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HYPERTONIC GLUCOSE INHIBITS THE PRODUCTION OF OXYGEN-DERIVED FREE RADICALS BY RAT NEUTROPHILS

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Summary

We studied the influence of graded degrees of hypertonic glucose or sucrose on the generation of oxygen-derived free radicals by rat neutrophils. Hypertonic glucose and sucrose exerted dose- and time-dependent inhibition of chemiluminescence amplified by luciferin analog (CLA-DCL) and luminol (L-DCL) in response to fMLP. Hypertonic glucose was more effective to this chemiluminescence inhibition than hypertonic sucrose was. This inhibition of hypertonicity was more effective in CLA-DCL than in L-DCL. Although the production of superoxide anion measured by the reduction of ferricytochrome c was more inhibited by hypertonic glucose than by hypertonic sucrose, the myeloperoxidase activity was not affected by either glucose or sucrose hyperosmolarity. These data suggest that hyperosmotic state by itself and an additional direct glucose-toxicity may contribute to the impaired neutrophil function in the diabetic state.

In this decade it is becoming clear that medium osmolarity and induced-cell volume changes play an important direct role in the intracellular regulation of metabolism and function (1-3). Cell swelling due to medium hyposmolarity or permeant molecules stimulates exocytosis (4-7) and intracellular metabolism (3). In contrast, cell shrink due to medium hyperosmolarity suppresses exocytosis (8). In this phenomenon, osmotic force are suggested to be a main factor, however it becomes clear that second messenger systems are also involved (8).

On the other hand, despite the great improvement brought by insulin and antimicrobial agents, bacterial infection still accounts for an important causes of morbidity and mortality in diabetic patients (9). Neutrophils play a critical role in the host defence mechanism against various bacterial infections, and it is suggested that the impaired neutrophil functions (*e.g.* chemotaxis, phagocytosis, and bactericidal functions) cause the susceptibility to infections in diabetic patients (9-12). Recently, we (13,14) and others (15-17) have demonstrated that an impaired production of oxygen-derived free radicals (*e.g.* superoxide anion (O_2^-) and hydrogen peroxide-myeloperoxidase-halide (H_2O_2-MPO-Cl^-) antimicrobial system) by neutrophils from poorly controlled diabetic patients and streptozotocin (STZ)-induced diabetic rats. However, the mechanism by which the generation of oxygen-derived

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free radicals is decreased in diabetic neutrophils is still unclear.

To know the possibility that glucose hyperosmolarity may affect the generation of oxygen-derived free radicals in neutrophils, we studied the effect of hypertonic glucose or sucrose on the production of oxygen-derived free radicals; those were monitored by (i) chemiluminescence amplified by either luciferin analog (CLA; 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]-pyrazin-3-one) or luminol (L; 5-amino-2,3-dihydro-1,4-phthalazine-dione) in response to formyl-metionyl-leucyl-phenylalanine (fMLP), (ii) O_2^- generation measured by the reduction of added ferricytochrome c, and (iii) MPO activity.

Materials and Methods

The leukocytes were collected from male Wistar rats (200-250 g) by the method previously described (4). The obtained cells consisted of 95-98 % neutrophils and suspended in Hanks buffer ($1x10^6$ neutrophils/ml). Hypertonic sucrose or glucose solution was made by the addition of sucrose or glucose in Hanks buffer (isotonic=300 mOsm, pH=7.4).

CLA-DCL and L-DCL of prepared neutrophils were measured by the method previously described (13). Briefly, 50 μl of CLA or L (final concentration: 50 μM) were added into the sample tube containing 100 μl of the neutrophils-suspended solution and 1800 μl of Hanks buffer. After the preincubation for 2 min, the neutrophils were stimulated by 50 μl of fMLP (Sigma) solution (final concentration: 30 nM, which is ED_{50} in this system). Chemiluminescence from neutrophils was measured by Luminescence Reader (Aloka, Inc., Model BLP 102, Tokyo, Japan). The CLA-DCL and L-DCL were assessed with the peak value as KC/min/10^6 cells.

The O_2^- generation was measured by the reduction of added ferricytochrome c, as described before (18). The standard reaction mixture, which consisted of 10^5 neutrophils and 50 μM ferricytochrome c, was agitated in an incubator at 37 C. 10 min after 30 nM fMLP stimulation, the tubes were placed on ice and centrifuged at 1500 G for 5 min in the cold room. Absorbance of the supernatant solutions was determined spectrophotometrically at 550 nm.

MPO activity was measured by guaiacol method, as described before (4). 0.2 mM phosphate buffer, 40 mM guaiacol, and 0.02 % setaburon solution were added to the $1x10^4$ cells disrupted solution. Following the addition of 0.5 mM H_2O_2, changes of absorbance after the production of tetraguayacol were measured using spectrophotometer.

Statistical analysis was made with Newman-Keuls and Student's *t* test. $P<0.05$ was considered statistically significant. Viability of cells measured by Trypan blue staining was >97 % at both the beginning and end of the experiments. All values are expressed as mean $\pm$ S.E. (N=5). Experiments were repeated twice with essentially identical results each time.

Results

CLA-DCL and L-DCL in response to fMLP by control neutrophils were 5.5 $\pm$ 0.6 and 20.4 $\pm$ 1.8 KC/min/10^6 cells, respectively. Three minutes-incubation of rat neutrophils with graded concentration of medium hyperosmolarity (305, 310, 320, 340, 380, 460, 620, and 940 mOsm; isotonic = 300 mOsm) due to sucrose (FIG.1, left panel) or glucose (FIG.1, right panel) suppressed fMLP-induced CLA-DCL and L-DCL in a dose-dependent manner. In CLA-DCL, the minimum effective dose of hypertonic sucrose and glucose were both 320 mOsm, and IC_{50}s were 375 mOsm and 358 mOsm, respectively. In L-DCL, the minimum effective dose of sucrose and glucose were

both 340 mOsm, and IC_{50}s were 481 mOsm and 461 mOsm, respectively. With the analysis of IC_{50}s, both sucrose and glucose hyperosmolarity clearly more suppressed CLA-DCL than L-DCL.

The suppressive effect of 380 mOsm hypertonic sucrose or glucose on CLA-DCL (FIG. 2, left panel) and L-DCL (FIG. 2, right panel) was clearly time-dependent. Hypertonic glucose was more effective in this suppression than hypertonic sucrose was ($P < 0.05$, after 10 min incubation).

FIG. 1

Effect of graded degrees of sucrose (left panel) or glucose (right panel) hyperosmolarity on CLA-DCL and L-DCL in response to fMLP by rat neutrophils. Neutrophils were incubated in the various concentrations of hypertonic sucrose or glucose [300(=isotonic), 310, 320, 340, 380, 460, 620, and 940 mOsm) for 3 min before 30 nM fMLP stimulation. CLA-DCL and L-DCL are expressed as the percent changes of those of control (isotonic condition).

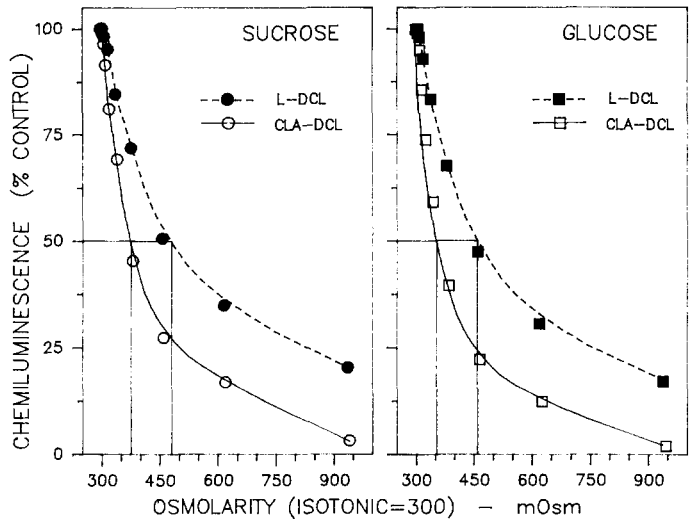
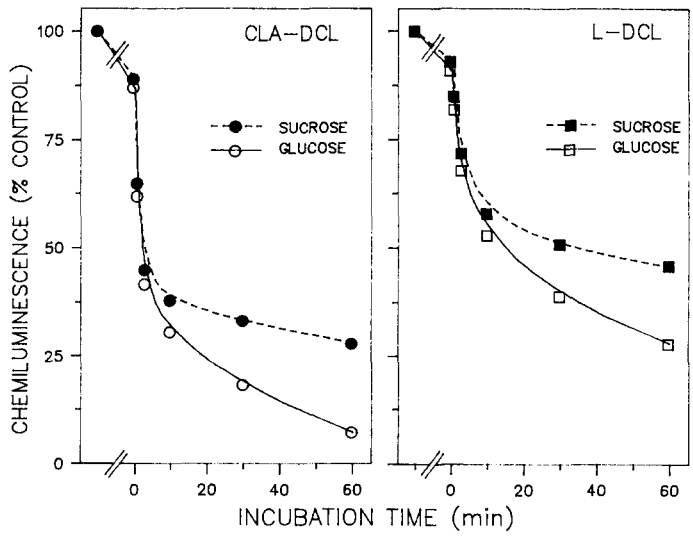


FIG. 2

Effect of incubation-time with hypertonic sucrose or glucose on CLA-DCL (left panel) and L-DCL (right panel). Neutrophils were incubated with 460 mOsm hypertonic sucrose or glucose for various time (0, 3, 5, 10, 30, and 60 min) before 30 nM fMLP stimulation. CLA-DCL and L-DCL are expressed as the percent changes of those of control (isotonic condition).



When O_2^- generation was measured directly by the reduction of added ferricytochrome c, both hypertonic sucrose and glucose suppressed O_2^- generation in a dose-dependent manner (FIG. 3). Hypertonic glucose clearly more inhibited O_2^- generation than hypertonic sucrose, when neutrophils were incubated under hypertonic condition > 460 mOsm.

MPO activity of control (intact) neutrophils was 594 ± 53 nmol/min/10^6 cells and the incubation with 620 mOsm hypertonic sucrose or glucose for 60 min did not significantly change the MPO activity (FIG. 4).

FIG. 3

Effect of graded degrees of sucrose or glucose hyperosmolarity on O_2^- generation. Neutrophils were incubated in the various concentrations of hypertonic sucrose or glucose [300 (=isotonic), 320, 340, 380, 460, 620, 940 mOsm] for 30 min before 30 nM fMLP. O_2^- generation was more inhibited by hypertonic glucose than by hypertonic sucrose under the condition of 460, 620, and 940 mOsm (*;$P<0.05$, Student's t test).

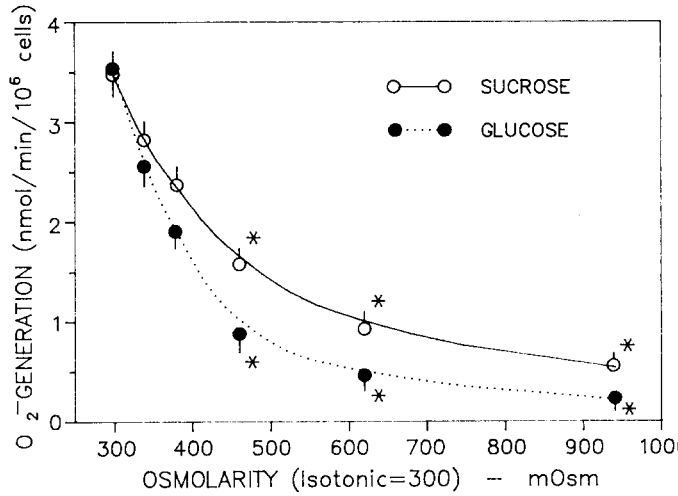
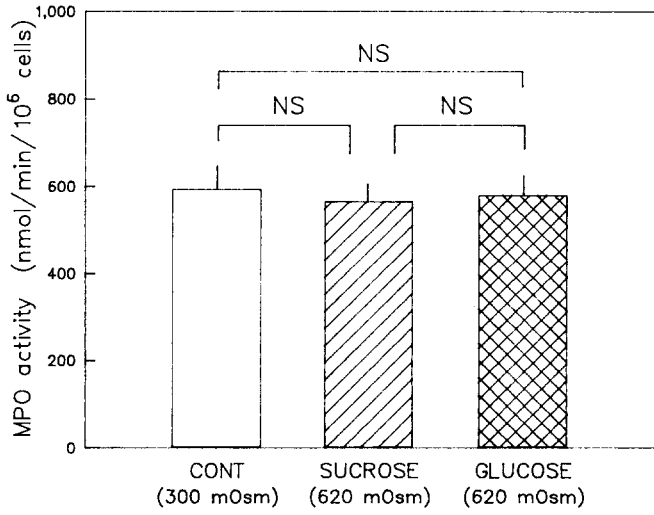


FIG. 4

Effect of hypertonic sucrose or glucose on MPO activity. MPO activity were measured from neutrophils incubated with either isotonic, 620 mOsm hypertonic sucrose, or 620 mOsm hypertonic glucose for 60 min. NS indicates that there is not significant difference between two groups (Newman-Keuls test).



Discussion

Previous studies have shown that the generation of oxygen-derived free radicals are reduced in the neutrophils from poorly controlled diabetic patients (9,16,17) and streptozotocin-induced diabetic rats (14). This impaired neutrophil function is suggested to account for the susceptibility to infections in diabetic states (9-12). It is reported that this impaired generation of oxygen-derived free radicals may be dependent on (i) the decreased production of NADPH, which is affected by insulin-dependent enzymes (14), (ii) the decreased MPO activity (13), and (iii) ketone bodies (18). However, there are very limited data about the effect of osmolarity, while (i) hyperosmotic states occur frequently in diabetes mellitus and (ii) extracellular osmolarity and induced cell volume changes exert an important direct influence on cell functions (1). The present data provide the first evidence that medium hyperosmolarity by itself inhibited the generation of oxygen-derived free radicals in neutrophils.

Most of oxygen uptake by stimulated neutrophils is utilized to form O_2^-, which is a major intermediate in the formation of H_2O_2. This generated H_2O_2 participates in the well established H_2O_2-MPO-Cl^- system (19,20). CLA-DCL and L-DCL appears to be highly dependent upon the generation of O_2^- (21,22) and a H_2O_2-MPO-Cl^- system (20,23-25), respectively. In this study, both CLA-DCL and L-DCL were clearly suppressed by medium hyperosmolarity due to either glucose or sucrose, and this inhibition was more effective in CLA-DCL than in L-DCL. Thus, our chemiluminescence (CLA-DCL and L-DCL) data suggest that medium hyperosmolarity inhibits mainly the generation of O_2^- and that a subsequent reduction of H_2O_2 generation affects a H_2O_2-MPO-Cl^- system. The H_2O_2-MPO-Cl^- system may not be directly altered by extracellular hyperosmolarity or glucose by itself. This idea is supported by the present results that hypertonic glucose or sucrose suppressed O_2^- generation, which was directly measured by cytochrome c, and it did not change the MPO activity.

O_2^- is generated from O_2 under the existence of NADPH oxidase and H_2O_2 is generated from O_2^- under the existence of superoxide dismutase. NADPH is mainly supplied from hexose monophosphate shunt. This *in vitro* experiment suggests that medium hyperosmolarity may reduce either NADPH oxidase activity or the generation of NADPH, and that this reduced O_2^- generation is not dependent on the impaired effect of insulin on insulin-dependent enzymes in the hexose monophosphate shunt.

Another interesting point in this study is that the generation of oxygen-derived free radicals was more inhibited by hypertonic glucose than hypertonic sucrose. This phenomenon became more significant when neutrophils were incubated under hypertonic condition for longer than 30 min. This result suggests that not only the medium hyperosmolarity but also glucose by itself reduced the generation of oxygen-derived free radicals in the neutrophils. The reason why hypertonic glucose is more effective in this phenomenon is not clear in the present study. The results are compatible with the entity of glucose toxicity (26). Finally, the present study suggests that keeping a good control of both blood glucose level and blood tonicity is an important factor to protect diabetic patients against frequent bacterial infections.

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