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Original Research

Dual effect of glucose on macrophage NADPH oxidase activity: a possible link between diabetes and tuberculosis

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Abstract

Glucose is known to stimulate NADPH oxidase, an enzyme responsible for respiratory burst and intracellular killing of pathogens; but hyperglycemic state is associated with enhanced infections by invading microorganisms. In the present work we have examined the effect of glucose on macrophage NADPH oxidase activity. THP-1 cells and monocytes obtained from normal human subjects are incubated in different concentrations of glucose for a given period before and after conversion into macrophages by PMA treatment. Macrophage NADPH oxidase activity is measured and glycation of the membrane fraction is estimated in both the cases. The derived macrophages show enhanced glycation and significantly less enzyme activity when it is incubated in glucose for four days but the enzyme activity increases significantly when the macrophage precursor cells are incubated in high concentrations of glucose. This is supported by cell free experiment.

INTRODUCTION

The association between diabetes mellitus and tuberculosis is a well known fact. Epidemiological research has proved strong positive association between occurrence of highly glycated hemoglobin (a marker of protein glycation and chronicity of diabetes) and pulmonary tuberculosis [1]. It is also proved that glucose stimulates NADPH oxidase activity of phagocytic cells which contributes to oxidative stress in diabetes by release of superoxide in the circulation [2-5]. This enzyme is responsible for killing of the intracellular pathogens by generation of superoxide.

In diabetic animal model, the peritoneal macrophages are reported to show enhanced NADPH oxidase activity as compared to the normal controls [6]. Therefore it is expected that in diabetic state; more intracellular pathogens will be killed by glucose-induced enhanced NADPH oxidase activity of the phagocytic cells, as NADPH oxidase activity is directly proportional to the killing of intracellular bacteria via generation of superoxide inside the phagosomes. On the contrary, diabetes mellitus is well known to be associated with increased infections in general, and tuberculosis in particular. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, resides within the phagocytic cell (particularly macrophages) in the course of pathogenesis. If in the hyperglycemic state, NADPH-oxidase activity is enhanced universally, the increased association of diabetes and tuberculosis is a paradox. This problem is addressed recently by Banerjee *et al* [8]. They have found by literature search and by *in silico* analysis that there is a chance of glycation induced inhibition of macrophage NADPH-oxidase activity that may account for the increased association of tuberculosis in a diabetic state. However, such hypothesis has not been experimentally verified so far. In this work, we have shown the dual effect of glucose on monocyte and macrophage NADPH oxidase activity that may account for increased association of

enhanced occurrence of tuberculosis in diabetic state, in-spite of hyperglycemia induced stimulation of phagocytic cell NADPH oxidase activity.

MATERIALS AND METHODS

Chemicals and supplies

Nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome C, flavin adenine dinucleotide (FAD), adenosin triphosphat (ATP), guanosine gamma thio-phosphate (GTPγS), ethylene glycol tetraacetic acid (EGTA), sodium pyruvate, trypsin, sucrose, glucose-free RPMI-1640 media, leupeptin, micro-cyctin, chymostatin, 4-(2-aminoethyl)-benzylsulphonyl flluoride, phenylmethylsulfonyl fluoride (PMSF), and superoxide dismutase (SOD; human RBC) were obtained from Sigma. Phorbol myristate acetate (PMA) and Hank's Buffered Salt Solution (HBSS) were purchased from Hi-Media. All other reagents used in the study were of analytical grade.

Cell culture and treatment (1st and 2nd experiments)

THP-1 cells (human acute monocytic leukemia cell line) were obtained from National Centre for Cell Sciences (NCCS, Pune, India). They were sub-cultured in glucose-free RPMI-1640 supplemented with 10% FBS (fetal bovine serum) and 5 mM glucose and 15 mM mannitol at 37ºC and 5% CO_2 [5]. They were used between 3rd and 6th passages with 90% confluency for all the experiments. Two sets of experiments were designed:

In the first set, the cells were cultured (1 x 10^6 cells/ml) in varying concentrations of glucose: 5 mM glucose and 15 mM mannitol, 10 mM glucose, 15 mM glucose, and 20 mM glucose in different wells for three days. Glucose was added daily along with fresh media to maintain a gradual high concentration of glucose in the different wells. The wells which were containing 5 mM glucose, *i.e.* glucose at physiological concentration were always supplemented with 15 mM mannitol to serve the purpose of osmotic control. After three days incubation PMA was added in all the wells to reach a final concentration of 20mM and the cells were incubated with PMA for 18 hours. PMA is the key activator that converts THP-1 cells to macrophages forming a monolayer over the well surface. The monolayer was washed twice with HBSS and then trypsinized to form a macrophage suspension [12]. Macrophages were subsequently washed with HBSA, supplemented with 5 mM EDTA and finally with HBE buffer containing 250 mM sucrose/0.5 mM EGTA/20 mM HEPES/KOH (pH 7.2) by centrifugation (300g for 3 min) [13]. Subsequently membrane and cytosolic fraction of these macrophages were isolated.

For the second set of experiments, THP-1 cells (1 10^6 cells/ml) maintained in different wells in glucose-free RPMI-1640 media supplemented with 10% heat inactivated FBS at 37ºC and 5% CO_2 atmosphere containing 5 mM glucose and 15 mM mannitol; they were converted to macrophages by addition of 20 nM PMA and incubating it for 18 h. After that various concentrations of glucose were added to different wells daily for four days so that the macrophages were exposed to various glucose concentrations for longer duration. The concentrations were similar to the first set of experiment. After the 4th day, the monolayer was washed, trypsinized and cell wash was done as before. Membrane and cytosolic fractions of these macrophages were isolated.

Blood from healthy human volunteers was drawn into heparinized tubes and diluted 3:5 with sterile phosphate-buffered saline (PBS) at 37ºC before being layered onto a ficoll-plaque gradient. Gradients were centrifuged at 1,400g for 30 min. The buffy coat was removed carefully and washed twice in sterile PBS (phosphate buffered saline) and once in glucose-free RPMI-1640 media. Monocytes were isolated [14]. These monocytes were subcultered in glucose free RPMI as THP-1 cells were sub cultured and first and second set of experiments were repeated as before.

Membrane and cytosolic fraction isolation and NADPH oxidase activity assay

Macrophages were resuspended in 2 ml relaxation buffer and disrupted by sonication in ice. The sonicates were centrifuged at 1,300g for 10 min at 4ºC. The supernatant layers on a discontinuous sucrose gradient and were centrifuged for separation and isolation of the membrane and cytosolic fractions of macrophages were done [15]. Mixtures containing 50 mM Kpi (pH 7.0), 100 μM FAD, 1 mM EGTA, 15 μM $MgCl_2$, 1 μM GTPγS, a 1:25 ration of membrane (1.5 μg) to cytosolic fraction (37.5 μg) and 100 μM SDS are incubated at 25ºC for 30 min followed by addition of 200 μM NADPH. The reaction was stopped at 2 min by adding 1% (vol/vol) Triton-X. Absorbance of cytochrome C was read at 550 nm and corrected for background observed in the presence of SOD. NADPH oxidase activity was expressed as μmol of superoxide per minute per mg of membrane protein [16].

Membrane fraction/cytosolic fraction incubation (cell free) experiment

The remaining membrane fraction and cytosolic fractions which were incubated with 5 mM glucose in the first set of experiments were pooled in the ratio of 1:25 membrane protein and cytosolic protein. These were incubated in HBE buffer supplemented with varying concentrations of 5 mM, 10 mM, 15 mM, and 20 mM in different aliquots in the presence and absence of 10 mM sodium pyruvate. The final mixture contained 2 mg/ml of protein. The incubation was

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continued for 72 h followed by activity measurement of the NADPH oxidase method.

Protein estimation was done by the bicinchoninic acid assay [17]. Glycation percentage of the proteins in the membrane fractions were assayed by the established procedure [18] after precipitating the membrane proteins by chilled ethanol [19]. All values are expressed as mean ± standard deviation of six separate observations (n = 6) and Student *t*-test was utilized to analyze the results; a 'p' value less than 0.05 was accepted as significant.

RESULTS

Effect of glucose on macrophage NADPH oxidase activity after incubating macrophage precursor cells in glucose

Circulating monocytes are the precursors of tissue macrophages [20] and its NADPH oxidase gene expression is known to be increased under high glucose conditions [3, 21]. Here, we examined the effect of glucose on macrophage NADPH oxidase enzyme activity after incubating monocytes and THP-1 cells, a standard monocytic cell line [22], in various concentrations of glucose followed by conversion to macrophages by PMA treatment. The results are present in Fig.1. It is observed that when monocytes and THP-1 cells are incubated with increasing concentrations of glucose, the macrophages derived from them showed enhanced NADPH oxidase activity. The enzyme activity increased significantly ($p < 0.05$) with increasing concentrations of glucose in a dose dependent manner.

Effect of glucose on macrophage NADPH oxidase activity after incubating macrophage cells in glucose.

Here, the macrophage precursor cells are maintained at physiological concentration of glucose and subsequently converted to macrophages by PMA treatment. Then the derived macrophages from its precursor cells are incubated in different concentrations of glucose for four days and its NADPH oxidase activity is estimated. The results are presented in Fig .2. It is observed that macrophages derived from both the sources show significant ($p < 0.05$) decrease in the enzyme activity with the increasing concentrations of glucose in a dose dependent manner.

Analysis of macrophage membrane fraction glycation

Glycation is known to inhibit many enzyme activities [23]. After PKC activation, the cytosolic components of NADPH oxidase are translocated in the cell membrane of the macrophages and the enzyme gets stimulated [24]. So, the glycation of the membrane fraction of the macrophages is estimated and the results are presented in Fig.3. In one set of experiments the

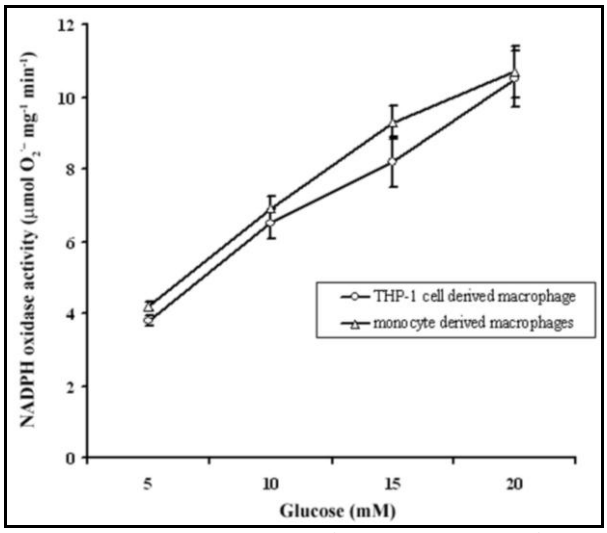

Figure 1. NADPH oxidase activity of macrophages derived from its precursor THP-1 cell or monocytes maintained at varying concentration of glucose are plotted. The enzyme activity of the macrophages increases significantly with the increasing incubation concentration of its precursor cells monocytes and THP-1 cells in *in vitro* cell culture system. The NADPH oxidase activity values of macrophages derived from THP-1 cell are represented as circles and the enzyme activity values of macrophages derived from monocytes isolated from normal human subjects are represented as triangles .The values are represented as mean ± standard deviation of six independent observations.

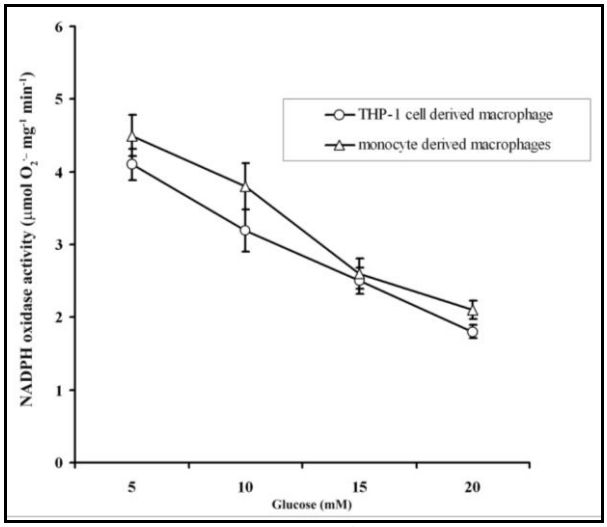

Figure 2. NADPH oxidase activities of macrophages maintained at varying concentrations of glucose which are derived from its precursors (monocytes and THP-1 cell) cultured at physiological concentration of glucose are plotted. The enzyme activity of the macrophages decreases significantly with increasing incubation concentration of glucose. The NADPH oxidase activity values of macrophages derived from THP-1 cell are represented as circles and the enzyme activity values of macrophages derived from monocytes isolated from normal human subjects are represented as triangles The values are represented as mean ± standard deviation of six independent observations.

macrophage precursor cells are incubated in various concentrations of glucose and after that they are converted into macrophages. In another set of experiments the macrophages are incubated in various concentrations of glucose. Here the macrophage precursor cells are maintained at physiological concentration of glucose before conversion to macrophages. In both the cases the macrophage membrane fraction glycation increased with the increasing concentrations of glucose. The increase in glycation in the membrane fraction is much more when the macrophages itself are incubated in different fractions of glucose which are obtained from THP-1 cells and monocytes isolated from healthy human subjects maintained at physiological concentration of glucose.

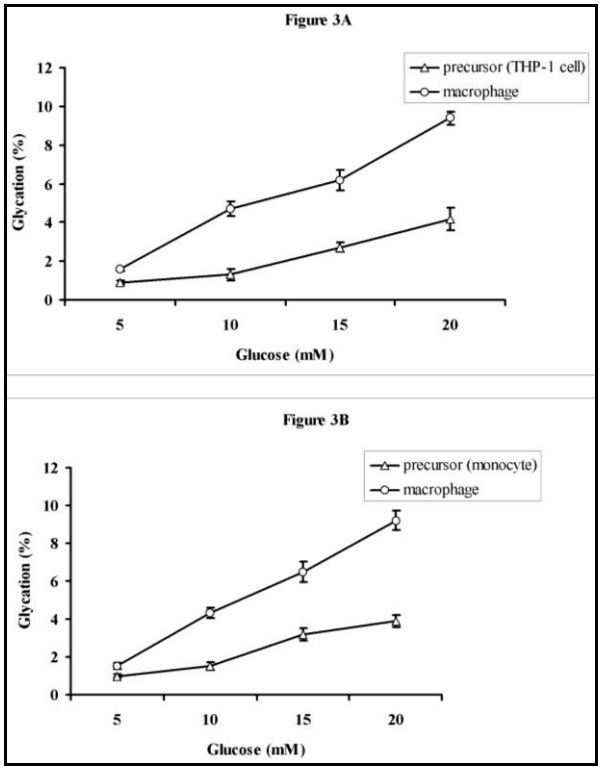

Figure 3. Membrane fraction glycation of macrophages. **3A:** THP-1 cell derived macrophage values. The values are represented by triangles for the experiments where THP-1 cells are incubated in varying concentrations of glucose and then converted to macrophages by PMA treatment followed by membrane fraction isolation and analysis of the glycation % of the macrophage membrane proteins. The values are represented by circles for the experiments where THP-1 cells maintained at physiological concentration of glucose, converted to macrophages by PMA treatment, and then incubated at varying concentrations of glucose followed by membrane fraction isolation and analysis of glycation % of the macrophage membrane proteins. **3B:** monocytes isolated from normal human subject derived macrophage values. Here similar experiments are performed as in 3A, only replacing THP-1 cells with monocytes isolated from normal human subjects. The values are represented by triangles for the experiments where monocytes are incubated in varying concentrations of glucose and then converted to macrophages by PMA treatment followed by membrane fraction isolation and analysis of the glycation % of the macrophage membrane proteins. The values are represented by circles for the experiments where monocytes isolated from normal human subjects are maintained at physiological concentration of glucose and converted to macrophages by PMA treatment and then incubated at varying concentrations of glucose followed by membrane fraction isolation and analysis of glycation % of the macrophage membrane proteins.
Percentage of glycation of macrophage membrane proteins are estimated by determination of concentration of glycated membrane protein with respect to concentration of total protein in the membrane fraction. The values are represented as mean ± standard deviation of six independent observations.

Effect of glucose on NADPH oxidase activity after incubating macrophage membrane and cytosolic fraction in various concentrations of glucose in a cell-free system in presence and absence of sodium pyruvate (glycation inhibitor)

Pyruvate is known agent to prevent glycation [25]. Here, in the cell-free system we checked the NADPH oxidase activity after incubating macrophage (obtained from THP-1 cells) derived membrane and cytosolic fractions in the increasing glucose concentrations, in the presence and absence of pyruvate. The NADPH oxidase activity decreased significantly ($p < 0.05$) with increasing concentrations of glucose in a dose dependent manner. The enzyme activity reduction in increasing concentration of glucose is significantly prevented in the presence of pyruvate. There is also significant protein glycation in the cell free system with increasing concentrations of glucose which is also prevented in the presence of pyruvate (Fig.4).

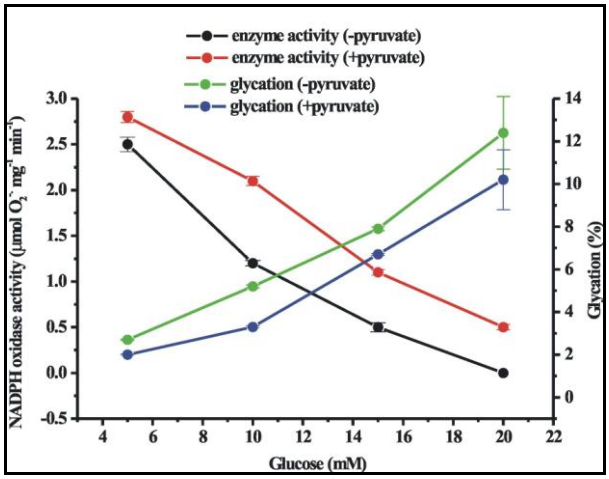

Figure 4. Graphical representation of NADPH oxidase activity in a cell free system obtained from macrophages when the cytosolic and membrane fraction of macrophages are incubated in different glucose concentrations in presence and absence of pyruvate (known glycation inhibitor). The black and red lines indicate the enzyme activity in absence and presence of pyruvate respectively in different glucose concentrations. Similarly, the green and blue lines indicate glycation percentage of membrane fraction in respect of concentration of total protein in membrane fraction in absence and presence of pyruvate respectively in different glucose concentrations.

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DISCUSSION

NADPH oxidase activity is an important determinant of killing of intracellular pathogens [7] and in congenital deficiency of NADPH oxidase activity, intracellular infections are more common [8-11]. With reference to the above mentioned facts the paradoxical relation of occurrence of enhanced tuberculosis in diabetic state [8] requires further study. In this context the observations recorded here are significant.

Previously, we have shown by *in silico* analysis that there may be glycation induced inhibition of NADPH oxidase activity [8]. In this work, we have conclusively shown that glycation inhibits NADPH oxidase activity. Particularly the macrophage NADPH oxidase activity is observed to be reduced as a function of glucose concentration available in the microenvironment of macrophages for long duration. At the same time dose dependent significant increase of glycation of macrophage membrane fraction is observed. However, when the macrophage precursor cells are incubated with glucose for the same duration it is observed that there is a dose dependent increment of macrophage NADPH oxidase activity just after conversion of monocytes (or THP-1cells) to macrophages. Here the macrophage membrane fraction glycation is comparatively less than the experiment where macrophages itself are incubated in various concentrations of glucose. This definitely explains why a chronic diabetic patient is more susceptible to tuberculosis in spite of hyperglycemia induced stimulation of NADPH oxidase activity of some phagocytic cells.

A question arises that why the glycation of macrophage membrane fraction is different in the two sets of experiment. This possibility is also addressed earlier [8]. Briefly, the macrophage and its precursor are different cells with obvious different properties [20, 26]. It is also conclusively known that mature macrophages concentrate more glucose compared to its precursor cells probably due to slower rate of glucose metabolism [27]. Consequently macrophages express more glucose transporters compared to its precursor monocytes [28]. Therefore in a microenvironment where glucose concentration is high, macrophages are expected to concentrate more glucose compared to the precursor monocytes. This explains the enhanced glycation of macrophage membrane fraction proteins in the first case compared to the second case as a function of concentration of glucose in the microenvironment of macrophage (and not its precursor) for long duration. It is needless to emphasize that it is impossible for NADPH oxidase to escape such possibility of glycation since the various subunits of NADPH oxidase are primarily membrane bound proteins [28, 29].

Glycation is a possible mechanism of NADPH oxidase inhibition which is proved by the results observed in the cell-free experiments which demonstrated that there is a glucose concentration dependent glycation of macrophage proteins in a cell-free system and corresponding inhibition of NADPH oxidase activity. The above view is attested by the observation that an established glycation inhibitor prevented the glycation of proteins in the cell free experiment and also preserved the enzyme activity.

One thing is clear from the above experiments that there is enough possibility of glycation induced inhibition of NADPH oxidase activity and exposure of the macrophage to increased concentrations of glucose for long duration causes significant glycation of the protein sufficient to inhibit the enzyme activity, but exposure of macrophage precursor cells like THP-1 cells or monocytes to glucose just before conversion to macrophages cause increase in macrophage NADPH oxidase activity and, in this case, the glycation of membrane fraction is comparatively less. So at hyperglycemic state of a diabetic individual the newly formed macrophages may have enhanced NADPH oxidase activity but with time in presence of persistent hyperglycemia there may be enhanced glycation induced inhibition of the enzyme activity and thus explaining the more incidences of infections by pathogens residing inside macrophages in chronic diabetic state. The above view is supported by previously published *in vivo* experiments in mice model of streptozotocin induced diabetes demonstrating decreased peritoneal macrophage NADPH oxidase activity after 12 weeks of hyperglycemic state but enhanced macrophage NADPH oxidase activity in earlier phase of diabetes [8].

It is expected that the dual effect of glucose that is observed in macrophages occurs at hyperglycemic state and cause diabetic complications. Hyperglycemia induced stimulation of monocyte NADPH oxidase activity that remains enhanced just after its conversion to macrophages must be contributing to oxidative stress via secretion of superoxide in circulation. On the other hand, glycation induced inhibition of macrophage NADPH oxidase activity is making the diabetic host susceptible to intracellular infections including tuberculosis. Therefore control of hyperglycemia in diabetic state will rescue the diabetic host from this deleterious dual effect of glucose. We recommend further verification of these results in *in vivo* systems.

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