Elevated Glucose Concentrations Promote Receptor-Independent Activation of Adherent Human Neutrophils: An Experimental and Computational Approach

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ABSTRACT Neutrophil activation plays integral roles in host tissue damage and resistance to infectious diseases. As glucose uptake and NADPH availability are required for reactive oxygen metabolite production by neutrophils, we tested the hypothesis that pathological glucose levels (≥12 mM) are sufficient to activate metabolism and reactive oxygen metabolite production in normal adherent neutrophils. We demonstrate that elevated glucose concentrations increase the neutrophil's metabolic oscillation frequency and hexose monophosphate shunt activity. In parallel, substantially increased rates of NO and superoxide formation were observed. However, these changes were not observed for sorbitol, a nonmetabolizable carbohydrate. Glucose transport appears to be important in this process as phloretin interferes with the glucose-specific receptor-independent activation of neutrophils. However, LY83583, an activator of glucose flux, promoted these changes at 1 mM glucose. The data suggest that at pathophysiologic concentrations, glucose uptake by mass action is sufficient to activate neutrophils, thus circumventing the normal receptor transduction mechanism. To enable us to mechanistically understand these dynamic metabolic changes, mathematical simulations were performed. A model for glycolysis in neutrophils was created. The results indicated that the frequency change in NAD(P)H oscillations can result from the activation of the hexose monophosphate shunt, which competes with glycolysis for glucose-6-phosphate. Experimental confirmation of these simulations was performed by measuring the effect of glucose concentrations on flavoprotein autofluorescence, an indicator of the rate of mitochondrial electron transport. Moreover, after prolonged exposure to elevated glucose levels, neutrophils return to a "nonactivated" phenotype and are refractile to immunologic stimulation. Our findings suggest that pathologic glucose levels promote the transient activation of neutrophils followed by the suppression of cell activity, which may contribute to nonspecific tissue damage and increased susceptibility to infections, respectively.

INTRODUCTION

Glucose is required for the survival, proliferation, and functions of most mammalian cells. In contrast to bacteria, which actively take up glucose to overcome high concentration gradients, mammalian cells take up glucose passively via facilitated diffusion. Human neutrophils, a type of terminally differentiated peripheral blood cell, utilize glucose for the housekeeping functions of glycolysis, and for activation of the hexose monophosphate shunt (HMS), which is necessary to generate large quantities of reactive oxygen metabolites (ROMs). Glucose uptake is required for superoxide anion production by neutrophils (1–3). Glucose is first metabolized by hexokinase to form glucose-6-phosphate, which can enter glycolysis or the HMS. The HMS is the primary source of NADPH, which, in turn, is used by the NADPH oxidase to produce superoxide anions (4). Most of the superoxide goes on to form other ROMs, including H_2O_2, hydroxyl radicals, and HOCl. The production of reactive nitrogen species (RNS) begins with the NO synthase, which also requires NADPH from the HMS. This enzyme produces NO, which, in combination with ROMs, produces peroxynitrite (5). Glucose metabolism leading to the production of ROMs and RNSs is therefore an important factor in host defense against infections, although it may also contribute to the host's collateral tissue damage.

As glucose is taken into cells by facilitated diffusion, heightened extracellular glucose concentrations will increase intracellular levels and may thereby nonspecifically stimulate neutrophil metabolism, and such changes may perturb normal biochemical pathways. For example, the elevated serum glucose levels associated with diabetes could influence neutrophil function. Unfortunately, apparently inconsistent results have been reported. Several reports indicate a significant decrease in the respiratory burst of normal neutrophils during exposure to ≥12 mM (6). On the other hand, others have reported that unstimulated leukocytes from diabetic patients produce enhanced levels oxidants (7). Furthermore, upon stimulation, diabetic neutrophils or diabetic levels of extracellular glucose lead to enhanced levels of superoxide production (8,9). It has also been reported that neutrophils from poorly controlled diabetics exhibit aberrant chemotaxis, bacterial killing, leukotriene production, lysosomal enzyme release, proinflammatory cytokine expression, respiratory burst, and superoxide production (10–27). It would seem that aberrant glucose concentrations

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affect neutrophil function, although a comprehensive model accounting for these divergent observations has not yet emerged.

Because infectious disease is a major contributor to the morbidity and mortality of diabetic patients (28,29) and impaired host defense is likely a key factor (29), a better understanding of the mechanism of glucose-mediated receptor-independent changes in neutrophil function is important. As neutrophils damage tissues and mediate host defense while adherent, we have studied the properties of adherent neutrophils. Nathan (30) has shown that the phenotype of adherent neutrophils differs from that of nonadherent neutrophils: adherent neutrophils generate far more ROMs than their nonadherent counterparts. In this study, we show that elevated glucose levels activate the HMS to promote ROM production by adherent neutrophils. These experimental studies were confirmed by using computational modeling of the underlying biochemical network. A model of neutrophil glycolysis shows that competition for glucose-6-phosphate by the HMS leads to the observed changes in metabolic dynamics and mitochondrial activity. However, this effect was limited, as longer incubation periods led to neutrophil exhaustion, an inability to produce normal levels of ROMs. We suggest that elevated glucose nonspecifically activates human neutrophils, which after prolonged exposure, leads to diminished cell function. This mechanism may contribute to the greater risk of infectious diseases in diabetes as well as greater oxidant stress on normal tissues.

RESEARCH DESIGN AND METHODS

Materials

Hydroethidine (HE), *n*-formyl-methionyl-leucyl-phenylalanine (FMLP), phloretin, and LY83583 were obtained from Sigma Chemical (St. Louis, MO). Anti-Glut1 Ab was obtained from Chemicon International (Temecula, CA). Buffers were purchased from Life Technologies (Rockville, MD) or prepared such that they could be mixed to achieve the desired concentration of glucose.

Neutrophil isolation

Peripheral blood neutrophils were obtained from normal healthy adults using two Ficoll-Hypaque solutions (Histopaque 1077 and 1119, Sigma Chemical) and centrifugation. Cells were washed and resuspended in HBSS, then examined for viability using trypan blue. Viability was found to be >95%.

Hexose monophosphate shunt (HMS) activity

HMS activity was measured using previously described procedures (31–32). Cells ($\sim 2 \times 10^6$) were incubated in a total volume of 0.8 ml in media consisting of either [D-1-^{14}C] or [D-6-^{14}C]-labeled glucose (American Radiolabeled Chemicals, St. Louis, MO) at 0.5 μCi/ml with 1 mM or 14 mM glucose in PBS. The produced $^{14}CO_2$ was captured in a center well containing 0.5ml hyamine hydroxide (Research Products, Mount Prospect, IL) and a strip of filter paper. Incubations were performed in sealed containers at 37°C in a shaking water bath for 4 h. CO_2 was released from the solution by addition of 1 ml of 0.7 N trichloroacetic acid followed by incubation for 1 h. Lastly, 3 ml of scintillation fluid was added to the center well followed by counting.

Microscopy

Cells were observed using an Axiovert fluorescence microscope (Carl Zeiss, New York, NY) with mercury illumination interfaced to a computer using Scion image processing software (33). DIC (differential interference contrast) and fluorescence images were collected as described previously (34,35). A narrow bandpass discriminating filter set (Omega Optical, Brattleboro, VT) was used with excitation at 485/22 nm and emission at 530/30 nm for FITC, and an excitation of 540/20 nm and emission at 590/30 nm for TRITC. Long-pass dichroic mirrors of 510 and 560 nm were used for FITC and TRITC, respectively. TMR was detected using a 540DF20 nm and 590DF30 nm filter set with 560 long-pass dichroic mirror. HE is oxidized to ethidium bromide, a fluorescent molecule, by superoxide. Ethidium bromide is then detected using a 540DF20nm and 590DF30nm filter set with a 560 nm dichroic mirror. The fluorescence images were collected with an intensified charge-coupled device camera (Princeton Instruments, Princeton, NJ).

Microscopy-based oxidant assays

Since DAF-2 DA fluoresces when exposed to NO (but not to peroxide or hydrogen peroxide (36)), 2% gelatin matrices in a fluid phase (at 45°C) were mixed with 15 μM DAF-2 DA then allowed to cool to a semisolid state at 37°C as described (33). For ROM studies, HE was employed at 3 μM in these matrices.

Detection of metabolic dynamics

NAD(P)H autofluorescence oscillations were detected as described previously (35,37). As the autofluorescence of NADH and NADPH cannot be distinguished spectroscopically, they are referred to as NAD(P)H. NAD(P)H autofluorescence is a well-established noninvasive method to study cell and tissue metabolism (38,39). In some cases, an LED operating at 365 nm (a Rapp ElectroOptic) was used to minimize illumination noise (both intensity fluctuations and out-of-band light noise). For flavoprotein fluorescence imaging, a filter set comprised of a 455DF70 nm excitation filter, a 520DF40 nm emission filter, and a 495 nm long-pass dichroic reflector was used. An iris diaphragm was adjusted to exclude light from neighboring cells. A cooled photomultiplier tube held in a model D104 detection system (Photon Technology International, Lawrenceville, NJ) attached to a Zeiss microscope was used. Dynamic changes in autofluorescence intensity were recorded and smoothed using Felix software (Photon Technology International). All experiments were conducted at 37°C.

Calcium studies

Neutrophils were labeled with indo-1 (Molecular Probes, Eugene, OR), then observed as described (40).

Computational methods

Computational modeling and simulation was done using the software Madonna (University of California at Berkeley, Berkeley, CA) and Copasi (EML Research, Heidelberg, Germany and VBI, Blacksburg, VA, http://www.copasi.org). The numerical routines for integration were the Rosenbrock and LSODA algorithms, respectively.

RESULTS

Effect of stimulus on NADPH, NO, and O_2^- production

Fig. 1 illustrates the dynamic cellular responses to FMLP and glucose-active reagents for adherent neutrophils. NADPH oscillations, NO, and superoxide production are shown in

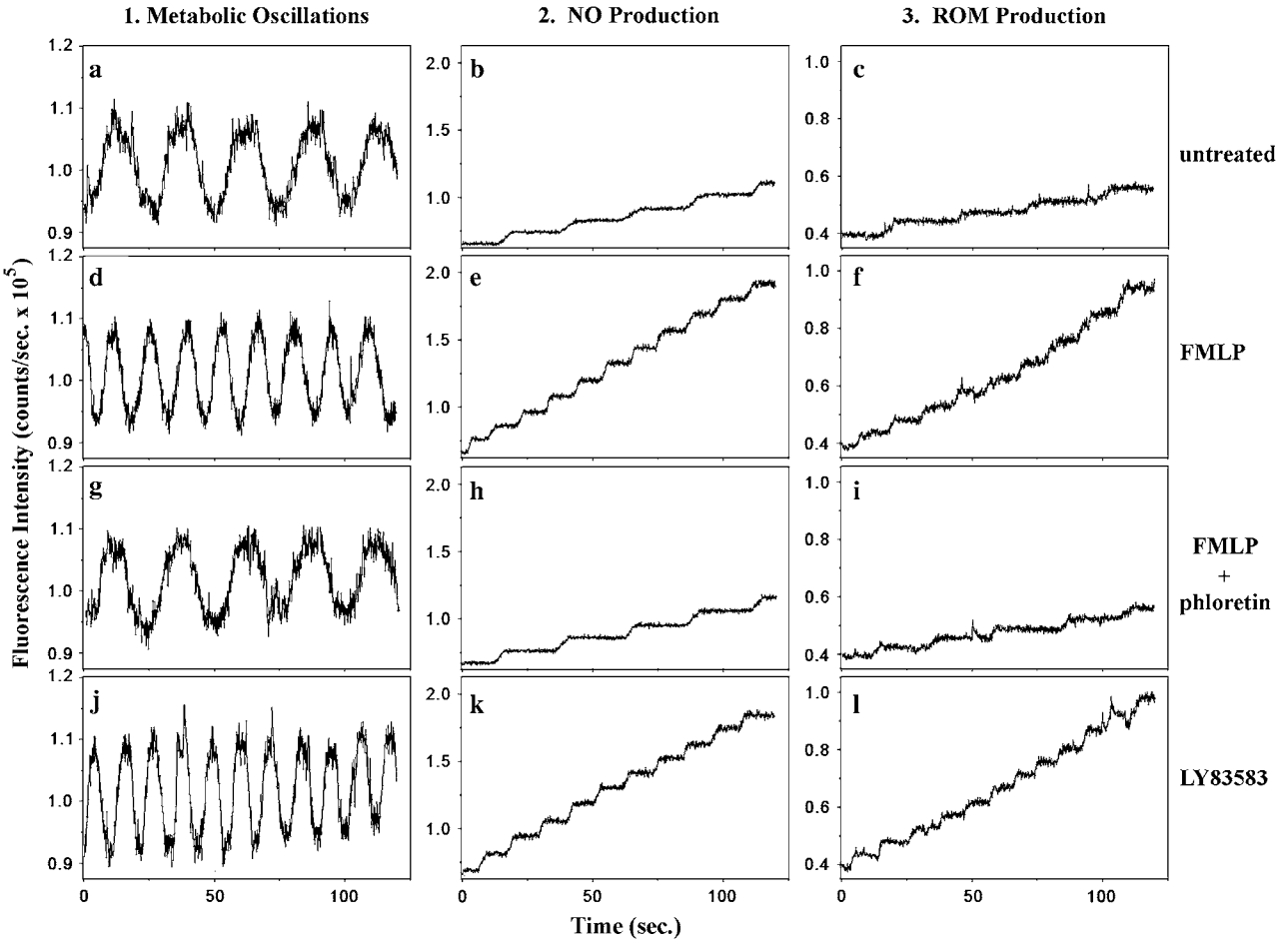

FIGURE 1 Representative kinetic traces NAD(P)H autofluorescence oscillations (column 1), NO release (column 2), and ROM production (column 3) of human neutrophils during several experimental conditions. NO and ROM release were detected by including DAF-2DA and HE, respectively, in the extracellular matrix. These traces show fluorescence intensity (*ordinate*) versus time (*abscissa*); to conserve space, only a few oscillations are shown. Polarized cells were studied on glass slides at 37°C. Untreated cells demonstrated NAD(P)H oscillations with a period of ≅20 s (trace *a*). Low rates of NO and ROM production were observed (traces *b* and *c*). As previously reported (33), neutrophil activation with FMLP leads to high frequency NAD(P)H oscillations ($\tau \cong$ 10 s) (*traces d–f*). However, pretreatment with 0.5 mM phloretin, which reduces glucose influx, blocks these increases in NAD(P)H frequency and oxidant release (traces *g–i*). To further address this mechanism, we nonspecifically activated glucose transport using LY83583 (traces *j–l*). In this case the perturbation in glucose transport directly led to metabolic changes and enhanced oxidant release ($n = 3$).

columns 1, 2, and 3, respectively. In these experiments, NO and superoxide were detected using the probes 15 μM DAF-2 DA and 3 μM HE, respectively. Individual morphologically polarized untreated neutrophils demonstrated ~20 s interval oscillations with little NO and O_2^- release. However, the addition of the activator 100 nM FMLP causes a doubling effect of the NAD(P)H oscillation frequency (Fig. 1 *d*) (~20–~10 s). These metabolic changes are accompanied by a greater rate of NO and O_2^- production (Fig. 1, *e* and *f*) compared with control conditions (row 1). On the other hand, if cells are exposed to FMLP after treatment with agents decreasing glucose uptake including 0.5 mM phloretin (Fig. 1, *g–i*) or with 10 μg/mL anti-GLUT1 antibody (data not shown), no change in the metabolic frequency or oxidant production were found. Furthermore, the receptor-independent enhancement of glucose flux using LY83583 causes a similar increase in metabolic frequency and the oxidant release (Fig. 1, *j–l*). These findings are consistent with the role of glucose influx in metabolic frequency changes and oxidant production, both of which have been associated with HMS activation.

Effect of glucose on NADPH, NO, and O_2^- production

As glucose influx appears to be a key parameter in cell activation and glucose influx can be enhanced by increasing the external glucose concentration, we assessed metabolic and oxidative responses as a function of glucose concentration. Glucose concentration of 1 and 5 mM had no significant effect on NAD(P)H dynamics or oxidant release by polarized

human neutrophils (Fig. 2, *a–f*). However, we found that glucose concentrations of 12–15 mM, similar to hyperglycemic levels, promote metabolic frequency doubling and greater ROM and NO production (Fig. 2, *g–i*). In contrast, 15 mM sorbitol had no effect on metabolic or oxidant oscillations. Detailed dose-response studies are given in Fig. 3, which show that 95% of the adherent or polarized cells have lost the resting phenotype (20-s NAD(P)H oscillations) at 20 mM glucose and display high frequency oscillations (data not shown). This increase in frequency could be inhibited with 0.5 mM phloretin (data not shown), suggesting a role for glucose transporters. We conclude that heightened glucose concentrations are sufficient to activate adherent neutrophils.

Kinetics of cellular responses

We next sought to characterize the timeframe of glucose-induced changes. The doubling effect appears in <6 min after glucose addition (Fig. 4), as all of cells displayed a 10-s metabolic oscillation period. Fig. 4 also illustrates that after 2.5 h of incubation, glucose's ability to activate metabolic oscillations begins to rapidly decline and reaches background levels in a few hours. Importantly, these changes in metabolic activity of the neutrophil population parallel the changes seen after activation with 100ng/ml LPS (Fig. 4).

Effect of 14 mM glucose on HMS activity

Although several previous studies (e.g., (33–35)) have linked 10 s metabolic oscillations to activation of the HMS, we sought to provide additional biochemical evidence for glucose-mediated activation of the shunt. To ascertain HMS activity, we measured the formation of $^{14}CO_2$ from 1-^{14}C-glucose and 6-^{14}C-glucose in the presence of 1 mM or 14 mM glucose (Fig. 5). In this determination, the amount of $^{14}CO_2$ formed during incubation with 6-^{14}C-glucose was subtracted from that formed in parallel experiments from 1-^{14}C-glucose (31). Fig. 5 shows the HMS activity in leukocytes at two glucose concentrations. A substantially higher level of HMS activity is present at 14 mM glucose. Therefore, HMS activity is upregulated by an increase in glucose concentrations. Moreover, cells incubated with 14 mM glucose for 8 h were refractive to FMLP-mediated activation, whereas similarly treated cells incubated with 1 mM glucose could be stimulated (data not shown). These findings suggest that glucose rapidly activates neutrophils followed by a gradual inactivation to a refractory state.

Computational modeling of neutrophil metabolism

To enable us to better understand the mechanism(s) responsible for the metabolic changes noted in the above experiments, we

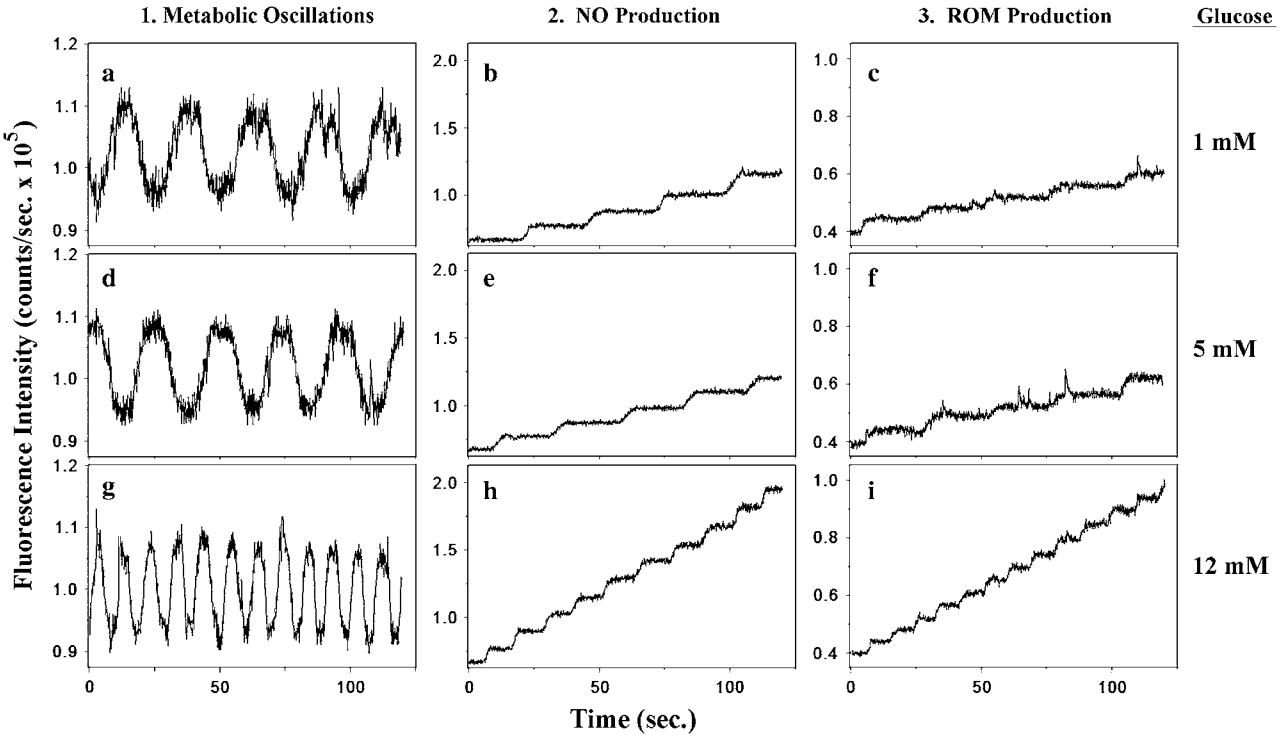

FIGURE 2 The effect of extracellular glucose concentrations on neutrophil metabolism and oxidant release. NAD(P)H oscillations, NO, and ROM production were monitored as noted in Fig. 1. At concentrations of 1 mM and 5 mM, extracellular glucose had no effect on neutrophil properties (*traces a–f*). However, in the absence of any other stimulus, 12 mM glucose triggered the activated phenotype of neutrophils as illustrated by the frequency of metabolic oscillations (*g*), NO release (*h*), and ROM production (*i*).

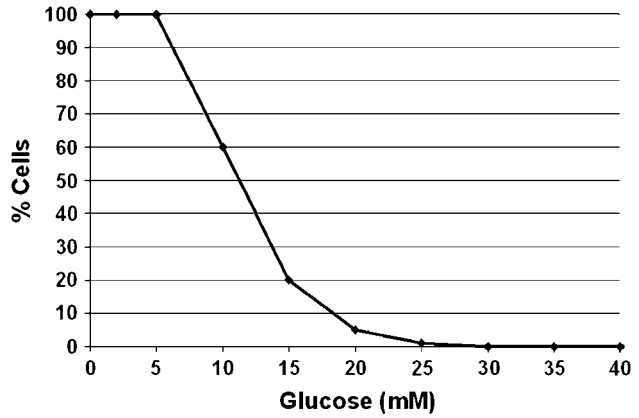

FIGURE 3 Dose-response studies of the effects of extracellular glucose concentrations on the metabolic phenotype of human neutrophils. The percentage of cells showing a 20 s NAD(P)H oscillatory period (*ordinate*) versus glucose concentration (*abscissa*) is shown.

performed computer simulations of the metabolic system thought to play the key role in this process. We created a model for the upper part of glycolysis in neutrophils based on a prior model for pancreatic cells (41). In addition, our new model contains a simplified term for the HMS. Key elements of the model are summarized in Table 1 (reactions) and Table 2 (kinetic equations). The equation for hexose kinase is simplified compared to Westermark and Lansner (41), neglecting the sigmoidal behavior of this enzyme assumed for pancreatic cells. Inclusion of such behavior does not change the computational findings described below. Thus, the kinetics is assumed irreversible and saturated with MgATP for simplification:

$$v_{\mathrm{hk}} = V_{\mathrm{hk}} \times \frac{glc}{(K_{\mathrm{mhk}} + glc)}. \tag{1}$$

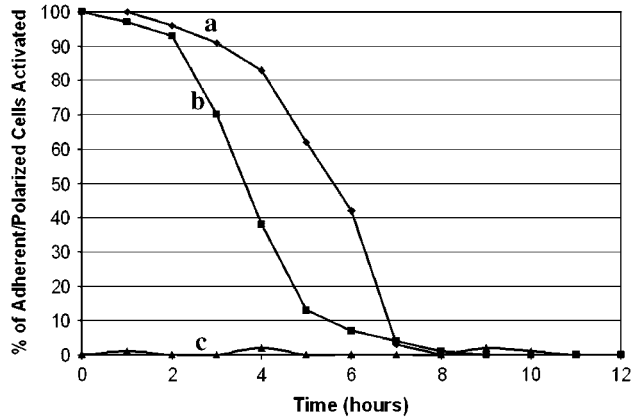

FIGURE 4 Glucose-mediated metabolic activation diminishes over time. Although all cells are initially activated by 20 mM glucose (*trace a*), as judged by the frequency of metabolic oscillations, this decays over time to reach background levels by ~7 h. Similar changes were noted for LPS (*trace b*), a well-known neutrophil activator. No changes were found in control samples incubated in the presence of 1 mM glucose (*trace c*).

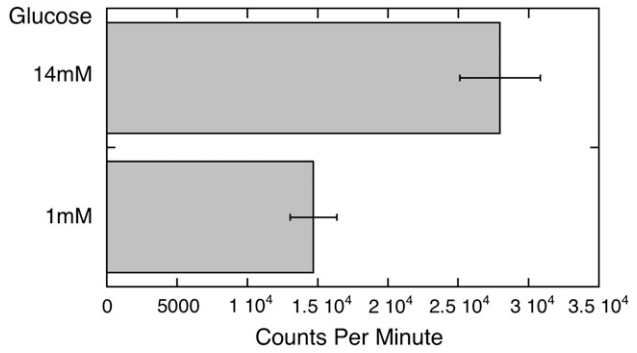

FIGURE 5 HMS activity in human neutrophils in the presence of 1 mM and 14 mM glucose. The metabolism of 1-^{14}C-glucose and 6-^{14}C-glucose to $^{14}CO_2$ was measured as described (27,28). The cpm of $^{14}CO_2$ formed from 6-^{14}C-glucose was subtracted from that formed from 1-^{14}C-glucose. The cpm (HMS activity) is listed on the graph (mean ± SE). Data were corrected for the amount of unlabeled glucose present. Heightened extracellular glucose substantially increases HMS activity in cells ($P < 0.001$ using the paired two-tailed *t*-test) ($n = 3$).

Glucose phosphate isomerase, which is only implicitly modeled in Westermark and Lansner (41), is explicitly described. However, it is simplified to a fast equilibrium reaction:

$$v_{\mathrm{gpi}} = k_{\mathrm{gpi}} \times g6p - k_{\mathrm{gpir}} \times f6p. \tag{2}$$

Phosphofructokinase and aldolase are modeled as described in Westermark and Lansner (41) and depicted in Table 2.

GAPDH is described as a Michaelis-Menten equation for two substrates. In contrast to Westermark et al. (41), we do not assume NAD+ and NADH to be constant. However, we also assume irreversibility and a constant concentration of phosphate. This is a strong simplification. However, as there is no additional feedback on or via GAPDH, the reaction has only limited influence on the oscillatory behavior of the system:

$$v_{\mathrm{gapdh}} = V_{\mathrm{gapdh}} \frac{gap * nad}{(K_{\mathrm{igap}} \times K_{\mathrm{nad}} + K_{\mathrm{gap}} \times nad + K_{\mathrm{nad}} \times gap + nad \times gap)}. \tag{3}$$

Apart from these reactions, glucose-6-phosphate is consumed by a simple linear term accounting for the HMS, which produces NADPH. NAD^+ is recycled from NADH by another simple linear term. The model is truncated at this point. This means that we do not account for all NADH produced during glycolysis. Kinetic parameters if available were taken from the literature. Thus, K_{m} values for hexokinase, aldolase, and GAPDH were taken from Mulquiney and Kuchel (42), whereas K_{m} values for PFK are close to the values reported in Westermark and Lansner (41). Maximal velocities and other rates were adjusted for the whole system to maintain steady state (albeit this steady state is dynamically instable as seen below). When computational simulations were performed, metabolic oscillations in NAD(P)H concentration were seen, as shown in Fig. 6. These oscillations are mainly due to the kinetic properties of phosphofructokinase as proposed by many authors and described by Westermark and Lansner (41). Hence, this model predicts oscillations similar to

TABLE 1 Reaction terms

Reaction number	Simplified reaction equation	Kinetics
R1	Glucose → Glucose-6-phosphate	$Vhk\,\frac{glc}{(K_{glc}+glc)}$
R2	Glucose-6-phosphate → Fructose-6-phosphate	$k_{gpi}\times g6p - k_{gpir}\times f6p$
R3	Fructose-6-phosphate → Fructose-bisphosphate	$V_{pfk}\,\frac{f6p^{h}}{(f6p^{h}+K_{f6p}^{h}\times\frac{(1+(k_x\times fbp)^{hx})}{(1+alpha^{h}\times(k_x\times fbp)^{hx})}}$ $h = 2.5 - scorr\times\frac{fbp}{(Kfbp+fbp)}$
R4	Fructose-bisphosphate → 2 Glyceraldehyde-3-phosphate	$V_{fba}\,\frac{fbp}{(K_{fbp}+fbp)}$
R5	Glyceraldehyde-3-phosphate + NAD^+ → Bisphosphoglycerate + NADH	$V_{gapdh}\,\frac{gap*nad}{(K_{igap}\times K_{nad}+K_{gap}\times nad+K_{nad}\times gap+nad\times gap)}$

those observed experimentally, albeit with a somewhat higher frequency.

Several hypotheses were tested using this model to simulate high glucose and HMS activation on metabolic oscillations. If HMS is activated due to the cells being exposed to high glucose accompanied by heightened glucose influx, there is substantial competition for glucose-6-phosphate, and the oscillatory frequency increases (Fig. 6). This effect is observed, if the HMS is activated while the glucose concentration is in the normal range as well as when raising the glucose concentration to the diabetic level. Given that the model is only semiquantitative in nature and that parameters, like the maximal velocity of the individual enzymatic reactions, including the enzyme concentrations, are undetermined for neutrophils, we studied the robustness of the observed qualitative effect by varying the individual parameter values within the oscillatory regime displaying simple periodic oscillations. Most parameters have no or little effect on the frequency response as depicted in Table 3. Some parameters are able to diminish the effect in a certain range. However, only if the system is close to the bifurcation from steady state to oscillations and exhibiting very low amplitude oscillations before HMS activation, the frequency change can be negligible or even reverted to some extent. Obviously, such very low amplitudes are not observed experimentally. The only exception to this is the maximal velocity of the aldolase. This parameter exhibits a regime where the frequency decreases with competing HMS. These results show that the initially observed frequency effect is robust with respect to most parameter changes and does not depend upon the exact set of parameters chosen. Of course, this does not imply that there could not be any combination of parameters that reverts the situation more dramatically, since the above analysis is only able to study the effect of local parameter changes starting from one initial set of parameters. The question whether the frequency increases or decreases with decreased flux through glycolysis seems to be tightly linked with the nonlinear characteristics of PFK. Thus, in a completely different model for glycolytic oscillations in yeast (43), which we studied as well, the competition for glucose-6-phosphate by the HMS also leads to an increased oscillation frequency (data not shown). Here, the characteristics of the PFK are different from the one used in the above model and influenced by the inhibition by ATP. This effect might also occur in neutrophilic PFK (44) and therefore, the qualitative result that the frequency also increases in this model when HMS competition occurs is of interest for the current study. However, if the sigmoidal characteristics of

TABLE 2 Systems equations

Metabolite	Right-hand side of equation
Glucose-6-phosphate	R1 − R2 − k_{hms} * *g6p*
Fructose-6-phosphate	R2 − R3
Fructose-bisphosphate	R3 − R4
Glyceraldehydes-3-phosphate	2 * R4 − R5
NAD^+	−R5 + k_{nadh} * *nadh*
NADH	R5 − k_{nadh} * *nadh*
NADPH	2 * k_{hms} * *g6p* − k_{nadph} * *nadph*

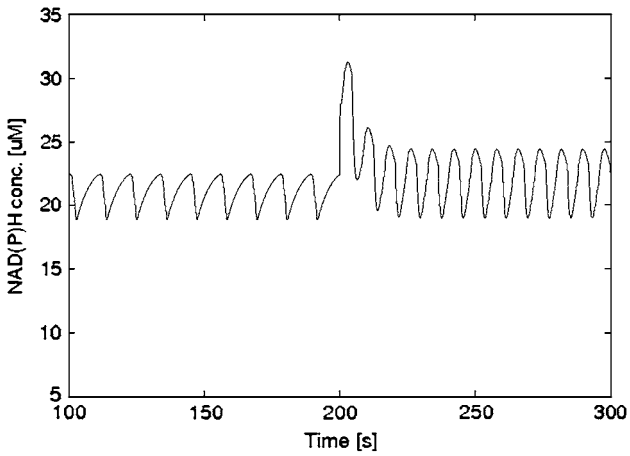

FIGURE 6 Simulated time course of NAD(P)H concentration (being the sum of NADH and NADPH). At $t = 200$ s, the HMS is activated from 0 to $k_{hms} = 0.5$/s. Parameters: $V_{hk} = 179$ μM/s; $K_{glc} = 47$ μM; $k_{gpi} = 1800$/s; $k_{gpir} = 2100$/s; $V_{pfk} = 2220$ μM/s; $K_{f6p} = 3400$ μM; $k_x = 2$/μM; $h_x = 2.5$; $alpha = 5$; $scorr = 1.5$; $K_{fbp} = 5$ μM; $V_{fba} = 220$ μM/s; $V_{gapdh} = 1100$ μM/s; $K_{igap} = 3210$ μM; $K_{nad} = 50$ μM; $K_{gap} = 98$ μM; $k_{nadh} = 16.8$/s; and $k_{nadph} = 15$/s. Initial concentrations: $glc = 5000$ μM; $g6p = 37$ μM; $f6p = 12$ μM; $fbp = 2$ μM; $gap = 1$ μM; $nadh = 70$ μM; $nad = 30$ μM; and $nadph = 1$ μM.

TABLE 3 Robustness analysis

Parameter	Range
V_{hk}	a.b. 160–190
K_{glc}	0–900 a.b.
k_{gpi}	1400–2400 s.a.
k_{gpir}	s.a. 1000–2600 a.b.
V_{pfk}	s.a. 1700–16,000 s.a.
K_{f6p}	1800–4200 a.b.
k_x	a.b. 1–5.6
h_x	0.5–3.9 a.b.
alpha	3.5–7.9 s.a.
scorr	s.a.1.3–1.4 and 1.5–2.4 s.a.
K_{fbp}	a.b. 2.8–6.7 s.a.
V_{fba}	210–250 rev.
V_{gapdh}	800–indef.
K_{igap}	a.b. 200–6500
K_{nad}	0–90
K_{gap}	0–12,700 s.a.
k_{nadh}	7.4–90
k_{nadph}	0.1–indef.

Starting from the parameter values listed in Fig. 6, individual parameters are changed and the range of values depicted in the table for which the system shows simple periodic oscillations with frequency increase with competing HMS. This effect can differ in strength. *a.b.* on one side of the range means that there is an additional bifurcation on this end, whereas *s.a.* stands for small amplitudes in the oscillations approaching steady state, which is not in accordance with experimental findings. *Rev.* indicates a regime on this side of the parameter range where the frequency effect is reverted. Parameter units of μM and s.

PFK are heavily altered, e.g., by neglecting FBP influence in the above-described model, the model will not show the increase in frequency and even exhibit reverse behavior. Therefore, and as described below, our predictions have to be verified experimentally. As a summary of our computational studies, we conclude that the change in frequency observed could be due to a lack of glucose-6-phosphate available to enter glycolysis as a result of competition from the HMS upstream.

Calcium oscillations are not responsible for glucose-mediated metabolic changes

As calcium oscillations increase in frequency during cell activation (45), calcium might drive the metabolic oscillations toward higher frequencies independent of cell metabolism. To test this mechanism, calcium and NAD(P)H concentrations were measured simultaneously (Fig. 7). When neutrophils are exposed to a high concentration of glucose, the frequency of their metabolic oscillations change first, which is followed by a change in the frequency of their calcium oscillations second. On the other hand, if the calcium signal changed first, then it should have approached the back of the preceding metabolic oscillation, not the metabolic oscillation approaching the back of calcium signal. As the order of frequency increase could be dependent upon the timing of glucose addition relative to the phase of intracellular oscillators, we also applied glucose at other points in the oscillatory metabolic cycle with identical results (data not shown). Thus, in this case, metabolism seems

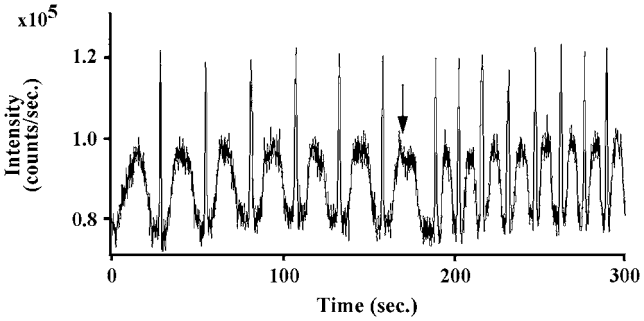

FIGURE 7 Simultaneous measurements of neutrophil calcium spikes and NAD(P)H oscillations. The fluorescence intensity (*ordinate*) is shown for a single cell versus time (*abscissa*). As the excitation and emission spectra of indo-1 and NAD(P)H are similar, the concentration of indo-1 was adjusted such that the intensities of NAD(P)H oscillations and calcium spikes were similar in magnitude so that a single emission channel could be used. Cells were suspended in HBSS containing 5 mM glucose. After glucose addition, the final concentration of glucose was 15 mM. A definite phase relationship exists between these two oscillators, which is perturbed immediately after glucose addition.

to drive calcium oscillations by a yet unknown mechanism and the decreased flux through glycolysis is apparently the major cause for the frequency change.

Response of mitochondrial flavoproteins to glucose

We next performed experiments to verify or falsify the somewhat counterintuitive modeling prediction that increasing glucose concentrations result in a decreased flux through glycolysis. To do this, we measured the autofluorescence of flavoproteins in neutrophils both before and after glucose exposure. In neutrophils, flavoproteins are found in mitochondria (α-lipoamide dehydrogenase and the electron transport dehydrogenase) and within a small subpopulation of granules containing the NADPH oxidase (46). As the amount of flavoprotein autofluorescence is inversely related to electron transport rate (47), it can be used as an indicator of metabolic activity. Fig. 8 shows representative traces of flavoprotein intensity versus time derived from individual neutrophils. In Fig. 8 *A*, 14 mM glucose was added at the indicated time point. The flavoprotein autofluorescence briefly dips, suggesting an increase in electron transport activity, then rises abruptly to a new steady-state level. The increase in the steady-state autofluorescence level reflects a decrease in mitochondrial activity. As a negative control, 14 mM sorbitol was added at the indicated time point in Fig. 8 *B* while the glucose concentration remained constant. In this case, the flavoprotein autofluorescence level remained constant, indicating that the glucose effect could not be accounted for by some nonspecific effect, such as osmotic pressure, on cell metabolism. Hence, mitochondrial metabolic activity is reduced by these pathophysiologic glucose levels.

If the reduction in mitochondrial activity is due to activation of the HMS, it should be possible to reconfirm our findings by normalizing metabolic activity by treating cells

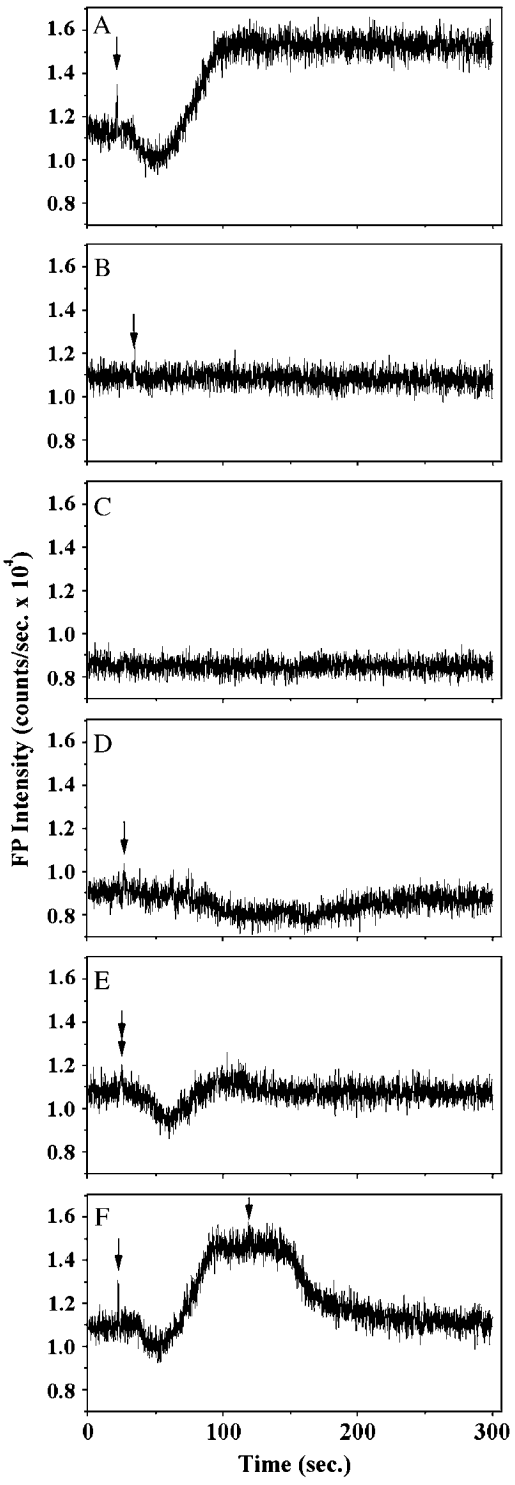

FIGURE 8 Flavoprotein responses to glucose. The autofluorescence of mitochondrial flavoproteins is inversely proportional to electron transport activity. When 14 mM glucose is added to cells, the autofluorescence briefly decreases in intensity, then quickly reaches a higher steady-state level (*A*). Although glucose perturbed mitochondrial activity, the nonmetabolizable sugar sorbitol at 14 mM had no effect (*B*). Addition of the HMS inhibitor 6-AN alone had no effect on cells (*C*). When 14 mM glucose is added to 1 mM 6-AN-treated cells, only a small change is noted in autofluorescence intensity (*D*), which is consistent with the role of the HMS in these changes. To confirm these changes, we altered the order of addition of glucose and 6-AN. When 14 mM glucose and 10 mM 6-AN are simultaneously added, a small dip in flavoprotein fluorescence is observed followed by a return to the pretreatment level (*E*). Furthermore, if 6-AN is added after the autofluorescence stabilizes at the higher level in the presence of 14 mM glucose, 6-AN is able to dramatically reduce the intensity of this steady-state level (*F*). These data suggest that greater levels of glucose reduce mitochondrial activity.

with 6-AN. If cells are treated with 1 mM 6-AN for 20 min, then observed by microfluorometry, a low and constant level of autofluorescence is observed (Fig. 8 *C*). If 14 mM glucose is added to these 6-AN-pretreated cells, a slight reduction in flavoprotein fluorescence is observed (Fig. 8 *D*). To follow the acute effects of HMS inhibition, we used a higher dose of 6-AN. In Fig. 8 *E*, 14 mM glucose and 10 mM 6-AN were simultaneously added to cells at the indicated time point. Under these conditions, there is a slight drop in autofluorescence intensity and the dramatic glucose-mediated increase is dramatically blocked. In Fig. 8 *F* we changed the order of addition such that glucose was added first then followed by 6-AN after the change in autofluorescence intensity. As this trace shows, 14 mM glucose (*first arrow*) caused a slight dip then large increase in intensity, that could be reduced by addition of 10 mM 6-AN at the second point indicated on the trace. These findings further confirm that the nonspecific activation of the HMS at high glucose levels reduces metabolic flux through the glycolytic pathway and mitochondria.

DISCUSSION

In this study, we have combined experimental and computational methods to better understand how glucose, especially pathophysiologic glucose concentrations, affects metabolic dynamics and cell functions of human neutrophils. Both methods we used have confirmed and extended the findings of the other. In the absence of other biological factors, such as receptor agonists, our results show that glucose has the ability to activate adherent human neutrophils. At 14 mM, glucose alone was found to be capable of:

1. Increasing the frequency of metabolic oscillations.
2. Enhancing the production of ROMs and NO.
3. Activating the HMS as judged by radiolabeled carbon flux.

These effects were not observed at low glucose concentrations. The cellular activation found at high glucose levels paralleled that previously observed for receptor-mediated activation of neutrophils, such at that mediated by FMLP (e.g., 44). The key role of glucose is consistent with earlier findings indicating that glucose is required for the neutrophil's respiratory burst (1,2) and that an upregulation of glucose transporter activity is associated with leukocyte activation (3). To further link these previous studies with our own, we found that the glucose transport inhibitor phloretin and anti-GLUT1 antibodies inhibit neutrophil activation. Moreover, an activator of glucose transport, LY83583, activates cells

in the absence of a receptor agonist. As glucose enters eukaryotic cells via facilitated diffusion, we suggest that greater extracellular glucose concentrations lead to greater intracellular levels of glucose and its downstream products, especially glucose-6-phosphate. Consequently, elevated glucose concentrations may simply bypass the normal regulatory signaling pathways of leukocytes that upregulates glucose transport to nonspecifically activate the HMS and ROM/RNS production.

We have previously demonstrated that adherent neutrophils exhibit NAD(P)H oscillations with a period of ~20 s, that is reduced to ~10 s in the presence of activating substances such as FMLP, LPS, interleukin-8, etc. (45). This correlation between period and HMS activity was confirmed using inhibitors of the HMS, such as 6-AN and dexamethasone, which block the formation of 10 s oscillations (45,48). However, the mechanism underlying the change in period has remained unknown. Our computational studies now show that the shorter metabolic oscillation period is due to the perturbation of glycolysis caused by activation of the HMS—specifically, the competition between glycolysis and the HMS for glucose-6-phosphate. Therefore, the frequency changes are not due to oscillations of the shunt, but rather by changes in glycolysis precipitated by activation of the shunt.

When the external glucose concentration is raised, glucose flux across the plasma membrane will increase until the glucose transporters become saturated. Although the concentration of glucose is increased, this apparently does not lead to an increased flux though glycolysis as might be expected. As our computational modeling studies indicate, the change in frequency is likely due to a decreased rather than an increased flux through glycolysis. This is a bit counterintuitive, but easy to understand if one assumes the competition by the HMS to be strong enough. To test this computational conclusion, further experiments were performed. Previous studies have shown that the autofluorescence of mitochondrial flavoproteins is inversely proportional to rate of mitochondrial electron transport (47). Using this approach, we have shown that the autofluorescence of neutrophils increases dramatically after addition of 14 mM glucose (Fig. 8), indicating a decrease in mitochondrial electron transport. As the NADPH oxidase contains a flavoprotein, it may be a factor in the total flavoprotein emission. However, as electron transport through the NADPH oxidase is increased by cell activation, this would reduce, rather than enhance, cell fluorescence. Hence, the enhanced autofluorescence intensity is an underestimate of the glucose-induced changes. We conclude that the reduction in metabolism predicted by the computational model has been experimentally confirmed. In addition, this is consistent with the conjecture of Esmann (49) that a reduction in cellular ATP levels due to a disturbance in carbohydrate metabolism takes place. Although our computational work is relatively new in this field, it is particularly important because it mechanistically explained our previous work on frequency changes and predicted our subsequent discoveries of changes in mitochondrial activity.

Elevated glucose concentrations were found to activate neutrophils, which is consistent with certain earlier studies (7–9). However, activation was not permanent. After ~2.5 h, the percentage of activated neutrophils began to fall and reached baseline levels within 8 h. Furthermore, glucose-exhausted neutrophils are no longer capable of becoming activated. These findings are consistent with other work suggesting that extended stimulation leads to a refractory state of neutrophils (50). Our findings are also consistent with the fact that neutrophils isolated from diabetic patients can have an ineffective respiratory burst (6), as most of these cells have been exposed to high levels of serum glucose for many hours. Thus, depending upon the experimental conditions, an enhancement of reduction in metabolic activity may be observed for glucose exposure, which may account for the variability in prior experimental results.

Our model in vitro experiments may provide clinically relevant insights. The glucose concentrations we have focused upon in these in vitro studies, 12–15 mM, correspond to the levels of glucose found in the peripheral blood of uncontrolled diabetics. In addition, oxidative stress, likely due to glucose, is thought to be an important factor in tissue damage in diabetes (51), although the mechanism responsible for this has not been established with certainty. We propose that one avenue of oxidant-mediated tissue damage may be the acute nonspecific activation of the neutrophil's respiratory burst. On the other hand, prolonged incubation with glucose leads to cell exhaustion. This, in turn, would lead to a greater risk for infectious disease, which is observed for the diabetic patient. We suggest that the length of time exposed to elevated glucose plays in key role in determining the behavior of neutrophils. Therefore, as neutrophils reach the circulation, they may be transiently activated to cause nonspecific oxidative tissue damage followed by a refractory phase in which they cannot mount a normal host defense. Our experimental and computational studies have also revealed the unexpected finding that mitochondrial metabolism is reduced at high glucose levels. This provides another potential route involving reduced energy resources for tissue damage during diabetes. These, and further biophysical/clinical studies may help explain the regulation of neutrophil activity in diabetes and control its detrimental effects in patients.

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