determined using a Bio-Rad kit. Cell lysate was mixed with Laemmli buffer and boiled for 5 min. Electrophoresis was carried out by adding equal amounts of protein (60 μg/lane) to a 7.5% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane and incubated with blocking solution (5% fat-free milk in PBS with 0.1% Tween 20, PBST) at 4°C for 2 h. The membranes were then incubated with purified polyclonal rabbit IgG antibody against murine iNOS at 1:500 dilution in the above solution overnight at 4°C. Blots were washed three times in PBST. Blots were further incubated for 1 h with the goat anti-rabbit IgG antibody conjugated to horseradish peroxidase at 1:2500 dilution in PBST. The enhanced chemiluminescence kit was used for detection and exposed to hyperfilm MP.

2.6. Measurement of hydrogen peroxide and superoxide produced by granulocytes using a fluorescent method

This method utilized the conversion of non-fluorescent 4-hydroxyphenylacetic acid (4-HPAA) into fluorescent 6,6′-dihydroxy-(1,1′-biphenyl)-3,3′-diacetic acid in the presence of horseradish peroxidase and H_2O_2. Since superoxide radicals could be quickly converted into H_2O_2 by excess superoxide dismutase (SOD), one could measure only H_2O_2 or both H_2O_2 and superoxide by not adding SOD or adding excess SOD, respectively. To measure the generation of H_2O_2 and superoxide, a modified protocol of Hyslop and Sklar [15] was employed: to 1 ml of granulocyte suspension containing approximately 3×10^5 granulocytes, 100 μl of PMA (0.1 μg/ml), 40 μl of NaN_3 (5 mM), and 40 μl of horseradish peroxidase (400 IU/ml) were added with or without 40 μl of SOD (1000 IU/ml). This reaction mixture was preincubated in a 37°C water bath with constant shaking for 5 min and 40 μl of 4-HPAA (50 mM) was then added, vortexed and incubated with constant shaking in a 37°C water bath. At appropriate time intervals, 250 μl of the reaction mixture were taken into a micro-centrifuge tube and centrifuged at 12 000 rpm for 5 min. After centrifugation, 200 μl of the supernatant was pipetted into a test tube containing 0.5 ml of 60 mM potassium phosphate buffer, pH 7.4 and incubated in the dark at 37°C with constant shaking. After incubating for 30 min, 1.5 ml of chilled 2 mM KCN in 0.1 M Na_2CO_3 was added to the supernatant. Fluorescence representing the amount of H_2O_2 and superoxide was then measured with excitation at 318 nm and emission at 405 nm using a Perkin-Elmer MPF-3 spectrofluorometer.

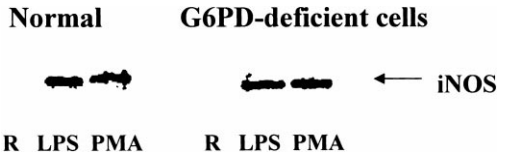

Fig. 1. LPS-induced iNOS protein expression in granulocytes. Normal (left panel) and G6PD-deficient cells were incubated with LPS (5 μg/ml) for 24 h. Cells were harvested at 24 h for Western blot analysis. This is a representative assay from two separate experiments.

3. Results

3.1. G6PD activity in normal and G6PD-deficient blood cells

All G6PD-deficient individuals carried the Taiwan-Hakka variant ($G6PD^{1376T}$) as confirmed by molecular analysis as previously described [8,9]. This means that they all had the same molecular defect with a mutation of G to T in cDNA 1376 of their G6PD gene. As shown in Table 1, G6PD activity in red blood cells (RBCs) of these G6PD-deficient individuals was not totally absent but much lower (28.0 ± 1.4 U/10^{12} cells) than that in normal RBCs (213.5 ± 6.4 U/10^{12} cells) and these data were similar to those reported previously [11]. G6PD activity of granulocytes from G6PD-deficient individuals ($14\,491 \pm 2345$ U/10^{12} cells) was also significantly ($P < 0.001$) lower than that found in normal granulocytes ($42\,529 \pm 2162$ U/10^{12} cells) but the degree of deficiency was not as severe as in RBCs.

3.2. NO production

Having reconfirmed the much lower G6PD activity in granulocytes of the G6PD-deficient individuals, we checked NO production in the granulocytes of these G6PD-deficient individuals. The production of NO in normal granulocytes was increased greatly upon stimulation with either LPS or PMA as seen in Table 2 where with 5 μg/ml of LPS or 10 μM of PMA, the production of NO in normal granulocytes was shown to increase by 252 ± 135% and 239 ± 72% of the resting stage, respectively. In contrast, the production of NO in G6PD-deficient granulocytes upon stimulation with LPS (5 μg/ml) or PMA (10 μM) was only 70 ± 26% and 76 ± 25% of the resting stage, respectively (Table 2).

Table 1
G6PD activity in normal and G6PD-deficient blood cells

Sample	G6PD activity in WBCs (U/10^{12} cells)	G6PD activity in RBCs (U/10^{12} cells)
Normal ($n = 4$)	$42\,529 \pm 2162$	213.5 ± 6.4
G6PD-deficient ($n = 3$)	$14\,491 \pm 2345$	28.0 ± 1.4

The results are shown as mean ± S.D.

Table 2
Nitrite production and NOS activity in LPS- or PMA-stimulated normal and G6PD-deficient human granulocytes

Sample	Nitrite production (% of resting)		NOS activity (% of resting)	
	LPS (5 μg/ml)	PMA (10 μM)	LPS (5 μg/ml)	PMA (10 μM)
Normal ($n = 4$)	252 ± 135	239 ± 72	328 ± 23	545 ± 79
G6PD-deficient ($n = 3$)	70 ± 26	76 ± 25	130 ± 37	86 ± 29

The results are shown as mean ± S.D.

Table 3
Generation of H_2O_2 and superoxide in PMA-stimulated[a] normal and G6PD-deficient human granulocytes

Sample	H_2O_2 generation (nmol/10^5 granulocytes)		Superoxide generation (nmol/10^5 granulocytes)	
	−PMA	+PMA	−PMA	+PMA
Control ($n=12$)	UD[b]	2.26 ± 0.96	0.13 ± 0.11	2.44 ± 0.36
G6PD-deficient ($n=4$)	UD	0.81 ± 0.92	0.16 ± 0.23	0.90 ± 0.53
CGD ($n=1$)	UD	UD	UD	UD

The results are shown as mean ± S.D.
[a]PMA stimulation was carried out for 15 min.
[b]UD: undetectable.

3.3. Western blot analysis of NOS

To determine whether the impaired NO production is due to inadequate expression of NOS upon stimulation by LPS or PMA, we performed Western blot analysis of NOS. Our data clearly indicate that G6PD-deficient granulocytes had normal expression of NOS upon stimulation (Fig. 1). In response to LPS (5 μg/ml) or PMA (10 μM), an inducible Ca^{+2}-independent 130 kDa NOS was produced in the normal granulocytes. Likewise, G6PD-deficient granulocytes also produced a similar amount of this Ca^{+2}-independent 130 kDa NOS upon stimulation with LPS (5 μg/ml) or PMA (10 μM).

3.4. Generation of hydrogen peroxide and superoxide induced by PMA

As shown above, the generation of NO, an NADPH-dependent reaction, was severely affected in G6PD-deficient granulocytes. We checked whether the production of H_2O_2 and superoxide, which is also dependent on NADPH, was affected by G6PD deficiency. After 15 min of stimulation, normal granulocytes produced 2.256 ± 0.955 nmol of H_2O_2 and 2.440 ± 0.362 nmol of superoxide per 10^5 cells, whereas G6PD-deficient granulocytes produced significantly less ($P < 0.05$): only 0.805 ± 0.924 nmol of H_2O_2 and 0.902 ± 0.525 nmol of superoxide per 10^5 cells (Table 3). For comparison, we also examined the production of H_2O_2 and superoxide in granulocytes from a patient with CGD. The granulocytes in this patient had no functional NADPH oxidase and hence produced no detectable H_2O_2 and superoxide (Table 3). With longer PMA induction times (30 min and 60 min), both normal and G6PD-deficient granulocytes produced more H_2O_2 and superoxide, but the increases in G6PD-deficient granulocytes were again substantially lower than in normal granulocytes (Figs. 2 and 3). Granulocytes from the patient with CGD produced virtually no detectable H_2O_2 and superoxide at any PMA induction time point (Figs. 2 and 3).

4. Discussion

We demonstrate here that G6PD-deficient granulocytes, as in RBCs, have lower G6PD activity. This decreased G6PD activity in granulocytes apparently leads to the impaired production of nitric oxide as well as H_2O_2 and superoxide upon stimulation by LPS or PMA. As far as we know, this is the first report on impaired NO production in G6PD-deficient cells.

As is known, lower G6PD activity in RBCs is a hallmark of G6PD deficiency. However, very little information is available concerning the activity of this enzyme in white blood cells (WBCs) with different G6PD variants. In view of the low G6PD activity in RBCs, it is natural to expect a low G6PD activity in WBCs. It is interesting to note, therefore, that granulocytes from individuals with G6PD^{1376T} maintain one third of the normal granulocyte G6PD activity level, while the RBCs from these same individuals have only 12% of normal RBC G6PD activity (Table 1). These findings concerning G6PD activity in G6PD-deficient granulocytes and RBCs are similar to those reported for an African G6PD variant, Gd(minus) Matam [16]. This difference in G6PD activity between granulocytes and RBCs may explain, in part, why acute clinical problems are largely related to RBCs in G6PD deficiency.

Through its participation in the pentose phosphate shunt,

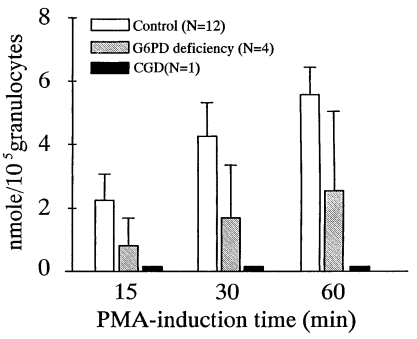

Fig. 2. PMA-induced H_2O_2 production in granulocytes. H_2O_2 production by normal, G6PD-deficient or CGD granulocytes was measured using a fluorescence method. Standard deviations are shown as bars in each set of experiments. No standard deviation is shown for the CGD granulocytes because only one case is reported in our study.

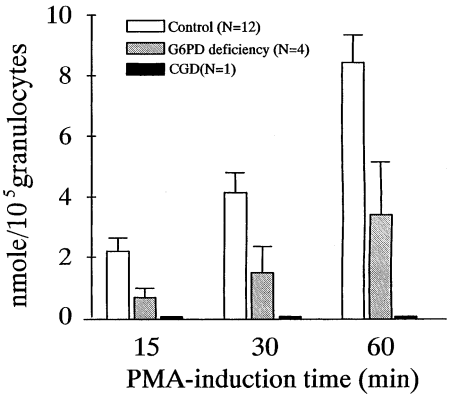

Fig. 3. PMA-induced superoxide production in granulocytes. Superoxide production by normal, G6PD-deficient or CGD granulocytes was measured using a fluorescence method. Mean and standard deviation are shown for each set of experiment. No standard deviation is shown for the CGD granulocytes because only one case is reported in our study.

G6PD provides a constant source of NADPH as reducing equivalents that are essential to a variety of metabolic functions. This is highlighted by the fact that NADPH is a cofactor for NOS activity. It has been observed that G6PD and NOS co-localize in brush cells of the gastric and pancreatic duct epithelial cells [17]. An increase in NO synthesis was found to parallel an increase in G6PD activity in activated bone marrow macrophages [18]. This finding could be of relevance to our observation that G6PD-deficient cells undergo no change in NO production upon stimulation by LPS or PMA.

Our finding that G6PD-deficient granulocytes produce far less NO than normal granulocytes upon stimulation with either LPS or PMA (Table 2) may have pathophysiologic implications in G6PD deficiency. NO can react with superoxide to form peroxynitrite [19], which is an important bactericidal agent during phagocytosis [20]. In fact, a study using activated human neutrophils indicates that the kinetics of NO and superoxide release favor the formation of peroxynitrite [20]. An impaired production of NO may render the production of peroxynitrite in neutrophils abnormal leading to defective microbicidal action. This finding may help to explain the earlier observations that severe G6PD deficiency can exhibit neutrophil dysfunction and increased susceptibility to infection [11,12]. In addition, NO is known to play very important roles in the cardiovascular system, including regulation of basal blood pressure, inhibition of smooth muscle contraction, inhibition of platelet aggregation and many others [13]. Hence, impairment in the production of NO under a stress condition in G6PD-deficient cells may render G6PD-deficient individuals more susceptible to certain cardiovascular diseases. A preliminary study from our laboratory indicates that among adult males with high blood pressure, there is a higher incidence (6.1%) of G6PD deficiency than in the general male population (3.0%) in Taiwan (unpublished observation), suggesting that G6PD deficiency may predispose affected individuals to high blood pressure. Whether G6PD deficiency also causes an impaired production of NO in endothelial cells leading to high blood pressure remains to be established.

In addition to decreased NO production, G6PD-deficient granulocytes also exhibit an impairment in the generation of H_2O_2 and superoxide upon stimulation with PMA (Table 3). The decreased superoxide production upon stimulation is similar to that reported by Pascale et al. [21] who found that polymorphonuclear leukocytes (PMNs) from individuals carrying the Mediterranean variant of G6PD exhibit a 58% decrease in superoxide formation upon stimulation by 12-*O*-tetradecanoyl phorbol 13-acetate as compared to normal PMNs. H_2O_2 was not monitored in their study. It should be pointed out that both the Mediterranean variant and the Taiwan-Hakka variant are class II variants as defined by the World Health Organization [22]. Since these G6PD-deficient granulocytes still maintain more than 30% of the normal amount of G6PD activity (Table 1), the reduction in H_2O_2 and superoxide production is not as severe as that in CGD granulocytes, where no detectable H_2O_2 and superoxide production was observed (Figs. 2 and 3). Nevertheless, the decrease in generation of H_2O_2 and superoxide by G6PD-deficient WBCs may help to further clarify the earlier observations that severe G6PD deficiency is associated with neutrophil dysfunction and increased susceptibility to infection [11,12], because these ROS are important microbicidal agents in phagocytes.

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