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EFFECTS OF PHYSIOLOGIC CONCENTRATIONS OF LACTATE, PYRUVATE AND ASCORBATE ON GLUCOSE METABOLISM IN UNSTRESSED AND OXIDATIVELY STRESSED HUMAN RED BLOOD CELLS

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Abstract—Glucose metabolism was studied in human red blood cells incubated in the presence of physiologic concentrations of ascorbate (0.1 mM) and/or lactate (2 mM) plus pyruvate (0.1 mM). The total flux through glycolysis, as measured by ^{14}C-labeling of glycolytic intermediates, was increased about 15% by ascorbate, 30% by lactate plus pyruvate, and 40% by ascorbate plus lactate plus pyruvate. We found, however, that physiologic concentrations of ascorbate and/or lactate plus pyruvate had no effect on flux of glucose or recycling of pentoses through the hexose monophosphate shunt. Increased formation of lactate accounted for most of the observed increase in glycolysis with little change in pyruvate formation, indicating that the increased flux of reducing equivalents from glucose was stored as lactate rather than being consumed by red cell metabolism. In all experiments, there was a net increase with time in the absolute amount of both lactate and pyruvate in red cell suspensions, indicating that lactate or pyruvate present at zero time did not function as a stoichiometric source or sink for reducing equivalents. There was little effect on steady-state levels of ATP or 2,3-diphosphoglycerate. Equilibration of ascorbate between red cells and the medium was complete before the addition of ^{14}C-labeled glucose to the medium. Glucose metabolism prevented net oxidation of ascorbate in the incubation medium. Physiologic concentrations of ascorbate, lactate and pyruvate appear to increase flux through glycolysis by increasing the turnover of ATP and/or 2,3-diphosphoglycerate. Red cells were exposed to mild oxidative stress by incubation with 0.27 mM 6-hydroxydopamine, 0.27 mM 6-aminodopamine, 0.13 mM 1,4-naphthoquinone-2-sulfonic acid or 0.27 mM phenylhydrazine. The metabolic response to oxidative stress was determined by measuring the formation of methemoglobin, pyruvate, lactate and CO_2 in the presence and absence of physiologic concentrations of lactate, pyruvate and ascorbate. Lactate, pyruvate and ascorbate had no effect on the net methemoglobin accumulation but rather on the distribution of the metabolic sources of reducing equivalents and on the flux of reducing equivalents to oxygen. Physiologic lactate and pyruvate allowed increased flow of reducing equivalents from glycolysis to methemoglobin and ultimately oxygen without the necessity of increased flux through glycolysis. This was accomplished by a decrease in the ratio of newly formed lactate to newly formed pyruvate with no increase in total lactate plus pyruvate. Ascorbate increased cycling between the quinone and quinol derivatives of oxidative agents, leading to increased production of reduced metabolites of oxygen and increased flux through the hexose monophosphate shunt. The effects of lactate plus pyruvate and ascorbate were superimposable without any apparent inhibition or synergism. Under mild oxidative stress, glycolysis and the hexose monophosphate shunt were equally important as sources of reducing equivalents in the red cell.

Ascorbate, lactate and pyruvate are normal constituents of the plasma. When red cells are incubated with high concentrations of these metabolites, alterations of the hexose monophosphate shunt [1,2] and glycolysis [3–5] are observed. Although these reports have contributed much to understanding possible interactions between the red cell and ascorbate, lactate and pyruvate, the behavior of red cells incubated with physiologic (plasma) concentrations has not been studied adequately. We have previously presented studies on the role of physiologic concentrations of these metabolites on methemoglobin reduction [6]. In this report, we examine the effects of physiologic concentrations of ascorbate, lactate and pyruvate on red cell glucose metabolism. The results show that in contrast to high concentrations of ascorbate, lactate and pyruvate, which cause striking alterations in flux through the hexose monophosphate shunt, physiologic concentrations of these metabolites caused increased flux through glycolysis with no effect on the hexose monophosphate shunt.

We have also investigated the role of physiologic lactate, pyruvate and ascorbate in the metabolic response of the red cell to oxidative stress. The metabolic response of red cells was gauged by measurements of the formation of methemoglobin, pyruvate, lactate and CO_2 by red cells exposed to relatively mild oxidative stress. Oxidative stress was provided by the addition to the suspending medium

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of one of four oxidative drugs: 6-hydroxydopamine, 6-aminodopamine, 1,4-naphthoquinone-2-sulfonic acid, and phenylhydrazine. Concentrations of each drug were chosen to produce mild oxidative stress such that after 1 hr at 37° there was no lysis of red cells, no formation of hemoglobin metabolites other than methemoglobin, and the flux of glucose through the hexose monophosphate shunt was accelerated no more than 7-fold. 6-Hydroxydopamine, a quinol, is a reducing agent which undergoes autoxidation to its semiquinone and quinone [7,8]. Superoxide, H_2O_2, hydroxyl radical and singlet oxygen are formed during autoxidation [9,10]. The quinone may be reduced to the quinol by products of glucose metabolism, including reduced ascorbate, giving rise to cycles of autoxidation [11–14]. Superoxide catalysis of 6-hydroxydopamine autoxidation [15] should be of minor importance in the presence of reactive reducing substances like ascorbate [14]. 6-Aminodopamine autoxidation is similar to that of 6-hydroxydopamine except that the reaction between the parent quinol and O_2 is faster and apparently requires no catalyst. The reaction between the quinone and ascorbate is very fast such that under certain conditions ascorbate is able to maintain the quinone/quinol ratio close to zero [14]. A quinone/quinol ratio close to zero prevents the immediate intracyclization and polymerization of the quinone of 6-aminodopamine [14]. 1,4-Naphthoquinone-2-sulfonic acid is an oxidizing agent which reacts with reducing agents to yield its semiquinone and quinol derivatives [16]. 1,4-Naphthoquinone-2-sulfonic acid reacts with oxyhemoglobin to generate superoxide, H_2O_2 and methemoglobin [16, 17]. Phenylhydrazine is oxidized by oxyhemoglobin to phenyldiazine which further reacts to generate superoxide, H_2O_2, methemoglobin and organic free radical derivatives of phenyldiazine [18]. In spite of the varied oxidative reactions of these four agents within red cells [17–23], the effects of physiologic lactate, pyruvate and ascorbate on the metabolic response of the red cell are similar in the presence of each agent.

MATERIALS AND METHODS

Red cell preparations. Adult human blood was drawn daily into a test tube containing a small amount of 3.8% sodium citrate solution. After centrifugation, plasma and white cells were removed, and red cells were washed three times with phosphate-buffered saline (139 mM NaCl, 10 mM KH_2PO_4/K_2HPO_4, pH 7.4). Red cells containing methemoglobin were prepared by suspending packed red cells in an equal volume of 0.5% $NaNO_2$ in half-concentrated phosphate-buffered saline and incubating for 10 min at 25°. The red cells were washed five times with phosphate-buffered saline to remove the NO_2^-.

Incubation conditions. Incubations were carried out using 25% (v/v) red cell suspensions in Krebs-Ringer glycylglycine buffer (120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 50 mM glycylglycine, pH 7.4). Erlenmeyer flasks (10 or 25 ml) containing 1 or 2 ml of red cell suspension, respectively, and closed with rubber stoppers, were incubated in a shaking water bath at 37° for 1 hr. Measurements of metabolic intermediates or products were made at zero time and 1 hr. Variable additions included 5 mM D-glucose and 0.1 mM L-ascorbate in the medium compartment and 2 mM L-lactate and 0.1 mM pyruvate in the cellular and medium compartments. Lactate and pyruvate were added together because they are linked by lactate dehydrogenase and are quickly equilibrated over medium and red cell compartments by a membrane carrier specific for monocarboxylates [24]. Stock solutions of ascorbate, lactate and pyruvate were prepared daily and brought to pH 7.4 before addition to red cell suspensions.

Red cell suspensions were oxidatively stressed by the addition to the medium compartment of 0.27 mM 6-hydroxydopamine (Aldrich Chemical Co., Milwaukee, WI), 0.27 mM 6-aminodopamine (a gift from Dr. Edward Engelhardt, Merck Sharp & Dohme, West Point, PA), 0.13 mM 1,4-naphthoquinone-2-sulfonic acid (Eastman Kodak, Rochester, NY) or 0.27 mM phenylhydrazine (Sigma Chemical Co., St. Louis, MO). Stock solutions of 6-hydroxydopamine and 6-aminodopamine were prepared in 140 mM NaCl, 1 mM KCl, pH 2.0, to inhibit autoxidation until addition to red cell suspensions. Equal volumes of 140 mM NaCl, 1 mM KCl, pH 2.0, without drug were added to red cell suspensions as controls.

Assays. Flux through the hexose monophosphate shunt was measured by following $^{14}CO_2$ formation from [U-^{14}C]glucose (total shunt activity), [1-^{14}C]glucose (initial entry into the shunt) and [2-^{14}C]glucose (first recycling) as described by Kelman *et al.* [25]. Formation of total glycolytic intermediates (fructose-6-phosphate through lactate) was determined by measuring ^{14}C-labeled glycolytic intermediates by the technique of Pescarmona *et al.* [26] as modified by Kelman *et al.* [25]. Pyruvate, lactate, ATP and 2,3-diphosphoglycerate were measured by techniques described by Beutler [27]. Total ascorbate (defined as reduced ascorbate plus dehydroascorbate plus diketogulonate) in medium and red cell compartments was measured as the 2,4-dinitrophenylhydrazone derivative by the method of Bessey *et al.* [28]. Reduced ascorbate in the medium compartment was measured by spectrophotometric titration at A_{515} with 2,6-dichlorophenolindophenol. Oxyhemoglobin, methemoglobin and intact hemoglobin (defined as the sum, oxyhemoglobin plus methemoglobin) were measured by a modification of the technique of Harley and Mauer [29] as previously described [22]. No decrease of intact hemoglobin was observed in any experiment.

RESULTS

Flux of glucose through glycolysis and the hexose monophosphate shunt. Table 1 shows the effects of incubation with physiologic concentrations of lactate, pyruvate and ascorbate on rates of formation of glycolytic intermediates. The total flux of glucose into glycolytic intermediates was increased about 30% by lactate plus pyruvate, 15% by ascorbate, and 40% by lactate plus pyruvate plus ascorbate. The effect of ascorbate on overall glycolytic flux

Table 1. Effects of ascorbate, lactate and pyruvate on formation of glycolytic intermediates*

Additions to incubation medium	3-carbon units [μmoles · hr^{-1} · (ml red cell)$^{-1}$]			
	Fructose-6-phosphate through phosphoenolpyruvate	Pyruvate	Lactate	Fructose-6-phosphate through lactate
Glucose	1.40	0.35 ± 0.01 (5)	1.62 ± 0.13 (5)	3.37 ± 0.40 (5)
Glucose + lactate + pyruvate	1.69	0.37 ± 0.04† (5)	2.30 ± 0.08‡ (5)	4.36 ± 0.52† (5)
Glucose + ascorbate	1.63	0.48 ± 0.06† (5)	1.72 ± 0.11†§ (5)	3.83 ± 0.35† (5)
Glucose + lactate + pyruvate + ascorbate	1.92	0.33 ± 0.04† (5)	2.47 ± 0.18‖ (5)	4.72 ± 0.57‡ (5)

* Red cell suspensions (25%, v/v) were incubated for 1 hr at 37° in the presence of various substrates. Concentrations in suspending medium were: glucose, 5 mM; lactate, 2 mM; pyruvate, 0.1 mM; and ascorbate, 0.1 mM. The formation of pyruvate, lactate and total glycolytic intermediates in 1 hr at 37° was determined as described in Materials and Methods. Formation of glycolytic intermediates was measured in ten independent experiments. In five experiments, the formation of all glycolytic intermediates from fructose-6-phosphate through lactate was measured by using radiolabeled glucose. In five experiments, the formation of pyruvate and lactate was measured by an enzymatic technique. Formation of fructose-6-phosphate through phosphoenolpyruvate was determined by calculation. Data are reported as mean ± S.E. with the number of experiments in parentheses. Level of significance was determined using a paired t-test.

† Not significant, $P > 0.05$ vs glucose alone.

‡ $P < 0.05$ vs glucose alone.

§ $P < 0.05$ vs glucose alone, significance of formation of pyruvate plus lactate.

‖ $P < 0.01$ vs glucose alone.

Table 2. Effects of ascorbate, lactate and pyruvate on the flux of glucose through the hexose monophosphate shunt*

Additions to incubation medium	CO_2 [μmoles · hr^{-1} · (ml red cell)$^{-1}$]			
	Carbon 1	Carbon 2	Carbons 3 through 6	Carbons 1 through 6
Glucose	0.041 ± 0.002 (3)	0.013 ± 0.003 (3)	0.003	0.057 ± 0.005 (3)
Glucose + lactate + pyruvate	0.039 ± 0.004 (3)	0.014 ± 0.002 (3)	0.002	0.056 ± 0.003 (3)
Glucose + ascorbate	0.042 ± 0.002 (3)	0.013 ± 0.002 (3)	0.000	0.055 ± 0.003 (3)
Glucose + lactate + pyruvate + ascorbate	0.042 ± 0.002 (3)	0.013 ± 0.001 (3)	0.008	0.063 ± 0.003 (3)

* Incubations were carried out as described in Table 1. Flux of glucose through the hexose monophosphate shunt was measured in three independent experiments. Formation of CO_2 from carbons 1, 2 and 1 through 6 was measured using [1-^{14}C]-, [2-^{14}C]- and [U-^{14}C]glucose, respectively, as described in Materials and Methods. Formation of CO_2 from carbons 3 through 6 was determined by calculation. Data are reported as mean ± S.E. with the number of experiments in parentheses.

Table 3. Lactate and pyruvate concentrations of red cell suspensions before and after incubation with physiologic concentrations of ascorbate, lactate and pyruvate*

Additions to incubation medium	Lactate (μmoles/ml red cell)		Pyruvate (μmoles/ml red cell)		Ratio of newly formed products ([lactate]/[pyruvate])
	0 time	1 hr	0 time	1 hr	
Glucose	0.62	2.23	0.24	0.59	4.6
Glucose + lactate + pyruvate	9.89	12.19	0.60	0.96	6.4
Glucose + ascorbate	0.62	2.34	0.24	0.72	3.6
Glucose + lactate + pyruvate + ascorbate	9.89	12.35	0.60	0.92	7.7

* Incubations were carried out as described in Table 1. Lactate and pyruvate were measured in 25% (v/v) red cell suspensions before and after a 1 hr incubation at 37° as described in Materials and Methods. Data are reported as the means of five independent experiments.

resulted from a significant increase in the sum of lactate and pyruvate formation. In contrast, increases in overall flux caused by incubation with lactate plus pyruvate or lactate plus pyruvate plus ascorbate reflected increased lactate formation with no change in pyruvate. Increases in intracellular pools of glycolytic intermediates other than lactate and pyruvate appeared to contribute to the increase in overall flux.

No change was observed in the intracellular pool of 2,3-diphosphoglycerate either at zero time or after 1 hr of incubation with any combination of glucose, lactate plus pyruvate and ascorbate (range: 3.0 to 3.6 μmoles/ml red cell). No change was observed in the intracellular pool of ATP either at zero time or after 1 hr of incubation with any combination of lactate plus pyruvate and ascorbate (ranges: 0.86 to 0.97 μmoles/ml red cell after 1 hr in the absence of glucose; 1.33 to 1.41 μmoles/ml red cell at zero time or after 1 hr in the presence of glucose).

Table 2 shows the flux of glucose through the hexose monophosphate shunt in the presence of various combinations of physiologic concentrations of lactate plus pyruvate and ascorbate. Lactate plus pyruvate and ascorbate had no effect on total flux, initial flux of glucose-6-phosphate (CO_2 from carbon 1), or recycling of pentoses (CO_2 from carbons 2 through 6) through the hexose monophosphate shunt.

Relationship between lactate and pyruvate pools. Table 3 shows the total content of lactate and pyruvate in red cell suspensions (red cells plus medium) before and after incubation with physiologic concentrations of glucose, lactate plus pyruvate and ascorbate. Under all conditions there was a net increase in both lactate and pyruvate content during 1 hr of incubation at 37°. This result indicates that 2 mM lactate added to the medium did not serve as a stoichiometric source of reducing equivalents. Incubation with ascorbate increased the content of both lactate and pyruvate and slightly shifted the ratio of new products in favor of pyruvate. Incubation with lactate plus pyruvate or lactate plus pyruvate plus ascorbate markedly shifted the ratio of new products in favor of lactate.

Table 4 shows the effects of lactate plus pyruvate and ascorbate on lactate and pyruvate formation in oxyhemoglobin- and methemoglobin-containing red cells. Methemoglobin-containing red cells had increased flux of glucose through glycolysis, resulting in increased formation of both lactate and pyruvate. The ratio of products ([lactate]/[pyruvate]) was shifted in favor of pyruvate. Incubation with lactate plus pyruvate increased the formation of new lactate plus pyruvate about the same extent as in oxyhemoglobin-containing red cells. In oxyhemoglobin-containing red cells, the overall increase in flux reflected increased lactate formation with no change in pyruvate; in methemoglobin-containing red cells a significant increase in pyruvate was observed, coupled with a less consistent increase in lactate. In methemoglobin-containing red cells, incubation with lactate plus pyruvate increased formation of products but had no effect on the ratio of products ([lactate]/[pyruvate]). Ascorbate had no significant effect on flux in methemoglobin-containing red cells.

Ascorbate pools. Total ascorbate (defined as reduced ascorbate plus dehydroascorbate plus diketogulonate) was measured in medium and red cell compartments before and after 1 hr of incubation with various combinations of glucose, lactate plus pyruvate and ascorbate (Table 5). A steady state between medium and red cell ascorbate was reached before the zero time of the incubation (approximately 5 min total elapsed before separation of medium and red cell compartments). No differences in total ascorbate occurred in medium or red cell compartments during the 1 hr incubation. The presence or absence of glucose and lactate plus pyruvate had no effect on the total ascorbate of either medium or red cell compartments. Incubation in the presence of 0.1 mM ascorbate increased red cell ascorbate content from about 0.05 mM (range: 0.048 to 0.059 mM) to about 0.08 mM (range: 0.072 to 0.084 mM). The presence of glucose but not lactate plus pyruvate in the medium maintained the ascorbate of the medium compartment in reduced form, whereas about 35% of ascorbate in the medium was oxidized in the absence of glucose.

Methemoglobin formation. The effects of lactate, pyruvate and ascorbate on the metabolic response

Table 4. Lactate and pyruvate in oxyhemoglobin and methemoglobin-containing red cells*

Additions to incubation medium	Lactate [μmoles · hr^{-1} · (ml red cell)$^{-1}$]		Pyruvate [μmoles · hr^{-1} · (ml red cell)$^{-1}$]		Ratio of products ([lactate]/[pyruvate])	
	HbO_2	MetHb	HbO_2	MetHb	HbO_2	MetHb
Glucose	1.62	1.92 ± 0.02 (4)	0.35	0.73 ± 0.01 (4)	4.6	2.6
Glucose + lactate + pyruvate	2.30	2.66 ± 0.39† (4)	0.37	1.00 ± 0.03‡ (4)	6.2	2.7
Glucose + ascorbate	1.72	1.97 ± 0.06† (4)	0.48	0.78 ± 0.04† (4)	3.6	2.5
Glucose + lactate + pyruvate + ascorbate	2.47	2.63 ± 0.36† (4)	0.33	1.14 ± 0.05‡ (4)	7.5	2.3

* Incubations were carried out as described in Table 1. Formation of lactate and pyruvate by methemoglobin (MetHb)-containing red cells was measured in four independent experiments as described in Materials and Methods. Data are reported as mean ± S.E. with the number of experiments in parentheses. Level of significance was determined using a paired *t*-test. Data with oxyhemoglobin (HbO_2)-containing red cells were taken from Table 1.

† Not significant, $P > 0.05$ vs glucose alone.

‡ $P < 0.01$ vs glucose alone.

Table 5. Ascorbate concentrations of medium and red cell compartments before and after incubation with physiologic concentrations of glucose, ascorbate, lactate and pyruvate*

Additions to incubation medium	Total ascorbate (mM)				Reduced ascorbate (mM)	
	Red cell		Medium		Medium	
	0 time	1 hr	0 time	1 hr	0 time	1 hr
None	0.059	0.048	0.000	0.000	0.000	0.000
Glucose		0.048		0.001		0.000
Lactate + pyruvate		0.052		0.003		0.000
Glucose + lactate + pyruvate		0.056		0.002		0.000
Ascorbate	0.084	0.072	0.098	0.100	0.090	0.067
Glucose + ascorbate		0.079		0.098		0.099
Lactate + pyruvate + ascorbate		0.080		0.099		0.060
Glucose + lactate + pyruvate + ascorbate		0.075		0.098		0.080

* Incubations were carried out as described in Table 1. Total ascorbate (reduced ascorbate plus dehydroascorbate plus diketogulonate) and reduced ascorbate were measured in 25% (v/v) red cell suspensions before and after a 1 hr incubation at 37° as described in Materials and Methods. The data are from a single representative experiment.

to oxidative stress were studied by incubating red cells in the presence of oxidative agents at concentrations which present a variety of oxidative stresses without causing lysis or loss of functional hemoglobin. Table 6 shows the methemoglobin accumulated in 1 hr at 37° in the presence of each oxidative agent and various substrates. Methemoglobin formation was less than 12% in all cases with 0.27 mM 6-hydroxydopamine or 0.27 mM phenylhydrazine. Methemoglobin formation was between 28 and 46% with 0.27 mM 6-aminodopamine or 0.13 mM 1,4-naphthoquinone-2-sulfonic acid. With each oxidative agent except phenylhydrazine, glucose inhibited methemoglobin accumulation to a greater extent than lactate plus pyruvate. The addition of lactate, pyruvate and ascorbate with glucose did not significantly decrease methemoglobin accumulation below that seen with glucose alone.

Effects of oxidative agents on flux through glycolysis and the hexose monophosphate shunt. Data on the effects of oxidative agents on formation of pyruvate and lactate are presented with each combination of physiologic substrates in the presence and absence of each oxidative agent (Tables 7–10). This is because physiologic lactate, pyruvate and ascorbate accelerated lactate formation and total glycolytic flux in the absence of an oxidative agent (Table 1). Physiologic lactate, pyruvate and ascorbate had no effect on flux through the hexose monophosphate shunt in the absence of an oxidative agent (Table 2) so that controls in the presence of glucose alone were carried out with each oxidative agent (Tables 7–10).

6-Hydroxydopamine (0.27 mM) caused a 20% increase in total glycolytic flux (pyruvate plus lactate) associated with a significant increase in pyruvate formation and a 500% increase in flux through the hexose monophosphate shunt (CO_2) (Table 7). In the presence of lactate plus pyruvate, 6-hydroxydopamine caused increased pyruvate formation without a significant increase in total glycolytic flux. In the presence of ascorbate, 6-hydroxydopamine increased both pyruvate and lactate formation. In the presence of all three physiologic constituents, lactate, pyruvate and ascorbate, the effects of 6-hydroxydopamine on glycolytic flux were essentially the same as with lactate plus pyruvate in the absence of ascorbate. Lactate, pyruvate and ascorbate had no effect on 6-hydroxydopamine-induced increases in flux through the hexose monophosphate shunt.

6-Aminodopamine (0.27 mM) caused a 20% increase in total glycolytic flux associated with a significant increase in pyruvate formation and a 300% increase in flux through the hexose monophosphate shunt (Table 8). In the presence of lactate plus pyruvate, 6-aminodopamine increased pyruvate formation and decreased lactate formation while causing no net change in total glycolytic flux. Ascorbate had no effect on 6-aminodopamine-induced alterations in glycolytic flux in the presence or absence of lactate plus pyruvate. Ascorbate but not lactate plus pyruvate caused increased 6-aminodopamine-induced flux through the hexose monophosphate shunt.

1,4-Naphthoquinone-2-sulfonic acid (0.13 mM) caused a 20% increase in total glycolytic flux associated with a significant increase in pyruvate formation and a 500% increase in flux through the hexose monophosphate shunt (Table 9). The effects of lactate, pyruvate and ascorbate on 1,4-naphthoquinone-2-sulfonic acid-induced changes in metabolic flux were qualitatively similar to their effects with 6-aminodopamine (compare Tables 8 and 9).

Phenylhydrazine (0.27 mM) caused a 15% increase in total glycolytic flux associated with a significant increase in pyruvate formation and a 100% increase in flux through the hexose monophosphate shunt (Table 10). In the presence of lactate plus pyruvate, phenylhydrazine caused increased pyruvate formation without a significant increase in total glycolytic flux. Ascorbate had no effect on phenylhydrazine-induced alterations in glycolytic flux in the presence or absence of lactate plus

Table 6. Formation of methemoglobin in red cells exposed to oxidative agents*

Additions to incubation medium	% Methemoglobin after 1 hr at 37° in the presence of:†			
	6-Hydroxydopamine	6-Aminodopamine	1,4-Naphthoquinone-2-sulfonic acid	Phenylhydrazine
None	11	39	46	7
Glucose	6	29	33	4
Lactate + pyruvate	9	38	40	4
Glucose + lactate + pyruvate	5	28	32	3
Ascorbate	10	39	42	7
Glucose + ascorbate	5	28	34	4
Lactate + pyruvate + ascorbate	9	38	40	5
Glucose + lactate + pyruvate + ascorbate	5	28	33	4

* Red cell suspensions (25% v/v) were incubated for 1 hr at 37° in the presence of various substrates and oxidative agents. Hemoglobin analyses were carried out at 1 hr as described in Materials and Methods. Concentrations in suspending medium were: glucose, 5 mM; lactate, 2 mM; pyruvate, 0.1 mM; ascorbate, 0.1 mM; 6-hydroxydopamine, 0.27 mM; 6-aminodopamine, 0.27 mM; 1,4-naphthoquinone-2-sulfonic acid, 0.13 mM; and phenylhydrazine, 0.27 mM.

† 1% Methemoglobin was present in the absence of oxidative agents.

pyruvate. Ascorbate but not lactate plus pyruvate caused increased phenylhydrazine-induced flux through the hexose monophosphate shunt.

Metabolic sources of reducing equivalents. Table 11 summarizes the oxidative metabolism stimulated by each oxidative agent in the presence of each combination of lactate, pyruvate and ascorbate. The results in Table 11 are expressed as the net flux of reducing equivalents to allow quantitative comparisons between hemoglobin oxidation and changes in oxidative flux through glycolysis and the hexose monophosphate shunt. In the presence of glucose, 0.27 mM 6-aminodopamine and 0.13 mM 1,4-naphthoquinone-2-sulfonic acid each resulted in formation of about 6 μmoles metheme per hr per ml red cells. Drug-induced increased utilization of reducing equivalents was about 50% greater from the hexose monophosphate shunt than glycolysis and increased utilization was about twice as great with 1,4-naphthoquinone-2-sulfonic acid than with 6-aminodopamine. At 0.27 mM, 6-hydroxydopamine caused formation of 1 μmole metheme per hr per ml red cells with 6-fold greater drug-induced utilization of reducing equivalents from the hexose monophosphate shunt than from glycolysis. A 0.27 mM concentration of phenylhydrazine caused formation of a 0.6 μmole metheme with 70% greater utilization of reducing equivalents from glycolysis than from the hexose monophosphate shunt. With each drug the decrease in metheme accumulation in the presence of glucose (Table 6) could be accounted for by the drug-induced increase in utilization of reducing equivalents through glycolysis and the hexose monophosphate shunt (Table 11). For example, in the presence of glucose, lactate, pyruvate and ascorbate, the decrease in μmoles heme oxidized versus the increase in μmoles of reducing equivalents utilized from glucose metabolism was: with 6-hydroxydopamine, 1.24 vs 2.15 μmoles; with 6-aminodopamine, 2.27 vs 2.66 μmoles; with 1,4-naphthoquinone-2-sulfonic acid, 2.68 vs 4.44 μmoles; with phenylhydrazine, 0.62 vs 1.41 μmoles.

The addition of lactate plus pyruvate caused increased utilization of reducing equivalents from glycolysis (increased accumulation of pyruvate) with all four oxidative agents with little effect on utilization of reducing equivalents from the hexose monophosphate shunt (Table 11). In contrast, the addition of ascorbate caused increased utilization of reducing equivalents from the hexose monophosphate shunt (except with 6-hydroxydopamine) with little effect on glycolysis. Simultaneous addition of lactate, pyruvate and ascorbate resulted in superimposition of the effects of each component on glycolysis and the hexose monophosphate shunt without apparent inhibition or synergism.

DISCUSSION

Incubation of red cells in a medium containing physiologic (plasma) levels of lactate (2 mM), pyruvate (0.1 mM) and ascorbate (0.1 mM) caused a 40% increase in the flux of glucose through glycolysis but had no effect on flux through the hexose monophosphate shunt. In contrast, these metabolites have

Table 7. Effects of 6-hydroxydopamine on flux through glycolysis and the hexose monophosphate shunt*

Additions to incubation medium	Pyruvate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	Lactate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	Pyruvate + lactate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	CO_2 [μmoles·hr^{-1}·(ml red cell)$^{-1}$]
Glucose	0.31 ± 0.04 (4)	2.13 ± 0.16 (4)	2.44 ± 0.17 (4)	0.068 ± 0.015 (4)
" + 6-hydroxydopamine	0.43 ± 0.06† (4)	2.54 ± 0.13‡ (4)	2.96 ± 0.19† (4)	0.429 ± 0.058 (4)
Glucose + lactate + pyruvate	0.28 ± 0.05 (4)	2.59 ± 0.26 (4)	2.87 ± 0.30 (4)	
" + 6-hydroxydopamine	0.62 ± 0.08† (4)	2.41 ± 0.19‡ (4)	3.02 ± 0.26‡ (4)	0.437 ± 0.060‡ (4)
Glucose + ascorbate	0.25 ± 0.05 (4)	2.19 ± 0.08 (4)	2.43 ± 0.12 (4)	
" + 6-hydroxydopamine	0.42 ± 0.06§ (4)	2.79 ± 0.13§ (4)	3.21 ± 0.18§ (4)	0.478 ± 0.076‡ (4)
Glucose + lactate + pyruvate + ascorbate	0.30 ± 0.03 (4)	2.58 ± 0.12 (4)	2.88 ± 0.15 (4)	
" + 6-*hydroxydopamine*	0.64 ± 0.05‖ (4)	2.69 ± 0.23‡ (4)	3.33 ± 0.27‡ (4)	0.436 ± 0.063‡ (4)

* Incubations were carried out as described in Table 6. The formation of pyruvate, lactate and CO_2 in 1 hr at 37° was determined as described in Materials and Methods. Data are reported as mean ± S.E. with the number of experiments in parentheses. Level of significance was determined using a paired *t*-test. For formation of pyruvate, lactate and pyruvate + lactate, statistical comparisons were made between paired incubations with and without the oxidative agent. For formation of CO_2, statistical comparisons were made between incubations containing glucose + oxidative agent and paired incubations containing glucose + oxidative agent + lactate, pyruvate and/or ascorbate.

† $P < 0.05$.

‡ $P > 0.05$, not significant.

§ $P < 0.01$.

‖ $P < 0.001$.

Table 8. Effects of 6-aminodopamine on flux through glycolysis and the hexose monophosphate shunt*

Additions to incubation medium	Pyruvate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	Lactate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	Pyruvate + lactate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	CO_2 [μmoles·hr^{-1}·(ml red cell)$^{-1}$]
Glucose	0.37 ± 0.04 (4)	2.43 ± 0.13 (4)	2.79 ± 0.13 (4)	0.073 ± 0.010 (4)
" + 6-aminodopamine	0.66 ± 0.03† (4)	2.63 ± 0.14‡ (4)	3.29 ± 0.15† (4)	0.292 ± 0.048 (4)
Glucose + lactate + pyruvate	0.26 ± 0.03 (4)	2.97 ± 0.29 (4)	3.23 ± 0.31 (4)	
" + 6-aminodopamine	0.98 ± 0.01§ (4)	2.26 ± 0.33‖ (4)	3.23 ± 0.33‡ (4)	0.307 ± 0.053‡ (4)
Glucose + ascorbate	0.34 ± 0.04 (4)	2.51 ± 0.16 (4)	2.84 ± 0.15 (4)	
" + 6-aminodopamine	0.61 ± 0.03† (4)	2.77 ± 0.10‡ (4)	3.39 ± 0.10‖ (4)	0.407 ± 0.078‖ (4)
Glucose + lactate + pyruvate + ascorbate	0.21 ± 0.06 (3)	3.33 ± 0.20 (3)	3.54 ± 0.14 (3)	
" + 6-aminodopamine	0.87 ± 0.02† (3)	2.77 ± 0.16‖ (3)	3.64 ± 0.16‡ (3)	0.408 ± 0.075‖ (4)

* Experimental details are described in the legend to Table 7.
† $P < 0.01$.
‡ $P > 0.05$, not significant.
§ $P < 0.001$.
‖ $P < 0.05$.

Table 9. Effects of 1,4-naphthoquinone-2-sulfonic acid on flux through glycolysis and the hexose monophosphate shunt*

Additions to incubation medium	Pyruvate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	Lactate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	Pyruvate + lactate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	CO_2 [μmoles·hr^{-1}·(ml red cell)$^{-1}$]
Glucose	0.34 ± 0.01 (4)	2.34 ± 0.13 (4)	2.68 ± 0.13 (4)	0.084 ± 0.004 (4)
" + 1,4-naphthoquinone-2-sulfonic acid	0.89 ± 0.08† (4)	2.27 ± 0.12‡ (4)	3.16 ± 0.19§ (4)	0.512 ± 0.067 (4)
Glucose + lactate + pyruvate	0.27 ± 0.03 (4)	2.81 ± 0.33 (4)	3.08 ± 0.33 (4)	
" + 1,4-naphthoquinone-2-sulfonic acid	1.79 ± 0.31§ (4)	2.01 ± 0.24† (4)	3.80 ± 0.44‡ (4)	0.514 ± 0.063‡ (4)
Glucose + ascorbate	0.32 ± 0.01 (4)	2.47 ± 0.13 (4)	2.78 ± 0.13 (4)	
" + 1,4-naphthoquinone-2-sulfonic acid	0.87 ± 0.07† (4)	2.42 ± 0.17‡ (4)	3.29 ± 0.23§ (4)	0.576 ± 0.065§ (4)
Glucose + lactate + pyruvate + ascorbate	0.24 ± 0.02 (4)	3.23 ± 0.23 (4)	3.46 ± 0.22 (4)	
" + 1,4-naphthoquinone-2-sulfonic acid	1.49 ± 0.11† (4)	2.28 ± 0.35† (4)	3.78 ± 0.36‡ (4)	0.568 ± 0.063§ (4)

* Experimental details are described in the legend to Table 7.
† $P < 0.01$.
‡ $P > 0.05$, not significant.
§ $P < 0.05$.

Table 10. Effects of phenylhydrazine on flux through glycolsis and the hexose monophosphate shunt*

Additions to incubation medium	Pyruvate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	Lactate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	Pyruvate + lactate [μmoles·hr^{-1}·(ml red cell)$^{-1}$]	CO_2 [μmoles·hr^{-1}·(ml red cell)$^{-1}$]
Glucose	0.33 ± 0.06 (4)	2.48 ± 0.11 (4)	2.81 ± 0.10 (4)	0.070 ± 0.010 (4)
" + phenylhydraziine	0.62 ± 0.05† (4)	2.60 ± 0.12‡ (4)	3.22 ± 0.10§ (4)	0.158 ± 0.022 (4)
Glucose + lactate + pyruvate	0.29 ± 0.06 (4)	3.03 ± 0.19 (4)	3.32 ± 0.18 (4)	
" + phenylhydrazine	0.75 ± 0.04‖ (4)	2.79 ± 0.19‡ (4)	3.53 ± 0.22‡ (4)	0.166 ± 0.023‡ (4)
Glucose + ascorbate	0.30 ± 0.04 (4)	2.51 ± 0.18 (4)	2.80 ± 0.15 (4)	
" + phenylhydrazine	0.55 ± 0.05‖ (4)	2.68 ± 0.12‡ (4)	3.23 ± 0.11§ (4)	0.190 ± 0.027§ (4)
Glucose + lactate + pyruvate + ascorbate	0.21 ± 0.04 (4)	3.22 ± 0.07 (4)	3.43 ± 0.06 (4)	
" + phenylhydrazine	0.66 ± 0.09‖ (4)	3.05 ± 0.07‡ (4)	3.71 ± 0.08‡ (4)	0.198 ± 0.027§ (4)

* Experimental details are described in the legend to Table 7.
† $P < 0.001$.
‡ $P > 0.05$, not significant.
§ $P < 0.05$.
‖ $P < 0.01$.

significant effects on the shunt when present in greater than physiologic concentration: 4 mM lactate caused about a 10% decrease in flux through the shunt (data not shown); 1 mM pyruvate caused about a 100% increase in flux [2]; and 12 mM ascorbate caused about a 600% increase in flux [1]. The increase in total flux through glycolysis caused by physiologic concentrations of lactate, pyruvate and ascorbate was mainly coupled to formation of lactate with no increase in pyruvate formation. This result indicates that no net NADH was made available to the red cell by the increase in glycolysis but rather the reducing equivalents from the increased flux were stored in newly formed lactate. Each 3-carbon unit (lactate or pyruvate) formed by the flux of glucose through glycolysis is coupled to formation of one equivalent of ATP from ADP and inorganic phosphate or alternatively to formation of one equivalent of 2,3-diphosphoglycerate. In the normally metabolizing red cell, most of the ATP/ADP couple is present as ATP, so that flux through glycolysis which requires ADP is tightly coupled to utilization of ATP by the red cell; alternatively, flux may be increased by increasing the pool size and/or turnover of 2,3-diphosphoglycerate [30]. Our results indicate that neither ATP nor 2,3-diphosphoglycerate pool sizes are altered by physiologic lactate, pyruvate and ascorbate. Increased turnover of ATP may account for the increased glycolytic flux since Momsen [31] has shown that 2,3-diphosphoglycerate turnover accounted for only 4–8% of flux through glycolysis under reducing conditions (high lactate/pyruvate ratio). Lactate has been shown to cause ATP breakdown in red cells [31] while pyruvate stimulated ATP synthesis [5,32]. Incubation with physiologic lactate, pyruvate and ascorbate caused increased formation of the sum of glycolytic intermediates from fructose-6-phosphate through phosphoenolpyruvate, consistent with the findings of others [31, 33] in red cells incubated under reducing conditions.

Incubation with physiologic lactate and pyruvate caused the same effects but with less magnitude than lactate and pyruvate in the presence of ascorbate. The link between physiologic lactate and pyruvate and increased glycolytic flux was unaffected by a superimposed need for reducing equivalents as shown by the results with methemoglobin-containing red cells. Methemoglobin itself increased the flux through glycolysis while shifting the ratio of new products (lactate/pyruvate) in favor of pyruvate, indicating utilization of reducing equivalents by the methemoglobin reductase system. The addition of physiologic lactate and pyruvate increased glycolysis by about the same absolute amount in methemoglobin-containing red cells as in oxyhemoglobin-containing red cells, but with the ratio of new products shifted in favor of pyruvate.

Addition of physiologic ascorbate alone caused an increase in glycolysis with an increase of both lactate and pyruvate formation. Incubation with ascorbate alone is therefore linked to both increased glycolytic flux and a small oxidative effect (without increased flux through the hexose monophosphate shunt). Ascorbate is slowly autoxidized in the medium followed by uptake of dehydroascorbate and reduction by glutathione [34] or by direct or transmembrane

Table 11. Metabolic sources of reducing equivalents in red cell incubations with oxidative agents*

Additions to incubation medium	Metabolic sources	Reducing equivalents [μmoles·hr^{-1}·(ml red cells)$^{-1}$] in the presence of: 6-Hydroxydopamine	6-Aminodopamine	1,4-Naphthoquinone-2-sulfonic acid	Phenylhydrazine
Glucose	Heme	1.03	5.77	6.59	0.62
	Glycolysis	0.24	0.58	1.10	0.58
	HMS	1.44	0.88	1.71	0.35
	Total	2.71	7.23	9.40	1.55
Glucose + lactate + pyruvate	Heme	0.82	5.56	6.39	0.41
	Glycolysis	0.68	1.44	3.04	0.92
	HMS	1.48	0.94	1.72	0.38
	Total	2.98	7.94	11.15	1.71
Glucose + ascorbate	Heme	0.82	5.56	6.80	0.62
	Glycolysis	0.34	0.54	1.10	0.50
	HMS	1.64	1.34	1.97	0.48
	Total	2.80	7.44	9.87	1.60
Glucose + lactate + pyruvate + ascorbate	Heme	0.82	5.56	6.59	0.62
	Glycolysis	0.68	1.32	2.50	0.90
	HMS	1.47	1.34	1.94	0.51
	Total	2.97	8.22	11.03	2.03

* Drug-induced increases in net flux of reducing equivalents (electrons) to oxygen or other electron sinks in 1 ml red cells in 1 hr at 37° are shown. The net flux is calculated from the drug-induced change in net accumulation of oxidized species (methemoglobin, pyruvate, CO_2) by 1 hr and does not include cycling between oxidised and reduced species. For example, oxidation of heme (Fe^{2+}) to heme (Fe^{3+}) followed by reduction to heme (Fe^{2+}) by NADH would appear as net flux through glycolysis not heme. Micromoles of reducing equivalents from heme = (percent methemoglobin accumulated in the presence of drug -1) $\times$ 0.206; μmoles of reducing equivalents from glycolysis = (pyruvate accumulated in the presence of drug − pyruvate accumulated in the absence of drug) $\times$ 2; μmoles of reducing equivalents from the hexose monophosphate shunt = (CO_2 accumulated in the presence of drug − CO_2 accumulated in the absence of drug) $\times$ 4. HMS, hexose monophosphate shunt.

reduction by NADH generated by the glyceraldehyde-3-phosphate dehydrogenase reaction [35]. In the presence of methemoglobin, no effect of ascorbate on glycolytic flux is observed as the flow of reducing equivalents through ascorbate may be exclusively utilized by the methemoglobin reductase system in these cells [6]. In oxyhemoglobin-containing red cells, the presence of physiologic lactate and pyruvate completely prevents the oxidative effect of physiologic ascorbate on the lactate/pyruvate products.

The most consistent effect of oxidative stress on glycolysis was the decrease in the ratio of the products, lactate/pyruvate. When lactate is accumulated as the final product of glycolysis, no net utilization of reducing equivalents by the red cell occurs; the accumulation of pyruvate implies utilization of two electron equivalents per mole of pyruvate. In the absence of exogenous lactate and pyruvate, we observed that increased total glycolytic flux and, with two of the oxidative agents, increased formation of lactate were coupled to methemoglobin reductase activity and accumulation of pyruvate. This can be explained by the requirement to establish intracellular and extracellular pools of lactate to support the activity of lactate dehydrogenase which is controlled by the redox ratios; lactate/pyruvate, $NADH/NAD^+$ and, through the methemoglobin reductase system, deoxyhemoglobin/methemoglobin. The presence at zero time of exogenous lactate and pyruvate, both at physiologic concentrations (lactate/pyruvate approximately 20/1), allowed a shift in accumulation of glycolytic products from lactate to pyruvate without a significant increase in lactate or total glycolytic flux (lactate plus pyruvate). It is important to note that exogenous lactate or pyruvate did not serve as a net electron source or sink since the total content in red cell suspensions of both pyruvate and lactate were increased by 1 hr at 37°. The importance of these findings to red cell metabolism lies in the fact that to increase total glycolytic flux, in the absence of exogenous lactate plus pyruvate, requires increased turnover of ATP and/or 2,3-diphosphoglycerate which might be deleterious to the red cell. Exogenous lactate and pyruvate allow for increased functioning of the methemoglobin reductase system with little or no increase in turnover of ATP or 2,3-diphosphoglycerate. In this way, the dual functions of glycolysis, production of ATP and NADH, are functionally uncoupled for maximum efficiency.

In the presence of an oxidative stress, physiologic levels of exogenous ascorbate increased flux through the hexose monophosphate shunt, but had no effect on glycolysis. Reducing equivalents from the hexose monophosphate shunt maintain ascorbate in the reduced form [6, 36] and ascorbate can, in turn, reduce quinones and semiquinones resulting in cycling of oxidative reactions and increased production of H_2O_2 [11–14]. Both increased H_2O_2 and increased cycling of ascorbate resulted in increased shunt activity. The exact role of exogenous ascorbate is complicated (e.g. the lack of significant effect in the presence of 6-hydroxydopamine) by the presence of 0.06 mM endogenous ascorbate remaining in washed red cells.

Methemoglobin accumulation by 1 hr at 37° in the presence of glucose was unaffected by the presence of lactate, pyruvate and ascorbate. With 6-hydroxydopamine, 6-aminodopamine and 1,4-naphthoquinone-2-sulfonic acid, the inhibition of methemoglobin formation in the presence of glucose was much greater than can be accounted for by the activity of the methemoglobin reductase system [6]. In the absence of oxidative stress, the reductase activity is usually the same with glucose as with lactate [6]. In the presence of oxidative stress, the increased inhibition of methemoglobin formation in the presence of glucose may be explained by prevention of methemoglobin formation or reduction of methemoglobin by semiquinones and quinols. The latter mechanism has been shown to operate with 6-hydroxydopamine [23], primaquine [37] and 1,4-naphthoquinone-2-sulfonic acid (data not shown). With these agents, reducing equivalents from the hexose monophosphate shunt reduce the quinone to the semiquinone and quinol which, in turn, reduce methemoglobin.

Studies with red cells containing various concentrations of methemoglobin demonstrated that availability of physiologic glucose, lactate, pyruvate and ascorbate resulted in increased flexibility in meeting the requirement of the red cell for continual methemoglobin reduction [6]. In the normal red cell, containing about 1% methemoglobin, and in the absence of oxidative stress, the availability of physiologic lactate, pyruvate and ascorbate resulted in no change in the hexose monophosphate shunt but an increase in glucose flux through glycolysis. The increase in glycolytic flux was coupled with the storage of reducing equivalents by increased formation of lactate with little change in pyruvate formation. This pattern of glucose metabolism allows increased flow of reducing equivalents from glycolysis in response to oxidative stress without increased total glycolytic flux. In mild oxidative stress, glycolysis and the hexose monophosphate shunt are equally important as sources of reducing equivalents.

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