Red Cell 2, 3-Diphosphoglycerate Levels Among Diabetic Patients with and Without Vascular Complications

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SUMMARY

There have been differences of opinion among authors concerning the levels of red cell 2,3-diphosphoglycerate (2,3-DPG) and nucleotides in nonacidotic diabetic patients. Our data suggest that abnormal levels of 2,3-DPG in diabetic patients are related to the presence of vascular complications and not to the duration of the disease per se. 2,3-DPG levels are normal in diabetic patients with no evidence of vascular complications (group A). In ambulatory patients with vascular complications (group B), significantly higher levels of 2,3-DPG are found than in normal subjects and patients in group A. In hospitalized diabetic patients with active peripheral vascular complications (group C), levels of 2,3-DPG are likewise significantly increased over those of normal subjects and patients of group A. 2,3-DPG was found to be significantly elevated in patients of group C as compared with group B. 2,3-DPG levels in venous blood from infected legs as compared with those of the peripheral venous blood were not significantly different, thereby ruling out local factors. There were no differences in the blood lactate levels in any of the groups studied. The elevation of the 2,3-DPG levels may be a reflection of attempted red blood cell compensation for tissue hypoxia in the diabetic with vascular disease. DIABETES *24*:724-29, August, 1975.

Since the discovery in 1967 by Chanutin and Curnish[1] and simultaneously by Benesch and Benesch[2] that 2,3-DPG and, to a lesser extent, ATP, influence the affinity of hemoglobin for oxygen and facilitate oxygen release, many conditions that change the levels of 2,3-diphosphoglycerate (2,3-DPG) have been described.[3] It has been shown that in both acute hypoxia[4,5] and chronic hypoxia,[6,7] the red blood cell (RBC) 2,3-DPG levels are elevated. This rise in 2,3-DPG levels is thought to be a physiologic adjustment to compensate for impairment in oxygen supply to the tissues.[7] There have been numerous studies with conflicting results regarding the levels of 2,3-DPG and other phosphate nucleotides in nonacidotic diabetic patients.[8-14] In these studies, no attempts were made to correlate the findings with the degree of vascular disease of the subjects.

In the current study, red blood cell total phosphate (P_t), nucleotide phosphate (P_n), and 2,3-diphosphoglyceric acid (2,3-DPG) as well as whole blood inorganic phosphate and extracellular (serum) inorganic phosphorus (P_i) and (P_{is}), respectively, were measured and compared in diabetic subjects grouped according to the degree of their vascular disease. Lactate levels were measured in an attempt to assess hypoxia, and ketones were measured to rule out significant ketosis.

The existence of tissue hypoxia secondary to microangiopathy, macrovascular disease, or metabolic insufficiency has been implicated as a causative factor in the development of peripheral tissue breakdown and gangrene in the diabetic subject. The current study was undertaken to assess the relationship between biochemical indicators of tissue hypoxia in the diabetic patient with varying degrees of demonstrated vascular disease.

METHODS

Sixty patients from the Diabetes Outpatient Clinics at Rancho Los Amigos Hospital (RLAH) and thirty patients from the Diabetes Inpatient Service at RLAH were studied. In all patients, anamnesis and physical examination were performed according to the same protocol. Diabetic retinopathy was determined in the Eye Clinic by an ophthalmologist. Thirty healthy, normal subjects served as controls.

The diabetics were subgrouped as follows:

Group A: Thirty ambulatory patients with well-controlled diabetes and no (1) microangiopathy (retinopathy, nephropathy); (2) large-vessel disease (peripheral pulses present); or history of myocardial infarction (MI) or cerebrovascular accident (CVA); or (3) peripheral neuropathy (no peripheral motor or sensory defects). Some patients did have moderate loss of

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vibratory sensation but maintained the Achilles reflex response.

Group B: Thirty ambulatory patients with history and findings of at least three of the following major complications: (1) healed amputation in the past due to ulceration and/or gangrene related to diabetes, (2) retinopathy, (3) nephropathy, and (4) neuropathy; or two of the above with evidence of previous myocardial infarction, cerebrovascular accident, or total absence of peripheral pulses in the lower extremities.

Group C: Thirty diabetic patients who met the criteria of patients in group B and in addition were hospitalized for ulcers or gangrene of the lower extremities or for surgical procedures such as amputations, bone resections, and débridement. Only six of the patients were febrile, with evidence of leukocytosis (WBC >10,000). All the others were afebrile and were without leukocytosis. Evidence of active infection was provided by positive lesion culture. In seven patients, radiographic evidence for osteomyelitis was present. In ten patients from this group, in addition to the sampling of antecubital venous blood, a second sample of femoral venous return from the affected leg was obtained, and the biochemical results were compared.

The blood samples were drawn in the morning with the patients fasting. Only nonobese patients with no evidence of pulmonary or other metabolic disease and with no clinical evidence of ketoacidosis were included.

Antecubital venous blood was drawn without stasis. Within two minutes, 5.0 ml. was placed into precooled, phosphate-free, heparinized tubes and transferred to an ice bath. Precisely 1.0 ml. of the heparinized blood was hemolyzed by adding to 4.0 ml. of cold, distilled, deionized water followed by vigorous mixing. The proteins were precipitated by adding 1.0 ml. of 30 per cent trichloracetic acid (TCA). The mixture was shaken and centrifuged at 1,500 RPM at 4° for ten minutes, and the clear supernate was filtered through glass wool plugs or filter paper (Whatman no. 1). The filtrate (about 4.0 ml.) was considered a 1:6 dilution of blood and was used for the phosphate determinations. From the filtrates, samples of 10 lambda in quadruplicate were taken for the determination of the total phosphate content (P_t) and the inorganic phosphate (P_i) by use of the Bessman phosphate analyzer.[15] Ten-lambda samples for P_t determinations were added to alternate tubes as they entered the semicircular ashing unit, and 10-lambda samples for P_i determinations were added to alternate tubes as they left the ashing unit.

The remaining filtrate was then shaken by vortex mixer with 100 mg. of activated charcoal. (The activated charcoal was prepared by boiling Nuchar charcoal in 1-N HCL for twenty minutes. The acid was decanted and the charcoal washed with distilled water until the pH of the washing water was above 5, and no phosphorus was detectable (about fifteen washings). The wet charcoal was then dried in an oven at 200° F.). After shaking for five minutes, the sample was centrifuged at 1,500 RPM at 4° for ten minutes, and the clear supernate was filtered through glass wool plugs. As shown by Dyce et al.,[16] virtually all the nucleotides are absorbed by the charcoal, but not 2,3-DPG. Thus, the filtrate was considered at this stage to be free of nucleotide phosphates. Ten-lambda samples in quadruplicate were taken for the measurement of the total phosphorus content (P'_t). Nucleotide phosphates (P_n) were calculated from the difference between the total phosphorus determinations before and after charcoal treatment ($P_n = P_t - P'_t$).

The 2,3-DPG levels were calculated as the difference between the total phosphorus (P_t) and the sum of the inorganic phosphate (P_i) plus the nucleotide phosphates (P_n) divided by two and using the formula $\text{2,3-DPG} = \frac{P_t - (P_n + P_i)}{2}$ (The results are divided by two to account for the two moles of phosphorus per mole of 2,3-DPG.) The results are given (after correction for hematocrit and dilutional factors) as millimols of inorganic phosphate per liter of red blood cells (mM/L.RBC) for the values of P_t, P_n, and P_i, and as mM/L.RBC of 2,3-DPG. Dyce and Bessman have shown the validity of the 2,3-DPG levels obtained by this method as against the enzymatic assay of 2,3-DPG.[16] For further validation, we compared the 2,3-DPG levels obtained from whole-blood samples of fifteen control subjects measured as above with the 2,3-DPG levels obtained from the same samples using column chromatographic separation.[17] The 2,3-DPG values obtained by the column chromatography method were found to be 96 per cent of the values obtained by the direct ashing method described above.

Blood lactate levels were obtained according to the method of Barker and Summerson.[18] Total ketone and acetoacetate levels were determined by the Bessman and Anderson modified method.[19] Determinations of total serum lipids were made by the Zellner and Kirsch[20] method, serum phospholipids by the Bauman[21] method, and serum triglycerides by Technicon semiautomated method.[22]

Determinations of hemoglobin and hematocrit as

TABLE 1

Biochemical parameters* (nonphosphate compounds)

	Hematocrit	Hemoglobin	BUN	Glucose	Cholesterol	Total lipid	Triglyceride	Phospholipid	Lactate	Total ketones	Acetoacetate
Control	40.6 ± 1.2	13.0 ± 0.2	14.1 ± 1.5	91.7 ± 2.2	187 ± 11				0.975 ±0.085	0.249 ± 0.020	0.138 ± 0.021
Group A	38.4 ± 0.8	12.4 ± 0.2	14.3 ± 1.7	142.9 ± 5.0†	207 ± 9.2	557 ± 44.6	126 ± 6.1	204 ± 7.2	1.011 ± 0.07	0.321 ± 0.04	0.193 ± 0.019
Group B	39 ± 1.1	11.7 ± 0.3	16.3 ± 2.2	201.5 ± 16.9†‡	240 ± 10.3	621 ± 19.4	166 ± 1.2	199 ± 6.8	1.171 ± 0.073	0.348 ± 0.033†	0.218 ± 0.016†
Group C	37.9 ± 1.5	11.2 ± 0.2	17.9 ± 2.1	210 ± 17.0†‡	207 ± 12.5	605 ± 22.7	148 ± 10	200 ± 5.1	1.049 ± 0.06	0.377 ± 0.023†	0.216 ± 0.015†

*Data given as mean ± S.E.M., hematocrit in per cent, hemoglobin in gm. per cent, lactate, total ketones, and acetoacetate in mEq./L., and other data in mg. per 100 ml.

†Significantly elevated above control ($P<0.005$).

‡Significantly elevated above Group A ($P<0.005$).

other hematologic data were obtained by the hospital laboratory by use of the Technicon Hemalog AutoAnalyzer. Serum glucose, urea nitrogen, phosphate, and cholesterol were assayed by the hospital laboratory using the SMA-12 Technicon multiple-channel AutoAnalyzer.

Statistical analysis was done by the Student *t*-test.

RESULTS

Table 1 details the levels of the hematocrit, serum urea nitrogen, glucose, cholesterol, total lipids, triglycerides, phospholipids, blood lactate, total ketones, and acetoacetate. There were no statistical differences (at the level of $P<0.05$) between control patients and groups A, B, and C (vide supra for definition of groups) in the serum urea nitrogen, cholesterol, lipid profile, and blood lactate levels. There was a statistically significant increase in the acetoacetate ($P<0.005$) and total ketone levels ($P<0.01$) of groups B and C over those of controls. However, there were no statistical differences in the lactate levels between the various groups. The serum glucose of all diabetic groups was significantly increased ($P<0.001$) over that of the control, and there were also significant differences in the glucose levels of groups B and C from those of group A ($P<0.005$). There were no significant differences between groups B and C. The hemoglobin and hematocrit levels were significantly higher in controls than in group C ($P<0.005$) and in controls than in group B ($P<0.01$). No significant differences in hemoglobin and hematocrit values were found between group A and groups B and C or controls.

Table 2 details the results of inorganic serum phosphorus levels (P_{is}), red blood cell inorganic phosphorus levels (P_i), and the various red cell organic phosphate compounds. P_{is} levels were significantly higher ($P<0.005$) in group B than in controls and in group C ($P<0.01$) than in controls. No statistical difference was found between P_{is} levels in group A and in controls or between the three diabetic patient groups. P_i levels were increased in groups B and C over those of the controls. No significant difference was found between these groups and group A. The increase in levels of total red cell phosphate (P_t) was statistically significant in groups B and C as compared both with controls and group A. The total nucleotide

TABLE 2

Levels of phosphate compounds

	P_{is}	P_i	P_t	2,3-DPG	P_n
Normal control (n=30)	3.13 ± 0.16	2.38 ± 0.05	18.09 ± 0.25	4.96 ± 0.12	5.59 ± 0.16
Group A (n=30)	3.26 ± 0.12	2.65 ± 0.11	18.31 ± 0.31	4.89 ± 0.11	5.70 ± 0.12
Group B (n=30)	3.90 ± 0.12	3.27 ± 0.10*	20.28 ± 0.55*†	5.80 ± 0.12*†	5.72 ± 0.31
Group C (n=30)	3.75 ± 0.15	3.05 ± 0.12*	21.37 ± 0.70*†	7.03 ± 0.19*†‡	6.01 ± 0.67

Data given as mean ± standard error of the mean (S.E.M.)

*Significant difference from control at level $P<0.005$.

†Significant difference from group A at level $P<0.005$.

‡Significant difference from group B at level $P<0.005$.

P_{is} = serum phosphorus (mg per 100 ml.)

P_i = inorganic phosphate (mM/L.RBC).

P_t = total phosphate (mM/L.RBC).

2,3-DPG = 2,3 diphosphoglycerate (mM/L.RBC).

P_n = total nucleotide phosphate (mM/L.RBC).

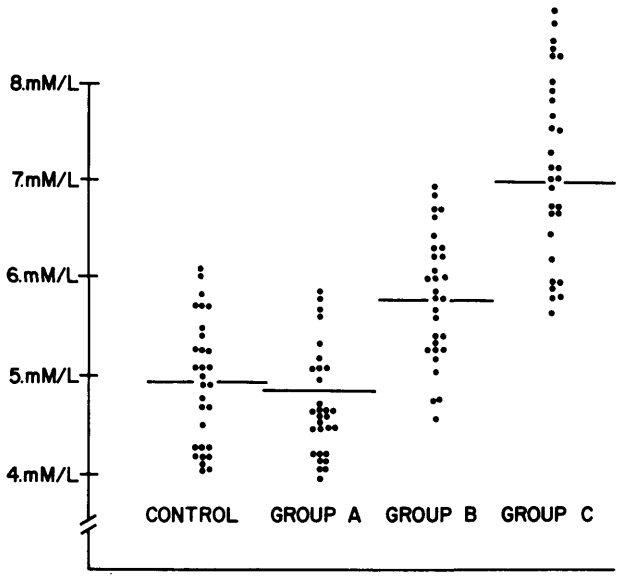

FIG. 1. Red cell 2,3-diphosphoglycerate levels among normal and diabetic subjects (see text for definition of groups).

phosphorus (P_n) levels were within the same range for all groups, with no statistical differences.

Levels of 2,3-DPG were significantly higher in groups B and C than in either controls or group A. In addition, the levels of 2,3-DPG in group C were significantly increased over those in group B (figure 1).

Table 3 shows the data of the venous return from the hand as compared with venous return from the infected gangrenous leg. The data reveal no significant differences between the corresponding levels of any of the parameters measured.

Table 4 compares levels of 2,3-DPG with urea nitrogen and glucose levels, sex, and duration of diabetes in groups B and C. There were no significant differences in blood glucose levels between these two groups. No correlation was found between the level of 2,3-DPG and the duration, per se, of the diabetes. No significant difference was found between the levels of 2,3-DPG of patients with normal BUN and those with BUN between 20 and 60. In group B, a significantly higher level of 2,3-DPG was found among females than among males. However, no significant differences of 2,3-DPG were found among males and females in groups A and C and the control groups. The pH and bicarbonate content of the venous blood as measured in about twenty patients of each group was within the normal limits, with no significant differences among the four groups studied.

DISCUSSION

Varied reports have been published on the RBC 2,3-DPG levels in nonacidotic diabetic subjects. Ditzel[8] showed that in ambulatory nonacidotic diabetics, the oxyhemoglobin dissociation curve might be shifted to the left as a result of a decrease in the total concentration of 2,3-DPG. Low levels of 2,3-DPG are known to occur in diabetic ketoacidosis.[11,24-26] Although Ditzel[8] early reported low levels of 2,3-DPG in uncontrolled diabetes, no significant changes in the 2,3-DPG levels in this type of patient were found by others.[9,10] 2,3-DPG fluctuations were described during hypoglycemia by Standal and Kolb.[27] However, they did not find significant differences between diabetic patients and normal controls. A rise in the levels of 2,3-DPG was found among normal and

TABLE 3
Comparison between affected leg and body venous blood*

	Lactate	Total ketones	Acetoacetate	P_t	P_i	2,3-DPG	P_n
Antecubital venous blood	0.965 ± 0.134	0.360 ± 0.064	0.232 ± 0.039	23.23 ± 1.11	3.18 ± 0.32	6.91 ± 0.17	5.85 ± 0.12
Femoral venous blood-affected leg	1.278 ± 0.160	0.398 ± 0.038	0.222 ± 0.034	22.42 ± 0.36	3.18 ± 0.35	6.84 ± 0.21	5.87 ± 0.23

*Data given as mean ± S.E.M., lactate, total ketones, and acetoacetate in mEq./L., phosphate compounds as in table 2.

TABLE 4
2,3-DPG levels in diabetic patients with vascular complications (correlation with other parameters)

	BUN Levels in mg/100ml		Sex differences		Duration of diabetes mellitus (yrs)			Serum glucose (mg/100ml)
	BUN 20	20 BUN 60	Males	Females	0-9	10-19	20-30	
Group B	5.27 ± 0.25 (n=15)	5.96 ± 0.28 (n=15)	5.6 ± 0.15 (n=17)	6.06 ± 0.18 (n=13)*	5.75 ± 0.2 (n=12)	5.99 ± 0.17 (n=10)	5.39 ± 0.1 (n=8)	201 ± 16.9 (n=30)
Group C	7.03 ± 0.24 (n=18)	7.16 ± 0.39 (n=12)	7.11 ± 0.27 (n=18)	7.07 ± 0.27 (n=12)	7.09 ± 0.33 (n=11)	7.06 ± 0.42 (n=9)	6.97 ± 0.30 (n=10)	210 ± 17.0 (n=30)

*Significantly different from males at $P<0.05$.

well-controlled diabetic pregnant women by Pang and Bleicher,[28] who related the elevation to a response to increased steroidal hormone level during pregnancy, since an identical effect was observed in patients who had been placed on estrogen-progesterone contraceptive medications.[28] Recently Ditzel, whose first report showed low levels of 2,3-DPG, reported that among nonacidotic diabetic children without signs of clinical microangiopathy,[12,23] the 2,3-DPG concentrations were significantly higher than those of a control group of children, and similar results have been obtained in adults.[13]

Our results demonstrate that significant elevations of the 2,3-DPG levels occur in ambulatory diabetic patients with vascular complications as compared to diabetics without such complications (figure 1). The studies also demonstrate that hospitalized patients with active peripheral vascular complications, such as foot ulcers, osteomyelitis, and/or gangrene, have even higher levels of 2,3-DPG than the diabetic patient with proved vascular disease but no evidence of tissue breakdown due to vascular insufficiency. Increased 2,3-DPG levels were obtained in febrile and afebrile patients with no significant difference between the two groups. Thus, the further elevation of 2,3-DPG levels in this group of patients (group C) compared to patients with evidence of vascular disease but no active peripheral vascular complications (group B) was not secondary to temperature elevation. Thus, our data may reflect an attempt by the RBC in the chronic diabetic to compensate for the impairment in the peripheral oxygen delivery caused by abnormalities in the vascular bed. These observations are at variance with earlier reports[8-10] but agree with reports on chronic hypoxemia,[6] cardiovascular insufficiency,[7] and ischemic heart disease [29,30] and with the recent studies among diabetics.[12-14,23]

Extracellular inorganic phosphorus levels did not show any significant differences between the various groups. Intracellular phosphorus levels (Pi) show significant elevations in groups B and C as compared with controls. Pt levels are also elevated in the diabetic subjects. However, in contrast to 2,3-DPG, which showed a progressive rise with increasingly severe manifestations of peripheral vascular disease (A<B<C), the increased levels of Pi and Pt were similar in patients with vascular disease with or without evidence of active peripheral vascular insufficiency.

Our data indicate that there is a gradual rise in 2,3-DPG levels in diabetic subjects with increasing evidence of vascular disease. Diabetic patients with no evidence of vascular disease (group A) have normal levels; patients with vascular complications (group B) have significant elevations of 2,3-DPG as compared with group A; and those patients with evidence of active peripheral vascular complications (group C) have the highest levels of 2,3-DPG. A possible explanation of our data is suggested by the studies of Asakura[31] and Yoshikawa.[32] These workers found that the glycolytic pathway in the red blood cell is stimulated by deoxygenation. Also, Ditzel[12,14] has shown that the increase in 2,3-DPG levels indicates a red blood cell response to tissue hypoxia among juvenile diabetics and an improvement in erythrocytic oxygen release following a dietary supplement of calcium diphosphate to diabetic and healthy children.[33]

Anemia is well known to be associated with a decreased affinity of hemoglobin for oxygen and with mild elevations in the 2,3-DPG levels within the red blood cell. As measured by Torrance[34] et al., for every gram per 100 ml. of hemoglobin decrease, there is a 2,3-DPG increase of 0.23 mM. In our study, the difference between the mean hemoglobin levels of group C and those of controls was 1.7 gm. per 100 ml. of hemoglobin, while the elevation in 2,3-DPG levels in group C above that of controls was 2.07 mM. All the groups in the study had mean hemoglobin levels above 11.2 gm. per cent. Thus, the effect of the small hemoglobin differences between the groups in our study is minimal, and the elevated 2,3-DPG levels seem to originate mostly from the stimulated red-cell glycolysis in the diabetic patients, presumably because of adaptive response to tissue anoxia. In the recent literature, other factors beside anoxia have been reported to elevate 2,3-DPG levels. The hormones aldosterone and cortisol,[35] androgens,[36,37] growth hormone,[38] and thyroid hormones[39,40] have been found to affect the 2,3-DPG levels and/or the oxygen affinity of the blood. Elevated levels of 2,3-DPG were recently found among nondiabetics with tissue damage and infected pressure sores, although the levels were below those found among diabetics with infection and gangrene (Kanter, Y., unpublished data).

Our data suggest that among nonacidotic chronic diabetic patients with vascular complications there are significantly elevated levels of 2,3-DPG. It appears that the 2,3-DPG is the red -cell phosphate intermediate most sensitive to the metabolic phenomena associated with progressive peripheral vascular insufficiency. Whether the 2,3-DPG changes represent a compensatory mechanism or play a contributory role in the pathogenesis of vascular complications in the diabetic is still unknown.

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