High glucose concentrations impair the activation of K⁺ channels and proteases in undifferentiated THP-1 monocytes.

Hiroshi Asaoka¹, Junko Kurokawa¹, Tetsushi Furukawa⁵, Kentaro Shimokado¹

¹) Department of Geriatrics and Vascular Medicine, Graduate School, Tokyo Medical and Dental University
²) Department of Bio-informational Pharmacology, Medical Research Institute, Tokyo Medical and Dental University
³) Department of Internal Medicine, International University of Health and Welfare, Mita Hospital

Original Article

Transient alkalization caused by activation of the BKCa channel has been reported to be essential for the activation of proteolytic enzymes and bacterial killing in the leukocytic phagosomes. We investigated the effects of high glucose concentrations on these processes in THP-1 cells. While E.coli transiently raised the pH of the phagosomes in THP-1 monocytes, high glucose concentrations impaired the transient rise of pH in a dose-and time-dependent manner. Electrophysiological studies confirmed that the bacteria elicited a transient K⁺ current, and that a high glucose concentration diminished the current. High glucose concentrations also inhibited the activation of cathepsin G in the THP-1 cells. NS1619, a BKCa channel opener, accentuated the transient alkalization induced by the bacteria, and reversed the inhibitory effect of high concentrations of glucose. However, electrophysiological study revealed that the membrane current differed from the BKCa current. Our findings indicate that high glucose concentrations impair K⁺ channel activation and the subsequent activation of proteolytic enzymes in THP-1 monocytes. The precise identity of the K⁺ channel remains unclear, although it dose not appear to be the BKCa. Suppression of the transient alkalization and activation of proteases may be one of the mechanisms for bacterial killing by phagocytes in diabetic patients.

Key words: diabetes, bacteria killing, cathepsin G, K⁺ channel, THP-1 monocytes

Introduction

Leukocyte dysfunction is an important component of the immune deficiency in diabetic patients. Hyperglycemia in diabetic patients alters neutrophil functions, including the bactericidal activity of these cells¹⁰. It has been reported that oxygen-burst-dependent bacterial killing may be impaired in diabetic patients because of myeloperoxidase (MPO) dysfunction and consequent reduction in the formation of oxygen free radicals⁶,⁷. However, the precise mechanisms underlying the impaired bactericidal activity of leukocytes in diabetic patients remain unknown.

Recently, the large-conductance Ca²⁺-activated K⁺ (BKCa) channel was reported to play a critical role in bacterial killing by human neutrophils and eosinophils⁸-¹⁰. When leukocytes phagocitize bacteria, the BKCa channel is transiently activated resulting in a potassium current inflow and alkalization of the phagocytic vacuole. The high potassium concentration in the phagosome releases and activates proteolytic enzymes from the matrix and activates them. Inhibitors of the BKCa channel abolish both the transient
alkalization and subsequent activation of the proteolytic enzymes in the phagosomes of the leukocytes induced by bacterial phagocytosis. Thus, inhibitors of these channels prevent bacterial-killing in the leukocytes without affecting the oxygen consumption and phagocytosis.

In this study, we addressed whether high glucose concentrations affected the transient alkalization of the phagocytic vacuole and consequent activation of proteolytic enzymes in undifferentiated THP-1 monocytes. We also studied the role of K⁺ channels in these processes.

**Materials and Methods**

Fluorescein-labeled *Escherichia coli* K-12 (Vybrant™ Phagocytosis Assay Kit) was obtained from Molecular Probes (OR, USA). NS1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one) was purchased from BIOMOL® (PA, USA), and cathepsin G (human leukocytes) was obtained from Sigma (MO, USA).

**THP-1 monocytes**

THP-1 cells were cultured in RPMI 1640 supplemented with 10% FCS, 1% penicillin-streptomycin, and 0.2% amphotericin B. The cells were suspended in culture media supplemented with various concentrations of glucose or mannitol, and plated on a 96-well plate at a density of 3×10⁴/cm². At the time points indicated, fluorescein-labeled *E.coli* was added, and the pH in the phagocytic vacuoles of the cells was determined.

**The pH in the phagocytic vacuoles**

The pH in the phagocytic vacuoles was determined using fluorescein-labeled *E.coli* in accordance with the manufacturer’s instructions. Briefly, at various time points after the addition of fluorescein-labeled *E.coli*, the culture plates were placed on ice, the supernatant was removed, and adherent cells were washed with ice-cold PBS. Fresh medium prewarmed at 37 °C was added, and the ratio of fluorescence intensities at λex 530 nm to λem 590 nm under excitation at λex 485 nm was determined by the fluorescence plate reader (CytoFluor II™, Applied Biosystems, CA, USA). The pH was calculated from a standard curve prepared by fluorescein-labeled *E.coli* incubated at known pH. In some experiments, a BKCa channel opener (NS1619, 30 μmol/l) was added 3 minutes before the addition of *E.coli*.

**Electrophysiological Study**

Membrane currents were measured using perforated patch-clamp procedures with Axopatch 200B amplifiers and the Pclamp 9.2 program (Axon Instruments, CA, USA). The THP-1 cells were dispersed to the Tyrode’s solution in Petri dishes that were placed on the stage of an inverted microscope (IX71, Olympus, Tokyo, Japan), and the cells attached to the bottom were used for the measurements. The control Tyrode’s solution contained 140 mmol/l NaCl, 2.5 mmol/l KCl, 0.5 mmol/l MgCl₂, 1.2 mmol/l CaCl₂, 5 mmol/l HEPES and 11 mmol/l glucose, and 22 mmol/l glycine or 22 mmol/l mannitol was adjusted to pH 7.4 with NaOH. For some experiments, glucose of 22 mmol/l or 22 mmol/l mannitol was added instead of 11 mmol/l glucose. All measurements were obtained at room temperature (22 °C). The tip resistance of the microelectrodes (borosilicate glass) was 2-4 MΩ when filled with the following internal solution: 140 mmol/l KCl, 10 mmol/l NaCl, 2 mmol/l MgCl₂, 0.7 mmol/l CaCl₂, 1 mmol/l EGTA and 10 mmol/l HEPES, pH 7.3 adjusted with KOH. For perforated patch recordings, amphotericin B was added to the above internal solution to yield a final concentration of 0.3 mg/ml. Adequate access resistance (6-12 MΩ) was usually attained within 10 minutes of seal formation. Cell capacitance (about 20 pF) was same in different external glucose concentrations.

In order to measure the time course of response to *E. coli*, total membrane currents were measured either during square pulses (400 ms) to 100 mV or during ramp pulses (400 ms) from -100 mV to 140 mV applied once every 2 s. Holding potentials of -30 mV were used for all recordings. After stable membrane currents were measured for 1 min, *E. coli* (1.6×10⁹/ml final) was applied from the extracellular side for 3-5 min. The amplitudes of membrane currents were obtained from end points of traces elicited by square pulses to 100 mV or from points at 100 mV of traces elicited by ramp pulses. Reversal potentials of elevated currents that responded to *E. coli* were obtained after subtraction of membrane currents before application of *E. coli*. Averaged data are shown as the mean ± S.E.M. for the electrophysiological experiments, because compound data from responsive and non-responsive cells to *E. coli* enlarge the SE values.

**Activity of cathepsin G**

THP-1 monocytes were stimulated with *E.coli* (1.5×10⁹/cm²) for 2 minutes. The cells were washed with ice-cold phosphate-buffered saline. The cell pellet...
was lysed in 500μl of assay buffer containing 50 mmol/l HEPES buffer, pH 7.4, 50 mmol/l NaCl and 0.1% Triton X-100. After removing debris, total protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA) and samples were kept at -70 °C until the assay. Cathepsin G was assayed as reported before using peptide-p-nitroanilide (pNA) as the substrate for cathepsin G.

Statistical analysis
The data are presented as the mean ± S.E.M. The statistical significance was analyzed by ANOVA, followed by Tukey’s or Dunnett’s test. Correlation coefficient was determined using simple regression analysis for glucose exposure time and concentrations. P<0.05 was considered statistically significant.

Results

Bacteria-induced alkalization of THP-1 cells was impaired by high glucose concentrations.

_E. coli_ raised the pH of phagocytic vacuole from 7.43 ± 0.097 to 7.90 ± 0.147. This rise returned to the base line by 4 minutes and gradually decreased to acidic pH. High glucose concentrations impaired the transient rise of pH in a dose-dependent fashion. NS1619, a BKCa opener, accentuated bacteria-induced transient alkalization (Figure 1). The effect became apparent after 48 hours, and became greater as the exposure time increased to 96 hours. The basic pH or acidification after 4 hours was not affected by glucose concentrations. The same concentrations of mannitol did not have an effect on it (Figure 1 and 2).

Electrophysiological study
To confirm that activation of potassium channels is impaired by high glucose concentrations, we conducted an electrophysiological study using a perforated patch-clamp technique. External application of _E. coli_ caused a robust increase of membrane currents in some (not all) THP-1 cells. The membrane currents elevated by _E. coli_ were appeared transiently within about 3-min (data not shown) and showed weak inward rectification. High potassium selectivity of the elevated membrane currents was confirmed that reversal potential of the currents (about -82 mV) was close to the equilibrium potential of the potassium ion (-103.4 mV). Averaged maximum response of membrane currents 3-min after application of _E. coli_ in control (11 mmol/l glucose) and negative control (11 mmol/l glucose) was 8.5

\[ \text{pH of phagocytic vacuole} \]

\[ \text{Time after phagocytosing E. coli (minutes)} \]

**Fig. 1.** High glucose concentrations reduced transient alkalization in phagocytic vacuole
THP-1 cells were cultured in media containing 11mmol/l glucose (control, open circle), 22 mmol/l glucose (open triangle) or 33mmol/l glucose (open square) for 48 hours, and stimulated with FITC-coated _E. coli_. NS1619 (30μmol/l) was added to cells cultured in media containing 11 mmol/l 3minutes before stimulation with _E. coli_ (closed circle). PH in the phagocitized vacuole was monitored as described in Methods. There was a significant difference when the four curves were compared using 1-way ANOVA and subsequent Dunnett’s test (p<0.05). Each value represents the mean ± S.E.M. of 5 independent experiments, *p<0.05 vs. control at 2 minutes.

**Fig. 2.** The effect of time of glucose exposure on transient alkalization of the phagocytic vacuole.
THP-1 cells were cultured in media containing 11mmol/l glucose (control, open circle), 22 mmol/l glucose (open triangle), 33mmol/l glucose (open square), 22 mmol/l mannitol (closed circle), or 33mmol/l mannitol (closed triangle) for various periods before stimulation with _E. coli_. The pH in the phagocytic vacuole was determined 2 minutes after stimulation. There was a significant difference when the five curves were compared using 1-way ANOVA and subsequent Dunnett’s test (p<0.05). Each value represents the mean ± S.E.M. of five independent experiments, *p<0.05 vs. control at 48 hours, *p<0.05 vs. control at 96 hours. Simple regression analysis revealed the correlation between glucose exposure time and pH in the phagocitized vacuole (glucose 22 mmol/l; R=0.84, p<0.01, glucose 33 mmol/l; R=0.78, p<0.01), and revealed the correlation between the glucose concentrations and pH in the phagocitized vacuole (glucose exposure time 48 h; R=0.61, p<0.01, glucose exposure time 96 h; R=0.94, p<0.01).
cose + 22 mmol/l mannitol) was decreased by high glucose concentrations without changing reversal potentials, confirming that activation of the potassium channel is impaired by high glucose concentrations (Figure 3A, 3B).

A BK_Ca channel opener abolished the effect of high glucose concentrations.

NS1619 enhanced the transient alkalization of the cells cultured in a low glucose concentration. When NS1619 was added to the culture with high concentrations of glucose, the impairment of alkalization was completely prevented (Figure 4).

High glucose concentrations inhibited bacteria-induced activation of cathepsin G.

To confirm that glucose-induced impairment of the alkalization is associated with impaired activation of proteolytic enzymes in the phagocytic vacuole, we measured the activity of cathepsin G in THP-1 monocytes cultured under various glucose concentrations for 48 hours. Bacteria-induced activation of the enzyme was significantly suppressed by 33 mmol/l glucose (Figure 5). NS1619 restored the suppression (Figure 6).

Fig. 3A.

High glucose concentrations reduced potassium channel currents activated by E. coli. Responses of total membrane currents to E. coli were compared between THP-1 cells cultured in media containing 11 mmol/l glucose (control), 33 mmol/l glucose (high glucose concentrations) or 11 mmol/l glucose + 22 mmol/l mannitol (negative control). (A) Culture with 33 mmol/l glucose media (Right) decreased membrane currents stimulated with E. coli compared with those from cells in control (Left). Shown are representative traces elicited by a ramp pulse (shown above the traces) from –100 mV to 140 mV before (open circles) and after (closed circles) stimulation with E. coli. Reversal potentials of the stimulated currents (Left: -85.6 mV, Right: -81.6 mV) imply high potassium ion selectivity. Dashed lines represent zero levels. Scale: 50 pA/pF, 100 ms. Arrows show reversal potential. (B) Summary of all pooled data before E. coli (open columns) and upon maximum response to E. coli stimulation (closed columns). Mean ± S.E.M. current densities at 100 mV are plotted for each glucose concentration as indicated below the bars. The numbers of experiments were as follows: 11 mmol/l glucose n = 8; 33 mmol/l glucose n = 15; 11 mmol/l glucose + 22 mmol/l mannitol n = 11. The results from the 1-way ANOVA and subsequent Tukey’s test showed a significant difference (p < 0.05) among the six groups. *p<0.05 vs. glucose 11 mmol/l without mannitol. †p<0.05 vs. glucose 33 mmol/l without mannitol.

Fig. 4.

NS1619 recovered transient alkalization impaired by high glucose concentrations. THP-1 cells were cultured in media containing 11 mmol/l, 22 mmol/l, or 33 mmol/l glucose in the presence of 30 μmol/l NS1619 that was added to the culture 3 minutes before stimulation with E. coli. The pH in the phagocitized vacuole was monitored as described in Methods. Each value represents the mean ± S.E.M. of five independent experiments. The results from the 2-way ANOVA and subsequent Tukey’s test showed a significant difference (p<0.05) between the samples of glucose 11 mmol/l, 22 mmol/l and 33 mmol/l.

Fig. 5.

NS1619 restored cathepsin G activity. THP-1 cells were cultured in media containing 11 mmol/l, 22 mmol/l, or 33 mmol/l glucose in the presence of 30 μmol/l NS1619 that was added to the culture 3 minutes before stimulation with E. coli. The results from the 2-way ANOVA and subsequent Tukey’s test showed a significant difference (p<0.05) between the samples of glucose 11 mmol/l, 22 mmol/l and 33 mmol/l.

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The present study showed that high glucose concentrations inhibit transient alkalization and activation of proteolytic enzymes in THP-1 monocytes. This alkalization is caused by activity of the potassium channel. Potassium current increased when cells phagocytized bacteria, and this increase was suppressed by high glucose concentrations. Contrary to the previous report, the role of BK Ca channel is minimal in THP-1 cells. Although the BK Ca opener restored the impairment of bacterial killing under high glucose conditions, the BKCa inhibitor did not inhibit the alkalization, and electrophysiological study detected no typical BK Ca current. Suppression of transient alkalization is a novel mechanism for impaired bacteria killing by leukocytes under high glucose conditions.

The transient alkalization was reported to be mediated by BKCa channel in neutrophils and eosinophils in previous studies. In agreement with their report, NS1619, a BKCa channel opener, accentuated transient alkalization in THP-1 cells. However, a BKCa channel inhibitor did not significantly abolish the alkalization. Furthermore, electrophysiological study showed that bacteria-stimulated membrane currents had weak inward rectification contradicting a property of the BKCa channel that had outward rectification, while the membrane currents were highly selective to permeating potassium ion. These results suggest that, upon stimulation with bacteria, potassium ions exit from the cytosol through potassium channels other than the BKCa channel in THP-1 cells. Precise identification of potassium channel subtypes remains to be elucidated.

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phagocytic activity of leukocytes. Thus, decreased alkalization could be attributed to decreased number of bacteria phagocitized under high glucose concentrations. However, we chose conditions with an extremely high bacteria/leukocyte ratio, and high glucose concentrations did not affect the number of bacteria phagocytized in our experiments. It took 48 hours for high glucose concentrations to affect the $K^+$ channel activity in THP-1 cells, and shorter exposures did not affect the alkalization suggesting that this was a secondary effect of high glucose concentrations.

Oxygen radical formation has been regarded as the major mechanism of bacteria killing by leukocyte\textsuperscript{14,15}. Recently it is advocated that the absence of neutrophil protease impairs intracellular killing of bacteria and fungus\textsuperscript{16-18}. Our findings together with those by other investigators suggest that reduced protease activity caused by hyperglycemia could be one of the mechanisms for immune deficiency in diabetic patients.

References