Inhibition of Polymorphonuclear Leukocyte Respiratory Burst by Elevated Glucose Concentrations in Vitro

CHRISTOPHER P. NIELSON AND DAVID A. HINDSON

Although impaired polymorphonuclear leukocyte (PMN) function may be a cause of infectious complications in diabetic patients, the mechanisms of altered cell function are not understood. Our studies of PMN function in healthy subjects demonstrated significant reduction in the respiratory burst after 30 min of in vitro cell exposure to glucose concentrations >11 mM (200 mg/dl). The respiratory burst was reduced 28 ± 5 and 74 ± 7% in PMNs incubated with 11 and 56 mM glucose, respectively. The impairment was independent of the cell stimulus (chemotactic peptide, calcium ionophore, or phorbol ester) and was not affected by sorbinil or *myo*-inositol. Because both D- and L-glucose had similar inhibitory effects, a nonenzymatic mechanism appeared to be the cause of impaired PMN function. Although mannitol and sorbitol did not affect cell function, monosaccharides (glucose, mannose, fructose) that form Schiff-base adducts with protein inhibited PMN function. These findings suggest a potential role for protein glycosylation in glucose-induced impairment of PMN function. *Diabetes* 38:1031–35, 1989

Infection in diabetic patients is a major cause of morbidity and mortality (1). Polymorphonuclear leukocytes (PMNs) from diabetic patients do not stimulate normally and have reduced bactericidal capacity (2). Because oxygen metabolites generated during the PMN respiratory burst are of major importance in host defense (3), an impairment in the respiratory burst may contribute to the pathogenesis of infectious complications in diabetes.

Although impaired PMN function in diabetes is well recognized, the cause of altered cell function is not understood (4,5). Because PMN impairment may correlate with the severity of hyperglycemia (6), we evaluated the effects of autologous plasma supplemented with glucose on PMN function in vitro.

RESEARCH DESIGN AND METHODS

Subjects. Blood samples from healthy male and female volunteers (aged 20–45 yr) were obtained at 0900. All subjects were fasting, had abstained from eating methylxanthine-containing foods for 12 h before study, and no subject was taking any medicine. Methylxanthine-containing foods were proscribed because of their potential for inhibiting PMN function. At the time of phlebotomy, mean (±SD) plasma glucose of the subjects was 5.2 (93 mg/100 ml) ± 0.04 mM. Mean (±SD) glycosylated hemoglobin was 7.2 ± 0.7%. The study protocol was approved by the University of Washington Human Subjects Committee.

Reagent preparation. Dulbecco's phosphate-buffered saline was prepared with 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The chemotactic peptide *N*-formylmethionyl-leucine-phenylalanine (FMLP) (1 μM), calcium ionophore A 23187 (0.2 μM), or the protein kinase C agonist phorbol myristic acetate (PMA) (1 nM) was used to initiate the PMN respiratory burst. A 23187 was dissolved in dimethyl sulfoxide at a concentration of 10 mM and stored at −20°C. PMA and FMLP were prepared in dimethyl sulfoxide immediately before use. Dimethyl sulfoxide was diluted at least 1:100 with buffer in the final reaction medium and did not alter PMN activation at this concentration.

PMN isolation. PMNs were isolated from venous blood that had been anticoagulated with 1 U/ml heparin via Ficoll-Hypaque centrifugation (7). Dextran (20 ml, 3% in normal saline) and EDTA (5 ml, 10 mg/ml) were added to 30 ml of whole blood, and erythrocytes were sedimented while blood was cooled to 4°C for 20 min. Leukocyte-rich plasma was then layered on a Ficoll-Hypaque gradient (sp gr 1.077) and centrifuged at 200 × *g* for 25 min. Bovine serum albumin (1 mg/ml) was included in both dextran and Ficoll-Hypaque solutions. PMNs were washed and contaminating erythro-

From the Research Unit, Veterans Administration Medical Center, Boise, Idaho; and the Division of Gerontology and Geriatric Medicine, and Division of Endocrinology, Department of Medicine, University of Washington, Seattle, Washington.

Address correspondence and reprint requests to Christopher Nielson, MD, Research Unit, Veterans Administration Medical Center, 500 West Fort Street, Boise, ID 83702.

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cytes removed with hypotonic lysis for 15 s in distilled water. PMNs were then resuspended in autologous plasma and kept on ice. The procedure yielded preparations that contained 95% PMNs that were at least 95% viable determined by trypan blue exclusion.

Measurement of PMN respiratory burst. Lucigenin-dependent luminescence was detected at 37°C with a Packard Instrument model 6500 Picolight luminometer. PMNs (200,000) were stimulated in 2 ml phosphate buffer with 1 μM FMLP, 1 nM PMA, or 0.2 μM A 23187; measurements of luminescence were then performed at 2-min intervals. Luminescence with lucigenin after oxidation of xanthine by xanthine oxidase (superoxide anion generation in cell-free system) was linearly related to xanthine concentration and superoxide concentration measured by cytochrome reduction. Lucigenin-dependent luminescence was inhibited by 100 U/ml superoxide dismutase and was not induced by hydrogen peroxide or hypochlorous acid. Thus, as previously reported (8), lucigenin-dependent luminescence appeared to indicate superoxide anion generation.

Control specimens demonstrated that glucose did not affect the lucigenin-dependent luminescence measurement of the respiratory burst. When introduced immediately before PMN stimulation, glucose, at concentrations of up to 65 mM (maximum tested), did not affect lucigenin-dependent luminescence. To determine whether a glucose reaction to either buffer or plasma components would affect the luminescence measurement, glucose (without PMN) was incubated at 37°C in buffer or plasma. Neither buffer nor plasma affected the respiratory burst when added immediately before cell stimulation. Finally, to determine whether the osmotic effects of glucose would affect cell function or luminescence measurement, PMNs were incubated with mannitol (60 mM) for 30 min and then stimulated to induce the respiratory burst. PMN incubated with mannitol generated the same magnitude of lucigenin-dependent luminescence as PMN did when incubated without mannitol.

Data analysis. Luminescence was integrated over 2–10 min after PMN activation. All comparisons of glucose effects were paired with PMN from the same blood sample, with activation and measurement of luminescence performed simultaneously under each experimental condition. Experiments were usually repeated five times with specimens from different donors. Statistical evaluation was performed with analysis of variance (ANOVA) and the Student-Newman-Keuls test.

RESULTS

Because impaired PMN function has been associated with poor glucose control in diabetic patients (9), the effects of PMN exposure to increased glucose concentrations were evaluated. Cells were initially exposed to glucose only during stimulation and without preincubation. When PMNs were stimulated by the chemotactic peptide FMLP in the presence of glucose at concentrations of 5–60 mM (90–1100 mg/dl), no effects of glucose on the PMN respiratory burst were apparent (Fig. 1; ANOVA $F_{5,24} = 1.9$, $P > .05$). Thus, increased glucose concentrations during the respiratory burst may not affect PMN function. However, previous studies have demonstrated impaired function in vitro in PMN exposed to hyperglycemia in vivo (5,10). Therefore, we evaluated the effects of PMN exposure to glucose before stimulation of the respiratory burst.

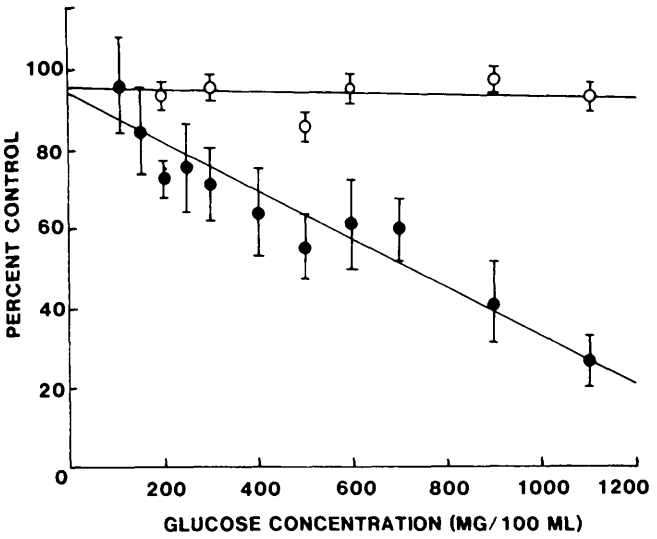

FIG. 1. Glucose effects on polymorphonuclear leukocyte (PMN) respiratory burst induced by 1 μM *N*-formylmethionyl-leucine-phenylalanine. D-Glucose was introduced at cell activation or 30 min before cell activation as indicated. Oxygen metabolite generation was measured with lucigenin-dependent luminescence at 2–4 min after activation. PMN function is indicated as percentage of luminescence from cells not exposed to added glucose (control specimen). Controls were separated, incubated, and stimulated parallel to specimens exposed to glucose. Data are means ± SE with specimens of 2 × 10^5 PMN from each of 5 subjects. ○, No incubation; ●, 30-min incubation.

PMNs (2 × 10^5 cells) were suspended in 40 μl of autologous plasma supplemented with glucose to concentrations of 5–60 mM (90–1100 mg/dl). The cells were then incubated at 37°C with gentle agitation. After 30 min, 1960 μl of phosphate buffer (37°C) containing 5 mM (90 mg/dl) glucose was added to each specimen. Lucigenin (1 μM) was then added, and the cells were stimulated with 1 μM FMLP. With these methods, the concentration of glucose to which cells were exposed during the respiratory burst (5–6 mM) was nearly uniform between specimens incubated with or without glucose. PMNs incubated with higher glucose concentrations had a marked reduction in the magnitude of respiratory burst induced by FMLP (Fig. 1; ANOVA $F_{10,40} = 11.55$, $P < .005$). The respiratory burst was impaired in all specimens of PMNs incubated in plasma containing any concentration of glucose >11 mM (200 mg/dl) (Student-Newman-Keuls test, $P < .05$).

When induced by chemotactic peptide, the PMN respiratory burst is dependent on receptor binding of the peptide, membrane transduction of the stimulus, and phosphatidylinositol hydrolysis (11). To determine whether glucose affected the initial membrane-dependent components of stimulus-response coupling, PMNs were stimulated with A 23187 or PMA. A 23187 directly mobilizes intracellular calcium, and PMA is a protein kinase C agonist. Although calcium and protein kinase C are partially independent pathways of stimulus-response coupling in PMN, both pathways probably activate the same NADPH oxidase responsible for the respiratory burst (12). These studies were also of interest because protein kinase C agonists have been reported to improve nerve tissue impairment in diabetes (13). PMNs stimulated by either FMLP, A 23187, or PMA were similarly inhibited by incubation with glucose (Fig. 2; 59, 64, and 64% inhibition by 56 mM glucose when PMNs stimulated by FMLP,

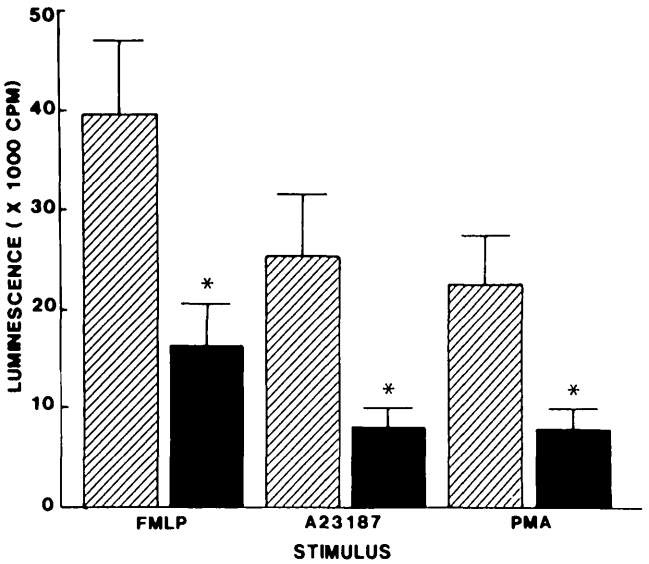

FIG. 2. Glucose (*solid bars*) effects on polymorphonuclear leukocyte (PMN) respiratory burst induced by 1 μM *N*-formylmethionyl-leucine-phenylalanine, 0.2 μM calcium ionophore A 23187, or 1 μM phorbol myristic acetate. Cells were incubated before stimulation for 30 min at 37°C in autologous plasma supplemented with 56 mM glucose or without glucose (control, *hatched bars*) as indicated. Respiratory burst was evaluated with lucigenin-dependent luminescence for 10 min after stimulation and is indicated with counts per minute (CPM), relative unit of luminescence from Packard Picolight luminometer. Data are means ± SE with specimens of 2 × 10^5 PMN from each of 5 subjects.

A 23187, and PMA, respectively; ANOVA $F_{2,12} = 0.23$, $P > .5$). To include the periods of maximal cell response to each stimulus, these measurements reflect average luminescence over 10 min after initial stimulation. The respiratory burst appeared to be suppressed throughout the period of cell activation. No change in the time of initial response or duration of the respiratory burst was apparent with any stimulus. Thus, inhibition of the PMN respiratory burst by glucose did not appear to be caused by an effect on membrane transduction of the cell stimulus.

Both sorbinil, an aldose reductase inhibitor, and *myo*-inositol have been shown to prevent or reverse some complications of diabetes (14). To determine whether glucose metabolism through the aldose reductase pathway contributed to the glucose-induced impairment of PMN function, either sorbinil or *myo*-inositol was included during PMN incubations with glucose. Concentrations of each agent were selected to be in excess of the concentration required to prevent or reverse diabetic complications in past studies. Improvement in neuron function has been reported after *myo*-inositol supplementation that increased plasma *myo*-inositol from 30 to 250 μM (15). Sorbinil inhibits aldose reductase by 50% at concentrations <1 μM (16). Plasma concentrations achieved with sorbinil doses that reverse diabetic neuropathy are <10 μM (17,18). However, neither 300 μM sorbinil nor 300 μM *myo*-inositol affected the impairment of PMN function induced by incubation with glucose. The PMN respiratory burst was reduced 62 ± 19 and 63 ± 9% by 56 mM glucose (30 min at 37°C in autologous plasma) despite inclusion of 300 μM sorbinil or 300 μM *myo*-inositol, respectively (both $P > .05$ vs. control without sorbinil or *myo*-inositol, $n = 5$).

Because enzyme binding to substrate is stereospecific, effects of glucose mediated by enzymatic processes are stereospecific. To determine whether PMN inhibition by glucose metabolism was dependent on an enzymatic reaction, effects of the stereoisomers D- and L-glucose on the respiratory burst were studied. Both D- and L-glucose induced similar PMN inhibition (Fig. 3, $P > .05$). Thus, a nonenzymatic process appeared to mediate PMN inhibition by glucose.

Nonenzymatic protein glycosylation is well recognized in diabetes (19). Glycosylation is the process of Schiff-base formation between a monosaccharide and protein followed by an Amadori rearrangement and, finally, protein cross-linking. Because Amadori product formation and protein cross-linking occur over days or weeks, glycosylation is often used clinically as a measurement of chronic diabetic glucose control (20). However, Schiff-base formation may occur much more rapidly (10–20 mM glucose incorporated into each mole of hemoglobin per hour with 300 mg/dl glucose) (21). The Schiff base can be formed between protein and most monosaccharides. Therefore, the effects of mannose and fructose, which may glycosylate protein, were compared with effects of mannitol and sorbitol, which are structurally similar to mannose and glucose but lack the aldehyde function necessary for glycosylation. PMNs incubated with either mannose or fructose (56 mM) were inhibited, whereas cells incubated with mannitol or sorbitol (56 mM) were minimally affected (Fig. 4). Both fructose and mannose caused significant inhibition compared with control, mannitol, or sorbitol ($P < .05$), although neither mannitol nor sorbitol caused significant inhibition compared with control ($P > .05$).

DISCUSSION

These studies demonstrate that the PMN respiratory burst is impaired by cell incubation with glucose at concentrations that commonly occur in diabetic patients. The results are

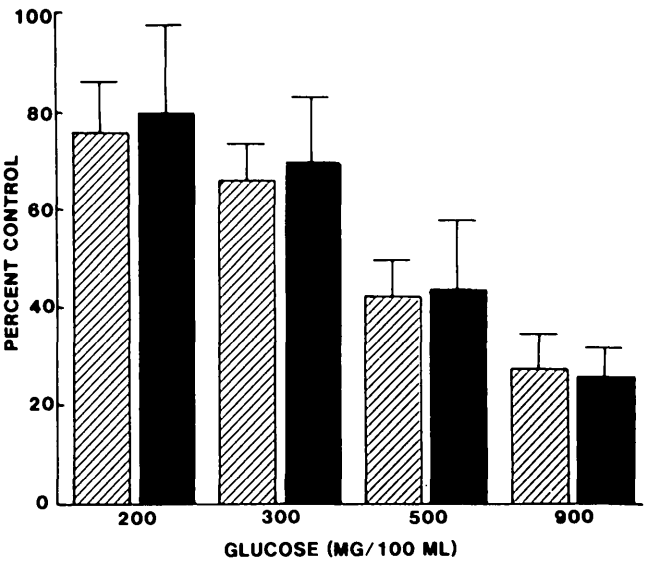

FIG. 3. Effects of polymorphonuclear leukocyte (PMN) incubation with D- (*hatched bars*) or L- (*solid bars*) glucose before induction of respiratory burst by 1 μM *N*-formylmethionyl-leucine-phenylalanine. Oxygen metabolite generation was measured with lucigenin-dependent luminescence for 4 min after cell activation. PMN function is indicated as percentage of luminescence from cells not exposed to added glucose (controls). These controls were separated, incubated, and stimulated parallel to specimens exposed to glucose. Data are means ± SE with specimens of 2 × 10^5 PMN from each of 3 subjects.

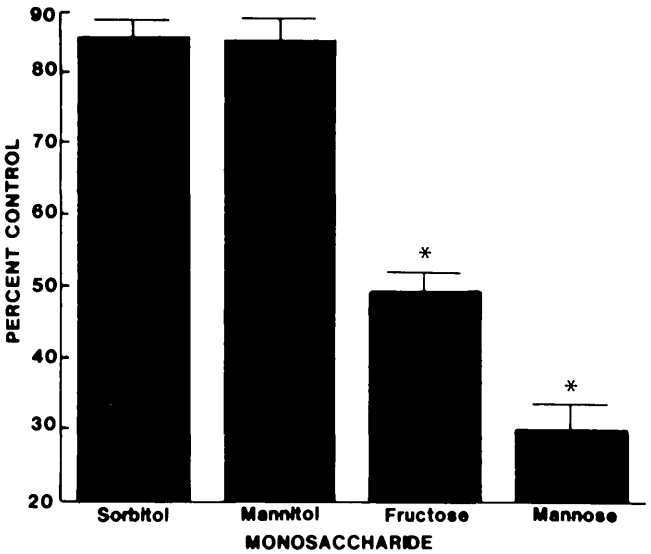

FIG. 4. Effects of sorbitol, mannitol, mannose, and fructose on the polymorphonuclear leukocyte (PMN) respiratory burst induced by 1 μM *N*-formylmethionyl-leucine-phenylalanine. Cells were incubated in autologous plasma supplemented with 56 mM sugar each as indicated. Oxygen metabolite generation was measured with lucigenin-dependent luminescence for 4 min after cell activation. PMN function is indicated as percentage of luminescence from cells not exposed to added sugar (controls). These controls were separated, incubated, and stimulated parallel to specimens exposed to sugars. Data are means ± SE with specimens of 2 × 10^5 PMN from each of 5 subjects.

particularly remarkable because of the short period of relatively modest elevation in glucose required to induce significant cell dysfunction. Elevation of plasma glucose >200 mg/dl (11 mM) is common in diabetic patients. These results support previous work that suggests hyperglycemia may predispose to infectious complications in diabetes (1,2).

Multiple cell types and numerous cell actions maintain host defense. The specific ramifications of an impairment in the PMN respiratory burst are unclear. However, disease states and pharmacological agents that reduce PMN chemiluminescence have been associated with reduced microbicidal activity in vitro (22) and with an increased incidence of infection (23). The reduction in chemiluminescence induced by many pharmacological agents correlates closely with a reduction in bactericidal activity (22). PMNs from diabetic subjects (229–312 mg/dl glucose) demonstrate a 5–45% decrease in staphylococcal killing (1). This impairment is in the same range as the 28% reduction in chemiluminescence induced by 300 mg/dl glucose in this study. Thus, a glucose-induced abnormality in the PMN respiratory burst may be of clinical significance.

Data have been reported that suggest PMN dysfunction in diabetes may be caused by reduced phosphofructokinase activity (24), intracellular sorbitol accumulation (25), and altered arachidonic acid metabolism (26). Although impaired PMN function in diabetes may be induced by multiple mechanisms, our results suggest that the rapid impairment in respiratory burst induced by glucose in vitro is caused by glycosylation. The experiments with glucose isomers, sorbinil, *myo*-inositol, and alternative stimuli of the respiratory burst argue against a detrimental effect of glucose mediated by either glycolytic or aldose reductase (polyol) pathways of glucose metabolism.

When tissues are exposed to hyperglycemia, increased conversion of glucose to sorbitol by aldose reductase is associated with accumulation of sorbitol and depletion of cellular *myo*-inositol (27). Because PMN stimulation by chemotactic peptide induces phosphatidylinositol hydrolysis (11), a depletion of *myo*-inositol could impair PMN response to chemotactic peptide. Although sorbinil blocks glucose metabolism by aldose reductase (28), and increased extracellular *myo*-inositol prevents reduction of intracellular *myo*-inositol (14), neither sorbinil nor *myo*-inositol prevented the impairment of PMN function induced by glucose. Thus, PMN function did not appear to be impaired by an effect mediated by glucose metabolism through the aldose reductase (polyol) pathway.

Although the optical isomers of glucose have identical chemical properties, only the D-isomer is a substrate for most enzymes. Because both D- and L-glucose caused similar impairment of PMN function, the effect appeared to be mediated by a nonenzymatic process. The nonenzymatic process most often implicated in diabetic complications is protein glycosylation (29).

Protein glycosylation results from formation of a Schiff base (aldimine) between the aldehyde function of a monosaccharide and a free amino group (usually ε-amino group of lysine) of protein (30,31). The Schiff base is labile and increases rapidly in amount. Slowly, an Amadori rearrangement occurs that leads to a stable ketoamine linkage and, finally, protein cross-linking may occur. Schiff-base formation reaches equilibrium within minutes to hours, whereas Amadori product formation requires hours to days (21). Various monosaccharides, including mannose and fructose, will condense with protein (32). In contrast, compounds that lack the aldehyde function are unable to form a Schiff base. Mannitol and sorbitol are structurally identical to mannose and glucose but lack the aldehyde function. Our studies demonstrated that glucose, mannose, and fructose would inhibit the PMN respiratory burst, whereas mannitol and sorbitol had minimal effect. These results demonstrated that a simple osmotic effect could not be responsible for inhibition of PMN function and further suggested that protein glycosylation may be the mechanism of impaired cell function.

Most studies of glycosylation have measured the stable Amadori or protein cross-linked products of glucose condensation (33,34). The distinction of which pathologic effects are mediated by cross-linked protein, Amadori products, or Schiff-base formation is not well established. In most circumstances, the relative amounts of each product will be closely correlated to one another. However, because PMN function impairment was induced by glucose within 30 min, effects of glycosylation were likely to have been mediated by Schiff-base formation.

A principal result of our study is the characterization of an in vitro model of the glucose-mediated impairment of PMN function. Initial results with this model suggest that glucose impairs PMN function independent of the pathway of stimulus-response coupling through a nonenzymatic mechanism that may involve protein glycosylation. Further studies, including direct measurement of glycosylation products and evaluation of the activities of specific enzymes, will be required before the mechanism of impaired PMN function in diabetes can be clearly defined. Although multiple factors,

including insulin deficiency and secondary effects of chronic hyperglycemia, may contribute to the impairment of host defense in diabetes, demonstration that PMN function is rapidly altered by modest elevation of plasma glucose may be relevant in the evaluation and management of diabetic patients.

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REFERENCES

1. Rayfield EJ, Ault MJ, Keusch GT, Brothers MJ, Nechemias C, Smith H: Infection and diabetes: the case for glucose control. *Am J Med* 72:439–50, 1982
2. Repine JE, Clawson CC, Goetz FC: Bactericidal function of neutrophils from patients with acute bacterial infections and from diabetics. *J Infect Dis* 142:869–75, 1980
3. Klebanoff SJ: Oxygen metabolism and the toxic properties of phagocytes. *Ann Intern Med* 93:480–89, 1980
4. Bagdade JD, Walters E: Impaired granulocyte adherence in mildly diabetic patients: effects of tolazamide treatment. *Diabetes* 29:309–11, 1980
5. Mowat AG, Baum J: Chemotaxis of polymorphonuclear leukocytes from patients with diabetes mellitus. *N Engl J Med* 284:621–27, 1971
6. Bagdade JD, Stewart M, Walters E: Impaired granulocyte adherence: a reversible defect in host defense in patients with poorly controlled diabetes. *Diabetes* 27:677–81, 1978
7. Boyum A: Separation of leukocytes from blood and bone marrow. *Scand J Clin Lab Invest Suppl* 21:1–89, 1968
8. Gyllenhammar H: Lucigenin chemiluminescence in the assessment of neutrophil superoxide production. *J Immunol Methods* 97:209–13, 1987
9. Bagdade JD, Root RK, Bulger RJ: Impaired leukocyte function in patients with poorly controlled diabetes. *Diabetes* 23:9–15, 1974
10. Shah SV, Wallin JD, Eilen SD: Chemiluminescence and superoxide anion production by leukocytes from diabetic patients. *J Clin Endocrinol Metab* 57:402–409, 1983
11. Takenawa T, Homma Y, Nagai Y: Role of Ca^{2+} in phosphatidylinositol response and arachidonic acid release in formylated tripeptide- or Ca^{2+} ionophore A 23187-stimulated guinea pig neutrophils. *J Immunol* 130:2849–55, 1983
12. McPhail LC, Snyderman R: Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli: evidence that the same oxidase is activated by different transductional mechanisms. *J Clin Invest* 72:192–200, 1983
13. Greene DA, Lattimer SA: Protein kinase C agonists acutely normalize decreased ouabain-inhibitable respiration in diabetic rabbit nerve. *Diabetes* 35:242–45, 1986
14. Winegrad AI: Does a common mechanism induce the diverse complications of diabetes? *Diabetes* 36:396–406, 1987
15. Greene DA, Lewis RA, Lattimer SA, Brown MJ: Selective effects of *myo*-inositol administration on sciatic and tibial motor nerve conduction parameters in the streptozocin-diabetic rat. *Diabetes* 31:573–78, 1982
16. Sarges R, Peterson MJ: Sorbinil: a member of the novel class of spirohydantoin aldose reductase inhibitors. *Metabolism* 35:101–104, 1986
17. Foulds G, O'Brien MM, Bianchine JR, Gabbay KH: Kinetics of an orally absorbed aldose reductase inhibitor, sorbinil. *Clin Pharmacol Ther* 30:693–700, 1981
18. Judzewitsch RG, Jaspan JB, Polonsky KS, Weinberg CR, Halter JB, Halar E, Pfeifer MA, Vukadinovic C, Bernstein L, Schneider M, Liang KY, Gabbay KH, Rubenstein AH, Porte D Jr: Aldose reductase inhibition improves nerve conduction velocity in diabetic patients. *N Engl J Med* 308:119–25, 1983
19. Cohen MP: *Diabetes and Protein Glycosylation*. New York, Springer-Verlag, 1986, p. 1–111
20. Brownlee M, Cerami A, Vlassara H: Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 318:1315–21, 1988
21. Higgins PJ, Bunn HF: Kinetic analysis of the nonenzymatic glycosylation of hemoglobin. *J Biol Chem* 256:5204–208, 1981
22. Horan TD, English D, McPherson TA: Association of neutrophil chemiluminescence with microbicidal activity. *Clin Immunol Immunopathol* 22:259–69, 1982
23. Rosen H, Klebanoff SJ: Chemiluminescence and superoxide production by myeloperoxidase-deficient leukocytes. *J Clin Invest* 58:50–60, 1976
24. Esmann V: The polymorphonuclear leukocyte in diabetes mellitus. *J Clin Chem Clin Biochem* 21:561–67, 1983
25. Wilson RM, Tomlinson DR, Reeves WG: Neutrophil sorbitol production impairs oxidative killing in diabetes. *Diabetic Med* 4:37–40, 1987
26. Qvist R, Larkins RG: Diminished production of thromboxane B_2 and prostaglandin E by stimulated polymorphonuclear leukocytes from insulin-treated diabetic subjects. *Diabetes* 32:622–26, 1983
27. Greene DA, Lattimer SA: Altered sorbitol and *myo*-inositol metabolism as the basis for detective protein kinase C and (Na,K)-ATPase regulation in diabetic neuropathy. *Ann NY Acad Sci* 488:334–40, 1986
28. Greene DA, Lattimer SA, Sima AA: Sorbitol, phosphoinositides, and sodium-potassium-ATPase in the pathogenesis of diabetic complications. *N Engl J Med* 316:599–606, 1987
29. Brownlee M, Vlassara H, Cerami A: Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Intern Med* 101:527–37, 1984
30. Garlick RL, Mazer JS: The principal site of nonenzymatic glycosylation of human serum albumin in vivo. *J Biol Chem* 258:6142–46, 1983
31. Garlick RL, Mazer JS, Higgins PJ, Bunn HF: Characterization of glycosylated hemoglobins. *J Clin Invest* 71:1062–72, 1983
32. Dolhofer R, Wieland OH: In vitro glycosylation of hemoglobin by different sugars and sugar phosphates. *FEBS Lett* 85:86–90, 1978
33. Brownlee M, Pongor S, Cerami A: Covalent attachment of soluble proteins by nonenzymatically glycosylated collagen. *J Exp Med* 158:1739–44, 1988
34. Brownlee M, Vlassara H, Kooney A, Ulrich P, Cerami A: Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232:1629–32, 1986